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Characterization of O-antigen delivered by Generalized Modules for Membrane Antigens (GMMA) vaccine candidates against nontyphoidal *Salmonella*

G. De Benedetto [a,b], R. Alfini [a], P. Cescutti [b], M. Caboni [c], L. Lanzilao [a], F. Necchi [a], A. Saul [a], C.A. MacLennan [d], S. Rondini [a], F. Micoli [a,*]

[a] GSK Vaccines Institute for Global Health (GVGH) S.r.l. (former Novartis Vaccines Institute for Global Health, NVGH), Via Fiorentina 1, 53100 Siena, Italy
[b] Dipartimento di Scienze della Vita, Ed. C11, Università degli Studi di Trieste, via L. Giorgieri 1, 34127 Trieste, Italy
[c] Antimicrobial Discovery Center, Department of Biology, 360 Huntington Ave., Boston, MA 02115, United States
[d] Jenner Institute, Nuffield Department of Medicine, University of Oxford, Old Road Campus Research Building, Roosevelt Drive, Oxford OX3 7DQ, UK

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ABSTRACT

Invasive nontyphoidal *Salmonella* disease (iNTS) is a leading cause of death and morbidity in Africa. The most common pathogens are *Salmonella enterica* serovars Typhimurium and Enteritidis. The O-antigen portion of their lipopolysaccharide is a target of protective immunity and vaccines targeting O-antigen are currently in development. Here we investigate the use of Generalized Modules for Membrane Antigens (GMMA) as delivery system for *S.* Typhimurium and *S.* Enteritidis O-antigen. Gram-negative bacteria naturally shed outer membrane in a blebbing process. By deletion of the *tolR* gene, the level of shedding was greatly enhanced. Further genetic modifications were introduced into the GMMA-producing strains in order to reduce reactogenicity, by detoxifying the lipid A moiety of lipopolysaccharide. We found that genetic mutations can impact on expression of O-antigen chains. All *S.* Enteritidis GMMA characterized had an O-antigen to protein w/w ratio higher than 0.6, while the ratio was 0.7 for *S.* Typhimurium Δ*tolR* GMMA, but decreased to less than 0.1 when further mutations for lipid A detoxification were introduced. Changes were also observed in O-antigen chain length and level and/or position of O-acetylation. When tested in mice, the GMMA induced high levels of anti-O-antigen-specific IgG functional antibodies, despite variation in density and O-antigen structural modifications.

In conclusion, simplicity of manufacturing process and low costs of production, coupled with encouraging immunogenicity data, make GMMA an attractive strategy to further investigate for the development of a vaccine against iNTS.

1. Introduction

Salmonella enterica Typhimurium and Enteritidis are the most common serovars responsible for invasive nontyphoidal *Salmonella* disease (iNTS) in Africa [1–4], resulting in case-fatality rates of around 20% [5]. iNTS is closely associated with malaria and malnutrition among African infants and children, and with HIV infection in all age groups [6]. Antibiotics are not always available in rural African settings, and increasing levels of multidrug-resistance are limiting their effectiveness [7–9], making this disease a high priority for vaccine development. Currently, there are no licensed vaccines against iNTS and efforts are ongoing to identify protective antigens and best strategies for vaccine development [10,11].

Antibodies directed against the O-antigen (OAg) portion of *Salmonella* lipopolysaccharide (LPS) have been shown to be able to mediate killing [12–16] and protect against infection in animal models [14,15,17]. *S.* Typhimurium and *S.* Enteritidis OAg share a common backbone consisting of galactose (Gal), rhamnose (Rha), and mannose (Man), which serologically constitutes epitope O:12 [18]. A different 3,6-dideoxy-hexose residue is linked to Man in these two serovars: abequose (Abe), conferring O:4 specificity to *S.* Typhimurium, or tyvelose (Tyv), conferring O:9 specificity to *S.* Enteritidis (Fig. 1) [19,20]. *Salmonella* OAg can demonstrate high levels of heterogeneity in terms of chain length and variation in O-acetylation and glucosylation of the repeating units [14,21–24]. For *S.* Typhimurium, Abe may be O-acetylated at

* Corresponding author.
E-mail address: francesca.x.micoli@gsk.com (F. Micoli).

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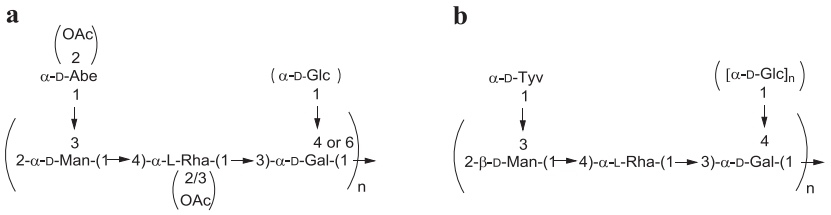


Fig. 1. OAg repeating unit structure of (a) *S.* Typhimurium (O:4,5) and (b) *S.* Enteritidis (O:9).

position C-2, which adds the O:5 specificity [25]. The additional presence of O-acetyl groups at C-2 and C-3 of Rha has also been reported [22,26]. OAg chains can also be variably glucosylated, with glucose (Glc) linked at C-4 (O:12_2 specificity) or C-6 (O:1 specificity) to Gal [21,27]. Studies in mice indicated that all these structural modifications can impact the immunogenicity of the corresponding glycoconjugate vaccines [14,23].

