Modulation of Endotoxicity of Shigella Generalized Modules for Membrane Antigens (GMMA) by Genetic Lipid A Modifications

**RELATIVE ACTIVATION OF TLR4 AND TLR2 PATHWAYS IN DIFFERENT MUTANTS**

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**Background:** GMMA from Gram-negative bacteria are an attractive vaccine technology, but lipopolysaccharide (LPS) reactogenicity limits use.

**Results:** Genetic LPS modification resulting in penta-acylation reduced *Shigella* GMMA reactogenicity to a TLR2-mediated limit. Modifications resulting in palmityloleoylated hexa-acylated LPS triggered higher TLR4-mediated reactogenicity.

**Conclusion:** Use of GMMA as vaccines will likely require LPS penta-acylation.

**Significance:** Understanding the relative contribution of TLR activation guides GMMA vaccine development.

Outer membrane particles from Gram-negative bacteria are attractive vaccine candidates as they present surface antigens in their natural context. We previously developed a high yield production process for genetically derived particles, called generalized modules for membrane antigens (GMMA), from *Shigella*. As GMMA are derived from the outer membrane, they contain immunostimulatory components, especially lipopolysaccharide (LPS). We examined ways of reducing their reactogenicity by modifying lipid A, the endotoxic part of LPS, through deletion of late acyltransferase genes, *msbB* or *htrB*, in GMMA-producing *Shigella sonnei* and *Shigella flexneri* strains. GMMA with resulting penta-acylated lipid A from the *msbB* mutants showed a 600-fold reduced ability, and GMMA from the *S. sonnei* Δ*htrB* mutant showed a 60,000-fold reduced ability compared with GMMA with wild-type lipid A to stimulate human Toll-like receptor 4 (TLR4) in a reporter cell line. In human peripheral blood mononuclear cells, GMMA with penta-acylated lipid A showed a marked reduction in induction of inflammatory cytokines (*S. sonnei* Δ*htrB*, 800-fold; Δ*msbB* mutants, 300-fold). We found that the residual activity of these GMMA is largely due to non-lipid A-related TLR2 activation. In contrast, in the *S. flexneri* Δ*htrB* mutant, a compensatory lipid A palmitoylation resulted in GMMA with hexa-acylated lipid A with ~10-fold higher activity to stimulate peripheral blood mononuclear cells than GMMA with penta-acylated lipid A, mostly due to retained TLR4 activity. Thus, for use as vaccines, GMMA will likely require lipid A penta-acylation. The results identify the relative contributions of TLR4 and TLR2 activation by GMMA, which need to be taken into consideration for GMMA vaccine development.

Gram-negative bacteria naturally shed particles that consist of outer membrane lipids, outer membrane proteins, and soluble periplasmic components. These particles, called native outer membrane vesicles, have been proposed for use as vaccines (1). However, the yield is usually too low for a practical vaccine production. We have developed genetic modification of bacteria to induce high level shedding of particles called generalized modules for membrane antigens (GMMA) (2, 3) and the corresponding industrial processes required for a practical vaccine platform (2). In the case of *Shigella*, the required genetic modification is a deletion of the *tolR* gene whose corresponding protein is involved with linking the inner and outer membranes. This development is part of a program to develop an effective and affordable vaccine for the causative agents of shigellosis, a global human health problem, especially in developing countries and in children younger than 5 years (4), with more than 125 million cases (5) and 100,000 deaths per year (6). *Shigella* are Gram-negative bacteria divided into 50 different serotypes based on the carbohydrate composition of the O antigen of their lipopolysaccharide (LPS) (7). A limited number of serotypes contributes to the global burden of shigellosis, but the leading disease-causing serotypes vary between regions (8). The current globally dominant serotypes are *Shigella sonnei* and *Shigella flexneri* 2a, which account for more than 20% of shigellosis cases each (9, 10).

GMMA are highly immunogenic (2), in part, probably because of strong self-adjuvanticity. Because they are derived from the outer membrane of Gram-negative bacteria, they have high levels of LPS and lipoproteins, molecules that are strong activators of the innate immune response through recognition by different pattern recognition receptors, including Toll-like receptors (TLRs), a widely expressed sets of molecules present in mammalian cells (11). The receptors of particular impor-

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2 The abbreviations used are: GMMA, generalized modules for membrane antigen; PBMC, peripheral blood mononuclear cell; TLR, Toll-like receptor; KDO, 2-keto-3-deoxyoctonate; OAg, O antigen.
Lipid A Modification in Shigella GMMA

Strains and Generation of Mutations—S. sonnei 53G (28) and S. flexneri 2a 2457T (29) were chosen as parent strains. The list of Shigella mutant strains used in this study and their abbreviated identifications are listed in Table 1. As all strains used in this study are GMMA-producing strains, the abbreviated names only refer to their additional mutations and characteristics. For generation of mutants from S. flexneri 2a without virulence plasmid, a white colony was selected by white appearance on Congo red agar before the start of the genetic modification. The curing of the virulence plasmid (pINV) was confirmed by the absence of the origin of replication (ori) and the plasmid-encoded genes, virG and ospD, using PCR. The primers are listed in Table 2. To generate the tolR deletion in S. flexneri 2a and plasmid-cured S. flexneri 2a-pINV, the same strategy and primers as described previously for the generation of the S. sonnei ΔtolR mutant (2) were used.

