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Glycoconjugate vaccine strategies for protection against invasive Salmonella infections

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Salmonella enterica **serovars Typhi and Paratyphi A and B and certain non-typhoidal *Salmonella enterica* (NTS) serovars are important causes of invasive Salmonella disease worldwide. NTS serovars Typhimurium and Enteritidis typically cause gastroenteritis in healthy children and adults in industrialized countries but in certain hosts (e.g., young infants, the elderly, immuno compromised individuals) they also cause invasive infections. These two serovars also cause invasive disease in infants and young children in sub-Saharan Africa. Whereas Salmonella surface polysaccharides are poor immunogens in animal models and do not generate immunologic memory, conjugation with carrier proteins overcomes these limitations. S. Typhi expresses a Vi polysaccharide capsule; Vi either alone or as a glycoconjugate protects humans from typhoid fever. In contrast, S. Paratyphi A and B and NTS (with rare exceptions) do not express capsular polysaccharides. Rather, their surface polysaccharides are the O polysaccharide (OPS) of lipopolysaccharide. In animal studies, immunization with Salmonella COPS (core polysaccharide-OPS) conjugated with carrier proteins generates functional immunity and protects against fatal Salmonella challenge. Conjugating to Salmonella proteins (flagellin, porins) may extend immune responses to another relevant target for antibody generation and enhance the glyconjugate's efficacy.**

Key words: salmonella, conjugate vaccine, polysaccharide vaccine, Vi, O polysaccharide, typhoid, paratyphoid, non-typhoidal salmonella

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Introduction

A relatively restricted number of the > 2,500 serovars of Salmonella are associated with invasive disease such as bacteremia, septicemia and meningitis. Four fairly distinct clinico-epidemiologic patterns of invasive Salmonella disease are recognized and are caused by distinct serovars: enteric fever; metastatic purulent infections; invasive disease in high risk hosts in industrialized and developing countries; invasive disease in young children in sub-Saharan Africa.

Three human-host-restricted enteric fever serovars (also called "typhoidal" serovars), *Salmonella enterica* serovar Typhi (S. Typhi), S. Paratyphi A and S. Paratyphi B, cause enteric (typhoid or paratyphoid) fever, manifested by persisting fever, abdominal discomfort and headache. If not treated promptly with effective antibiotics, typhoid and paratyphoid fever may lead to complications and death. In the pre-antibiotic era the case fatality rate of typhoid fever was ~15%. In infants, S. Typhi and S. Paratyphi bacteremic infections may be either clinically mild (with the bacteremia clearing spontaneously),[1] or severe.[2] Two serovars, S. Choleraesuis and S. Paratyphi C, cause metastatic purulent infections, an uncommon clinical form of invasive disease.[3-5]

In the US and Europe, gastroenteritis due to NTS serovars, a common disease, may occasionally be accompanied by invasive bacteremic disease. Susceptible hosts for invasive NTS disease include infants < 3 mo of age,[6-9] the elderly,[9] persons

with hemoglobinopathies and those with immunocompromise (inadequately treated HIV infection, etc.).[9] The most common NTS serovars associated with invasive disease in the US include S. Typhimurium, S. Enteritidis, S. Heidelberg, S. Dublin and S. Schwarzengrund.[9]

Finally, it has also become recognized that NTS commonly cause invasive bacterial disease among children < 3 y of age in many regions of sub-Saharan Africa.[10-16] Prior to the introduction of programmatic immunization with *Hemophilus influenzae* type b (Hib) or *Streptococcus pneumoniae* conjugate vaccines in countries in sub-Saharan Africa, invasive NTS disease was as common as invasive Hib or pneumococcal disease.[10-16] Of these clinico-epidemiologic syndromes caused by different serovars, all represent a sufficiently large burden as to be considered as targets for control by vaccines (except for S. Choleraesuis and S. Paratyphi C metastatic purulent infections, which are relatively rare). Whereas licensed vaccines are available to prevent typhoid fever, no specific licensed vaccines are available against S. Paratyphi A or B or NTS serovars.

