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Salmonella enterica Serovar Enteritidis Core O Polysaccharide Conjugated to H:g,m Flagellin as a Candidate Vaccine for Protection against Invasive Infection with *S.* Enteritidis▽†

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Nontyphoidal *Salmonella enterica* serovars Enteritidis and Typhimurium are a common cause of gastroenteritis but also cause invasive infections and enteric fever in certain hosts (young children in sub-Saharan Africa, the elderly, and immunocompromised individuals). *Salmonella* O polysaccharides (OPS) and flagellar proteins are virulence factors and protective antigens. The surface polysaccharides of *Salmonella* are poorly immunogenic and do not confer immunologic memory, limitations overcome by covalently attaching them to carrier proteins. We conjugated core polysaccharide-OPS (COPS) of *Salmonella* Enteritidis lipopolysaccharide (LPS) to flagellin protein from the homologous strain. COPS and flagellin were purified from a genetically attenuated (Δ*guaBA*) "reagent strain" (derived from an isolate from a patient with clinical bacteremia) engineered for increased flagellin production (Δ*clpPX*). Conjugates were constructed by linking flagellin monomers or polymers at random COPS hydroxyls with various polysaccharide/protein ratios by 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) or at the 3-deoxy-D-manno-octulosonic acid (KDO) terminus by thioether chemistry. Mice immunized on days 0, 28, and 56 with COPS-flagellin conjugates mounted higher anti-LPS IgG levels than mice receiving unconjugated COPS and exhibited high antiflagellin IgG; anti-LPS and antiflagellin IgG levels increased following booster doses. Antibodies generated by COPS-flagellin conjugates mediated opsonophagocytosis of *S.* Enteritidis cells into mouse macrophages. Mice immunized with flagellin alone, COPS-CRM_{197}, or COPS-flagellin conjugates were significantly protected from lethal challenge with wild-type *S.* Enteritidis (80 to 100% vaccine efficacy).

In a small subset of cases in the United States, primarily in susceptible populations with immature or weak immune systems (e.g., young infants, the elderly, and immunocompromised individuals), nontyphoidal *Salmonella* (NTS), which normally produces gastroenteritis in healthy adults and older children, can manifest as a lethal invasive disease (27). In sub-Saharan Africa, hospital- and clinic-based surveillance for blood-borne bacterial disease instituted primarily to quantify the burden of invasive *Haemophilus influenzae* type b (Hib) and *Streptococcus pneumoniae* (pneumococcal) disease discovered that invasive NTS infections rivaled Hib and pneumococcus as causes of bacteremia in infants and young children (4, 5, 16, 22, 26, 34, 39, 46, 56, 68). Some reports noted that approximately two-thirds of these young African children with invasive NTS disease did not present with or have a history of gastroenteritis (64), and clinical severity was high with case fatality rates of 15 to 30% (13). Two serovars, *Salmonella enterica* serovar Enteritidis (group D) and *Salmonella enterica* serovar Typhimurium (group B), accounted for 75 to 90% of reported cases (4, 5, 16, 22, 26, 34, 39, 46, 56, 64, 68), and most *Salmonella* bacteria carried resistance to multiple clinically relevant antibiotics. Most sub-Saharan *S.* Typhimurium bacteria were found to belong to an unusual multilocus sequence type (28).

On the basis of the epidemiological characteristics and severe clinical outcomes associated with these emerging invasive African NTS strains among some of the world's most disadvantaged pediatric populations, efforts have been initiated in several quarters to design intervention strategies to diminish this disease burden. Development of a safe and effective bivalent vaccine against *S.* Enteritidis and *S.* Typhimurium would constitute one practical public health tool to help achieve this goal.

Vaccines targeting the capsular and outer membrane polysaccharides of pathogenic bacteria have proven to be an effective strategy for protection from disease caused by multiple bacterial pathogens (18, 38, 48, 50, 51). Bacterial polysaccharides are generally T-independent antigens that are poorly immunogenic in infants and do not confer immunologic memory at any age (15, 51). The immunogenicity of polysaccharides can be enhanced by their covalent attachment to carrier pro-

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teins, resulting in higher antibody levels, predominance of different IgG subtypes, and T helper cell-induced immunologic memory (45, 51).

Salmonella bacterial outer membrane lipopolysaccharide (LPS) provides virulence functions to the bacterium. Structurally, it is characterized by a terminal lipid A group at the 3-deoxy-D-manno-octulosonic acid (KDO) terminus of the conserved core polysaccharide (19). The serovar-specific O polysaccharide (OPS) region extends as a repeating polymer from the distal end of the core (49, 53). The OPS of *Salmonella* groups A, B, and D have a common group 12 →2)-α-D-Man*p*-(1→4)-α-L-Rha*p*-(1→3)-α-D-Gal*p*-(1→ trisaccharide backbone. *S.* Enteritidis, like all serogroup D *Salmonella*, exhibits immunodominant antigen 9, characterized structurally by the α-3,6-tyvelose linked (1→3) to the mannose residue of the trisaccharide backbone (53). The structure of *Salmonella* OPS influences the activity of the alternative arm of the complement cascade, resulting in resistance to bactericidal killing and to uptake by phagocytes (23, 36). Long-chain LPS can also shield the bacterial surface from the complement system membrane attack complex (MAC), thus precluding direct bactericidal killing (17). These virulence properties of *Salmonella* LPS can be overcome by specific antibody against the polysaccharide of LPS. Conjugates consisting of *S.* Typhimurium OPS linked to heterologous (e.g., tetanus toxoid, bovine serum albumin) (25, 62, 70) and homologous *Salmonella* (porin) carrier proteins (63) have protected mice against lethal *S.* Typhimurium challenge. Antibody elicited by these conjugates can mediate opsonophagocytic uptake of *Salmonella* into phagocytic cells and provide immunity following passive transfer into naïve hosts (25, 62, 63, 70).

Salmonella flagella are virulence factors (24, 71) that extend from the outer membrane to provide motility and are comprised almost entirely of polymers of the ~50-kDa FliC flagellin protein (7). The *S.* Enteritidis genome encodes only a phase 1 flagellin, FliC, which exhibits the H:g,m epitopes. In the murine typhoid model, flagellin has been reported as a major target of the host adaptive immune response following systemic *S.* Typhimurium infection and is also a protective antigen (3, 40, 60, 61). Flagellin is also a target of the host innate immune Toll-like receptor 5 (TLR5) at regions that form the interior core of the flagellar polymer (59). Flagellar polymers thus activate TLR5 but to a lesser extent than flagellin monomers (59). A variety of heterologous prokaryotic, viral, and eukaryotic protein antigens have shown increased immunogenicity in mice following genetic fusion or chemical conjugation with flagellin (6, 43, 69).

Flagellin as a carrier protein for NTS OPS thus should both enhance the immunogenicity of the OPS hapten as well as provide immunity to a second homologous pathogen protective antigen. In this article, we report the synthesis, immunological characterization, and protective efficacy in mice, of a candidate *Salmonella* Enteritidis conjugate vaccine comprised of FliC conjugated to lipopolysaccharide-derived core-OPS (COPS).