The null mutation of msbB1 (26), htrB (20), and rfbG (essential for OAg biosynthesis in S. flexneri 2a (30)) was obtained by replacing the gene of interest ("gene") with an antibiotic resistance cassette, using the following strategy. The upstream and
downstream regions of gene were amplified using the primer pairs gene-U and gene-D or rfbF and rfc (for the rfg knock out). The deletion cassette used to replace gene was amplified using primer pairs EcoRV.Ery.F/EcoRV.Ery.R or EcoRV.Cm.F/EcoRV.Cm.R and used to transform recombination-prone htrB deletion strains of S. sonnei or S. flexneri as described previously (2). In S. sonnei, the htrB gene was replaced by the chloromphenicol resistance gene cat (31). In S. flexneri 2a, msbB1 and htrB were replaced by cat, and rfbG was replaced by the erythromycin resistance gene erm (32). In the rfgB knock out, also the flanking genes rfbF and rfc (30) were partially deleted. The rfbG deletion was introduced before the msbB or htrB deletion. The msbB1 mutation was only introduced into the plasmid-cured strain as the plasmid carries a second copy of msbB (msbB2 (26)). For simplicity, the mutant is referred to as ΔmsbB.

To complement strains carrying the htrB deletion, the htrB gene was amplified from S. sonnei 53G, including 239 bp upstream and 172 bp downstream using primers P1.htrBcompl-EcoRI and P2.htrBcompl-NcoI and inserted into low copy vector pACYC184 (New England Biolabs). The resulting plasmid pACYChtrB was introduced into electrocompetent S. sonnei or S. flexneri 2a ΔhtrB cells.

**GMMA Production and Purification**—Bacterial strains were routinely grown at 30 °C in liquid or on solid M9 medium supplemented with nicotinic acid (N\(_2\)HPO\(_4\) 7 g/liter, KH\(_2\)PO\(_4\) 3 g/liter, NaCl 0.5 g/liter, NH\(_4\)Cl 1 g/liter, 1 M MgSO\(_4\) 2 ml/liter, 1 M CaCl\(_2\) 0.1 ml/liter, glucose 0.4%, nicotinic acid 0.01 g/liter) or in chemically defined medium (SDM), with the same composition to the previously described SSDM (2) with the exception of the carbon source as follows: KH\(_2\)PO\(_4\) 13.3 g/liter, (NH\(_4\))\(_2\)HPO\(_4\) 4 g/liter, citric acid 1.7 g/liter, L-aspartic acid 2.5 g/liter, D-glucose 15 g/liter, CoCl\(_2\)6H\(_2\)O 0.0025 g/liter, MnCl\(_2\)4H\(_2\)O 0.015 g/liter, CuCl\(_2\)2H\(_2\)O 0.0015 g/liter, H\(_2\)BO\(_3\) 0.003 g/liter, Na\(_2\)MoO\(_4\)2H\(_2\)O 0.0025 g/liter, Zn(CH\(_3\)COO)\(_2\)2H\(_2\)O 0.0025 g/liter, ferric citrate 2 μM, MgSO\(_4\) 2 mM, thiamine 0.05 g/liter, nicotinic acid 0.01 g/liter, pH 6.7 (with NH\(_4\)OH). When required, kanamycin (30 μg/ml), chloramphenicol (20 μg/ml), erythromycin (100 μg/ml), or tetracycline (20 μg/ml) were added.

For GMMA production, overnight cultures were grown in the presence of the specific selective antibiotics and used to inoculate the production medium to an OD of 0.03–0.05. Production cultures were incubated at 30 °C and 200 rpm overnight. Culture supernatants were collected by a 10-min centrifugation at 5,000 × g followed by a 0.22-μm filtration. GMMA were concentrated using an Amicon stirrer cell with a regenerated cellulose filter with a 100-kDa nominal molecular mass limit (Amicon Ultracel) under nitrogen flow. The retentate was collected in a 70-ml ultracentrifuge propylene tube (Beckman Coulter) and ultracentrifuged at 186,000 × g using 45Ti rotor (Beckman Coulter) for 2 h at 4 °C. Pellets were resuspended in 4 ml of PBS followed by 0.22-μm filtration. GMMA were stored at 4 °C.

**GMMA Protein and KDO Quantification**—GMMA quantities were expressed as total protein present in GMMA. The protein quantity was determined using the DC protein assay (Bio-Rad) kit (Lowry assay) according to the manufacturer’s instructions.
instructions. Bovine serum albumin (Pierce) was used for the standard curve in the range 1–50 µg/assay. Measurements of GMMA were performed in two different dilutions, each in duplicate.

Core-reducing end 2-keto-3-deoxyoctonate (KDO) after lipid A cleavage was quantified using the semicarbazide/HPLC-size exclusion chromatography method as we reported previously (33). To apply the method to GMMA, 150 µg of GMMA were hydrolyzed in 1% acetic acid for 3 h at 100 °C and subsequently centrifuged for 15 min at 14,000 × g. Supernatants were collected and dried in a SpeedVac, and the pellets were dissolved in water. Samples and a standard of 4–40 µg/ml of KDO ammonium solution (Sigma, K2755) were derivatized using semicarbazide and analyzed by HPLC-size exclusion chromatography using a TSKgel G3000 PW-XL column (TOSOH, 808021). The amount of core reducing end KDO was calculated using the calibration curve built with the peak areas of derivatized KDO standard at 252 nm.

Negative Staining Transmission Electron Microscopy—A drop of 5 µl of GMMA suspension at a concentration of 100 µg/ml in PBS was adsorbed onto 300 mesh copper Formvar/carbon-coated grids for 5 min. Grids were then washed with a few drops of distilled water and dried by blotting with Whatman filter paper. For negative staining, grids were treated with 2% uranyl acetate in double distilled H2O for 1 min, blotted with Whatman filter paper, air-dried, and observed with a Tecnai 2 Spirit transmission electron microscope (FEI, Eindhoven, The Netherlands) operating at 80 kV. Electron micrographs were recorded at a nominal magnification of 105,000×. GMMA diameters were measured manually on printed copies of the electron micrographs in comparison with the scale bar. The nonparametric Kruskal-Wallis test was used to statistically compare the sizes of GMMA obtained from different strains.