Vi Based Conjugate Vaccines for Protection against S. Typhi Infections

Capsular polysaccharides of Hib, *S. pneumoniae* and *Neisseria meningitidis* have been linked to carrier proteins as the basis of well tolerated, immunogenic and efficacious licensed conjugate vaccines, documenting that the conjugate vaccine strategy is reliable, robust and flexible for polysaccharide-encapsulated pathogens that invade via the bloodstream. S. Typhi expresses a capsular polysaccharide, Vi antigen, which mediates resistance to bactericidal killing and opsonophagocytic uptake by the alternative arm of the complement system.[17] Serum IgG anti-Vi is a correlate of protection in humans.[17-19] Like most polysaccharides,[20] Vi is poorly immunogenic in infants and fails to induce immunologic memory.[21] However, conjugation of Vi to a carrier protein overcomes these limitations,[20,21] as has been documented through clinical trials with a pioneering conjugate vaccine consisting of Vi conjugated to recombinant exoprotein A of *Pseudomonas aeruginosa* (Vi-rEPA) developed at the US National Institute of Child Health and Human Development. Vi-rEPA was tested in clinical trials in a high typhoid incidence area in Vietnam where, following demonstration of safety and immunogenicity in older children and adults,[22-25] it was evaluated for efficacy in a randomized, controlled phase 3 field trial in pre-school children.[23,24] A high level of protection was observed over 46 mo of follow-up.[23,24] Vi-rEPA is immunogenic in Vietnamese infants when administered concomitantly with other pediatric vaccines that are part of the Vietnamese Expanded Program on Immunization (EPI).[26] Several investigators proposed a minimal threshold protective level of serum IgG anti-Vi that can facilitate the clinical development of new Vi conjugates.[19,23,26] Carrier proteins utilized in Vi conjugates include diphtheria toxoid (DT),[27] tetanus toxoid (TT), and CRM_{197}.[28] Phase 1 and 2 clinical trials with Vi-CRM_{197} have shown its' safety and immunogenicity in adults and teenagers. Vi-CRM_{197} elicited comparable levels of antibody at 1/20th of the standard dose of unconjugated Vi polysaccharide vaccine.[29] One Vi-TT conjugate has been licensed in India but no peer review publications have presented the safety and immunogenicity data generated with this vaccine. The paucity of published data on this specific conjugate has led to some controversy in India.[30-32]

S. Paratyphi C and some clones of S. Dublin also express Vi capsular polysaccharide but no field data have documented the efficacy of Vi conjugate vaccines against these serovars. Some have raised the theoretical concern that widespread use of Vi-based parenteral vaccines exert immunologic pressure selecting for the emergence of Vi-negative strains of S. Typhi.[33,34] Vi-negative strains are generally rare but one study using molecular diagnostics convincingly detected Vi-negative S. Typhi uncommonly in blood.[35]

Salmonella O Antigens and Relevance for Developing Vaccines to Prevent Invasive NTS Disease and Paratyphoid Fever

Since NTS and S. Paratyphi A and B do not express capsular polysaccharides, investigators have studied vaccines that contain the repeating polymer of O-polysaccharide (OPS) as the basis of eliciting antibody-based protection in a manner analogous to what Vi polysaccharide and Vi conjugates have been able to accomplish in preventing S. Typhi disease. The lipopolysaccharide (LPS) of Salmonella is comprised of lipid A (endotoxin) attached to a highly conserved core polysaccharide and a repeating OPS polymer. The overwhelmingly majority of invasive Salmonella isolates from humans fall into Salmonella groups A, B, C or D. OPS of Salmonella groups A, B and D are similar in overall structure. They share a common trisaccharide backbone →2)-α-D-Man*p*-(1→4)-α-L-Rha*p*-(1→3)-α-D-Gal*p*-(1→ (which serologically constitutes epitope 12). A dideoxy hexose saccharide linked α-(3→6) at the mannose of the repeating trisaccharide[36] results in an immunodominant epitope that confers Salmonella group identity. Thus, if the dideoxy hexose linked to the mannose is a paratose, this provides immunodominant epitope 2, specifying a Group A Salmonella. If the α-(3→6)-linked dideoxyhexose is an abequose, immunodominant epitope 4 specificity is conferred, indicative of Group B. If the α-(3→6)-linked dideoxyhexose is a tyvelose, immunodominant epitope 9 results, putting the isolate into Group D. The rhamnose in the backbone →2)-α-D-Man*p*-(1→4)-α-L-Rha*p*-(1→3)-α-D-Gal*p*-(1→ trisaccharide repeat of S. Paratyphi A is also partially O-acetylated; however, there is no antigenic epitope recognized in association with this modification.[37,38]

