Chimeric oligosaccharide conjugate induces opsonic antibodies against *Streptococcus pneumoniae* serotypes 19A and 19F†

Someswara Rao Sanapala,‡a Bruna M. S. Secoa,‡b Ju Yuel Baeka, Shahid I. Awan,a Claney L. Pereiraab and Peter H. Seebergerid,‡ab

*A* *Streptococcus pneumoniae* 19A (ST19A) and 19F (ST19F) are among the prevalent serotypes causing pneumococcal disease worldwide even after introduction of a 13-valent pneumococcal conjugate vaccine (PCV13). Synthetic glycoconjugate vaccines have defined chemical structures rather than isolated polysaccharide mixtures utilized in marketed vaccines. Ideally, a minimal number of synthetic antigens would cover as many bacterial serotypes to lower cost of goods and minimize the response to carrier proteins. To demonstrate that a chimeric oligosaccharide antigen can induce a protective immune response against multiple serotypes, we synthesized a chimeric antigen (ST19AF) that is comprised of a repeating unit of ST19A and ST19F capsular polysaccharide each. Synthetic glycan epitopes representing only ST19A, and ST19F were prepared for comparison. Semisynthetic glycoconjugates containing chimeric antigen ST19AF induced high antibody titers able to recognize native CPS from ST19A and ST19F in rabbits. The antibodies were able to kill both strains of *pneumococci*. Chimeric antigens are an attractive means to induce an immune response against multiple bacterial serotypes.

Introduction

Invasive pneumococcal diseases (IPD) caused by *Streptococcus pneumoniae* (Sp), are a major cause of morbidity and mortality in toddlers and older adults worldwide.‡a Despite the introduction of a 13-valent pneumococcal conjugate vaccine (PCV13) in 2010, ST19A remains a major pathogen worldwide‡a that is associated with 14% of all IPD cases.‡a Since the efficacy of PCV 13 against ST19A is being debated‡a,‡b the development of new vaccine approaches covering ST19A and ST19F is desirable.

Currently glycoconjugate vaccines contain polysaccharides isolated from bacterial cell cultures, that are conjugated to carrier proteins. To demonstrate that a chimeric oligosaccharide antigen can induce a protective immune response against multiple serotypes, we synthesized a chimeric antigen (ST19AF) that is comprised of a repeating unit of ST19A and ST19F capsular polysaccharide each. Synthetic glycan epitopes representing only ST19A, and ST19F were prepared for comparison. Semisynthetic glycoconjugates containing chimeric antigen ST19AF induced high antibody titers able to recognize native CPS from ST19A and ST19F in rabbits. The antibodies were able to kill both strains of *pneumococci*. Chimeric antigens are an attractive means to induce an immune response against multiple bacterial serotypes.