SDS-PAGE and Peptide Mass Fingerprinting—SDS-PAGE of GMMA was performed using 12% (w/v) polyacrylamide gels (Bio-Rad) as described previously (2). The gels were stained using Coomassie Blue stain (Sigma) according to the manufacturer’s instructions for proteins.

Protein spots were excised from the gel and processed as described previously (34). Briefly, mass spectra were acquired on an Ultraflex MALDI TOF-TOF mass spectrometer (Bruker Daltonics) in reflectron, positive mode, in the mass range of 900–3,500 Da. Spectra were externally calibrated by using a combination of standards pre-spotted on the target (Bruker Daltonics). MS spectra were analyzed by peptide mass fingerprinting with FlexAnalysis (FlexAnalysis version 2.4, Bruker Daltonics). Peaks were annotated with FlexAnalysis default parameters and manually revised. Protein identification was carried from the generated peak list using the Mascot program (Mascot server version 2.2.01, Matrix Science). Mascot was run on a database containing protein sequences deduced from sequenced Shigella genomes (2).

MALDI-TOF and MS/MS Analysis on Lipid A—Lipid A was precipitated from GMMA using mild acid hydrolysis with 1% acetic acid for 2 h at 100 °C (35). Samples were centrifuged at 14,000 × g for 15 min; the pellets were resuspended in water and washed twice with water. The pellets were dried overnight using a SpeedVac and resuspended in chloroform/methanol 4:1 and mixed with an equal volume of Super DHB solution (Sigma). 2 µl of the mixture were loaded to the target plate (MTP 384 target plate ground steel BC, Bruker Daltonics) and analyzed by Ultraflex MALDI-TOF (Bruker Daltonics) in reflectron ion-negative mode. A peptide calibration standard (Bruker Daltonics), mixed with the Super DHB solution, was included in each analysis. For MS/MS analysis of lipid A, main peaks from the linear mode analysis were selected for collision-induced dissociation, and the resulting fragments were detected by MALDI TOF-TOF in ion negative mode. For each sample, spectra represent the integration of the analysis of 20 different areas of the spot by 50 single laser shots. The m/z ratios were determined by Flex Analysis software in comparison with the peptide standard.

Quantitative Real Time PCR and RNA Isolation—RNA was purified using RNeasy Plus mini kit (Qiagen) from 2 ml of bacteria grown at 30 °C to an optical density of 1. Reverse transcription (2 µg of RNA/reaction) was performed using Superscript II, and the product was purified by QIAquick PCR purification kit (Qiagen). Quantitative RT-PCR for genes msbB and lpxP and was performed using 10 ng of cDNA, SYBR Green kit (Invitrogen), and 0.4 µM primers msbB.F/msbB.R and lpxP.F/lpxP.R in thermocycler MX3005P (Stratagene) with 40 cycles (95 °C, 15 s; 60 °C, 60 s). Fold induction was calculated as 2−ΔΔCt, where ΔCt is the difference between the numbers of cycle of amplification needed to reach the threshold (fluorescence dRn = 0.018) for lpxP versus the cycle of amplification of the late acyltransferase gene msbB of the constitutive lipid A biosynthesis pathway (36) in same strain. ΔCt represents the difference in the Ct of lpxP in S.2a_p − OAg ΔhtrB compared with S._p − OAg ΔhtrB.

NF-κB Luciferase Reporter Assay—TLR-specific activation assays were performed using human embryonic kidney 293 (HEK293) cells expressing luciferase under control of the NF-κB promoter and stably transfected with either TLR4, MD2, and CD14 (TLR4-HEK293) or TLR2 (TLR2-HEK293) (37). HEK293-transfected cells were maintained in DMEM complemented with 4.5 g/liter glucose and HEPES (Invitrogen), and 0.4 µg/ml puromycin and 4.0 g/ml hygromycin (Invitrogen). The product was purified by QIAquick PCR purification kit (Qiagen) from 2 ml of bacteria grown at 30 °C to an optical density of 1. Reverse transcription (2 µg of RNA/reaction) was performed using Superscript II, and the product was purified by QIAquick PCR purification kit (Qiagen). Quantitative RT-PCR for genes msbB and lpxP and was performed using 10 ng of cDNA, SYBR Green kit (Invitrogen), and 0.4 µM primers msbB.F/msbB.R and lpxP.F/lpxP.R in thermocycler MX3005P (Stratagene) with 40 cycles (95 °C, 15 s; 60 °C, 60 s). Fold induction was calculated as 2−ΔΔCt, where ΔCt is the difference between the numbers of cycle of amplification needed to reach the threshold (fluorescence dRn = 0.018) for lpxP versus the cycle of amplification of the late acyltransferase gene msbB of the constitutive lipid A biosynthesis pathway (36) in same strain. ΔCt represents the difference in the Ct of lpxP in S.2a_p − OAg ΔhtrB compared with S._p − OAg ΔhtrB.

For the NF-κB luciferase assay, 25,000 cells/well were seeded in 90 µl of complete DMEM without antibiotics in 96-well µClear® luciferase plates (PBI International) and incubated for 24 h at 37 °C. 10 µl of serial 5-fold dilutions of GMMA in PBS (0.0001–1,000 ng/ml final concentration in the assay) were added. All GMMA concentrations were tested in duplicate. After incubation for 5 h at 37 °C, supernatants were aspirated from each well, and cells were lysed for 20 min at room temperature using 20 µl/well of 1:5 diluted “passive lysis buffer” (Promega). Produced luciferase was detected using 100 µl/well luciferase assay reagent (Promega), and emitted light was immediately quantified using a luminometer Lmax II184 (Molecular Devices). NF-κB activation of cells stimulated with GMMA is expressed as fold-increase of emitted light over the average result of PBS-stimulated control cells. GMMA concen-
Lipid A Modification in Shigella GMMA

MaxiSorp 96-well plates were coated overnight at 4 °C with 2 ng/ml GMMA. Concentrations of the different GMMA mutants were recovered after centrifugation of the plates at 4,000 × g and stored at −70 °C until analysis.

