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Ontwikkeling van nieuwe synthetische oligosacharide vaccins

De immunogeniciteit van oligosacharide-CRM_{197} neoconjugaten en oligosacharide/peptide hybride goudnanodeeltjes gebaseerd op de polysacharide structuur van *Streptococcus pneumoniae* type 14 (met een samenvatting in het Nederlands)

Pengembangan vaksin oligosakarida sintetik baru

Imunogenisitas dari neokonjugat oligosakarida-CRM_{197} dan hibrida oligosakarida/peptida nanopartikel emas berdasarkan struktur polisakarida dari *Streptococcus pneumoniae* type 14 (dengan ringkasan dalam bahasa Indonesia)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 30 november 2010 des ochtends te 10.30 uur

door

Dodi Safari

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For my beloved family: Teti, Nisa, Khayla

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2. Neeltje Carpaij

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Chapter 1

General Introduction

Streptococcus pneumoniae

Pneumonia kills more children than any other illness – more than AIDS, malaria, and measles combined (112). *Streptococcus pneumoniae* (*S. pneumoniae* or referred to as the pneumococcus) is a leading cause of bacterial pneumonia, meningitis, and sepsis in children worldwide and it is estimated that 1.6 million people die from these infections each year, of whom one million are children (15,117). In elderly, pneumonia caused by this micro-organism is a major health care problem. Incidence of invasive pneumococcus disease varies substantially by age, genetic background, socioeconomic status, immune status, and geographical location (105). Therefore effective vaccines against pneumococcal disease are of great benefit especially for the young and old.

S. pneumoniae are lancet-shaped gram-positive, alpha-hemolytic bacteria that colonize the mucosal surfaces of the upper respiratory tract (48). Three phase variants of *S. pneumoniae* as seen on agar plates have been identified: transparent, opaque, and intermediate but variation is independent of capsule expression which is related to the interaction of the pneumococcus with its host (114). Most clinical isolates from the bloodstream and nasopharynx consist of heterogeneous populations of at least two colony phenotypes i.e. opaque and transparent (54,94). Three major surface layers can be distinguished from the inside to the outside: the plasma membrane, the cell wall, and the capsule (26) (Figure 1). The cell wall consists of a triple-layered peptidoglycan backbone that anchors the capsular polysaccharide, the cell wall polysaccharide, and also various proteins. The capsule is the thickest layer, completely concealing the inner structures of exponentially growing *S. pneumoniae* bacteria.

Three clusters of surface proteins of *S. pneumoniae* can be distinguished by genome analysis. Firstly the lipoproteins including putative proteinase maturation protein A (PpmA), streptococcal lipoprotein rotamase A (SlrA), the pneumococcal surface adhesion A (PsaA) protein, and the iron uptake ABC transporter lipoproteins (PaA and PiuA). A second cluster is formed by the choline-binding proteins (CBPs) family as there are autolysins, pneumococcal surface protein A (PspA) and C (PspC) and phosphorylcholine esterase (Pce). Thirdly, proteins with an LPxTG motif namely neuraminidase A (NanA), IgA1-protease, zinc metalloproteases (Zmp), surface-exposed serine protease (PrtA) and high-temperature requirement A (HtrA) protease (7) (Figure 1). The most important virulence factors determinants include the antiphagocytic and adherence properties of capsular polysaccharides, adherence factors, invasion genes, iron and other heavy-metal transporters, oxidative stress

protection, host-defence evasion, pneumolysin production, bacteriocin production, and quorum sensing, and biofilm formation (105).

Capsular polysaccharide

Capsular polysaccharides are well known as the major virulence factors of *S. pneumoniae*. Today more than 90 serotypes have been identified based on the different chemical structures of these polysaccharides (51,76). This diversity determines the ability of the serotypes to survive in the bloodstream and very likely the ability to cause invasive disease, especially in the respiratory tract (26,51). The most prevalent serotypes are more heavily encapsulated which endows them with greater resistance to neutrophil-mediated killing (113). Recently, new *S. pneumoniae* serotypes has been identified, e.g. serotype 6C (76), 6D (12,73), and 11E (17).

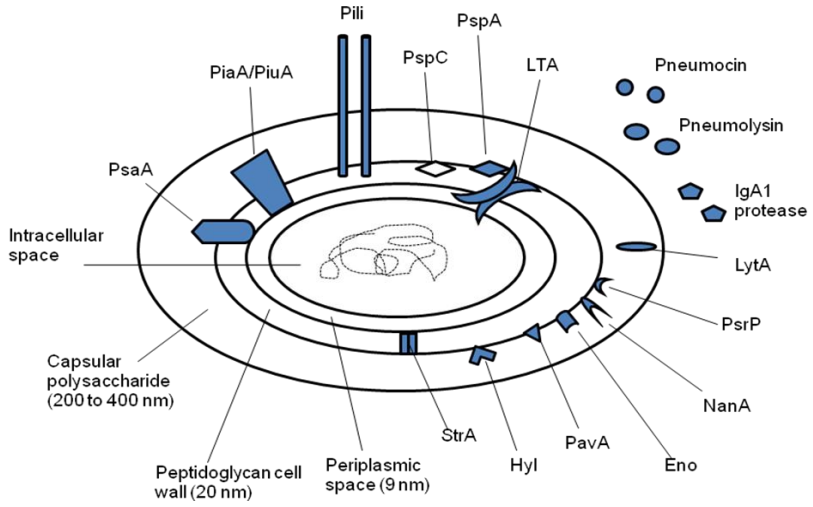

Figure 1. Schematic structure of S. pneumoniae. Adopted from van der Poll, T. and Opal, S.M. (105) and de Velasco, E.A. et al (26). StrA=sortase A. Hyl=hyluronate lyase. PavA=pneumococcal adhesion and virulence. Eno=enolase. NanA=neuraminidase. PsrP=pneumococcal serine-rich repeat protein. LytA=autolysin. LTA=lipoteichoic acid. PspA=pneumococcal surface protein A. PspC=pneumococcal surface protein C. PiaA/PiuA=pneumococcal iron acquisition and uptake. PsaA=pneumococcal surface antigen A.

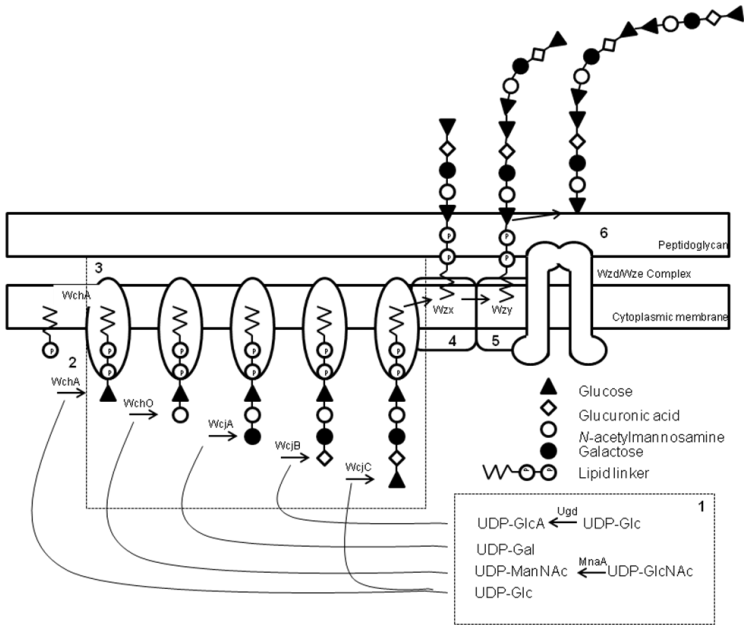

Figure 2. Representation of the Wzx/Wzy-dependent pathway for Biosynthesis of CPS 9A as described by Bentley. S.D. et al (6). Representation of the Wzx/Wzy-Dependent Pathway Pictured is a hypothetical model for capsule biosynthesis in *S. pneumoniae* based on a mixture of experimental evidence and speculation.

1. Non-housekeeping nucleotide sugar biosynthesis.
2. The initial transferase (WchA in this case) links the initial sugar as a sugar phosphate(Glc-P) to a membrane-associated lipid carrier (widely assumed to be undecaprenyl phosphate).
3. Glycosyl transferases sequentially link further sugars to generate repeat unit.
4. Wzx flippase transports the repeat unit across the cytoplasmic membrane.
5. Wzy polymerase links individual repeat units to form lipid-linked CPS.
6. Wzd/Wze complex translocates mature CPS to the cell surface and may be responsible for the attachment to peptidoglycan.

Capsular polysaccharides are large (0.5-2x10^6 Da) polymers, composed of multiple repeating units of up to eight sugar residues (26). The capsular polysaccharides are generally synthesized by the Wzx/Wzy-dependent pathway, except for type 3 and 37 which are synthesized by the synthase pathway (6,67). It consists of a number of steps. Input is provided by non-housekeeping nucleotide sugar biosynthesis. The initial transferase (WchA in this case) links the initial sugar as a sugar phosphate (Glc-P) to a membrane-associated lipid carrier (widely assumed to be undecaprenyl

phosphate). Glycosyl transferases sequentially link further sugars to generate repeat unit. The Wzx flippase transports the repeat unit across the cytoplasmic membrane. Then Wzy polymerase links individual repeat units to form lipid-linked CPS. Finally, Wzd/Wze complex translocates mature CPS to the cell surface and may be responsible for the attachment to peptidoglycan (Figure 2) (6). In the synthase pathway capsule is produced through processive transferase activity (2,61).

S. pneumoniae type 14 polysaccharide (Pn14PS) consists of tetrasaccharide repeating units: {6)-[β-D-Gal*p*-(1→4)-]β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→}n (60). It was estimated that the molecular size of the native polysaccharide of Pn14PS is 2,000 kDa, corresponding to 2,500 repeating units (115). *S. pneumoniae* serotype 14 bacteria is one of the major serotype to cause invasive disease in young children worldwide (29,37). Pn14PS was included in the current and new development pneumococcal vaccine (Table 1). Pn14PS is structurally related to with group B streptococcus type III polysaccharide (GBSIIIPS) which differ only in the absence (in Pn14PS) or presence (in GBSIIIPS) of the (α2→3)-linked sialic acid N-acetylneuraminic acid (Neu5Ac) in their side chains (49).