MATERIALS AND METHODS

Bacterial strains. *Salmonella enterica* serovar Enteritidis R11 was isolated from blood from a toddler in Mali (35), and its serotype was confirmed by the Clinical Microbiology Unit of the Center for Vaccine Development (CVD). Strain CVD 1941 (R11 Δ*guaBA* Δ*clpP*) is an attenuated derivative of *S.* Enteritidis R11 that hyperexpresses flagella (64a) and was used as a "reagent strain" for safe and efficient bulk purification of LPS and flagellin proteins. Mutant derivatives of R11 deficient in the *invA*, *fliC*, and *rfaL* genes were constructed by lambda red mutagenesis (12) with primers as described in the supplemental material (Table S1). *S.* Enteritidis was grown in or on animal product-free LB Lennox medium (Athena ES, Baltimore, MD) supplemented with 0.005% guanine (wt/vol) as necessary (for growth of CVD 1941) at 37°C. *Salmonella* cells used for *in vitro* analyses and *in vivo* challenge experiments were grown to mid-log phase on solid medium, harvested, and suspended in sterile phosphate-buffered saline (PBS).

Cell culture. J774 murine macrophage cells and human epithelial kidney cells stably expressing the firefly luciferase reporter under the control of an NF-κB dependent promoter (HEK293-Luc, generously provided by S. Ghosh, Yale University, New Haven, CT) (72) were maintained in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) fetal bovine serum, 1 mM sodium pyruvate, and 100 μg/ml penicillin-streptomycin. The cells were incubated at 37°C in a 5% CO_2 atmosphere.

Polysaccharide and protein analyses. Polysaccharide concentration was assessed using the resorcinol sulfuric acid assay (44). Protein concentration was determined by absorbance at 280 nm using the calculated extinction coefficients of *S.* Enteritidis flagellin or CRM_{197} determined by the Expasy Protparam tool (http://www.expasy.ch/tools/protparam.html) with protein sequences obtained from the NCBI database (www.ncbi.nlm.nih.gov). For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, samples were fractionated by 4 to 20% Tris-glycine acrylamide gels (Invitrogen) and stained with Coomassie GelCode blue (ThermoFisher). For polysaccharide staining, gels were stained using the Pro-Q staining system (Invitrogen) per the manufacturer's instructions.

Purification of flagellin polymers. *Salmonella* cell-associated flagella were isolated as described previously (57). Flagellar protein was assessed for identity by Western blotting with flagellin-specific monoclonal antibody 15D8 (Bioveris) (see Fig. S1C in the supplemental material).

Preparation of flagellin polymers and monomers for conjugation. Monomers for construction of O:H 1:1 lot 1 conjugate were generated as previously described by heating flagellar filaments at 70°C for 15 min (59). For preparation of polymers and monomers for conjugation in all other flagellin-based conjugates, a modified version of the method of Ibrahim et al. (21) was utilized. Dispersion of purified flagellar filaments into monomers was accomplished by lowering the pH to 2 for 30 min with stirring. For flagellar purification, flagellin in solution at pH 2 was subjected to 100,000 × *g* centrifugation for 3 h at 4°C in order to pellet LPS vesicles. The supernatant was then adjusted to 2.67 M ammonium sulfate and incubated overnight at 4°C with stirring. The precipitate was recovered by 10,000 × *g* centrifugation, resuspended in water, and dialyzed against saline in 10-kDa molecular weight cutoff (MWCO) dialysis cassettes (Thermo). Depolymerized flagellin monomers prepared by either method were filtered through a 100-kDa MWCO ultrafiltration (UF) centrifugal device (Amicon) to remove any remaining high-molecular-weight flagellar polymers and LPS vesicles, followed by concentration with 30-kDa MWCO UF and adjustment to pH 7.2 with 10× concentrated PBS. The residual endotoxin level was measured with the chromogenic endpoint *Limulus* Amebocyte Lysate assay (Lonza). These purification methods effectively removed >99% of contaminating LPS, leaving <50 endotoxin units per μg of protein. Flagellin monomers were assessed for size compared to flagellar polymers (see Fig. S1D in the supplemental material).

Preparation of *Salmonella* LPS. Strain CVD 1941 was grown in medium supplemented with 0.005% guanine (wt/vol) and 1 mM $MgCl_2$ to supplement the *gua* deficiency and enhance production of long-chain LPS (R. K. Ernst and S. I. Miller, unpublished data), respectively, overnight in flasks at 37°C and 250 rpm. Cells were harvested by centrifugation at 7,000 × *g* for 20 min at 4°C, and LPS was isolated by the method of Darveau and Hancock essentially as described with the addition of a final phenol purification step (11, 20). Purified LPS was resuspended in pyrogen-free water (PFW) to 20 mg/ml for subsequent manipulation.

Isolation of *Salmonella* core/outer membrane polysaccharide (COPS). *Salmonella* COPS was isolated from purified LPS as described previously (70). Briefly, LPS was brought to 10 mg/ml in 1% acetic acid and incubated for 1.5 h at 100°C. Insoluble lipid A was removed by ultracentrifugation for 5 h at 100,000 × *g* and discarded in the pelleted fraction. The supernatant fraction was concentrated by lyophilization and resuspended in PFW. High-molecular-weight COPS was fractionated by size exclusion chromatography on a Superdex 75 10/300 GL column (GE/Amersham) with an AKTA Explorer (GE/Amersham) run at 0.5 ml/min in saline solution with 1-ml fractions taken. Fractions were monitored for polysaccharide using the resorcinol sulfuric acid assay. High-molecular-weight fractions used for conjugation were pooled, concentrated by lyophilization, and resuspended in PFW. The final COPS concentration was determined with the resorcinol sulfuric acid assay using purified CVD 1941 LPS as standards. The endo-

toxin level was assessed by LAL to be <1 endotoxin unit per μg of polysaccharide.

Synthesis of COPS conjugates. (i) Conjugation by CDAP. Direct conjugation between COPS at random points along the polysaccharide chain with either flagellin monomers, flagellar polymers, or CRM_{197} (a generous gift from L. Martin, Novartis Vaccine Institute for Global Health, Sienna, Italy) was accomplished using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) (Research Organics) (32, 55). Briefly, random activation of hydroxyl groups on COPS was accomplished by addition of 100 mg/ml CDAP in acetonitrile to an equivalent amount (wt/wt) of either 4 mg/ml (for O:H 1:1 lot 1 conjugate) or 10 mg/ml (for all other CDAP-linked conjugates) COPS in 10 mM sodium borate buffer, pH 9 (pH goes down to 8 to 8.5). After 30 s, 1/100th volume of triethylamine (Sigma) was added to bring the pH to 9 to 10. After 2 min, protein (3 mg/ml flagellin monomers, 12 mg/ml flagellar polymers, 13 mg/ml CRM_{197}) was added at predefined ratios of polysaccharide to protein (1:1 or 10:1, wt/wt). The conjugation reaction mixture was incubated at room temperature for 2 h with tumbling rotation and then at 4°C for 3 days with tumbling rotation, at which point the reaction was quenched by bringing the solution to 10 mM glycine and assayed for conjugation.