Cytokine Analysis by ELISA and 7-Plex Mesoscale—Nunc MaxiSorp 96-well plates were coated overnight at 4 °C with 2 µg/ml human IL-6 capture antibody (eBioscience 14-7069) in PBS, subsequently washed three times with PBS with 0.05% Tween 20 (PBST), blocked for 1 h with PBS with 1% BSA at room temperature, and washed three times with PBST. 50 µl of supernatants from PBMC experiments, diluted 1:4 with PBS, were incubated for 2 h at room temperature. A 2-fold dilution series of recombinant human IL-6 (eBioscience 39-8069) of 31.24 to 4,000 pg/ml in RPMI 1640 medium with 10% FBS was included as standard curve on each plate. Plates were washed three times with PBST. Bound IL-6 was detected using 2 µg/ml biotin-conjugated anti-human IL-6 (eBioscience 13-7068) in PBST with 0.1% BSA for 2 h at room temperature, followed by three washes with PBST, 20 min of incubation at room temperature with streptavidin/horseradish peroxidase (R&D Systems, DY998) diluted 1:200 in PBST with 0.1% BSA, three washes with PBST, and a color reaction with 100 µl/well substrate (R&D Systems, DY999) for 8 min at room temperature in the dark. The reaction was stopped by adding 50 µl/well of 12.5% sulfuric acid. The plates were read at 450 and 630 nm and the A450–630 nm, was determined. IL-6 concentrations in the samples were calculated in comparison with the standard. Results below the detection limit were assigned half of the detection limit.

Mesoscale 7-spot (MSD Technology) analysis for cytokines IL-6, IL-8, IL-1β, TNF-α, IL-10, IL-12, and IFN-γ was performed with 25 µl of supernatants from PBMC according to the manufacturer’s instructions. Concentrations of the different cytokines in the samples were determined in comparison with the preloaded standard in the plates.

For the analysis of the cytokine release by PBMC, the average cytokine levels in the duplicate assays were plotted against the GMMA concentration. GMMA concentrations needed to obtain a 10-fold increase of IL-6 release over the average level obtained at the lowest GMMA concentration (background) were determined as x axis intercepts from the generated curves and used to compare the stimulatory activity of the different GMMA. The nonparametric Mann-Whitney test was used to statistically evaluate the results obtained with different GMMA.

For statistical analysis of the results of the TLR blocking experiments, the ratio of IL-6 produced by PBMC treated with anti-TLR4 or anti-TLR2 and the IL-6 produced by PBMC not treated with blocking antibodies, stimulated with the same concentration of GMMA, were calculated to normalize between the different experiments using PBMC from different donors. The ratio was determined for each replicate in the experiments. The nonparametric Wilcoxon signed rank test was used to assess whether the obtained ratios were significantly different from 1 (no effect by blocking).

RESULTS

Mutant Production and Conditions for GMMA Production—We previously reported the generation of a S. sonnei-pSS ΔtolR ΔmsbB mutant (Ss_p-ΔOAg ΔmsbB) that was able to grown to high optical densities in chemically defined medium developed for fermentation at 30 °C. The Ss_p-ΔOAg ΔmsbB strain was cured of the virulence plasmid (2) to remove a second copy of the msbB gene (msbB2 (26)) and the OAg biosynthesis genes (39) encoded on the plasmid. Thus, we chose a plasmid-deficient background to generate the htrB mutant of S. sonnei (Ss_p-ΔOAg ΔhtrB) and also used a plasmid- and OAg-deficient background in S. flexneri 2a to compare GMMA from the respective ΔmsbB and ΔhtrB mutants to the GMMA from the S. sonnei strains.

Similar to the Ss_p-ΔOAg ΔmsbB mutant (2), the Ss_p-ΔOAg ΔmsbB mutant retained the ability to grow in complex media at 37 °C but at a slower rate (2-fold duplication time) than a single ΔtolR mutant. In contrast, ΔhtrB strains only grew in chemically defined or minimal media and only at 30 °C. The duplication time of the ΔhtrB or ΔmsbB strains increased from 30 min to ~2 h, but all the strains were able to reach high ODs (OD 10) after overnight incubation in chemically defined medium at 30 °C. Thus 30 °C and chemically defined medium were chosen as the standard growth conditions. All strains with lipid A modifications yielded more than 50 mg/liter GMMA protein.

GMMA from Shigella strains carrying different mutations showed similar morphology by electron microscopy (Fig. 1A) with average sizes of 30–32 nm in all six strains and a size distribution of 17–53 nm, measured with 30 GMMA per strain. A comparison of the GMMA sizes from all strains gave no significant difference (p = 0.90). To characterize whether the genetic lipid A modifications might alter the protein composition of GMMA, the protein pattern of GMMA from the different mutants was evaluated by SDS-PAGE (Fig. 1B). Although the overall pattern remained similar, four protein bands, iden-
Lipid A Modification in Shigella GMMA

dified as pyruvate dehydrogenase, glutamine synthetase, ketol-acid reductoisomerase, and d-3-phosphoglycerate dehydrogenase (Fig. 1B) by peptide mass fingerprinting, were found to be up-regulated in GMMA from Sf2a-p-OAg ΔhtrB. As these proteins are cytoplasmic proteins, no effect on the reactogenicity studies was expected.