Immunogenicity of capsular polysaccharides

Many studies have demonstrated that antibodies directed against the capsular polysaccharide are essential for protection against pneumococcal disease (13,82,84). However, the native capsular polysaccharides are well-known thymus-independent type-2 (TI-2) antigens that lack T-helper epitopes and therefore mainly induce IgM antibodies, and to a lesser degree IgG (31). They generally stimulate short lived B cell responses by cross-linking the B-cell receptor to their repeating units (epitopes), which drives the differentiation of B cells to antibody producing plasma cells (80).

TI-2 antigens are able to activate B lymphocytes in the almost complete absence of T lymphocytes, although T lymphocytes are able to improve the antibody response. It is unclear however, how a TI-2 antigen (in the context of this manuscript: a capsular polysaccharide) can be presented to T lymphocytes. Class I or class II MHC molecules cannot do this. CD1, a class I MHC-like molecule is able to present glycolipids, a variety of nonpeptide antigens, and probably also polysaccharides (47). Which category of T lymphocytes can be activated by polysaccharides presented in CD1 molecules on APC also has not been established. They can be either classical $CD4^+$ Th lymphocytes, but studies in knock-out mice also suggest that a subset of

$CD8^+$ cells are required to provide helper function for the production of antibodies against polysaccharide (57). Polysaccharide have also been reported to induce memory B cells that are phenotypically distinct from those elicited by protein antigens; an inducement that is regulated by anti-specific IgG antibodies (72). Recently it has been shown that *S. pneumoniae* generates long-lived bone marrow (BM) plasma cells whose numbers can be increased by the use of CpG oligodeoxynucleotides (ODN) (101). The TI-2 characteristics of polysaccharides can be altered by conjugation of polysaccharide to a protein carrier (glycoconjugate) resulting in a switch to an anti-polysaccharide antibody response with characteristics of a T-cell-dependent response. This is reflected by the generation of memory B and T cells and the induction of high titers of anti-polysccharide IgG antibodies after booster immunization (1).

It should be noted that not all polysaccharides behave as TI-2 antigens. Zwitterionic polysaccharides such as *S.pneumoniae* type 1 polysaccharide: [→3)-α-AATGal-(1→4)-α-D-Gal*p*A-(1→3)-α-D-Gal*p*A-(1→]$_n$ with a right-handed helix with repeated zwitterionically charged grooves elicit potent T cell responses *in vivo* and *in vitro* (19,50).

Development of pneumococcal vaccines

Although the first pneumococcal vaccines, including the application of the principle of conjugate vaccination, were already initiated in the beginning of the previous century, most of these developments stopped when antibiotics were introduced. Existing vaccines were even withdrawn from the market. By now, in many parts of the world, the antibiotic resistance of *S. pneumoniae* bacteria has increased : America (52,104), Africa (111), Europe (98,110), Asia (41,96,119), and Australia (36). This makes treatment of pneumococcal infections more difficult and stresses the importance of the development of effective vaccines as a strategy to reduce morbidity and mortality caused by *S. pneumoniae* infection worldwide.

Pneumococcal polysaccharide-based vaccines.

Today, two vaccine types against *S. pneumoniae* are commercially available: a pneumococcal polysaccharide vaccine (PPV) and a pneumococcal conjugate vaccine (PCV) (3). The first multivalent pneumococcal polysaccharide vaccine (PPV) contains 23 purified capsular polysaccharides (25 µg of each capsule type; Pneumovax®, PPV23, Table 1) which is licensed for use in adults and children older

than 2 years of age (70). This vaccine was shown to be moderately effective in young adults (99) but not in young children (27) and elderly (75) and also not in immunocompromised patients, e.g HIV infected people(102,107). Inhaled delivery of PPV23 vaccine does not result in enhanced pulmonary mucosal immunoglobulin responses compared to intramuscular injection of vaccine(35).

In early 2000, a polysaccharide-protein conjugate vaccine targeting seven pneumococcal serotypes was licensed in the United States for use in young children (Prevnar®, PCV7, Table 1). The polysaccharides are conjugated to the non-toxic cross reactive material from diphtheria toxin, CRM_{197} and each dose contains 2µg of each capsule type, except for 6B, for which 4 µg is included in every vaccine dose(116). The aim of the PCV7 is preventing disease in young children, for whom the vaccine is indicated, and maybe reducing the rate and severity of disease in adults. The vaccine provides an effective new tool for reducing disease caused by drug-resistant strains (116). The PCV7 vaccine produces a significant effect regarding prevention of invasive pneumococcal disease in children younger than 24 months (based on a meta-analysis of published data from trials on pneumococcal vaccine) (77). The PCV7 vaccine is safe and prevents acute otitis media (AOM) in infants by serotypes included in the vaccine (28). PCV7 is unable to prevent subsequent episodes of AOM in children who already suffer from recurrent AOM (106).

Large scale introduction of PCV7 has resulted in an overall decline in infectious pneumococcal disease (IPD). However, IPD caused by the non-vaccine serotypes serotypes 1, 19A, 3, 6A, and 7F has increased (replacement disease), highlighting the need for inclusion of these serotypes in future improved vaccine formulations (42). The virtual disappearance of vaccine serotypes in *S. pneumoniae* carriage has occurred in young children, with rapid replacement with penicillin-nonsusceptible nonvaccine serotypes, particularly 19A and 35B after PCV7 introduction in USA (40).

Apart from the CRM_{197} based PCV7, several new candidate pneumococcal conjugate vaccines have been developed to cover more serotypes with different protein carriers and most of them are in clinical trials, e.g. PCV9, PCV10, PCV11, PCV13, and PCV5 (Table 1).

Table 1. Development of pneumococcal polysaccharide vaccines

Vaccines	Polysaccharide serotypes included in the vaccine	Protein carrier	Ref.
PPV23	1, 2, 3, 4, 5, 6B, 7, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 15F, 18C, 19A, 19F, 20, 22F, 23F, 33F	None	(35,75)
PCV7	4, 6B, 9V, 14, 18C, 19F, 23F	CRM_{197}	(28,77,116)
PCV9	1, 4, 5, 6B, 9V, 18C, 19F, 23F,	CRM_{197}	(20,55,58)
PCV10	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F	*H. influenzae*-derived protein D DT[1] for serotype 19F TT[2] for serotype 18C)	(8,56)
PCV11-protein D	1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F	*H. influenzae*-derived protein D	(82,83)
PCV11-DT	1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F	DT	(63)
PCV13	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F	CRM_{197}	(93)
PCV5	1, 5, 6B, 19F, 23F	PspA	(69)

[1]DT = Diphtheria toxoid; [2]TT = Tetanus toxoid

Pneumococcal protein-based vaccines

An alternative vaccine strategy focuses on the use of pneumococcal surface-associated proteins which are to be assumed to elicit protection in all age groups against all, or nearly all, pneumococcal serotypes. Protection induced by the proteins should be serotype-independent and possibly cheaper and thus within reach of developing countries (9). Currently, several surface pneumococcal proteins are investigated as a candidate vaccine against *S. pneumoniae* infection with single or combination of recombinant proteins (Table 2).

Table 2. Development of pneumococcal protein-based vaccine

Type of protein	Model study	Immunogenicity/ protection	Ref.
PspA family fusion proteins	Mouse	Enhancing protection against multiple strains of *S. pneumonaie*	(118)
Sortase A	Mouse	Confers protection in mice against *S. pneumoniae upon* intraperitoneal challenge	(34)
Pilus subunits	Mouse	Affords protection against lethal challenge with the *S. pneumoniae* serotype 4 strain TIGR4	(33)
PiuA and PiaA	Mouse	Protects against intranasal challenge with *S. pneumoniae*	(14,47)
Polyamine transport protein D (PotD)	Mouse	Generates both mucosal and systemic immune responses and prevents establishment of nasopharyngeal carriage by multiple pneumococcal serotypes	(95)
Pneumolysin (Ply); PspA1 and PspA2	Pregnant women in Papua New Guinea	High correlation between maternal and cord blood antibody titers to Ply, PspA1, and PspA2	(30)
Pneumococcal histidine triad protein D (PhtD), choline binding protein A (CbpA), and lysozyme (LytC)	Pregnant women and their infants in the Philippines	Development of antibody response in young children to PhtD, CbpA, and LytC	(39)

Pneumococcal synthetic oligosaccharide-based vaccines

The polysaccharide conjugate vaccines listed in Table 1 all are based on natural polysaccharides, purified form bacterial cultures. Synthetic oligosaccharide–protein conjugates (neoglycoconjugate), involving functional mimics of the natural polysaccharide antigens have emerged as an attractive option (81). The advantages of synthetic oligosaccharide based vaccines (neoglycoconjugates) are well-defined chemical structures (chain length, epitope conformation, and carbohydrate/protein ratio) as well as a lack of the impurities present in polysaccharides obtained from bacterial cultures (5,44).

The chemical synthesis of oligosaccharide fragments however is complex. According to the sequence in the natural polysaccharide, monosaccharide residues have to be linked in such a way that they form an oligosaccharide with the required stereospecificity (epitope). Various methodologies and strategies for synthesis of carbohydrates have successfully been used for production of experimental neoglycoconjugates, as reviewed by Kamerling (51). In 2001, the first automated synthesis of oligosaccharides was reported by Plante, O.J. et al (79).