(ii) Conjugation at the KDO terminus by aminooxyoxime thioether chemistry. Conjugation at the carboxyl group present at the KDO terminus on COPS with flagellin monomers was accomplished with aminooxy chemistry (33). COPS was suspended to 10 mg/ml in 100 mM morpholineethanesulfonic acid (MES) (pH 5)–28 mg/ml ethylcarbodiimide hydrochloride (EDC) (Sigma)–11 mg/ml diaminooxy cysteamine (33) (custom synthesis from Solulink, CA) and incubated first for 3 h at room temperature and then overnight at 4°C on a rotating mixer. The derivatized COPS was brought to 250 mM dithiothreitol (DTT) the next day and dialyzed in 3-kDa MWCO dialysis cassettes (Thermo) against 10 mM sodium acetate–10 mM EDTA (pH 6) overnight at 4°C. Activated flagellin monomers (3 mg/ml) were prepared by incubation with 1.5 mg/ml *N*-(γ-maleimidobutyloxy)-sulfosuccinimide ester (sulfo-GMBS) (Molecular BioSciences) in 25 mM HEPES (pH 7.5) for 3 h at room temperature. The reaction was then brought to 100 mM sodium acetate (pH 5) and incubated overnight at 4°C. The derivatized protein was dialyzed the next day in 3-kDa MWCO dialysis cassettes (Thermo) against 10 mM sodium acetate–10 mM EDTA (pH 6) overnight at 4°C. The thiol-labeled COPS polysaccharide was added to sulfo-GMBS derivatized flagellin monomer protein at a ratio of 3:1 (wt/wt), and the pH was incrementally raised to 6.8 with 10× PBS (pH 7.4). Conjugation was allowed to proceed for 3 days at 4°C with tumbling before the reaction was assayed for conjugation.

Purification and characterization of COPS conjugates. (i) Purification by size exclusion chromatography (SEC). The conjugation reaction was concentrated with 30-kDa UF and then fractionated on a Superdex 200 10/300 GL column (GE/Amersham) with an AKTA Explorer (GE/Amersham) run at 0.5 ml/min in 10 mM Tris (pH 7.5) with monitoring at 280 nm. High-molecular-weight conjugate eluted fractions were assessed by the optical density at 280 nm (OD_{280}) trace and SDS-PAGE with Coomassie blue staining for fractions. Conjugate-containing fractions were pooled and concentrated by 30-kDa UF.

(ii) Purification by anion-exchange chromatography (IEX). Some conjugates were further purified by membrane anion-exchange chromatography (R. Simon and A. Lees, unpublished data). Briefly, flagellin monomer and CRM_{197} conjugates purified by SEC or unpurified flagellar polymer conjugates in 10 mM Tris (pH 7.5) were applied to a 3-ml Sartobind Nano-Q anion-exchange membrane (Sartorius) with an AKTA Explorer (GE/Amersham) at 1 ml/min with monitoring at 280 nm. The membrane was washed first with 15 ml of 10 mM Tris (pH 7.5) and then with 30 ml of either 10 mM Tris–80 mM NaCl (pH 7.5) for flagellin-containing conjugates or 10 mM Tris–50 mM NaCl (pH 7.5) for CRM_{197} conjugates. The membranes were eluted with a gradient in 24 ml of the indicated wash buffer to 10 mM Tris–1 M NaCl (pH 7.5). The eluate fraction was pooled and concentrated by 30-kDa MWCO UF and used for immunogenicity experiments in mice.

(iii) Characterization of purified conjugates. Polysaccharide concentration in conjugate constructs was assessed with the resorcinol sulfuric acid assay with *Salmonella* CVD 1941 LPS as standards. The protein concentration in conjugates synthesized with CDAP was assessed by bicinchoninic acid (BCA) assay kit (Thermo) with unconjugated protein as standards. Conjugates prepared by thioether chemistry were assessed for protein concentration by measurement of absorbance at 280 nm using the calculated extinction coefficient of *S.* Enteritidis flagellin.

Flagellin stimulation of epithelial cells. Monolayers of HEK293-Luc cells seeded at 1.7×10^4 cells/well in 96-well plates for NF-κB activation analyses were treated for 4 h with purified flagellin protein, conjugate, or LPS. Extracts were prepared, and luciferase activity was assessed by the Luciferase Assay system (Promega) according to the manufacturer's instructions using a Versamax II plate luminometer. Normalization to the total protein level was determined by Bradford reagent (Bio-Rad).

Mice. Female outbred CD-1 and inbred BALB/c mice (8 to 10 weeks old) were purchased from Charles River Laboratories (Wilmington, MA) and maintained in the University of Maryland School of Medicine animal facility. All animal experimental protocols were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

Immunizations. Mice were injected intramuscularly (IM) in the right hind limb with antigen suspended in 50 μl of sterile PBS. For immunizations with conjugate vaccines, mice were immunized at 0, 28, and 56 days, and serum samples were obtained 21 or 22 days following each immunization and stored at −20°C until use. For immunization with purified flagellin monomers or flagellar polymers, mice were immunized at 0 and 10 days.

Challenge. CD-1 mice immunized with conjugate vaccines were challenged by the intraperitoneal (IP) route 28 days after the third immunization (on day 84), with either 5×10^5, 1×10^6, or 5×10^6 CFU of virulent wild-type *S.* Enteritidis strain R11. Mice immunized with purified flagellin were challenged 14 days following the second dose (on day 24) with 5×10^5 CFU of virulent wild-type *S.* Enteritidis strain R11. The IP 50% lethal dose (LD_{50}) of *S.* Enteritidis R11 in CD-1 mice was determined to be 2.2×10^5 CFU. Mice were monitored for 21 days postchallenge, recording overall health, weight loss, and mortality. As alternative endpoints, moribund mice exhibiting symptoms including lethargy, nonresponsiveness, and >20% weight loss were euthanized and recorded as dead.

Serum antibody analysis. (i) ELISA. Serum IgG levels against LPS, flagellin protein, or CRM_{197} protein were measured by ELISA. Briefly, 96-well plates were coated with *S.* Enteritidis LPS (5 μg/ml), H polymer (2 μg/ml) in 0.05 M sodium carbonate (pH 9.6), and CRM_{197} (5 μg/ml) in PBS (pH 7.4) for 3 h at 37°C and blocked overnight with 10% dried milk in PBS. After each incubation, the plates were washed with PBS containing 0.05% Tween 20 (PBST) (Sigma). Sera were tested in serial dilutions in PBST containing 10% milk. Specific antibodies were detected using peroxidase-labeled anti-mouse IgG (KPL) diluted 1:1,000 in PBST containing 10% dried milk. 3,3′,5,5′-Tetramethylbenzidine (TMB) was used as the substrate (KPL). Test and control sera were run in duplicate. Titers were calculated by interpolation of absorbance values of test samples into the linear regression curve of a calibrated control (reference serum). The endpoint titers reported as ELISA units (EU) represent the inverse of the serum dilution that produces an absorbance value of 0.2 above the blank. Seroconversion in vaccinated mice was defined as a 4-fold increase in the antibody titer after immunization compared to control mice immunized with PBS.