In some Group B serovars such as S. Typhimurium, phage conversion modifies the galactose of the trisaccharide backbone epitope 12 so that it becomes α-(1→6) glucosylated and minor epitope 1 can be detected.[39] Some Group B serovars also express minor epitope 5, resulting from a chromosomal gene product that

acetylates the 2-hydroxyl group of the abequose residue.[40,41]

OPS of Salmonella serogroups C are structurally and serologically distinct from Groups A, B and D.[36,39] Salmonella isolates with OPS exhibiting immunodominant epitopes O:6,7 characterize Salmonella Group C_1. Isolates lysogenized with phage 14, resulting in the antigen pattern O:6,7,14, used to be designated group C_4 but are presently considered as members of Group C_1. Salmonella isolates bearing immunodominant O:8 comprise Group C_2, whether or not they also express epitope 6. In older typing regimens, isolates bearing O:6,8 were referred to as C_2 to distinguish them from isolates bearing only O:8, which were designated C_3.

The critical issues revolving around the use of OPS-based conjugate vaccines to prevent invasive NTS disease and paratyphoid fever include whether O antibodies to NTS and Paratyphi A and B serovars in humans can mediate protection, the biological activities of anti-LPS antibodies in humans and whether antibodies to an OPS-based vaccine made with purified OPS from one serovar cross-protect against other serovars within the same O serogroup, as would be expected.

Biological Activity of anti-O Antibodies

Although Salmonella are intracellular pathogens, they are vulnerable while extracellular when IgG and IgM directed against the surface polysaccharides of Salmonella can bind them leading to bacteriolysis or opsonophagocytosis. The importance of serum immunity is underscored by the increased virulence seen for Salmonella that can evade the alternative pathway of complement through alteration in the length and structure of their OPS and expression of the resistance to complement killing (*rck*) gene.[42-45] Antibodies to Salmonella surface carbohydrates mediate opsonophagocytosis through Fc receptors on phagocytes that can kill by oxidative burst.[46] Activation of the antibody mediated complement pathway by IgM and IgG can also kill directly via formation of the C9 membrane attack complex; surface deposition of C3b also enhances opsonophagocytosis.[47,48]

Evidence that Salmonella OPS Antibodies can Protect Animals and Humans

Passively transferred IgG or IgM monoclonal antibodies specific for S. Typhimurium OPS protected mice against S. Typhimurium challenge.[49] A study to assess the protection related to specific epitopes within OPS suggests that antibodies to the immunodominant group-specific epitope constitute the primary protective species; IgG or IgM specific for epitope 4 protected to a greater extent than an IgG to epitope 12.[50] A monoclonal IgA directed against epitope 5 has also been shown to prevent mucosal infection with S. Typhimurium given to mice by oral challenge.[40,51] Polyclonal antibodies elicited by COPS conjugates in rabbits and mice also provide passive immunity against fatal NTS challenge in mice.[52,53]

While the protective efficacy of antibody against NTS OPS and COPS is well documented in animal studies, the functionality of anti-COPS in humans is less clear. Antibody to S. Typhimurium LPS from HIV positive individuals in Africa was shown to interfere with complement mediated bactericidal killing of a serum sensitive prototype African S. Typhimurium strain.[54] Anti-LPS IgG however does not interfere with opsonophagocytosis and oxidative burst in human neutrophils with either complement resistant or sensitive S. Typhimurium strains.[46] NTS isolates from the blood also frequently display marked resistance to complement mediated bactericidal killing.[55] Further work is needed to better define the role of anti-OPS in serum bactericidal and opsonophagocytic killing in immunity to invasive NTS infection in humans.