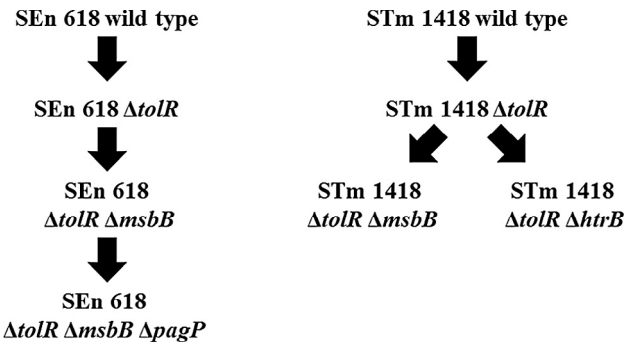
We are investigating a Generalized Modules for Membrane Antigens (GMMA) [28] approach to the development of a bivalent vaccine against *S.* Typhimurium and *S.* Enteritidis [29]. Gram-negative bacteria naturally shed outer membrane as blebs [30,31]. The release of blebs can be greatly increased by genetic manipulation of the bacteria resulting in GMMA. In *Salmonella*, deletion of the *tolR* gene affects the stability of the linkage between the inner and the outer membrane, and results in an enhanced shedding process [28,29,32,33]. GMMA derived from the surface of Gram-negative bacteria contain potent immunostimulatory components, such as lipoproteins and LPS, which can contribute to their immunogenicity, but also to reactogenicity [30,31]. In GMMA vaccine development, removal or modification of such components may alter the balance between reactogenicity and immunogenicity. One way to reduce the reactogenicity of LPS is to modify its acylation pathway, for example by deletion of *msbB*, *pagP* and *htrB* genes [29].

In this study, we report that mutations introduced to increase GMMA release and decrease reactogenicity are associated with changes in the structure of surface OAg. We investigate the impact of these changes on the antibody response to GMMA in mice and serum bactericidal activity of these antibodies *in vitro*.

2. Materials and methods

2.1. Strains

Salmonella enterica serovar Typhimurium isolate SGSC1418 (STm 1418) (LT-2 collection [34], University of Calgary) and Enteritidis SA618 (SEn 618) (CEESA EASSA II collection [35] of Quotient Bioresearch Limited), both isolated from animals, were chosen as parent strains [23]. Mutants were generated as previously described [29] (Scheme 1).



Scheme 1. GMMA of *S.* Enteritidis and *S.* Typhimurium mutated strains chosen for characterization [29].

2.2. GMMA production and characterization

GMMA were produced and purified as described [29]. Total protein content was estimated by Lowry assay [36,37]. OAg sugar content was quantified by High-Performance Anion-Exchange Chromatography coupled to Pulsed Amperometric Detector (HPAEC-PAD) as previously described [21], after performing acid hydrolysis directly on GMMA. GMMA components did not interfere in the quantification of the OAg sugar monomers. The amount of core reducing end KDO (2-keto-3-deoxy-octonate) was assumed equal to the amount of lipid A and quantified by semicarbazide/High Performance Liquid Chromatography - Size Exclusion Chromatography (HPLC-SEC) method after sugar extraction [21]. The percentage of OAg chains was calculated as the molar ratio of their KDO divided by total KDO, including LPS molecules with just core. Lipid A structures were investigated by Matrix-Assisted Laser Desorption/Ionization-Mass Spectrometry (MALDI-MS) [29]. Protein pattern profile was analyzed by SDS-PAGE analysis (SI).

2.3. OAg purification and characterization

OAg extraction and purification from wild type bacteria was performed as previously described [38]. For extraction of the OAg from GMMA, a similar procedure was used as detailed in SI. Gel filtration chromatography with differential refractive index (dRI) detection was used to fractionate the OAg chains obtained after extraction from GMMA. Samples were run on Sephacryl S300 column (90 cm × 1.6 cm i.d.). The mobile phase was $NaNO_3$ 0.05 M at the flow rate of 8 mL/h. Fractions from Sephacryl S300 were desalted by gel filtration chromatography on Biogel P2 column (90 cm × 1.6 cm i.d., flow rate 8 mL/h) or on a Bioline preparative chromatographic system equipped with Superdex G30 column (90 cm × 1.0 cm i.d., flow rate 1.5 mL/min). OAg structural analysis was performed as previously reported [21]. OAg peak molecular weight (MP) was calculated by HPLC-SEC analysis by using dextrans as standards on TSK gel G3000 PWXL column (30 cm × 7.8 mm; particle size 7 μm; Cat. N. 808021) with TSK gel PWXL guard column (4.0 cm × 6.0 mm, particle size 12 μm; Cat. N. 808033) (Tosoh Bioscience) and with in-line dRI detector. 80 μL of samples 100 μg/mL polysaccharide content were injected and eluted at the flow rate of 0.5 mL/min with 0.1 NaCl, 0.1 NaH_2PO_4, 5% CH_3CN, pH 7.2 as mobile phase.

2.4. High-Performance Liquid Chromatography - Size Exclusion Chromatography/Multi-Angle Light Scattering (HPLC-SEC/MALS)

GMMA samples were analyzed by HPLC-SEC with Sepax SRT-C 2000-1000 columns in series (Cat. N. 235980-7830; 235950-7830) equilibrated in PBS and with in-line UV, fluorescence emission, dRI and MALS detectors. 80 μL of samples 100 μg/mL protein content were injected and eluted with a flow rate of 0.5 mL/min.