(ii) Opsonophagocytic uptake. Analysis of functional antibody was examined by opsonophagocytic assay measuring serum-mediated uptake into phagocytes. Briefly, logarithmic growth cultures of wild-type *S.* Enteritidis R11 cells were adjusted to 3×10^6 CFU/ml and incubated in 10% heat-inactivated serum for 20 min at room temperature. Opsonized R11 cells were added to monolayers of J774 cells in 24-well plates (1×10^5 cells/well) in antibiotic-free medium at a ratio of 1:1 (cell/cell) and centrifuged for 10 min at $1,000 \times g$ at room temperature. At 1 h after the addition, the growth medium was replaced with medium containing 100 μg/ml gentamicin (Gibco). At the 2-h time point, cell monolayers were washed three times with sterile PBS, lysed with PBS containing 0.5% Triton X-100 (Sigma), and assessed for viable CFU. Results are expressed as the percentage of phagocytosis as defined by the number of recovered CFU/number of CFU added to the J774 cells. For experiments involving uptake by immune sera of different *Salmonella* Enteritidis mutants, the fold uptake relative to control sera is shown to account for variations in the basal level of uptake between different mutants.

Statistical analysis. Statistical significance between geometric mean IgG titers from different experimental groups and comparative protection from mortality by different conjugate vaccines was assessed by Mann-Whitney rank sum analysis, *t* test, and Fisher's exact test (FET), respectively, using the Sigma-Stat software package.

RESULTS

Characterization of *S.* Enteritidis COPS. The LPS of *S. enterica* serovar Enteritidis was fractionated by SDS-PAGE into several isoforms, ranging from high-molecular-weight, very long species to low-molecular-weight, short species (Fig. 1A). Following removal of lipid A, COPS similarly separated into high- and low-molecular-weight species by size exclusion chromatography (SEC) (Fig. 1B). COPS with lipid A removed

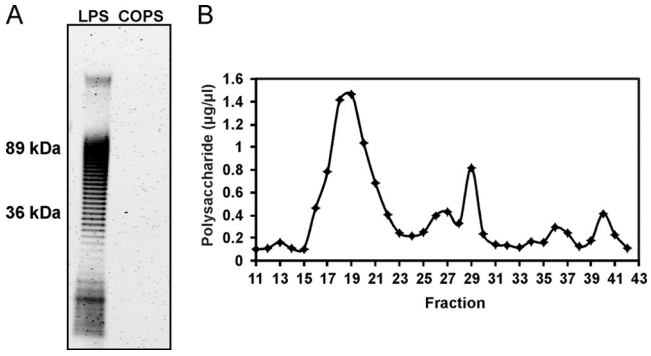

FIG. 1. Gel filtration profile of *S.* Enteritidis LPS and COPS. (A) SDS-PAGE analysis with Pro-Q staining for 10 μg of *S.* Enteritidis LPS and 10 μg of *S.* Enteritidis COPS. (B) Size exclusion gel filtration profile of *S.* Enteritidis COPS through Superdex 75 assessed by resorcinol assay for polysaccharide.

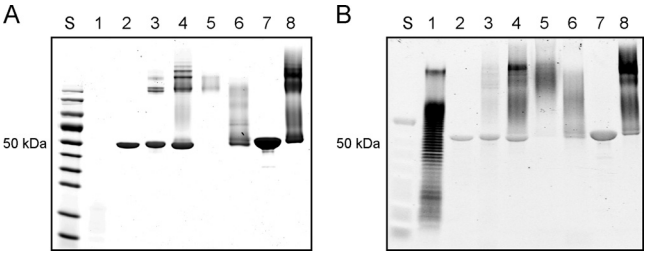

FIG. 2. Conjugation of *S.* Enteritidis COPS conjugates. (A and B) Four to 20% SDS-PAGE showing Coomassie blue (A) or Pro-Q (B) staining of COPS conjugates. Lanes S, protein standards; 1, 10 μg *S.* Enteritidis LPS; 2, 10 μg *S.* Enteritidis flagella; 3, 10 μg O:H polymer; 4, 10 μg O:H 1:1 lot 2 conjugate; 5, 10 μg O:H 10:1 conjugate; 6, 10 μg O:H Amox. conjugate (named Amox. for aminooxy); 7, 10 μg CRM_{197}; 8, 10 μg O:CRM_{197} conjugate.

did not enter an SDS-polyacrylamide gel, indicating removal of the negatively charged phosphate groups present in and around lipid A (Fig. 1A). Removal of lipid A in COPS preparations was further confirmed by *Limulus* Amebocyte Lysate (LAL) assay which showed <1 endotoxin unit per μg of COPS polysaccharide. For conjugation, pooled high-molecular-weight COPS species from SEC were used, since conjugates with long-chain OPS were previously shown to be more immunogenic than conjugates made with short-chain OPS (25).

Synthesis, purification, and characterization of COPS-protein carrier conjugates. To test the ability of COPS to conjugate with flagellin, a COPS-flagellin conjugate was constructed by 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) chemistry. This conjugate construct designated "O:H 1:1 lot 1" was used in immunogenicity experiments in BALB/c and CD-1 mice. Additional conjugates were subsequently prepared to test the contributions of various parameters known to influence the immunogenicity of polysaccharide-protein glycoconjugates (Table 1). The experimental parameters tested include various carrier proteins (flagellin monomer, flagellar polymer, and CRM_{197}), conjugation chemistries (CDAP or aminooxy thioether), and polysaccharide/protein ratios (1:1 or 10:1). These constructs were tested in CD-1 mice.

Conjugation of COPS with protein by CDAP chemistry generates a heterogeneous glycoconjugate population due to the generation of reactive cyano-esters on carbohydrate hydroxyl groups throughout the polysaccharide which can conjugate to several available lysine residues on the protein. This results in covalent links being formed at multiple potential points. Coupling by this method can produce conjugates consisting of a single protein molecule linked to one or more COPS molecules, as well as a lattice that can be formed by intermolecular linkage by a single COPS molecule with multiple protein subunits. We observed that conjugation of protein with COPS produced a spectrum of high-molecular-weight species that migrated above the molecular weight of unconjugated protein by SDS-PAGE and were readily visualized in conjugates constructed with a 1:1 equivalent polysaccharide/protein coupling ratio (Fig. 2A and B, lanes 3, 4, and 8). By comparison, the single weakly staining band observed by SDS-PAGE with the COPS-flagellin constructed at a 10:1 polysaccharide-to-protein coupling ratio (Fig. 2A and B, lanes 5) suggests that all of the protein was conjugated and that the majority of conjugates were likely linked in a lattice type structure that was too large to enter the 4 to 20% SDS-polyacrylamide gel. These high-molecular-weight COPS conjugates also stained positive for both protein (Fig. 2A) and polysaccharide (Fig. 2B), indicating the presence of both components in the conjugate.