Characterization of Lipid A by MALDI-TOF and MALDI-TOF/TOF—The lipid A of LPS of the mutants was extracted and analyzed by MALDI-TOF. The spectra are reported in Fig. 2, and the structures of lipid A corresponding to the main peaks were assigned on the basis of mass and by comparison of results with similar mutants of E. coli (Fig. 2L) (40). The main peaks in the mass spectra obtained by MALDI-TOF from lipid A purified from GMMA from S. sonnei and S. flexneri 2a strains with wild-type (WT) LPS (Fig. 2, Ss-p-OAg (A) and Sf2a-p-OAg (B)) had an m/z corresponding to the theoretical mass of the hexa-acylated lipid A of 1,798 Da. The main peaks obtained by mass spectrometry from the Ss-p-OAg ΔmsbB GMMA (Fig. 2C) and Sf2a-p-OAg ΔmsbB (Fig. 2D) GMMA corresponded, in both strains, to a penta-acylated lipid A lacking a myristoyl chain (theoretical mass 1,588 Da, 210 m/z shift to WT lipid A due to the absence of a C14 fatty acid chain) consistent with msbB knock outs.

The mass spectrum of lipid A from Ss-p-OAg ΔhtrB GMMA (Fig. 2E) showed a main peak corresponding to a penta-acylated lipid A lacking a lauroyl chain (theoretical mass 1,616 Da, corresponding to the absence of a C12 fatty acid chain giving a m/z shift of 182), consistent with an htrB knock out. The spectra obtained from GMMA of Sf2a-p-OAg ΔhtrB (Fig. 2F) also had the penta-acylated lipid A species with the deletion of a lauroyl chain but showed a new peak at m/z 1,850 (Fig. 2F), most likely corresponding to a hexa-acylated lipid A species with an m/z different from WT lipid A. The mass of this lipid A species corresponds to acylation by a palmitoleoyl chain (a C16:1 fatty acid chain, m/z shift of 236) of the penta-acylated lipid A (m/z 1,616) present in both the Ss-p-OAg ΔhtrB GMMA and of Sf2a-p-OAg ΔhtrB to give a hexa-acylated lipid A. Confirmation for the palmitoleoylation was obtained by MS/MS analysis using collision-induced decay to fragment the lipid A species present in the main peaks of the first dimension MS for GMMA from Ss-p-OAg ΔhtrB (m/z 1,615, Fig. 2E) and Sf2a-p-OAg ΔhtrB (m/z 1,850, Fig. 2F). The main difference observed when overlaying the MS/MS spectra was a peak with an m/z corresponding to a palmitoleoyl chain (m/z 252, highlighted in Fig. 2G) in Sf2a-p-OAg ΔhtrB. MALDI-TOF spectra of GMMA from the Ss-p-OAg ΔhtrB and Sf2a-p-OAg ΔhtrB strains complemented with pACYCChtrB (Fig. 2, H and I) showed in both cases a hexa-acylated WT lipid A as the main peak (observed, m/z 1,797; theoretical, m/z 1,797), and no hepta-acylated peak (theoretical m/z 2,034 for a wild-type with extra palmitoleoyl chain) was observed.

The palmitoleoylated hexa-acylated form (m/z 1,852) was also the main form in MALDI-TOF spectra of lipid A purified from GMMA from Sf2a-p + OAg (Fig. 2K), Sf2a-p + OAg′ and S. flexneri 3a and 6 ΔtolR ΔhtrB strains.3

To test whether the production of the palmitoleoylated hexa-acylated lipid A species (m/z 1,851) could be induced in S. sonnei under stress conditions, Ss-p-OAg ΔhtrB was grown at 12 °C to induce a cold stress response. In the corresponding lipid A analysis by MALDI-TOF, a small amount (signal intensity less than 5% of the main species) of the palmitoleoylated lipid A species was identified (Fig. 2F).

Palmitoleoylation of lipid A in the absence of the lauroyl chain is consistent with the activity by the late acyltransferase LpxP. Thus, the expression level of lpxP was quantified in

3 F. Citrolo, unpublished results.
Lipid A Modification in Shigella GMMA

$S_s-p-OAg \Delta htrB$ and $Sf2a-p-OAg \Delta htrB$ grown at 30 °C in comparison with the $msbB$ gene that is part of the constitutive lipid A pathway using real-time PCR. In three independent experiments, the ratio of transcript of $lpxP$ to $msbB$ was on average 7.3-fold higher (standard deviation 0.9) in $Sf2a-p-OAg \Delta htrB$ compared with $S_s-p-OAg \Delta htrB$.

FIGURE 2. MALDI-TOF spectra of lipid A preparations in reflectron ion-negative mode. Lipid A was extracted from GMMA from the following: A, $S_s-p-OAg$; B, $Sf2a-p-OAg$; C, $S_s-p-OAg \Delta msbB$; D, $Sf2a-p-OAg \Delta msbB$; E, $S_s-p-OAg \Delta htrB$; F, $Sf2a-p-OAg \Delta htrB$; G, overlay of negative ion LIFT MALDI-TOF/TOF spectra in the low m/z range of the dominant species in lipid A from $S_s-p-OAg \Delta htrB$ (E) and $Sf2a-p-OAg \Delta htrB$ (F) after collision-induced dissociation; H, lipid A structures with molecular weights corresponding to the observed main peaks.
TLR4-NF-κB Luciferase Reporter Assay—We stimulated HEK293 cells stably transfected to express only human TLR4 recognition complex and an NF-κB-inducible luciferase reporter gene (41) to characterize TLR4 stimulation by GMMA with the different lipid A species. The results obtained with different concentrations of GMMA are displayed in Fig. 3 and Table 3.