Neoglycoconjugates have been prepared for saccharides of different microorganisms. In 2004, Verez Bencomo et al., reported the large-scale synthesis and the introduction of a synthetic oligosaccharide vaccine for *Haemophilus influenza* type b for use in humans in Cuba (108). The immunogenicity of the synthetic oligosaccharide fragment of the O-specific polysaccharide (O-PS) of *Vibrio cholera* O1, serotype Ogawa, conjugated to bovine serum albumin has been investigated in a mouse model (89,90). A multimeric bivalent synthetic hexasaccharide fragment of the O-specific polysaccharide of *Vibrio cholera* O1, serotype Ogawa, in combination with Inaba:1 or a synthetic disaccharide tetrapeptide peptidoglycan fragment as adjuvant were prepared and conjugated to recombinant tetanus toxin H(C) fragment as protein carrier (11). The immunogenicity of synthetic oligosaccharides mimicking the O-antigen of the *Shigella flexneri* 2a lipopolysaccharide (LPS) was also investigated in mice (78,88). Immunization of mice with synthetic hexasaccharide of glycosylphosphatidylinositol malarial toxin conjugated to a protein carrier was reported to protect the mice from an otherwise lethal dose of malaria parasites (92). A fully synthetic carbohydrate-based antitumor candidate vaccine for the common T-synthase was recently reported (46).

Our group focuses on the development of neoglycoconjugates related to different *S. pneumoniae* serotypes (Table 3). Up till now, synthetic oligosaccharide-protein conjugates have been prepared for serotypes 3, 6A and 6B, 14, 17F, 19F and serogroup 9. The immunogenicity of those neoglycoconjugates has been demonstrated in mice (100). For a limited number of conjugates, protection against challenge with live pneumococci has been shown.

Table 3. Development of synthetic oligosaccharide-protein conjugates related to *S. pneumoniae*[]

Serotype	Synthetic oligosaccharide fragment	Protein carrier	Ref.
3	β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→O$(CH_2)_3NH_2$ β-D-Glc*p*-(1→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→O$(CH_2)_3NH_2$, β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-b-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→O$(CH_2)_3NH_2$	CRM_{197}	(5)
6A, 6B	α-L-Rha*p*-(1→4)-D-Rib-ol-[5→*P*→$(CH_2)_3NH_2$ D-Rib-ol-(5→*P*→2)-α-D-Gal*p*-(1→3)-a-D-Glc*p*-1→O$(CH_2)_3NH_2$ α-L-Rha*p*-(1→4)-D-Rib-ol-(5→*P*→2)-a-D-Gal*p*-(1→3)-α-D-Glc*p*-[1→O$(CH_2)_3NH_2$	KLH	(43)
14	β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-DGlc*p*NAc-(1→O$(CH_2)_3S(CH_2)_2NH_2$ β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-DGlc*p*NAc-(1→O$[CH_2]_3$O→4)-β-D-Glc*p*-(1→O$CH_2CH{=}CH_2$ β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→O$[CH_2]_3$O→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-(1→O$[CH_2]_3$O→4)-β-D-Glc*p*-(1→OCH_2-CH=CH_2	CRM_{197}	(68)
14	β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-β-D-Glc*p*NAc-$(CH_2)_6NH_2$ β-D-Glc*p*-(1→6)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-$(CH_2)_6NH_2$ β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-$(CH_2)_6NH_2$ β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-$(CH_2)_6NH_2$ β-D-Glc*p*-(1→6)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-$(CH_2)_6NH_2$ β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-$(CH_2)_6NH_2$ β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-$(CH_2)_6NH_2$ β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-$(CH_2)_6NH_2$ β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-$(CH_2)_6NH_2$ β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-$(CH_2)_6NH_2$ β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-$(CH_2)_6NH_2$ β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-	CRM_{197}	In this study (87)

	Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-$(CH_2)_6NH_2$ β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-$(CH_2)_6NH_2$ β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-$(CH_2)_6NH_2$ β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-$(CH_2)_3NH_2$ β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)]-β-D-Glc*p*NAc-$(CH_2)_3NH_2$		
17F	α-L-Rha*p*-(1→2)-D-Ara-ol-(1→*P*)-$(CH_2)_3NH_2$ α-L-Rha*p*-(1→2)-D-Ara-ol-(1→*P*→3)-L-β-Rha*p*-$(CH_2)_3NH_2$ α-L-Rha*p*-(1→2)-D-Ara-ol-(1→*P*→3)-L-β-Rha*p*-(1→4)-D-β-Glc*p*-$(CH_2)_3NH_2$	KLH	(25,45)
23F	β-L-Rha*p*-(1→4)-β-Glc*p*-(1→4)-β-D-Gal*p*-$(CH_2)_3NH_2$ β-D-Glc*p*-(1→4)-[α-L-Rha*p*-(1→2)][Gro-(2→P→3)]-β-D-Gal*p*-(1→4)-β-L-Rha*p*-$(CH_2)_3NH_2$	KLH	(24)
9A, 9L, 9N, 9V	α-D-Gal*p*-(1→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-$(CH_2)_2NH_2$ α-D-Glc*p*-(1→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-$(CH_2)_2NH_2$ α-D-Glc*p*A-(1→3)-α-D-Gal*p*-(1→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-$(CH_2)_2NH_2$ α-D-Glc*p*-(1→4)-α-D-Glc*p*A-(1→3)-α-D-Gal*p*-(1→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-$(CH_2)_2NH_2$	CRM_{197}	Unpublished data

[1]Modified from Snippe, H. et al (2008) (100).

Gold glyconanoparticles

The integration of biomolecules into nano structured materials has produced novel hybrid nanobiomaterials with synergistic properties and functions that have found applications in bioassays and bioelectronic devices (biosensing), as well as in therapeutics and diagnostics (53,120). The variety of materials that can be chosen for the inorganic core of these nanobiomaterials permits access to nanostructures possessing a range of optical, electronic, mechanical, and magnetic properties. On

the other hand, different biomolecules (ranging from genetic material, proteins, carbohydrates, antibodies, etc) can be tailored into the metallic nanocluster with different methodologies.

The design of multifunctional nanoparticles as an alternative system for drug and gene delivery has great potential for therapy in areas, such as cancer and neuropathological disease (91). Gold-biomolecule nanoparticles are perhaps the best known among these hybrid nanobiomaterials (10). The covalent binding between noble metals and biomolecules can be easily achieved by means of thiol chemistry, i.e. generating self-assembled monolayers (SAMs) of thiol-armed biomolecules onto the metal surface (62,103,109).

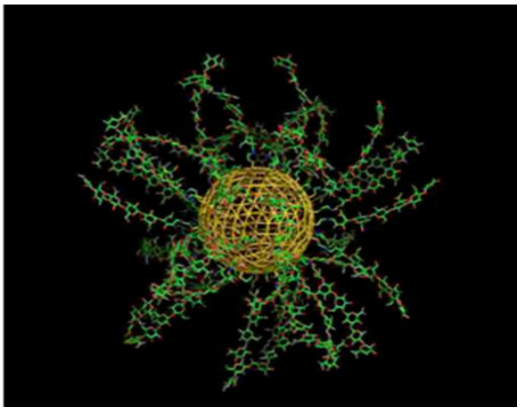

Figure 3. Illustration of a gold glyconanoparticle (GNP). The oligosaccharide fragments related to *S. pneumoniae* type 3 polysaccharide (5) coupled to the gold cluster with 2.18 nm mock up core shell and 10% coverage by PyMol program (http://pymol.sourceforge.net/). (Made by Dr. Jimmy Rossen at CNRS-CERMAV, Grenoble - France).

The gold surface can in fact be readily functionalized by using thiolated ligands, which form covalent bonds with the gold atoms. The formation of SAMs on two dimensional surfaces can be transformed to a three-dimensional surface of metal nanoclusters. DNA-functionalized gold nanoparticles have already been used in bio-barcode assays for real time detection of cancer (38). Peptides can be designed to form self-assembled monolayers on gold nanoparticles resulting in nanomaterials with some chemical properties analogous to those of proteins (peptide-capped nanoparticles as artificial protein) (59). By modification of the procedure of Burst et

al (16), gold nanoparticles which consist of self-assembled monolayers of carbohydrate antigens were constructed by reducing *in situ* a gold salt in the presence of thiol-armed glycosides (21). These nanomaterials were named gold glyconanoparticles (GNPs) and are multivalent systems that can incorporate carbohydrates with high polyvalency and control over ligand number and nanocluster size (core size ranging from 1 nm to 150 nm). GNPs are water soluble, have a long storage stability as well as resistance to enzymatic degradation, and they are nontoxic (86).

The GNPs with their globular shape, size range similar to many common biomolecules, and their chemically well-defined structure, provide a glycolyx-like surface that mimics carbohydrate presentation in glycoproteins or in glycosphingolipid patches at the cell surface (Figure 3) (23,97).

GNPs have already been discussed for studies with carbohydrate-based interactions, biolabels and biosensors, and could have a variety of applications in biomedicine and material science (23,32). GNPs were used as chemical tools to study carbohydrate-carbohydrate interaction by means of different techniques. In particular, the group of Penadés demonstrated the calcium-dependent self-aggregation of GNPs coated with the antigen determinant Lewis X (β-D-Gal-(1→4)-[α-L-Fuc-(1→3)]-D-GlcNAc, Le^X) by means of transmission electron microscopy (TEM), surface plasmon resonance (SPR), atomic force microscopy (AFM), and isothermal titration calorimetry (ITC) (22). The group of Kamerling investigated the carbohydrate-mediated self-recognition of marine sponge cells (18) and Russell and co-workers reported that lactose is able to cause calcium-mediated, dose-dependent and reversible aggregation when tailored on gold nanoclusters of ca. 16 nm diameter (85). GNPs were also used to study and intervene in carbohydrate-protein interactions (64). Carbohydrate-based anti-HIV agents were designed and constructed using GNPs (65,66). Glyconanoparticles presenting lactose have been used successfully to significantly reduce the progression of experimental metastasis in cancer (86).

Fluorescent glyconanoparticles have been prepared for labeling in biological test systems. They are water soluble, stable under physiological conditions and present an exceptionally small core size (4). Studies of nanotoxicology, including immunotoxicology of nanoparticles in general (71) and the application of multifunctional GNPs as potential platform for carbohydrate-based anticancer vaccines are ongoing and will be extended (74). No examples of nanoparticulate-

based immunization that elicits IgG antibodies towards a selected carbohydrate have been reported so far.