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results and discussion

Antigen construction of 3, 4, 5 requires the stereoselective installation of two 1,2-cis glycosidic linkages and the stereoselective formation of an α-phosphodiester linkage to the trisaccharide (Fig. 1B). Trisaccharides 6 or 7 can be synthesized via a linear synthetic approach from the reducing to the non-reducing end using the building blocks 8, 9, 10, 11, and 11. For the assembly of trisaccharide building block 6, glucosyl imidate 10 was coupled to rhamnosyl acceptor 8 to obtain α-linked disaccharide 12 (\(J_{C-H} = 170.5\) Hz) in 82% yield (Scheme 1A). Regioselective reductive ring opening of the benzylidene acetal in 12 using triethylsilane and trifluoroacetic acid delivered acceptor 13 in 69% yield. Disaccharide acceptor 13 was glycosylated with glucosyl imidate 11 to afford trisaccharide 14 in 83% yield (doublet, \(J = 8.0\) Hz, \(J_{C-H} = 167.9\) Hz). Selective levulinoyl ester (Lev) cleavage (92%) using hydrazine acetate followed by inversion of the 2-hydroxyl group was achieved by conversion of the corresponding sulfonyl diimidazole and subsequent nucleophilic displacement with tetrabutylammonium azide to obtain 16 (82% over two steps). The inversion of stereochemistry was confirmed by examination of the \(^1\)H-\(^1\)H and \(^1\)C-\(^1\)H coupling constants for H-1 at 1.4 4.3 Hz (doublet, \(J = 1.4\) Hz) and \(J_{C-H} = 163.2\) Hz. Conversion of the azide to the corresponding acetamido group was obtained by treatment with freshly activated Zn–Cu couple to give 17 in 74% yield. Removal of the p-methoxyphenyl group using ceric ammonium nitrite (CAN) gave hemiacetal 18. The installation of a stereo-selective H-phosphonate at the reducing end of phosphate diester 19 was achieved with phosphorous acid and an excess of sterically bulky 2-chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinane in pyridine at 50 °C to obtain thermodynamically more stable α-phosphonate 6 (\(^{31}\)P NMR: \(\delta = 0.91, J_{P,H} = 641.8, J_{P,H-1} = 7.5\) Hz & \(^3\)H NMR: \(\delta = 5.70, J_{C,H} = 176\) Hz). The selectivity may result from conversion of the more reactive β-glycosyl H-phosphonate to the thermodynamically more stable α-phosphonate by Sn2 displacement with excess phosphorous acid. The protected phosphodiester-linked trisaccharide 19 was prepared by coupling H-phosphonate 6 with 3-azido pentanol using pivaloyl chloride in pyridine as a condensing agent followed by oxidation with iodine in water–pyridine. Hydrogenation of protected disaccharide 20 passed through Dowex 50W X4 Na+ resin as a single diastereomer.

The synthesis of ST19A trisaccharide 4 followed a similar synthetic approach (Scheme 1B). Orthogonal glycosylation between glucosyl imidate 10 and n-pentenyl acceptor 9 gave α-linked disaccharide 20 (\(J_{C,H} = 172.0\) Hz), which was subjected

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Fig. 1 (A) Structures of the S. pneumoniae CPS repeating units of serotypes ST19F (1), ST19A (2) and synthetic oligosaccharide antigens 3, and 4 that resemble these repeating units as well as chimeric oligosaccharide 5; (B) Retrosynthetic analysis of 3, 4, and 5.
to reductive benzylidene acetal cleavage to obtain 21. Disaccharide 21 containing a 4-hydroxyl group was coupled with glycosyl imidate 11 to give trisaccharide 22 (δ 4.42, doublet, J = 8.0 Hz, 1JCH = 165.2 Hz). Cleavage of the Lev group followed by stereoselective inversion of the C2-hydroxyl group in 23 yielded the corresponding azide 24 (δ 4.29, singlet, 1JCH = 162.2 Hz).

Reduction of the azide to the corresponding acetamido derivative using Zn–Cu couple afforded 25. Hydrolysis of pentenyl glycoside delivered hemiacetal 26, which was converted into the azidopentyl linked phosphonate derivative 27 via the H-phosphonate method. Hydrogenation using Pd/C in EtOAc : MeOH : H2O (3 : 2 : 1) provided deprotected trisaccharide as triethylammonium salt that was converted into corresponding sodium salt using Dowex 50W X4 Na⁺ resin to obtain white solid 4 as a single diastereomer (δP = 1.92, 1JCH = 174.2 Hz).

With trisaccharides 25 and 6 in hand, the synthesis of the chimeric antigen 5 commenced (Scheme 1C). Reductive cleavage of the benzylidene acetal in trisaccharide 25 was achieved using triethylsilane and TFA to give 28, that was acetylated at the 4-OH to furnish 29. Hydrolysis of the pentenyl glycoside followed by H-phosphonate installation gave solely α-glycosyl phosphate 31 (δP = 2.64, 3JPHH = 8.1 Hz, 1JCH = 176.0 Hz). Acetate ester cleavage furnished acceptor 7 that was condensed with ST19F H-phosphonate 6 to afford hybrid ST19AF 32. Finally, hexasaccharide 32 was deprotected by hydrogenolysis to provide the disodium salt of antigen 5 following exchange with Dowex 50W X4 Na⁺ resin. The antigens 3, 4, and 5 were obtained as white solids following purification by reverse phase C18-column HPLC and lyophilization.