GMMA from Sf2a-p-OAg ΔmsbB and Ss-p-OAg ΔmsbB stimulated similar levels of NF-κB expression in the HEK293 TLR4 transfectant cells (Fig. 3) and required ~600-fold more GMMA than the parent GMMA (Table 3) to give a 3-fold increase in NF-κB activity. In contrast, GMMA from Ss-p-OAg ΔhtrB and Sf2a-p-OAg ΔhtrB gave very different results. NF-κB induction by Ss-p-OAg ΔhtrB GMMA was only detectable at a high concentration, requiring ~60,000-fold more GMMA than the GMMA with wild-type lipid A and 100-fold more than ΔmsbB GMMA to stimulate the same level of NF-κB activity (Fig. 3 and Table 3). In contrast, Sf2a-p-OAg ΔhtrB GMMA (Fig. 3 and Table 3) retained higher TLR4 stimulation and required 10-fold less GMMA (p = 0.0286) than ΔmsbB GMMA to result in a similar induction of NF-κB. Accordingly, the decrease of TLR4 stimulation compared with GMMA with wild-type lipid A was the smallest (50-fold) of all tested GMMA with lipid A modifications.

To ensure that the observed differences are the results of differences of the stimulatory activity of the lipid A and not caused by different amounts of lipid A present in GMMA, the molar amount of lipid A per mg of protein was determined by quantifying the LPS core sugar KDO. The amounts of lipid A were similar in all GMMA. In comparison with GMMA from Ss-p-OAg ΔhtrB with the lowest activity, GMMA from Ss-p-OAg contained 1.1-fold, Sf2a-p-OAg 2.0-fold, Sf2a-p-OAg ΔhtrB 0.3-fold, Ss-p-OAg ΔmsbB 0.8-fold, and Sf2a-p-OAg ΔmsbB 1.1-fold the amount of lipid A.

Cytokine Release from Human PBMC—In order measure the endotoxin level of GMMA in a more natural context, and in particular to examine whether the GMMA could stimulate additional pattern recognition receptors to TLR4, GMMA purified from different mutants were used to stimulate human PBMC in the monocyte activation test (Fig. 4). GMMA purified from Shigella strains without lipid A modification (both Ss-p-OAg and Sf2a-p-OAg) induced high levels of the pro-inflammatory cytokines interleukin 6 (IL-6), TNF-α, IL-1β, and INF-γ.

TABLE 3

| GMMA from strain: | GMMA concentration at 3-fold NF-κB induction [ng/mL] | GMMA with lipid A type: | Mean [ng/mL] | Fold difference to GMMA with WT lipid A | Fold difference to ΔmsbB GMMA |
|-------------------|---------------------------------------------------------|--------------------------|--------------|----------------------------------------|--------------------------|
|                   | 1            | 2            | 3            | 4            | Mean                                    |                          |
| Ss-p-OAg          | 0.003        | 0.003        | 0.010        | 0.005        | 0.005                                   | Wild-type 0.008          |
| Sf2a-p-OAg        | 0.004        | 0.004        | 0.021        | 0.010        | 0.010                                   | ΔmsbB 4.65               |
| Ss-p-OAg ΔmsbB    | -            | -            | 5.17         | 4.16         | 4.67                                    | ΔmsbB 581                |
| Sf2a-p-OAg ΔmsbB  | -            | -            | 1.56         | 7.71         | 4.64                                    | Ss-p-OAg ΔhtrB 500       |
| Ss-p-OAg ΔhtrB    | 418          | 324          | 362          | 897          | 500                                     | Sf2a-p-OAg ΔhtrB 0.40    |
| Sf2a-p-OAg ΔhtrB  | 0.39         | 0.16         | 0.59         | 0.44         | 0.40                                    | 50                       |

FIGURE 3. Activation of TLR4 reporter cell line by different GMMA. 25,000 TLR4-HEK293 cells/well were stimulated with 0.0001–1000 ng/ml (5-fold steps) of GMMA obtained from Ss-p-OAg, Sf2a-p-OAg, Ss-p-OAg ΔmsbB, Sf2a-p-OAg ΔmsbB, Ss-p-OAg ΔhtrB, and Sf2a-p-OAg ΔhtrB. After 5 h, luciferase expression was measured and expressed as fold-induction compared with cells incubated with PBS and plotted as averages of duplicates with standard deviations. GMMA concentrations that resulted in 3-fold increased activation of NF-κB (black dashed line) over background are shown as x axis intercepts (colored dashed lines). A representative result of four independent experiments is shown.
FIGURE 4. Cytokine release by human PBMC after stimulation with different types of GMMA. 200,000 human PBMC cells were stimulated for 4 h with 0.0001–1000 ng/ml (10-fold steps) of GMMA from Ss/H1002p/H1002OAg, Sf/H1002p/H1002OAg/H9004msbB, Ss/H1002p/H1002OAg/H9004htrB, and Sf/H1002p/H1002OAg/H9004htrB.

Release of the following: A, IL-6; B, TNF-α; C, IL-1β; D, IL-8; E, interferon-γ (IFN-γ); F, IL-10; and G, IL-12 p70, were measured by human pro-inflammatory 7-plex mesoscale and plotted as averages of duplicates with standard deviations. GMMA concentrations that resulted in 10-fold increase of IL-6 release over background are shown as x axis intercepts (colored dashed lines). Cytokine release by PBMC exposed to PBS was used as control.
IL-8, intermediate levels of IFN-γ, and low levels of IL-12 and IL-10 (Fig. 4). All GMMA with lipid A modifications resulted in substantially lower cytokine release (Fig. 4).

The GMMA concentration required to give a 10-fold increase in IL-6 release over background (Table 4) was used for comparing relative activity (42). The same analysis performed at other points within the linear part of the curve (3- and 30-fold over background, respectively) gave similar results (data not shown).