In this thesis, we synthesized conjugates of synthetic oligosaccharides and peptides on gold nanoparticles and analysed the evoked immuneresponse in mice following upon a particular vaccination schedule.

Scope of this thesis

The first goal of this thesis is to investigate the immunogenicity of synthetic oligosaccharide fragment-protein conjugates (neoglycoconjugates) related to *S. pneumoniae* type 14 polysaccharides (Pn14PS). **Chapter 1** provides a general introduction of *S. pneumoniae* bacteria and development of vaccines to prevent this infection. **Chapter 2** describes the immunogenicity of synthetic overlapping oligosaccharide fragments of Pn14PS that were conjugated to CRM_{197} protein and injected into mice to determine the smallest immunogenic structure. In **Chapter 3**, we investigated the effect of booster immunization on the outcome of sustained immunity to *S. pneumoniae* type 14. We further investigated the conditions which could contribute to optimal antibody- and cell-mediated immune responses after primary immunization with neoglyconjugate of Pn14PS by coadministration of a variety of adjuvants (**Chapter 4**). **Chapter 5** describes the use of a panel of mouse and human of anti-Pn14PS sera to investigate the potential immunogenic epitopes shared by Pn14PS and Group B streptococcus type III polysaccharide (GBSIIIPS) as they are identical apart from the (α2 $\rightarrow$3)-linked sialic acid in the side chain. Ultimately, in **Chapter 6** the immunogenicity of conjugates of synthetic oligosaccharides and peptides on gold glyconanoparticles (GNPs) in mice was investigated. The GNP preparations used consisted of synthetic branched tetrasaccharide fragments of Pn14PS, $OVA_{323\text{-}339}$-peptide and monosaccharide glucose as inert component. Finally **Chapter 7** summarizes the findings of this thesis and provides an outlook for further research into optimalization of synthetic oligosaccharide conjugates.

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Chapter 2

Identification of the smallest structure capable of evoking opsonophagocytic antibodies against *Streptococcus pneumoniae* type 14

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Abstract

Synthetic overlapping oligosaccharide fragments of *S. pneumonia* serotype 14 capsular polysaccharide (Pn14PS), {6)-[β-D-Gal*p*-(1→4)-]β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→}$_n$, were conjugated to CRM_{197} protein and injected into mice to determine the smallest immunogenic structure. The resulting antibodies were then tested for Pn14PS specificity and for their capacity to promote the phagocytosis of *S. pneumoniae* type 14 bacteria. Earlier studies have reported that the oligosaccharide corresponding to one structural repeating unit of Pn14PS; i.e. Gal-Glc-(Gal-)GlcNAc, induces a specific antibody response to Pn14PS. The broader study described in this paper, which evaluated 16 oligosaccharides, showed that the branched trisaccharide element Glc-(Gal-)GlcNAc is essential in inducing Pn14PS-specific antibodies and that the neighboring galactose unit at the non-reducing end contributes clearly to the immunogenicity of the epitope. Only the oligosaccharide conjugates that produce antibodies recognizing Pn14PS were capable of promoting the phagocytosis of *S. pneumoniae* type 14. In conclusion, the branched tetrasaccharide Gal-Glc-(Gal-)GlcNAc may be a serious candidate for a synthetic oligosaccharide conjugate vaccine against infections caused by *S. pneumoniae* type 14.

Introduction

Synthetic carbohydrate-based vaccines are being investigated by many researchers for the prevention of diseases caused by *Streptococcus pneumoniae* (12), *Haemophilus influenza* type b (7,33), meningococcus group C (8), *Vibrio cholerae* (27), etc. Advantages of synthetic carbohydrate-based vaccines include their well-defined chemical structures (chain length, epitope conformation, and carbohydrate/protein ratio) and a lack of the impurities present in polysaccharides obtained from bacterial isolation (2). A breakthrough for this type of vaccines was made in 2004 by Verez Bencomo et al. (33) when they reported the large-scale synthesis and the introduction of a synthetic oligosaccharide vaccine for *Haemophilus influenza* type b for human in Cuba.

Pneumococcal disease is a major public-health problem worldwide and it is estimated that 1.6 million people die from this infection each year, one million of whom are children (36). Capsular polysaccharides (PS) are well known as the major virulence factors of *S. pneumoniae*. Numerous studies have demonstrated that antibodies against PS are essential for protection against pneumococcal disease (3,24,26). Based on the diversity of the chemical structures of PS (16,23), more than 90 serotypes have been identified to date. This diversity determines the ability of the serotypes to survive in the bloodstream and possibly also the ability to cause invasive disease, especially in the respiratory tract (6,16). In general, PS are poorly immunogenic: they mainly induce IgM antibodies and, as a vaccine, they are only moderately protective in adults and ineffective in young children (1).

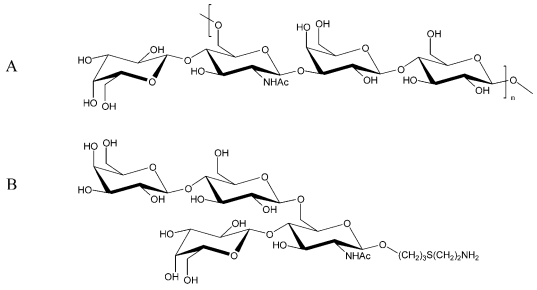


Figure 1. Stucture of (A) the biosynthetic tetrasaccharide repeating unit of Pn14PS (19) and (B) the branched tetrasaccharide unit Gal-Glc-(Gal-)GlcNAc, synthesized and studied by Mawas et al. (20).

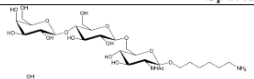
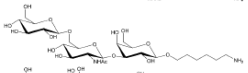
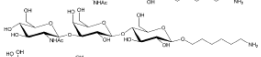
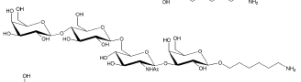
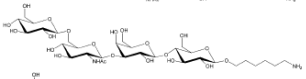
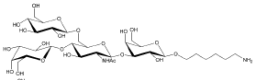
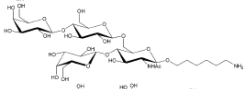
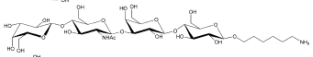
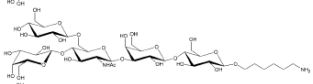
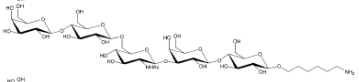
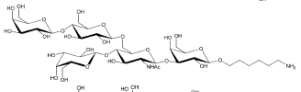
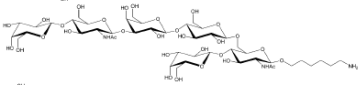
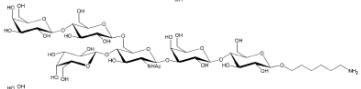
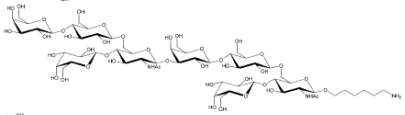
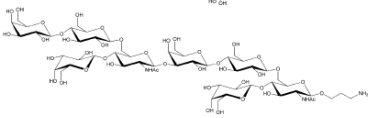
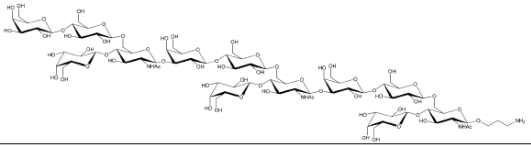
S. pneumoniae type 14 capsular polysaccharide (Pn14PS) consists of biosynthetic repeating units of the tetrasaccharide (19) {6)-[β-D-Gal*p*-(1→4)-]β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→}$_n$ (Figure 1A). Pn14PS is structurally related to and has cross-reactivity with group B streptococcus type III (9). The immunogenicity of this polysaccharide and its depolymerized oligosaccharide fragments conjugated to a protein has been reported to produce specific anti-Pn14PS antibodies in mice (17,34,35). In 2002, a synthetic branched tetrasaccharide, corresponding to a single structural repeating unit of Pn14PS conjugated to the cross-reactive material of diptheria toxoid (CRM_{197}), was found to induce anti-polysaccharide type 14 antibodies (20) (Figure 1B). Based on this result, the present study was set up to investigate how small the minimal structure in Pn14PS can be and still produce specific antibodies against polysaccharide type 14. To do this, a series of oligosaccharide fragments of Pn14PS, varying from tri- to dodecasaccharide, were synthesized (14,15,21,30). These oligosaccharide fragments were then conjugated to a protein carrier, i.e. either CRM_{197} or bovine serum albumin (BSA). The immunogenicity of the conjugates was subsequently studied in BALB/c mice.

Materials and Methods

Synthetic oligosaccharide fragments. Sixteen different oligosaccharide fragments, varying from tri- to dodecasaccharide and representing fragments of the Pn14PS, were synthesized. Fourteen had a 6-aminohexyl spacer (14,15,21) and two a 3-aminopropyl spacer (30) (Table 1).

Preparation of neoglycoconjugates. Diethyl squarate was employed to link the oligosaccharide fragments to a protein carrier (either CRM_{197} or BSA) as described previously (2,31). A total of 100 µl (4 µmol) stock solution containing 74.7 µl diethyl squarate in 12.8 ml ethanol was added to a solution of spacered oligosaccharide fragments (1 µmol) in 0.1 M sodium phosphate buffer (75 µl, pH 7.0). After stirring for 16 h, the ethanol was evaporated and the water layer was applied to a C18 cartridge (500 mg) (Alltech, Deerfield, IL, USA), that was preconditioned with methanol (5 ml) and followed by H_2O (5 ml). The elution was effected with H_2O (5 × 1 ml), ethyl acetate (5 × 1 ml), and methanol (5 × 1 ml). Fractions containing the pure desired compound were then concentrated to acquire the elongated oligosaccharide (80–99%), which was used for the conjugation reaction.