Conjugation of COPS by aminooxy thio-ether chemistry limits coupling to a single point at the terminal 3-deoxy-D-manno-octulosonic acid (KDO) region by activation of the KDO carboxyl and should result in conjugates consisting of COPS molecules conjugated at the polysaccharide terminus to one or more available lysine residues on the protein. Since coupling by this method precludes the formation of a lattice, it resulted in a comparatively less heterogeneous lower-molecular-weight

TABLE 1. Preparation and characterization of *S. enterica* serovar Enteritidis COPS-conjugates used for immunization experiments in mice

Conjugate lot designation	Carrier protein	Polysaccharide/protein conjugation ratio (wt/wt)[a]	Conjugation chemistry	Purification scheme	Final polysaccharide/protein ratio (wt/wt)[b]	Mouse
O:H 1:1 lot 1	Flagellin monomer	1:1	CDAP	SEC	0.78	BALB/c and CD-1
O:H 1:1 lot 2	Flagellin monomer	1:1	CDAP	SEC-IEX	0.26	CD-1
O:H polymer	Flagellar polymer	1:1	CDAP	IEX	0.56	CD-1
O:H 10:1	Flagellin monomer	10:1	CDAP	SEC-IEX	0.97	CD-1
O:H Amox.	Flagellin monomer	3:1	Aminooxy	SEC-IEX	0.45	CD-1
O:CRM_{197}	CRM_{197}	1:1	CDAP	SEC-IEX	0.55	CD-1

[a] Ratio of polysaccharide to protein used in the conjugation reaction.
[b] Final ratio of polysaccharide to protein in the purified conjugate.

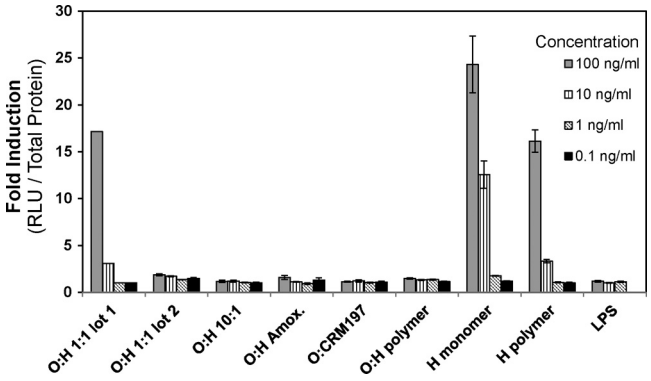

FIG. 3. NF-κB activation in epithelial cells by flagellin and COPS conjugates. HEK293 cells stably transformed with a firefly luciferase reporter gene under the control of an NF-κB-dependent promoter were treated in triplicate with increasing concentrations of COPS-based conjugate vaccines, *S.* Enteritidis flagellin monomer, *S.* Enteritidis flagellar polymer, or *S.* Enteritidis LPS. The cells were harvested at 4 h after treatment, and luciferase activity (in relative light units [RLU]) was determined. Luciferase activity was normalized to HEK293 cell extract protein. Values are averages ± standard errors (error bars) from three independent experiments, with the exception of the results shown with O:H 1:1 lot 1 conjugate, which were from one representative experiment of two independent experiments.

conjugate population compared to conjugates prepared by CDAP (Fig. 2A and B, lanes 6).

Unconjugated free polysaccharide is recognized as being deleterious to the efficacy of polysaccharide-protein conjugate vaccines and can impair antipolysaccharide immune responses (51). Unreacted polysaccharide and protein were removed by size exclusion chromatography (see Fig. S2A and S2B in the supplemental material). Fractionation of conjugates by SEC resulted in a clear separation of high-molecular-weight conjugated species (>75 kDa) from low-molecular-weight unreacted proteins (<75 kDa), with very high-molecular-weight fractions eluting in the column void volume (Fig. S2A and S2B).

As COPS is neutral or weakly negatively charged compared to the protein carrier (Fig. 1A), separation by ion exchange is possible as well. To remove residual unconjugated high-molecular-weight COPS that may remain following SEC purification, a second purification step utilizing separation by charge was accomplished by anion exchange (see Fig. S2C in the supplemental material). A novel membrane chromatography method was used to remove residual unconjugated carbohydrate. High-molecular-weight conjugates usually do not bind well to conventional chromatography resins due to limited accessibility to narrow pores within the resin beads (A. Lees, unpublished observations). Macroporous sorbents that utilize large channels such as monoliths and membranes can be used to purify conjugate vaccines (A. Lees, PCT application WO2011/017101). For the O:H flagellar polymer conjugate, membrane anion-exchange chromatography alone was used for removal of unreacted polysaccharide. Since CDAP reagent absorbs strongly at OD_{280}, unreacted CDAP-labeled polysaccharide intermediates also absorb strongly at OD_{280}. Following application of the SEC-purified conjugates to the anion-exchange membrane, unreacted CDAP-labeled polysaccharide was evident in the flowthrough and low-salt wash step, as indicated by both OD_{280} and the resorcinol assay (see Fig. S2C and S2D in the supplemental material). Negligible protein was detected by BCA assay in these fractions (Fig. S2D). High levels of both protein and polysaccharide were detected in the high-salt elution step, indicating successful elution of the conjugate and efficient removal of unreacted polysaccharide.

The final assessed polysaccharide/protein ratio varied between conjugate preparations as a function of the ratio of individual antigens used in the conjugation reaction (Table 1). Notably, conjugates constructed at a 10:1 polysaccharide-to-protein ratio maintained a higher final polysaccharide level than conjugates constructed with lower coupling ratios.

When assessed for activation of the transcription factor NF-κB in an epithelial cell line that is responsive to flagellin (57), the initial O:H 1:1 lot 1 conjugate retained significant bioactivity *in vitro*. In contrast, the subsequent conjugate constructs were virtually devoid of NF-κB stimulatory activity (Fig. 3). Conjugation by either of the chemistries utilized occurs at random lysine residues on flagellin, several of which appear in or near the TLR5 stimulatory region (see Fig. S1A in the supplemental material). Thus, the modified scale-up methods used in preparing the later lots of conjugates, which resulted in loss of NF-κB stimulatory activity, may be a result of conjugation at or near these residues, resulting in steric hindrance for receptor binding (1, 59).

Parenteral immunization with flagellin monomers or flagellar polymers protects CD-1 mice against challenge with wild-type *S.* Enteritidis. Outbred CD-1 mice were immunized intramuscularly (IM) on days 0 and 10 with either PBS, 2.5 μg of flagellin monomer, or 2.5 μg of flagellar polymer to assess the capacity of flagellin alone to serve as a protective vaccine antigen against fatal infection with wild-type *S.* Enteritidis. The use of CD-1 mice (instead of inbred mice) enables the assessment of candidate vaccines in a genetically heterogeneous model system that better resembles the genetic diversity of humans. CD-1 mice are also a better animal model for testing the protective efficacy of nonliving *Salmonella* vaccines than BALB/c mice because of their innate resistance to *Salmonella* infection (58). At 14 days following the second immunization (day 24), mice were challenged intraperitoneally (IP) with a lethal dose (5×10^5 CFU) of wild-type *S.* Enteritidis R11. Whereas high mortality (19/20) was seen in control mice immunized with PBS, strikingly, no mortality (0/20) was seen in mice immunized with either flagellin monomers or flagellar polymers (Table 2).