GMMA purified from Shigella strains carrying lipid A modification gave a similar rank order in reduction of IL-6 release upon stimulation in comparison with the parent strains with wild-type lipid A (Fig. 4 and Table 4), as observed in the TLR4-specific assay (Fig. 3 and Table 3) but with smaller differences than in the TLR4-specific assay. The amount of GMMA required to give a 10-fold increase in IL-6 release was as follows: Ss_p-OAg ΔhtrB (800X) > Ss_p-OAg ΔmsbB = Sf2a_p-OAg ΔmsbB (300X) > Sf2a_p-OAg ΔhtrB (50X) the amount of GMMA wild-type lipid A (Table 4).

TLR Blocking—With the objective of identifying the TLRs that contribute to the residual activation by GMMA with lipid A modification, PBMC were incubated with TLR2 and/or TLR4 blocking antibodies before stimulation with 1 and 10 ng/ml of GMMA, concentrations chosen to give a significant but not saturating increase of IL-6 (Fig. 5). The three GMMA with penta-acylated lipid A, Ss_p-OAg ΔhtrB (Fig. 5D), Ss_p-OAg ΔmsbB (Fig. 5B), and Sf2a_p-OAg ΔmsbB (Fig. 5C), gave similar results as follows: IL-6 production was substantially reduced following incubation with TLR2 blocking antibody (70–90%) but either no reduction or minimal reduction was observed with TLR4 blocking antibody (10–30%) suggesting residual activity was principally due to TLR2 activation. With the Ss_p-OAg ΔmsbB and Sf2a_p-OAg ΔmsbB GMMA, a small reduction was obtained with the TLR4 blocking antibody alone (Ss_p-OAg ΔmsbB, p = 0.0234; Sf2a_p-OAg ΔmsbB, p = 0.0078 in four experiments) suggesting that there was still residual TLR4 activation. Further reduction was achieved with the combination of TLR2 and TLR4 blocking antibodies compared with TLR2 blocking antibody alone. With the Ss_p-OAg ΔhtrB GMMA, no effect from TLR4 blocking was observed.

The GMMA with mostly hexa-acylated lipid A (Sf2a_p-OAg ΔhtrB, Fig. 5E) gave a substantial reduction with a TLR4 blocking antibody, i.e. 80% reduction at 1 ng/ml and 40% at 10 ng/ml GMMA concentration. TLR2 blocking showed no effect on the IL-6 release at 1 ng/ml GMMA concentration and resulted in ~35% reduction (average of three independent experiments) at the 10 ng/ml GMMA concentration. Incubation with both TLR2 and TLR4 blocking antibodies gave lower IL-6 production at 1 and 10 ng/ml GMMA suggesting that both TLR were still active but that the TLR4 activation was dominant especially at lower GMMA concentrations.

To confirm that the differences in the relative contribution of TLR4 and TLR2 to activation observed in the blocking experiments were primarily dependent on the differential TLR4 activation by the different GMMA, the ability of the GMMA to activate TLR2 was tested by stimulating HEK293-TLR2 transfectant cells. All four ΔmsbB or ΔhtrB GMMA required similar GMMA concentrations (2.6–4.9 ng/ml) to give a 10-fold NF-κB induction (Fig. 6).

DISCUSSION

GMMA are attractive candidates for vaccines as they present surface antigens in their natural environment and conforma-

tion. For use as vaccines, dependent on the dose, the reactoge-
nicity needs to be reduced, as GMMA contain LPS and other TLR stimulatory components, e.g. lipoproteins. Previously, we described a high yield production process for GMMA (2). The goal of this study was to demonstrate the impact of reducing the endotoxin potential of Shigella GMMA by genetic modification of the lipid A component of the GMMA LPS.
The results of deleting the msbB gene from both *S. sonnei* ΔtolR and *S. flexneri* 2a ΔtolR and by deleting the htrB gene from *S. sonnei* ΔtolR were as expected: conversion of a hexa-acylated lipid A to a penta-acylated lipid A through loss of a myristic acid (ΔmsbB) or lauric acid (ΔhtrB). However, we have demonstrated the presence of a palmitoleoyl chain in lipid A purified from *Sf* 2a Δ TolR GMMA. Palmitoleoylation in *lpxL* (ΔhtrB) mutants has previously been reported in *E. coli*. It was shown to be a moderately abundant species at 30 °C and as the dominant species at 37 °C (43). In *S. flexneri* 2a ΔhtrB, the signal of palmitoleoylated lipid A was the dominant species at 30 °C, with very little signal due to the penta-acylated species present. Similar results were obtained for the *S. flexneri* 3 and *S. flexneri* 6 strains tested.

The inability to detect a hepta-acetylated lipid A in either the *Sf* 2a Δ TolR GMMA or the *Sf* 2a Δ TolR ΔhtrB GMMA complemented with htrB expression from pACYCΔ htrB suggests that the palmitoleoylation is on the same site occupied by lauric acid in the wild-type lipid A, although further studies would be required to prove this. This would also be consistent with palmitoleoylation catalyzed by the action of LpxP, a late acyltransferase that acts at the HtrB site, as part of what has been described as a cold response in *E. coli* (23). Thus, the palmitoleoylation could be part of a stress response. Similarly, in the *E. coli* ΔlpxL mutant, the abundance of the palmitoleoylated lipid A increased with stress (growth at 37 °C (43)). In ΔtolR mutant strains, the tolR mutation could provide stress and thus result in higher abundance of palmitoleoylated lipid A.