Table 1. A series of spacered oligosaccharide fragments of Pn14PS

Oligosaccharide fragments	Spacered structure	Code
Gal-Glc-GlcNAc		JJ42
Glc-GlcNAc-Gal		JJ141
GlcNAc-Gal-Glc		JJ118
Gal-Glc-GlcNAc-Gal		DM65
Glc-GlcNAc-Gal-Glc		JJ153
Glc-(Gal-)GlcNAc-Gal		JJ5
Gal-Glc-(Gal-)GlcNAc		JJ1
Gal-GlcNAc-Gal-Glc		JJ9
Glc-(Gal-)GlcNAc-Gal-Glc		JJ6
Gal-Glc-GlcNAc-Gal-Glc		DM35
Gal-Glc-(Gal-)GlcNAc-Gal		DM66
Gal-GlcNAc-Gal-Glc-(Gal-)GlcNAc		JJ10
Gal-Glc-(Gal-)-GlcNAc-Gal-Glc		DM36
Gal-Glc-(Gal-)GlcNAc-Gal-Glc-(Gal-)-GlcNAc		JJ4
Gal-Glc-(Gal-)GlcNAc-Gal-Glc-(Gal-)-GlcNAc		ML1
Gal-Glc-(Gal-)GlcNAc-Gal-Glc-(Gal-)GlcNAc-Gal-Glc-(Gal-)GlcNAc		ML2

CRM_{197} conjugates. The elongated oligosaccharide fragments (1 µmol) were dissolved in 0.1 M sodium borate buffer (400 µl, pH 9.5), and 56 µl (0.05 µmol) stock solution containing 52.25 mg/ml CRM_{197} (Novartis Vaccine & Diagnostics, Siena, Italy) was added. Incubations were carried out for 3-4 days, until the desired loading was reached as determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Voyager-DE PRO mass spectrometer, Applied Biosystems, Foster City, CA, USA). The mixtures were then desalted in a 30-kDa MicrosepTM Microconcentrator (Filtron Technology Corp., Northborough, UK) and subsequently reconstituted with 0.05 M sodium phosphate buffer (pH 7.2, 5 × 0.5 ml).

BSA conjugates. After the elongated oligosaccharide fragments (1 µmol) were dissolved in 0.1 M sodium borate buffer (1.5 ml), a solution containing BSA (0.05 µmol) in 0.1 M sodium borate buffer pH 9.0 (0.6 ml) was added. The mixture was stirred for 2-3 days and then loaded in a 30-kDa MicrosepTM Microconcentrator (Filtron Technology Corp.) and washed with H_2O (5 × 2 ml). Then, the retained material was freeze-dried.

Determination of carbohydrate/protein molar ratios. The average molar ratio of carbohydrate and protein was determined by MALDI-TOF MS analysis. Samples were prepared by mixing 1 µl conjugate with 1 µl sinapic acid (20 mg) in 70% acetonitrile containing 0.1% trifluoroacetic acid as matrix solution. The carbohydrate/protein molar ratio of each of the conjugates is presented in Table 2.
Mice immunization studies. The mice vaccination study was approved by the Ethics Committee on Animal Experiments of University Medical Center Utrecht, Utrecht, The Netherlands. Inbred six-week-old female BALB/c mice were maintained at the animal laboratory of Utrecht University. Five mice per group were immunized intracutaneously with 2.5 µg carbohydrate at four different sites in the neighborhood of the lymphnodes of the axillae and the groins. Pn14PS conjugated to CRM_{197} (CRM_{197}-Pn14PS; Wyeth Research, Pearl River, NY, USA) was injected into one group of mice, which then served as the antibody-positive control mice. The other control mice were injected respectively with either saline (0.9%(w/v) NaCl in water), unconjugated PnPS14, or CRM_{197} protein. When adjuvants were applied on the priming day a fivefold lower dose of carbohydrate (0.5 µg) was injected. In these cases, the conjugates were mixed with adjuvants (10 µg monophosphoryl lipid-A [MPL derived from *S. minnesota* R595 LPS; Ribi ImmunoChem Research Inc., Hamilton, MT, USA] and 20 µg Quil-A [Superfos Biosector, Vedbaek, Denmark] per animal) and injected intracutaneously at the four sites, as mentioned above. A booster of 2.5 µg carbohydrate was given on day 35 without adjuvant. Using a retro-

orbital puncture, blood samples were taken by from isofluran-anaesthetized mice 1 week before the booster and 2 and 3 weeks after the booster.

Table 2. Carbohydrate/protein molar ratios within the CRM_{197} and BSA conjugates

Code	Carbohydrate/protein molar ratios [a]	
	CRM_{197} conjugate	BSA conjugate
JJ42	5.3	5.0
JJ118	6.3	6.0
JJ141	3.6	2.5
DM65	5.0	1.0
JJ153	6.2	3.0
JJ1	6.2	NT[b]
JJ9	6.0	2.0
JJ5	7.0	4.0
JJ6	6.5	1.0
DM35	5.0	3.7
DM66	5.5	5.0
JJ10	5.6	0.5
DM36	6.6	NT
JJ4	3.0	NT
ML1	2.6	NT
ML2	1.0	NT

[a] Ratios were determined by MALDI-TOF MS analysis

[b] NT, not tested

Measurement of Pn14PS-, protein carrier-, and spacer-specific antibodies by ELISA. Enzyme-linked immunosorbent assay (ELISA) was performed to measure the anti-Pn14PS antibodies, as described previously (2,18). Briefly, diluted sera were incubated for 1 h at 37°C in flat-bottom plates (Corning Inc., Corning, NY, USA) which were coated with Pn14PS (0.3 µg/well) and blocked with 3% gelatine. After washing, horseradish peroxidase-conjugated goat anti-mouse IgM or IgG (Nordic Immunology Laboratories, Tilburg, The Netherlands) were incubated for 1 h at 37°C. A mixture of 3,3',5,5'-tetramethylbenzidine (Sigma Chemicals Co, St. Louis, MO, USA) and H_2O_2 (Sigma Chemicals Co) was then added to visualize the amount of bound peroxidase; the reaction was stopped with the addition of 0.5 M H_2SO_4. Optical density (OD) values were obtained with a microtiter plate spectrophotometer at 450 nm (Bio-Rad, model 3550 UV, Bio-Rad Laboratories, Hercules, CA, USA). Antibody titers were expressed as the $\log_{10}$ of the dilution giving twice the OD obtained for control mice (immunized with saline) with a cutoff value of 0.2.

CRM_{197}- and BSA-mannose-coated plates (0.1 µg/well) were also used to measure anti-protein carrier and anti-spacer titers respectively. BSA-mannose was constructed by coupling 6-aminohexyl α-D-mannopyranoside, via diethyl squarate, to BSA.

Detection of oligosaccharide-specific antibodies. In order to investigate the immune response to the oligosaccharide fragments that did not induce anti-Pn14PS antibodies, ELISAs were performed after pre-incubating the sera with BSA-mannose to block the antibodies recognizing the spacer molecule (-C_6H_{12}-NH-C_4O_2-NH-). After the uncoated plates were blocked with 2% gelatin in PBS and washed with PBS/0.05% Tween 20 several times, diluted sera (1:100 in PBS supplemented with 0.05% Tween 20 and 3% Protifar) were incubated with BSA-mannose in a concentration ranging from 0 to 100 µg/ml for 1 h at 37°C and left overnight at 4°C. The mixtures were then transferred to three differently coated plates (0.1 µg/well): BSA-mannose, BSA-conjugates corresponding with CRM_{197} conjugates, and BSA-DM66 (for the structures, see Table 1). The amount of specific antibodies in these absorbed sera was detected by ELISA method as described above. The results were expressed as the OD changes of the sera incubated with BSA-mannose compared to sera alone.

Measurement of avidity. The antibody avidity of mice sera that recognized Pn14PS as the coating material was measured by ELISA using chaotropic sodium thiocyanate (NaSCN; Sigma Chemicals Co), as previously described (18,25). Briefly, Pn14PS-coated plates were incubated with diluted sera (1:25 in PBS, 0.05% Tween 20 and 3% Protifar) and washed. Series of NaSCN concentrations (0-3.0 M) were incubated on the plates at 37°C for 15 min. After washing five times, horseradish peroxidase-conjugated goat anti-mouse IgG (1:10.000) in PBS was added and the solution was incubated for 1 h at 37°C. The amount of antibodies left was detected by ELISA method as described above. In the present study, the avidity index (AI) is expressed as the concentration of NaSCN needed to reduce the OD_{450nm} by 50%. The absorbance value of sera without NaSCN should be at least 0.5.

Measurement of phagocytosis titer. The opsonic activity of mouse sera was determined as the uptake of *S. pneumoniae* type 14 by the mouse macrophage cell line J774A.1 (ATCC TIB67). The phagocytosis assay procedure that was used was described by de Velasco, E.A., et al. (5) and Lefeber et al. (18). Two-fold dilutions of heat-inactivated pooled sera in HBSS/1% BSA were added with 2% complement (guinea-pig serum) in round-bottom plates (Greiner bio-one, Frickenhausen, Germany). The assay was performed by mixing 20 µl J774A.1 cells (3.10^6 cells/ml)

and 20 µl FITC-labeled *S. pneumonie* type 14 (ATTC 634) (3.10^7 cells/ml) (11), followed by incubation at 37°C under vigorous shaking at 900 rpm for 50 min. The mixtures were then fixed with cold 2% paraformaldehyde and analyzed in a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA, USA). The percentage of FITC-positive J774A.1 cells was used as a measure of the phagocytic activity and corrected using the values found in the mixtures without sera. The phagocytosis titers are expressed as the $\log_{10}$ of the serum dilution during phagocytosis that resulted in 25% of the J774A.1 cells being positive for FITC(13).
Statistical methods. Unpaired t test was used to determine the differences in antibody titers and a P value of ≤ 0.05 was considered statistically significant. The $\log_{10}$ of the dilutions was used in all analyses.