Immunogenicity of COPS, flagellin, and O:H 1:1 lot 1 in BALB/c mice. To assess the ability of COPS conjugated to

TABLE 2. Efficacy of *Salmonella* Enteritidis FliC monomers or polymers in protecting CD-1 mice from lethal challenge with wild-type *S.* Enteritidis R11[a]

Vaccine	Mortality (no. of dead mice/total no. of mice)	*P* value[b]	Vaccine efficacy (%)
PBS	19/20		
Flagellin monomer	0/20	<0.0001	100
Flagellar polymer	0/20	<0.0001	100

[a] The mice were challenged with 5×10^5 CFU *S.* Enteritidis R11 intraperitoneally (IP). (LD_{50} of 2.2×10^5 CFU).

[b] The mortality rate of the vaccinated group compared to PBS-immunized control animals by two-tailed Fisher's exact test.

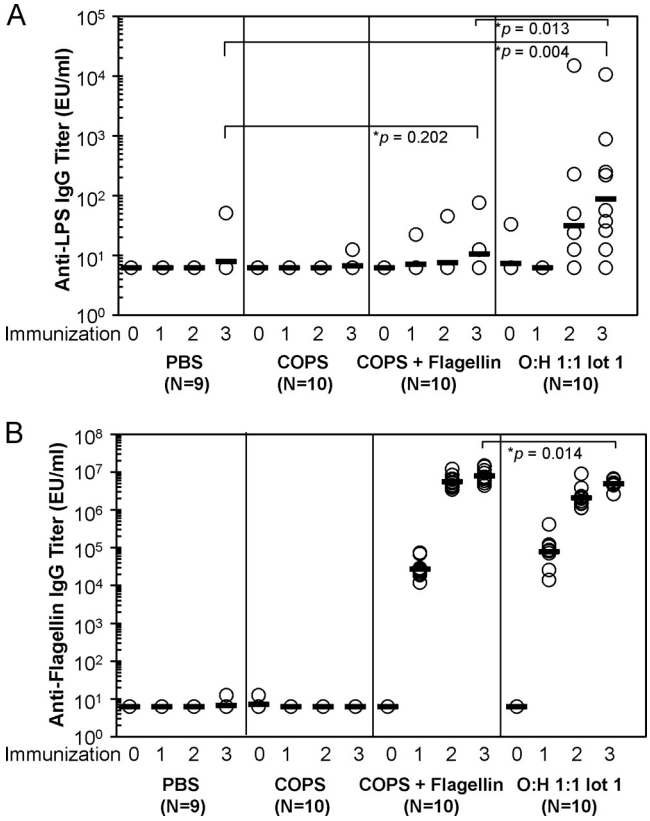

FIG. 4. Humoral immune responses in BALB/c mice following immunization with PBS (sham), *S.* Enteritidis COPS, COPS admixed with flagellin monomers, or O:H 1:1 lot 1 conjugate. Serum anti-LPS IgG (A) and antiflagellin IgG (B) levels in individual mice (○) and geometric means (short black bars) before immunization (time zero) and at 21 days following the first (1), second (2), or third (3) immunization. The asterisks before the *P* values indicate that the values were compared by the Mann-Whitney rank sum test.

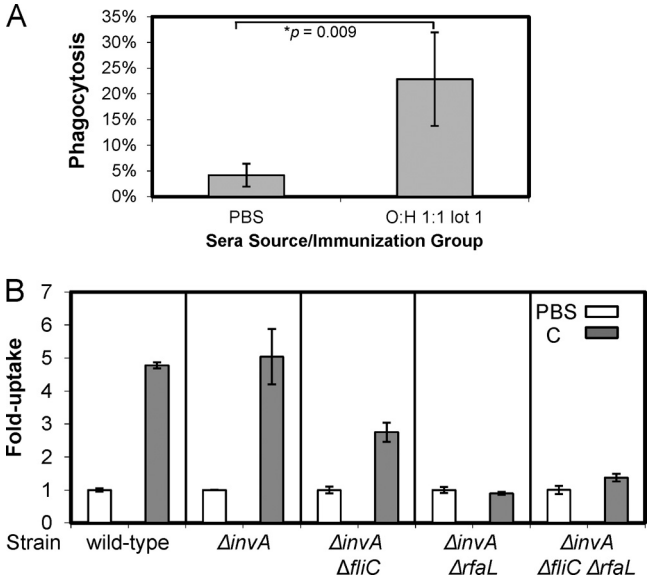

FIG. 5. Opsonophagocytic uptake of wild-type *S.* Enteritidis R11 by J774 mouse macrophages following treatment with sera from BALB/c mice immunized with O:H 1:1 lot 1 conjugate. (A) Bacterial uptake in the presence of individual PBS control ($n = 3$) or immune sera ($n = 6$) displaying high anti-LPS and antiflagellin IgG levels (averages ± standard deviations [error bars]; the asterisk indicates that statistical significance was assessed by *t* test). (B) Uptake by pooled sera from mice immunized with O:H 1:1 lot 1 conjugate (C) relative to PBS, of wild-type *S.* Enteritidis R11 and derivatives mutated in *invA*, *fliC*, and *rfaL* as indicated. Averages ± standard errors from duplicate wells and representative of two independent experiments are shown.

flagellin to stimulate anti-LPS and antiflagellum immune responses, inbred BALB/c mice were immunized intramuscularly at 0, 28, and 56 days with PBS or with 2.5 μg of COPS alone or admixed with purified flagellin monomers or with the O:H 1:1 lot 1 conjugate. BALB/c mice were used in these experiments, because this strain is genetically homogenous and animal-to-animal variability in antibody levels may be diminished. As shown in Fig. 4A, immunization with COPS alone or COPS admixed with flagellin failed to stimulate a significant increase in serum anti-LPS IgG titer. In contrast, anti-LPS was detected by 14 days following the second immunization in mice that received the conjugate vaccine, with higher levels achieved after the third immunization. LPS seroconversion (≥4-fold increase over baseline of 6 ELISA units [EU]/ml) was observed in 70% of mice that received the conjugate, and anti-LPS levels were significantly elevated relative to mice receiving PBS ($P < 0.05$).

The serum antiflagellin monomer IgG levels elicited in mice immunized with either purified flagellin admixed with COPS or with O:H 1:1 lot 1 conjugate were observed as early as 21 days after the first immunization (Fig. 4B). Antiflagellin antibodies reached the highest levels ($>10^6$ EU/ml) after only two doses of vaccine, and the individual responses within the groups were similar. Mice immunized with O:H 1:1 lot 1 conjugate exhibited antiflagellin IgG levels that were similar to those of mice immunized with COPS admixed with flagellin, suggesting that conjugation does not interfere with the ability to mount an antiflagellin immune response.

Serum opsonophagocytic assays were performed to confirm the presence of functional antibody in BALB/c mice immunized with COPS-flagellin conjugate. Individual serum samples from high responder animals immunized with O:H 1:1 lot 1 conjugate significantly increased the opsonophagocytic uptake of wild-type *S.* Enteritidis R11 by J774 mouse macrophages compared to negative-control sera from unvaccinated mice (Fig. 5A).