**FIGURE 5. TLR blocking experiments.** 200,000 human PBMC cells were stimulated with GMMA from different strains. After a 4-h incubation, IL-6 release was measured by ELISA and plotted as average of duplicates with standard deviation. A, IL-6 release after stimulation with 0.0001–1000 ng/ml (10-fold steps) of GMMA from different strains. The rectangle highlights the concentration of GMMA used in blocking experiments. B–E, cells were incubated with 25 μg/ml anti-TLR4 (dark red graphs), 15 μg/ml anti-TLR2 (green graphs), or both (violet graphs) for 30 min before exposure to 1 or 10 ng of GMMA from *Ss* Δ TolR ΔmsbB (B); *Sf* 2a Δ TolR ΔmsbB (C); *Ss* Δ TolR ΔhtrB (D); and *Sf* 2a Δ TolR ΔhtrB (E). The graphs from experiments without blocking are shown in the same color as in A. A representative result of three independent experiments is shown.
Lipid A Modification in Shigella GMMA

detect no residual TLR4 activity of the penta-acylated lipid A in S. sonnei ΔhtrB, indicated by a 60,000× increase in the amount of GMMA (500 ng/ml), required to give similar activation compared with GMMA with WT lipid A (0.008 ng/ml). GMMA from both S. sonnei and S. flexneri 2a ΔmsbB mutants gave similar TLR4 activation at comparable GMMA concentrations (4.7 and 4.6 ng/ml). Although this required 600× more GMMA compared with GMMA with WT lipid A, the amount was 100× less compared with GMMA from the S. sonnei ΔhtrB with penta-acyl lipid A, showing that the ΔmsbB penta-acyl lipid A retains some residual ability to activate human TLR4. Although the magnitude of difference in TLR4 activation between the Ss_p–OAg ΔhtrB and the ΔmsbB GMMA with penta-acylated lipid A was unexpected, minor modifications in penta-acylated lipid A have been shown to affect TLR4 stimulation (46). In S2a_p–OAg ΔhtrB GMMA, the substitution of a lauroyl chain with the longer palmitoleoyl chain in hexa-acylated lipid A also led to approximately a 50× decrease in the ability to simulate human TLR4 (0.4 ng/ml) compared with GMMA with WT lipid A. The mass spectrum showed a mixture of hexa-, penta-, and tetra-acylated lipid A, of which the palmitoleoylated hexa-acylated peak was by far the dominant peak present. Although the relative height of the peaks in this experiment is not necessarily strictly proportional to the abundance in the GMMA, it seems highly unlikely that a 50× reduction in activation could be due to the decrease in percentage of the hexa-acylated by the presence of these lower sized peaks but that the reduction of TLR4 stimulation is rather linked to the difference of the acyl chain composition. In PBMC, GMMA without lipid A modification predominantly stimulated the pro-inflammatory cytokines IL-6, TNF-α, IL-1β, and IL-8 as observed previously for Neisseria meningitidis native outer membrane vesicles (47). All GMMA with lipid A modification showed a marked decrease of cytokine release compared with GMMA with WT lipid A. IL-6 release was chosen for detailed comparison of the relative ability of GMMA to elicit cytokine release due to its role in fever pathogenesis (48). In contrast to the TLR4-specific assay, the difference in IL-6 stimulation by S. sonnei ΔhtrB GMMA and S. sonnei or S. flexneri 2a ΔmsbB GMMA was very small, only ~3-fold (S. sonnei ΔhtrB 800-fold and ΔmsbB 300-fold), compared with the 100-fold difference in TLR4 activation (S. sonnei ΔhtrB 60,000-fold and ΔmsbB 600-fold). This small difference is in accordance with the presence of additional TLR being stimulated by GMMA. It indicates that the decrease of GMMA reactogenicity obtained by either form of penta-acylated lipid A is close to the maximum decrease of GMMA reactogenicity achievable by lipid A modification, when assayed in a complex system as PBMC that is more relevant for estimating responses in humans (38). Based on the large reduction of in vitro cytokine release from PBMC, an S. sonnei ΔhtrB GMMA was chosen as a first candidate for vaccine development. Phase 1 clinical trials are currently underway and will give an important indication of the tolerability of GMMA from these constructs.

To characterize which TLR receptors contribute to the residual activity of GMMA, TLR blocking experiments in PBMC were performed. We demonstrated that the remaining activity of GMMA with penta-acylated lipid A was predominantly due to TLR2 activation. With GMMA from Ss_p–OAg ΔhtrB, no
effect from TLR4 blocking was observed, whereas GMMA resulting from a msbB deletion retained detectable TLR4 activity in line with the results of the TLR4-specific assay. However, the induction of IL-6 via lipid A by the ΔmsbB GMMA that was dependent on TLR4 was smaller than the induction via TLR2. In contrast, GMMA from S. flexneri 2a ΔhtrB with palmitoleoylated hexa-acylated lipid A was primarily stimulated TLR4. Further reduction of reactogenicity of GMMA from S. sonnei 53G and Shigella flexneri 2457T. In contrast, compensatory palmitoleoylation in the residual activity is probably due to non-lipid A-related TLR2 inflammatory cytokines from human PMBC for which the structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex (509). Thus, although N. meningitidis and Shigella are not closely related and e.g. have different LPS structures (49) modification of TLR2 activators is not expected to be required.

The broad aim of this study was to examine ways of reducing the reactogenicity of GMMA to make them suitable for use as a human vaccine, but it resulted in a surprisingly complex outcome. Deleting the htrB gene from S. sonnei or the msbB gene from S. sonnei and S. flexneri 2a resulted in GMMA with a penta-acyl lipid A with a marked reduction in induction of inflammatory cytokines from human PMBC for which the residual activity is probably due to non-lipid A-related TLR2 activation. In contrast, compensatory palmitoleoylation in the S. flexneri 2a ΔhtrB GMMA results in retained TLR4 activation. Although the level of reduction of GMMA reactogenicity required for an acceptable vaccine will depend on the dose required to give a strong immune response, which can only be determined in clinical trials, the data suggest that the use of GMMA as vaccines will likely require lipid A penta-acylation and that GMMA with palmitoleoylated hexa-acylated LPS are less likely to result in a useful vaccine.

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