Results

Specific antibodies against Pn14PS in immunized mice without adjuvant. Groups of five mice were immunized intracutaneously with CRM_{197}-oligosaccharide conjugates (2.5 µg carbohydrate per mouse) without adjuvant. All mice were boosted on day 35 with the same conjugate at the same dose used for the primary immunization. For the positive control, mice were immunized with CRM_{197}-Pn14PS; for the negative control, mice were mock-immunized with saline solution. Small amounts of IgM antibodies that bound to Pn14PS were detected in the sera of the mock-immunized mice. These IgM antibody levels did not rise or fall after primary and booster immunization with conjugate (data not presented). After primary immunization only, low IgG antibody titers against PnPS14 were detected in mice from the groups receiving JJ1, DM66, DM36, JJ4, ML1, and ML2 (Table 1; Figure 2). One week after the booster injection on day 35, these antibody titers had increased threefold (Figure 2). Moreover, mice boosted with JJ5 and JJ10 also had a low IgG titer. The other groups of mice did not produce specific IgG antibodies against Pn14PS when it was used as the antigen-coating material. Additional experiments (data not shown) demonstrated that neither unconjugated Pn14PS nor the carrier protein CRM_{197} induced IgG specific antibodies against Pn14PS.

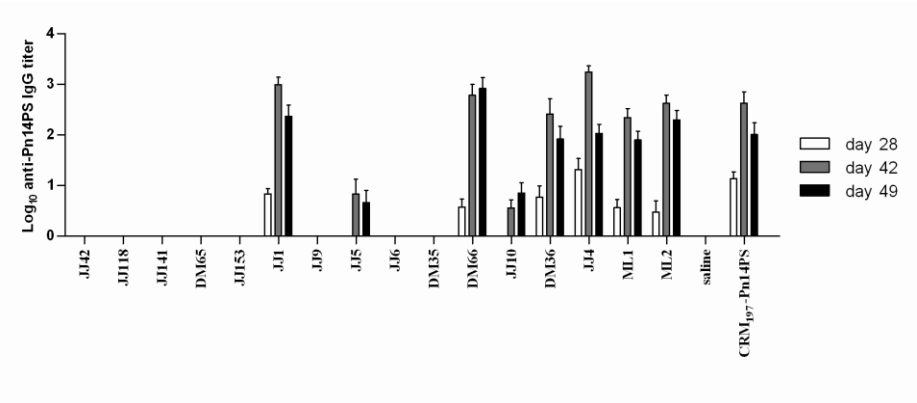


Figure 2. Total IgG antibody titers recognizing Pn14PS as coating material. Groups of mice (n=5) were immunized with the oligosaccharides conjugated to CRM_{197} and boosted on day 35 with the same preparation. Sera were collected on day 28, 42, and 49 and ELISA was employed to measure IgG antibody titers against PN14PS. Antibody titers were expressed as the $\log_{10}$ of the dilution giving twice the OD obtained for control mice (immunized with saline).

Structures of the branched tetrasaccharide Gal-Glc-(Gal-)GlcNAc with one (Gal, DM66: Gal-Glc-(Gal-)GlcNAc-Gal) or two (Gal-Glc, DM36: Gal-Glc-(Gal-)GlcNAc-Gal-Glc) extra monosaccharides at the reducing end also elicited high titers of IgG antibodies against Pn14PS, similar to that elicited by the branched tetrasaccharide JJ1 [Gal-Glc-(Gal-)GlcNAc)] (Figure 2). When two extra monosaccharides were added to the branched tetrasaccharide at the non-reducing end (Gal-GlcNAc, JJ10: Gal-GlcNAc-Gal-Glc-(Gal-)GlcNAc), specific antibody titers against Pn14PS were sharply reduced. Interestingly, a change in the position of the galactose unit of the glucose-linked galactose from the non-reducing (JJ1: Gal-Glc-(Gal-)GlcNAc) to the reducing (JJ5: Glc-(Gal-)GlcNAc-Gal) end resulted after immunization with a lower IgG titer against Pn14PS. The immunogenicity against Pn14PS was totally lost when one glucose residue was added to JJ5 at the reducing end, yielding JJ6: Glc-(Gal-)GlcNAc-Gal-Glc (Figure 2). The linear tetrasaccharide (JJ9: Gal-GlcNAc-Gal-Glc), which lacked the branching Gal at the GlcNAc unit, did not elicit any antibodies against Pn14PS even after co-administration of the adjuvant (Table 3). In addition, the conjugates of smaller fragments (JJ42 [Gal-Glc-GlcNAc], JJ118 [GlcNAc-Gal-Glc], and JJ141 [Glc-GlcNAc-Gal]) were not able to produce specific antibodies against Pn14PS (Figure 2). Finally, non-branched conjugates, such as DM65 (Gal-Glc-GlcNAc-Gal), JJ153 (Glc-GlcNAc-Gal-Glc), and DM35 (Gal-Glc-GlcNAc-Gal-Glc), did not produce antibodies against Pn14PS (Figure 2).

Table 3. The effect of adjuvant administration on the titers of specific IgG antibodies against Pn14PS[a]

Code	Log_{10} IgG titer two weeks after booster[b]	
	Without adjuvant	With adjuvant
JJ42	0.0	0.0
JJ141	0.0	0.0
JJ5	1.24 ± 0.29[c]	2.59 ± 0.25
JJ118	0.0	0.0
DM65	0.0	0.0
DM35	0.0	0.0
DM66	2.92 ± 0.48	3.67 ± 0.17
JJ1	2.10 ± 0.40	2.55 ± 0.37
JJ6	0.0	0.0
JJ10	1.03 ± 0.25	1.61 ± 0.67
CRM_{197}-Pn14PS	2.01 ± 0.53	2.85 ± 0.43

[a] Groups of mice (n=5) were immunized with conjugates only or in the presence of adjuvants (10 µg MPL and 20 µg Quil-A per mouse).
[b] A booster without adjuvant was given at day 35. Sera collected one week before booster showed no significant anti-Pn14PS IgG.
[c] Antibody titers (mean ± SD) were expressed as the log_{10} of the dilution giving twice the OD obtained for control mice (immunized with saline).

Effect of adjuvant administration on antibody levels against Pn14PS. To determine the effect of adjuvants on the immunogenicity and specificity of the conjugates, the adjuvants MPL and Quil-A were co-administered in combination with a lower conjugate dose (0.5 µg carbohydrate per mouse), as reported previously (18). On day 35, the groups of mice were boosted with 2.5 µg carbohydrate per mouse without adjuvant. The results are only presented for ten groups of mice and just the data from sera collected one week after booster are shown. In general, although anti-Pn14PS antibodies titers were higher than those in mice injected with the corresponding conjugates without adjuvant (1.6–2 fold), only mice immunized with JJ5 showed significantly increased anti-Pn14PS antibody titers (2-fold, P value <0.05) (Table 3). In contrast, those conjugates which were previously shown not to induce anti-Pn14PS antibodies (Figure 2) were still unable to do so after co-administration of adjuvant.

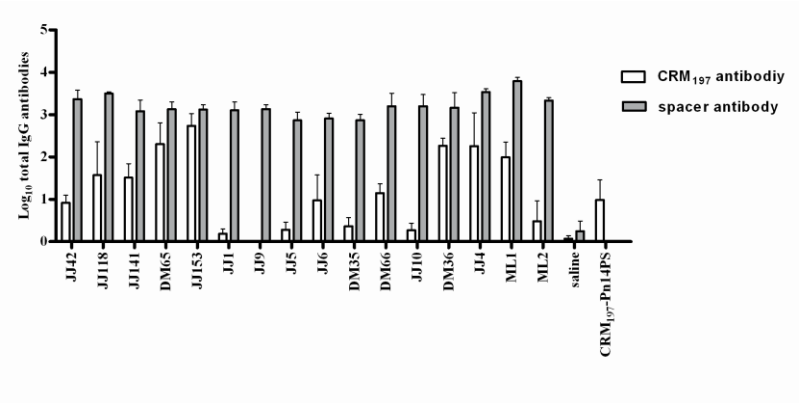


Figure 3. Antibodies recognizing the carrier CRM_{197} (white bar) or the spacer present in BSA-mannose (grey bar) as coating material. The mice sera (n=5) were obtained from two weeks after the booster. Antibody titers were expressed as the $\log_{10}$ of the dilution giving twice the absorbance value corrected by buffer.

Anti-carrier and anti-spacer antibodies within the conjugates. CRM_{197} protein was coated on microtiter plates in order to determine specific antibodies against the carrier protein. One week after the booster, IgG antibodies against CRM_{197} protein were detected in several sera (Figure 3). Specific antibodies were not detected in the sera of group of mice immunized with JJ1, JJ9, JJ5DM35 and JJ10. In order to determine the antibodies that recognized the spacer present in the conjugates, BSA-mannose was constructed containing the same spacer (-C_6H_{12}-NH-C_4O_2-NH-) that was present in all but the ML1 and ML2 CRM_{197} conjugates. These two conjugates had a shorter spacer (-C_3H_6-NH-C_4O_2-NH-). BSA-mannose was used as the coating material on the microtiter plates. All immunized mice produced specific IgG antibodies against the spacer, including the one for ML1 and ML2 (Figure 3). No anti-spacer antibodies were detected in the sera of mice immunized with saline or CRM_{197}-Pn14PS (Figure 3), which was constructed without such a spacer.

Specific antibodies against the oligosaccharide fragment of conjugates. ELISA techniques were employed to investigate whether the conjugates that were not able to produce Pn14PS antibodies (Figure 2) were capable of producing oligosaccharide-specific antibodies. Pooled sera were pre-incubated with a serial concentration of BSA-mannose to absorb anti-spacer antibodies and then transferred to three different coated plates: BSA-oligosaccharide conjugates corresponding to CRM_{197} conjugates, BSA-mannose, and BSA-DM66 as control.