Engineered defined mutants of *S.* Enteritidis were used in the opsonophagocytic assay to assess the role of antibodies to COPS and flagellin in mediating opsonophagocytosis. *S.* Enteritidis R11 cells lacking *fliC* (in a Δ*invA* background) were taken up at a slightly reduced level by pooled sera from mice immunized with O:H 1:1 lot 1 conjugate than wild-type bacteria (Fig. 5B). *S.* Enteritidis R11 cells that possess a deletion in *rfaL*, which is required for synthesis of long-chain OPS, showed no increased level of opsonophagocytosis by immune sera compared to uptake by sera from PBS-immunized control mice.

In contrast to the enhanced opsonophagocytic uptake mediated by sera from conjugate-immunized mice, the same sera did not exhibit evidence of complement-mediated serum bactericidal activity (SBA) against wild-type *S.* Enteritidis R11 (see Fig. S3 in the supplemental material). Similar SBA resis-

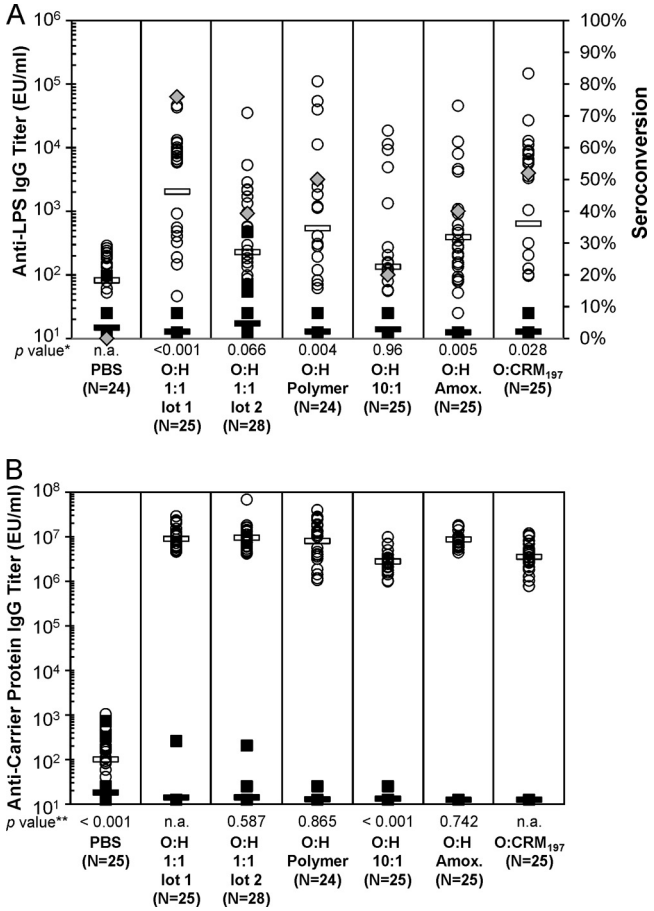

FIG. 6. Humoral immune response in CD-1 mice immunized with COPS conjugates. (A and B) Anti-LPS IgG (A) and anti-carrier protein (flagella or CRM_{197}) IgG (B) levels in preimmune sera (individual mice [black squares] and geometric means [short black bars]) and 21 days after the third immunization (individual mice [open circles] and geometric means [short white bars]) with the indicated *S.* Enteritidis COPS conjugate. Percent seroconversion to anti-LPS IgG is indicated by gray diamonds (≥4-fold increase over basal level of 100 EU/ml). In panel A, statistical significance (*P* value) for anti-LPS IgG was compared to the value for PBS (indicated by one asterisk), while in panel B, statistical significance compared the values for antiflagellum IgG compared to O:H 1:1 lot 1 conjugate (indicated by two asterisks). Statistical significance of the values for the groups were compared by Mann-Whitney rank sum test. n.a., not applicable.

tance was found against several other *S.* Enteritidis clinical isolates from Mali (data not shown).

Immunogenicity of definitive conjugates in CD-1 mice. On the basis of the encouraging results seen in inbred BALB/c mice with O:H 1:1 lot 1 conjugate, outbred CD1 mice were immunized to compare the immunogenicity of the conjugates that varied in carrier protein, polysaccharide/protein ratio, and conjugation chemistry, along with preliminary O:H 1:1 lot 1 conjugate serving as a bridge to the study in BALB/c mice. Immunization was performed as described above; 2.5 μg of conjugate was administered at 0, 28, and 56 days. Consistent with results seen in BALB/c mice, anti-LPS IgG levels exhibited significant animal-to-animal variability within individual experimental conjugate groups (Fig. 6A). The geometric mean titers (GMTs) and seroconversion rates differed between conjugate preparations. The highest overall anti-LPS levels were seen in mice immunized with O:H 1:1 lot 1 conjugate (76% seroconversion; GMT of 2,042 EU/ml), the lowest levels were seen in mice immunized with O:H 10:1 conjugate (20% seroconversion; GMT of 134 EU/ml). Intermediate and comparably elevated levels were seen for the other conjugates tested (39 to 52% seroconversion; GMTs of 227 to 640 EU/ml). All except one of the conjugates constructed with flagellin as the carrier protein stimulated comparably high antiflagellum IgG levels following immunization with three doses of conjugate (Fig. 6B); the exception was O:H 10:1 conjugate, which elicited an elevated but significantly lower titer of antiflagellin IgG ($P = < 0.001$) compared to O:H 1:1 lot 1 conjugate. High anti-CRM_{197} IgG levels were seen in mice immunized with the COPS-CRM_{197} conjugate. Sera from CD-1 mice immunized with the various conjugate preparations exhibited increased opsonophagocytic activity compared to sera from negative-control mice inoculated with PBS, with little difference noted in sera from groups of mice given the different conjugates (data not shown).

COPS-flagellin conjugates are protective in CD-1 mice against lethal wild-type *S.* Enteritidis challenge. CD-1 mice immunized with any of the COPS-based conjugate vaccines were significantly protected against IP challenge with 5×10^5 CFU *S.* Enteritidis R11, with low mortality observed in the vaccine groups compared to 100% mortality in the PBS-immunized group (Table 3). Somewhat higher mortality was seen in all conjugate vaccine groups following challenge with 1×10^6 CFU *S.* Enteritidis R11 IP; however, significant protection was still observed for all the conjugates, with the exception of the COPS-flagellin monomer 10:1 CDAP-linked conjugate. A small group of mice immunized with O:H 1:1 lot 2 conjugate

TABLE 3. Efficacy of COPS-protein conjugate vaccines in protecting CD-1 mice from lethal challenge with wild-type *S.* Enteritidis R11

Vaccine	Intraperitoneal challenge dose (CFU)[a]	Mortality (no. of dead mice/total no. of mice)	*P* value[b]	Vaccine efficacy (%)
PBS (control)	5×10^5	12/12		
Conjugate vaccines				
O:H 1:1 lot 1	5×10^5	0/12	<0.001	100.0
O:H 1:1 lot 2	5×10^5	1/12	<0.001	91.7
O:H polymer	5×10^5	2/12	<0.001	83.3
O:H 10:1	5×10^5	2/12	<0.001	83.3
O:H Amox.	5×10^5	1/12	<0.001	91.7
O:CRM_{197}	5×10^5	3/12	<0.001	75.0
PBS	1×10^6	12/13		
Conjugate vaccines				
O:H 1:1 lot 1	1×10^6	1/13	<0.001	91.7
O:H 1:1 lot 2	1×10^6	3/13	<0.01	75.0
O:H polymer	1×10^6	2/12	<0.001	81.9
O:H 10:1	1×10^6	8/13	0.16	33.3
O:H Amox.	1×10^6	2/13	<0.001	83.3
O:CRM_{197}	1×10^6	3/13	<0.01	75.0
O:H 1:1 lot 2	5×10^6	0/3		

[a] IP LD_{50} of 2.2×10^5 CFU.
[b] The mortality rate of the conjugate vaccine group was compared to that of the PBS-immunized control animals by two-tailed Fisher's exact test.

were challenged IP with a higher inoculum (5×10^6 CFU) and were also protected.