The synthetic antigens 3–5 were conjugated to CRM197 with an average of seven, five and five oligosaccharides attached per CRM197 molecule respectively as determined by MALDI analysis. Rabbits were immunized with conjugates containing the chimeric ST19AF, or the ST19A or ST19F antigens formulated with aluminum hydroxide. The conjugates induced strong anti-
glycan antibody titers after delivery of three vaccine doses at two week intervals. The long term immune response was confirmed following a boost three and a half months after the last immunization (day 133). High antibody titers were faster attained showing that memory B cells were activated and able to generate antibodies against the vaccine antigen (day 144) (Fig. 2). Rabbits immunized with Prevnar13®, a marketed vaccine containing both native CPS serotypes, produced higher antibody titers against the hybrid ST19AF when compared to ST19A and ST19F antigens (Fig. 2). Increased binding to the chimeric oligosaccharide may be a result of an ionic interaction with the phosphate diester connecting the two repeating units. Ionic protein–glycan interactions are known for cell recognition and antibody–glycan interactions. Rabbit antibodies raised in response to glycoconjugate vaccination were tested against native CPS of ST19A (CPS19A) and ST19F (CPS19F) to determine the cross-reactivity of antibodies produced against synthetic or native antigens. Chimeric ST19AF generated antibodies that recognize both CPS. Anti-CPS19A antibody titer was highest after three doses (day 35). ST19A antigen at the reducing-end of the chimeric structure may favor a better B-cell presentation due to the proximity to the carrier protein presented via MHC class II (Fig. 3). ST19F conjugate induced low antibody titers against CPS19F while ST19A did not generate antibodies recognizing native CPS19A although antibodies against the synthetic antigen were detected (Fig. 2). The absence of the phosphate diester in the shorter repeating units might impair the generation of antibodies that can recognize native CPS since Prevnar13®, that contains the native antigens might impair the generation of antibodies that can recognize native CPS (Fig. 2).

Antibodies produced in response to semisynthetic glycoconjugate ST19AF recognize native CPS19A and CPS19F. Zika rabbits (n = 5 per group) were immunized three times (days 0, 14, 28) intramuscularly with semisynthetic ST19A, ST19F or ST19AF glycoconjugate formulated with aluminum hydroxide as well as positive control Prevnar13® and negative control PBS + aluminum hydroxide. Polysaccharide-specific antibody titers were analyzed by ELISA. The 1:100 sera dilution was used in the analysis. a.u. = absorbance units.

**Conclusions**

We synthesized a chimeric oligosaccharide antigen containing the repeating units of ST19A and ST19F capsular polysaccharides as well as synthetic glycan epitopes resembling ST19A and ST19F. The chimeric antigen glycoconjugate induced an excellent immune response in rabbits. The antibodies produced in response to the chimeric antigen killed ST19A and ST19F bacteria, while the conjugates containing the other glycan epitopes failed to do so. With an increasing number of serotypes to be included in vaccines due to serotype replacement, ideally, each antigen can induce an immune response against more than one serotype, reducing the chemical steps needed for synthetic vaccine production. These findings will expedite glycoconjugate vaccine development as co-formulation studies containing chimeric antigens will help to reduce the amount of carrier protein in semi-synthetic glycoconjugate vaccines.
Materials and methods

Oligosaccharide antigens were synthesized using standard protocols and conjugated to CRM197. Synthetic antigens were printed on NHS-activated microarray slides. Animal experiment (project 49062) was performed in strict accordance with the NIH/OLAW Animal Welfare Assurance, identification number F16-00178 (AS755-01) and was authorized by LALLF MV (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern, Germany) in accordance to TierSchG 7221.3-2-002/19. The immune response was analyzed by glycan microarrays and ELISA. The functional attribute of the immune response was monitored by OPKA using HL-60 cells. Bacteria serotypes ST19A and ST19F were isolates from Charité Infektiologie und Pneumologie department, Universitätsmedizin Berlin. Detailed materials and methods can be found in ESI Appendix.†

Conflicts of interest

None of the authors declare a conflict of interest.

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