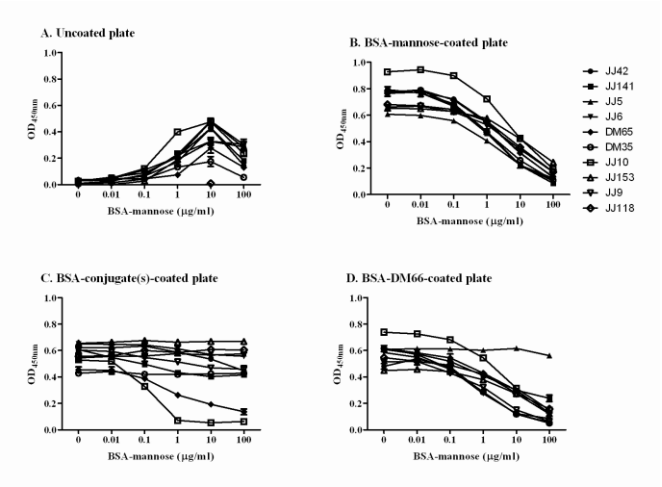


Figure 4. Antibodies recognizing the specific oligosaccharide fragments within the conjugates. Pooled sera (duplo) were combined with serial concentrations of BSA-mannose conjugate which could inhibit the binding of the antibodies to the spacer (in the conjugates) and incubated on an uncoated plate (A). After incubation, the mixture was transferred onto plates coated either with BSA-mannose (B) or BSA corresponding conjugates (C) and BSA-DM66 as control (D). The results are expressed as the OD changes of the sera incubated with BSA-mannose and compared to sera alone.

The immune complexes formed by BSA-mannose and the anti-spacer antibodies were detected as an immunoglobulin (IgG) precipitate on the uncoated plates, which also served as the pre-incubation plates (Figure 4A). Absorption of sera with BSA-mannose showed a dose-dependent response to BSA-mannose-coated plates (Figure 4B). When the BSA-mannose- absorbed sera were transferred to the BSA conjugate-coated plate (corresponding to CRM_{197} conjugate), just the sera immunized with JJ10 or DM65 showed a dose-dependent response (Figure 4C). This suggests that, in most sera, oligosaccharide-specific antibodies are present that do not recognize Pn14PS. When the BSA-mannose absorbed sera were transferred to the BSA-DM66-coated plates (DM66, Gal-Glc-(Gal-)GlcNAc-Gal), there was response to BSA-DM66 as observed with BSA-mannose (Figure 4D, B). This suggests the recognition of spacer-specific antibodies only. Sera obtained from JJ5-immunized mice were used throughout the absorption experiment as a control for the sera recognizing Pn14PS. After absorption with BSA-mannose, this serum displayed a dose-independent response when BSA-DM66 was used as coating material (Figure 4D).

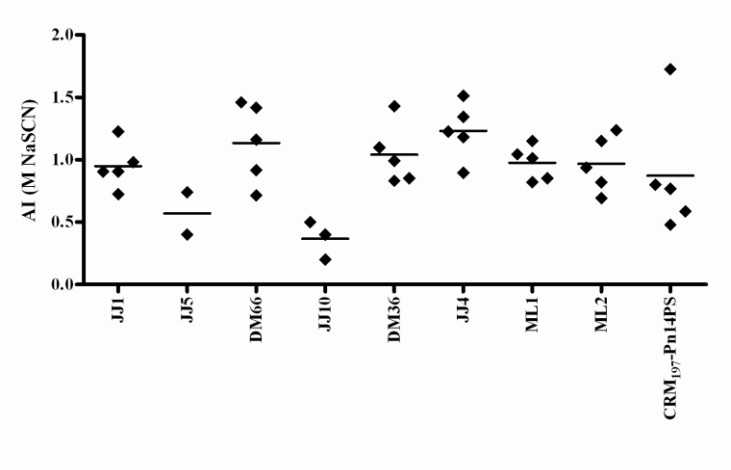


Figure 5. Avidity index (AI) of sera that recognize Pn14PS as coating material. Sera were obtained from mice one week after booster injection (day 42). The avidity index (AI) is expressed as the concentration of NaSCN needed to reduce the OD_{450nm} by 50%. The absorbance value of sera without NaSCN was at least 0.5 (18).

Antibody avidities of sera from mice receiving a booster injection. The thiocyanate elution ELISA was used to measure the anti-Pn14PS antibody avidity of the sera obtained 1 week after the booster (Figure 5). All conjugates that elicited anti-Pn14PS antibody titers were evaluated with regard to the avidity index (AI). Except for conjugates JJ5 and JJ10, the observed AI was not significantly different between conjugates, which could be a consequence of the lower anti-Pn14PS titer (Figure 5).

Phagocytic capacity of sera from mice receiving a booster injection. To analyze the phagocytic capacities of groups receiving different conjugates, pooled mouse sera obtained 1 week after the booster were tested in a phagocytosis assay using S. pneumoniae type 14 and the mouse macrophage cell line J774A.1. Except for JJ10, all conjugates that elicited antibodies against Pn14PS (Figure 2) were also capable of promoting the phagocytosis of S. pneumoniae type 14 (Figure 6). All conjugates that did not induce Pn14PS-specific antibodies were not able to promote the phagocytosis of S. pneumoniae type 14.

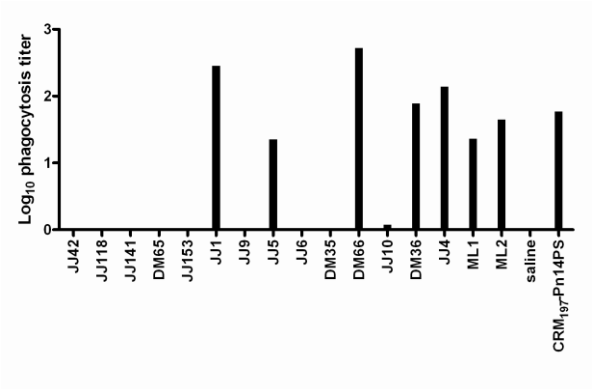


Figure 6. Phagocytosis titer in sera obtained one week after booster that were heat-inactivated and supplemented with 2% complement. The assay was performed with heat-inactivated FITC-labelled *S. pneumoniae* type 14 and J774A.1 cells by flow cytometry. The titers are expressed as the $\log_{10}$ of the serum dilution during phagocytosis resulting in 25% of J774 cells being positive for FITC.

Discussion

The smallest immunogenic structure of Pn14PS was identified in this study by testing 16 overlapping synthetic oligosaccharide fragments of Pn14PS (Table 1). The oligosaccharides were conjugated to the protein carrier CRM_{197}. CRM_{197}-Pn14PS, which contains of the entire *S. pneumoniae* type 14 polysaccharide and was used as the positive control, produced a specific antibody response to Pn14PS, as reported previously (32,34,35). The conjugate of one defined structural repeating unit of Pn14PS, namely, the branched tetrasaccharide Gal-Glc-(Gal-)GlcNAc (JJ1) produced a specific antibody response to Pn14PS, similarly to that reported by Mawas et al. (20). The conjugates of two and three repeating units of this branched tetrasaccharide, as represented by JJ4/ML1 and ML2, respectively, also produced antibodies against Pn14PS (Figure 2). No significant differences in immune response were observed between the conjugates JJ4 and ML1. These conjugates contain identical oligosaccharides, but differ in the length of the spacer.

Since several conjugates did not produce anti-Pn14PS antibodies, the question was raised whether oligosaccharide-specific antibodies could be demonstrated. BSA-mannose, which contains the spacer present in CRM_{197} and BSA conjugates, was employed to block the anti-spacer antibodies. Its use was based on the results shown in Figure 3, i.e. all conjugates produced specific antibodies to the spacer part of the

conjugates. Almost all conjugates induced specific antibodies to their own oligosaccharide fragments (Figure 4). It was noted, that JJ10 did not induce oligosaccharide-specific antibodies, while low amounts of Pn14PS antibodies could be detected in the sera (Figure 2, Table 3, Figure 4C); this will be discussed further on. DM65 also did not evoke oligosaccharide-specific antibodies (Figure 4C). Other studies have shown that anti-spacer antibodies can suppress the induction of antibodies against the carbohydrate antigen (4,22). In the present study, we found that almost all conjugates produced antibodies against the specific oligosaccharide of the conjugate that was not suppressed by the simultaneous production of anti-spacer and anti-carrier antibodies (Figure 3).

The biological relevance of the evoked antibodies was investigated by measuring the capacity of the sera to promote opsonophagocytosis of *S. pneumoniae* type 14. First, the antibody avidity of the sera was measured in order to evaluate the relative strength of the binding of antibodies to Pn14PS (25). Antibody avidity is an important characteristic of protection immune response (10) and strongly correlated with antibody titer and the ability to opsonize pneumococci *in vitro* and with the potency of an antibody to passively protect mice against pneumococcal infections (29). We found that the conjugates that elicited specific antibodies against Pn14PS have almost similar binding strengths as indicated by the avidity index except for JJ5 and JJ10. Apparently the antibodies (paratope) that were elicited by conjugates JJ5 and JJ10 might not fit precisely with the epitope of polysaccharide type 14. (Figure 5). Opsonophagocytosis was performed using heat-inactivated FITC-labeled *S. pneumoniae* type 14 and the mouse macrophage cell line J774A.1. We observed that the sera containing antibodies against Pn14PS were also capable of promoting the phagocytosis of *S. pneumoniae* type 14. There was one exception: JJ10 (the conjugate that induced lower antibody titers against Pn14PS in the other tests) sera did not promote phagocytosis (Figure 6). When whole-cell ELISA for *S. pneumoniae* type 14 was used, additional experiments with JJ10-specific antibodies did not detect any binding of these antibodies. This explains the inability of these antibodies to promote phagocytosis (data not shown). It is hypothesized and proven by additional experiments (data not shown) that JJ10 does not evoke antibodies against the branched structure Gal-Glc-(Gal-)GlcNAc present in PnPS14 but does evoke antibodies against the non-reducing end of the oligosaccharide Gal-GlcNAc-Gal-Glc. We concluded that the JJ10-specific antibodies may recognize an epitope that is exposed in purified PnPS14, but is hidden in the capsule of the bacterium. The other conjugates that did not evoke specific antibodies against polysaccharide type 14 also did not display phagocytic capacity. They did, however, elicit specific antibodies against their own oligosaccharide structure. We found that type-specific

anti-Pn14PS antibodies did not cross-reactive with different polysaccharides of *S. pneumoniae* (See appendix I).