Salmonella COPS and OPS as Vaccine Antigens in Humans and in Animal Models

Little is known regarding the immunogenicity of purified Salmonella COPS or OPS in humans administered parenterally as a polysaccharide vaccine. In the early 1960s, clinical studies assessed the clinical acceptability and immunogenicity of two LPS-based vaccines containing purified S. Typhi LPS. The efficacy of these parenteral vaccines was also examined in large-scale field trials that included killed whole cell S. Typhi vaccines, also administered parenterally.[56,57] Whereas the parenteral killed whole cell vaccines conferred a moderate level of protection against typhoid fever, the unconjugated LPS vaccines provided little or no protection. Vi PS expressed by wild type S. Typhi may have interfered with the ability of anti-LPS antibodies to bind to LPS on the bacteria present in blood, perhaps explaining the poor efficacy of these early LPS-based vaccines. However, evidence from studies in mice also suggests that Salmonella COPS as an isolated polysaccharide is a poor immunogen.[37,58,59] In contrast, conjugation of Salmonella COPS to protein carriers results in vaccines that have been effective in generating anti-OPS in animal models.[37,52,53,58,59] NTS COPS conjugate vaccines have also demonstrated protection against mortality in the mouse model of lethal Salmonella infection. In one study, conjugation of S. Typhimurium COPS to the homologous strain porin proteins elicited increased levels of anti-COPS IgG, and demonstrated protection against an LD_{100} challenge with virulent S. Typhimurium.[53] Antibodies elicited by this conjugate in mice, as well as an OPS conjugate with bovine serum albumin (BSA) in rabbits, exhibited functional opsonophagocytic antibody that could transfer protection by passive immunization.[52,53,60] Similar results were seen following immunization of mice with a conjugate of S. Typhimurium COPS with TT.[59] A S. Paratyphi A COPS-TT conjugate also increased the immunogenicity of COPS in mice, and elicited antibodies demonstrating complement-mediated bactericidal killing.[37] S. Paratyphi A COPS-TT was safe and immunogenic in humans in phase 1 and 2 clinical trials; serum from the vaccinated humans displayed functional bactericidal activity.[61] A conjugate vaccine

consisting of S. Enteritidis COPS linked to the homologous serovar flagellin FliC elicited LPS-specific IgG and protected mice against otherwise lethal challenge with virulent S. Enteritidis.[58]

Selecting the Carrier Protein for Salmonella OPS-based Conjugates

Salmonella OPS glyconjugates that use homologous pathogen protective antigens (e.g., flagellins, porins) as carrier proteins can enhance protection by concomitantly eliciting immune responses to a second relevant antigen, thereby providing greater protective efficacy than conjugates constructed with heterologous carrier proteins (e.g., tetanus toxoid, CRM_{197}). Mice immunized with conjugates of S. Typhimurium COPS with homologous strain porins displayed lower mortality to fatal Salmonella challenge than mice immunized with porins alone.[53] S. Enteritidis COPS-flagellin conjugates that elicited high titers of antibodies to both COPS and flagellin exhibited higher efficacy than conjugate antigens that elicited high antibodies to only one component.[58] There is interest to test in humans the hypothesis that antibodies directed toward a carrier protein derived from Salmonella may have an additive or synergistic effect on immunogenicity (and protection). Salmonella flagellins as vaccine antigens are particularly attractive as it is anticipated that they can be economically manufactured at large scale[58,62,63] and are amenable to several biochemical conjugation strategies.

Multivalent Salmonella Glycoconjugate Vaccine Formulations

If COPS-flagellin or COPS-porin conjugates prove to be well tolerated, immunogenic and efficacious against pilot serovars and if antibodies to the immunodominant O serogroup antigens demonstrate cross protection against other clinically important serovars within the same serogroup, one can envision a global multivalent conjugate vaccine. With ca. 5–6 conjugates, such a multivalent conjugate vaccine could offer protection against virtually all the serovars that presently cause invasive disease globally. Thus, for example, a multivalent vaccine formulation consisting of COPS conjugates from S. Paratyphi A (group A), S. Typhimurium (group B), S. Enteritidis (group D) and S. Choleraesuis (Group C), along with a Vi-conjugate, would constitute a broad-based vaccine covering almost all invasive Salmonella disease.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest with regard to this manuscript.

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