DISCUSSION

S. enterica serovars Typhimurium and Enteritidis are the two most common serovars responsible for invasive nontyphoidal *Salmonella* (NTS) disease in the United States (67), Europe, and sub-Saharan Africa. We investigated COPS-flagellin protein conjugates as a vaccine strategy to prevent invasive NTS disease, using *S.* Enteritidis as a prototype. Since purified *Salmonella* OPS functions as a T-independent antigen, previous workers pioneered the covalent linkage of *S.* Typhimurium OPS to carrier proteins such as tetanus toxoid (TT) to enhance its immunogenicity (25, 63, 70). Our results with *S.* Enteritidis corroborate that conjugating OPS to various carrier proteins enhances the serological responses that are directed to the OPS. We elected to use COPS (OPS linked to core PS) because of its ease of purification. However, since core PS is virtually identical across most *Salmonella* serovars, this may also provide some degree of cross protection across serovars.

We have established that both flagellin monomers and flagellar polymers by themselves constitute protective vaccine antigens when administered parenterally. Thus, using flagellin as the carrier protein to which COPS is linked in conjugate vaccines offers the potential to achieve enhanced protection by the additive effect of anti-OPS and antiflagellin immune responses. Nevertheless, the importance of anti-OPS must not be underestimated, since the COPS-CRM_{197} conjugate conferred significant protection against lethal challenge and the O:H 10:1 conjugate elicited low OPS seroconversion (measured by ELISA) and gave poor protection against challenge with the higher inoculum (1×10^6 CFU) of wild-type *S.* Enteritidis, despite eliciting high antiflagellin levels.

COPS-flagellin conjugates stimulated opsonophagocytic antibody and conferred protection, despite the inability to detect complement-mediated serum bactericidal killing *in vitro*. Indeed, the role of serum bactericidal antibodies in protection against NTS infection may be negligible, as resistance to complement- and serum-mediated bactericidal killing is likely a common trait among invasive *Salmonella* strains (52). Furthermore, prior studies in mice immunized with heat-killed bacteria have demonstrated that comparable protection was evident whether the mice were challenged IP with either complement-sensitive or complement-resistant *S.* Typhimurium or *S.* Enteritidis (47). Opsonophagocytosis mediated by anti-*Salmonella* antibodies and phagocytic cells from humans has also been shown to be an effective antibacterial mechanism against both complement-resistant and complement-sensitive NTS strains (14).

Phase 1 *S.* Enteritidis flagellin protein (H:g,m) is an attractive alternative to the use of TT, diphtheria toxoid (DT), and CRM_{197} as a carrier protein to link to *S.* Enteritidis COPS. Although TT, DT, and CRM_{197} have a strong track record of safety and immunogenicity when used in *Haemophilus influenzae* type b (Hib), pneumococcal, and meningococcal conjugate vaccines in humans, arguments have been raised that new carrier proteins are needed. The overuse and repetitive exposure to TT, DT, and CRM_{197} antigens as part of routine vaccination schedules can antagonize the antipolysaccharide immune response in TT- and DT-based conjugate vaccines (10). High levels of preexisting antibody levels and overly strong anti-carrier protein immune responses can interfere with the antipolysaccharide immune response through epitope suppression and bystander interference (10). Flagellin as a carrier protein avoids this concern.

The adjuvant activity of flagellin monomers is believed to result from signaling through TLR5 that causes upregulation of inflammatory cytokine production and the maturation of antigen-presenting cells (43). Surprisingly, only a modest difference was observed in anti-LPS IgG levels, and no difference was seen in the antiflagellum IgG levels in mice immunized with the COPS-flagellin conjugates that differed in innate immune bioactivity. This implies that TLR5 activity may be redundant for the powerful immunogenic properties of flagellin. Flagellin can also cause maturation and release of interleukin 1β (IL-1β) and IL-18 through intracellular recognition by NLRC4/Ipaf (41). However, since conjugate vaccines are extracellular antigens, activation of NLRC4/Ipaf may be negligible (41). Work in transgenic mice suggests that the immunogenicity and adjuvanticity of flagellin may be TLR independent (54). In these experiments, comparable antiflagellin antibody levels and a measurable adjuvant boost against coadministered ovalbumin were seen between TLR5-deficient and wild-type mice. In the absence of TLR5 signaling, high antiflagellin IgG levels may be due, in part, to partial activation of NLRC4/Ipaf (66) or highly efficient cross-linking of B-cell receptors due to the polymeric nature of the antigen or possibly through efficient antigen processing and presentation on major histocompatibility complex (MHC) molecules of flagellin epitopes (2, 3, 8, 54). Furthermore, as anti-LPS antibody levels were similar between flagellin- and CRM_{197}-based conjugates, in contrast to the powerful adjuvant boost that flagellin bestows on protein antigens, the comparative adjuvanticity toward polysaccharide antigens may be modest.

Results from a phase 1 clinical trial with a TLR5 stimulatory influenza hemagglutinin (HA)-flagellin genetic fusion protein vaccine suggest that flagellin may have a low dosage threshold for vaccine-associated reactogenicity. In these experiments, while immunogenicity was observed at low antigen doses without reactogenicity, dosages of greater than 1 μg of vaccine were found to elicit local and systemic adverse reactions (65). The lack of TLR5 activity observed with several formulations of our flagellin-based conjugate vaccines may be advantageous if they elicit clinically protective levels of anti-LPS and antiflagellum antibodies without stimulating the innate immune system, which can result in clinical adverse reactions.

Encouraging results have been generated with conjugate vaccines to prevent typhoid fever caused by *S. enterica* serovar Typhi. *S.* Typhi Vi capsular polysaccharide conjugated to recombinant exoprotein A (rEPA) from *Pseudomonas aeruginosa* demonstrated 89% efficacy against typhoid fever over 46 months of follow-up in a field trial in Vietnam (31, 37, 38). Other Vi conjugate vaccines in various stages of development utilize TT, DT, and CRM_{197} as carriers (9, 42).

Prior work has established the ability of OPS-based glycoconjugate vaccines to protect mice against otherwise lethal *S.* Typhimurium infection (63, 70). Conjugates of *S. enterica* serovar Paratyphi A OPS (29, 30) have also shown promise in mouse studies and in human clinical trials. These observations

indicate that a COPS-flagellin conjugate vaccine to prevent invasive *S.* Enteritidis and *S.* Typhimurium infections in infants and young children in sub-Saharan Africa is feasible.

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