Our findings (28) and the work of others (7,8,27,33) on synthetic oligosaccharide-protein conjugates should encourage more research on synthetic oligosaccharide-conjugate vaccines. Establishing whether animals and humans can produce antibodies against minimal synthetic oligosaccharides, however, is just the first step in the development of a synthetic pneumococcal vaccine. The next steps are to define the most immunogenic-protective epitopes on the PS and to optimize the presentation of these epitopes to the immune system.

In conclusion, the large number of *S. pneumoniae* serotypes should stimulate the generation of new synthetic saccharides. Then, natural PS can, when needed and when possible, be replaced sequentially by their (bio)synthetic oligosaccharide counterparts in future semi-synthetic pneumococcal vaccines. The present study has shown that the branched trisaccharide Glc-(Gal-)GlcNAc is the core structure in inducing Pn14PS-specific antibodies and that the neighboring galactose at the non-reducing end significantly contributes to the induction of phagocytosis-promoting (functional) antibodies. Furthermore, the branching element within Pn14PS plays an important role in the recognition of the PS of *S. pneumoniae* type 14. Taking into account the various results from the present work, the structure, originally described by Mawas et al. (20), is a serious candidate for a synthetic oligosaccharide conjugate vaccine against infections caused by *S. pneumoniae* type 14.

Acknowledgements

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Appendix I

Antibodies evoked by oligosaccharide fragments of Pn14PS do not cross-react with different polysaccharides of *Streptococcus pneumoniae*

Dodi Safari, Huberta A. Th. Dekker, Ben de Jong, Ger T. Rijkers, Johannis P. Kamerling, and Harm Snippe

A panel of sera from mice immunized with various neoglycoconjugates of oligosaccharides of serotype 14 pneumococcal polysaccharide (Pn14PS) containing antibodies (See Chapter 2 (5)) was investigated for potential cross-reactivity with eight different pneumococcal polysaccharides that were included in the commercial pneumococcal vaccines using a multiplex flow cytometric assay (Luminex). Sera obtained from mice immunized with two types of different commercial pneumococcal vaccines, a 23-valent pneumococcal polysaccharide vaccine, i.e. 1, 2, 3, 4, 5, 6B, 7, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 15F, 18C, 19A, 19F, 20, 22F, 23F, 33F, (PPV23, Merck, West Point, Pa.) and a 7-valent pneumococcal polysaccharide conjugate vaccine, i.e. 4, 6B, 9V, 14, 18C, 19F, 23F, (PCV7, Wyeth, Philadelphia, Pa.) served as controls.

Polysaccharides of type 3, 4, 6B, 9V, 14, 18C, 19F, 23F and cell wall polysaccharide (CWPS) (Table 1) were modified with adipic acid dihydrazide (ADH) and then coupled to polystyrene fluorescent beads of uniform size (5.6 micron; Luminex Corperation, Austin TX) according to the manufactures instructions at the Department of Medical Microbiology and Immunology, St. Antonius Hospital (Nieuwegein, the Netherlands).The fluorescent-particle-based multiplex flow cytometric immunoassay was performed as described previously (3). Diluted mice sera (1:250) and the control sera (1:25) were added to multiplex-polysaccharide beads (5,000 beads/polysaccharide/well) in a 96-well MV Multiscreen filter plate (Millipore, Billerica, MA) and incubated for 1 hour at room temperature (RT) on a plate shaker at 200 rpm in the dark. After three washes with PBS using a vacuum manifold, wells containing beads were incubated for 1 hr at RT in the dark with R-phycoerythrin-conjugated goat anti-mouse IgG (BD Biosciences Pharmingen, San Jose CA) diluted 1:100 in PBS. Following three washes the samples were measured on a Luminex 100 system (Bioplex). The median fluorescence intensity (MFI) was measured for minimally 50 beads per region, corresponding to a bead coupled with a specific polysaccharide. Data were analyzed using Bio-Plex Manager software, version 4.1.1 (Bio-Rad Laboratories, Hercules, CA).

Table 1. Structure of capsular polysaccharides of *S. pneumoniae*[1]

Type	Repeating unit of polysaccharide
3	→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→
4	→3)-β-D-Man*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→3)-α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*2,3(*S*)Pyr-(1→
6B	→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→4)-D-Rib-ol-(5→*P*→
9V	→4)-α-D-Glc*p*A-(1→3)-α-D-Gal*p*-(1→3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→ 2Ac (17%) 4Ac (6%) 2Ac (3%) 3Ac(25%) 6Ac (55%) 3Ac (4%)
14	→6)-[β-D-Gal*p*-(1→4)-]β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→
18C	→4)-β-D-Glc*p*-(1→4)-[α-D-Glc*p*6Ac0.3-(1→2)-[Gro-(1→*P*→3)-]β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→3)-β-L-Rha*p*-(1→
19F	→4)-β-D-Man*p*NAc-(1→4)-α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→*P*→
23F	→4)-β-D-Glc*p*-(1→4)-[α-L-Rha*p*-(1→2)-[Gro-(2→*P*→3)-]β-Gal*p*-(1→4)-β-L-Rha*p*-(1→
CWPS (6)	→6)-β-D-Glc*p*-(1→3)-α-AAT*p*-(1→4)-[Cho→*P*→6]α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→1)-D-Ribitol-5-P-(O→

[1]Adopted from Kamerling, 1999 (4).

Immunization of mice with the 23-valent pneumococcal polysaccharides (PCV23) resulted in low levels of IgG antibodies against the eight different polysaccharides tested , except type 3 and type 23F which elicit high level IgG antibodies. On the other hand, sera obtained from mice immunized with the 7-valent pneumococcal conjugate vaccine bound with high specificity to the seven type specific- and the cell wall polysaccharide-beads but not to type 3 (not present in the 7-valent pneumococcal conjugate vaccine [PCV7]). As expected, sera obtained from mice immunized with the polysaccharide type 14 conjugated to the cross-reactive material of diptheria toxoid (Pn14PS-CRM_{197}; See Chapter 2) identical with the component in the heptavalent conjugate vaccine, interacted with high specificity to Pn14PS-beads. High- (JJ1, JJ4, DM36, ML1, DM66, ML2) and low- (JJ5, JJ10) tittered anti Pn14PS sera also bound with Pn14PS-beads (Figure 1) but did not show cross reactivity with any of the other tested polysaccharides.

The pentasaccharide repeating units of the 4 serotypes of serogroup 15 of *S. pneumoniae* are identical, but polymerisation forms a linear polymer in 15A and 15F and a branched structure in 15B and 15C (Figure 2) (1). The serogroup 15 polysaccharides also share the common linear structure of polysaccharide with

Pn14PS: -β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*- (4,7). The potential cross-reactivity of this panel of anti Pn14PS sera with four different *S. pneumoniae* serotype 15 polysaccharides was investigated by ELISA using Pn15APS, Pn15BPS, Pn15CPS, and Pn15FPS coated plates as described previously (5). We found no cross reactivity between series of mice sera related to Pn14PS with these four different serotype 15 polysaccharides, except for sera of mice immunized with JJ1. JJ1 is composed of the branched tetrasaccharide fragment of Pn14PS and elicits high titer of anti-Pn14PS IgG antibodies has a low binding to polysaccharide type 15C (Figure 3).

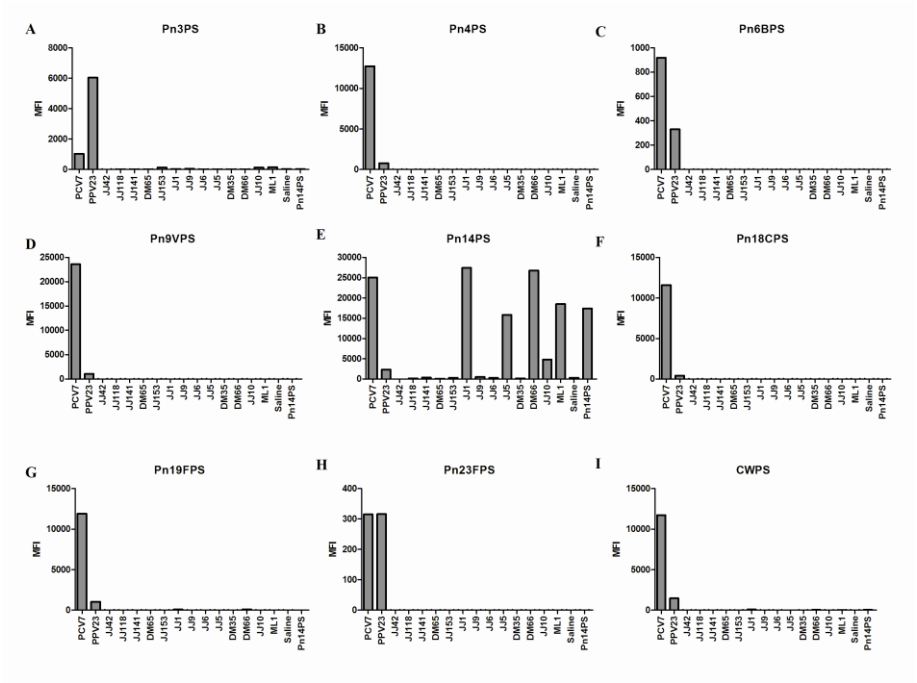

Figure 1. Lack of cross reactivity between type 14-specific mice sera and eight different polysaccharides of *S. pneumoniae*. Multiplex immunoassay was preformed to investigate the cross-reactivity between mice sera from neoglycoconjugates of Pn14PS immunization with eight other pneumococcal polysaccharide serotypes: Pn3PS (A), Pn4PS(B), Pn6BPS(C), Pn18CPS (D), Pn9VPS(E), Pn14PS (F), Pn19FPS (G) and Pn23FPS (H), and cell wall polysaccharide type I (CWPS) (I). The data were expressed as median fluorescence intensity (MFI)

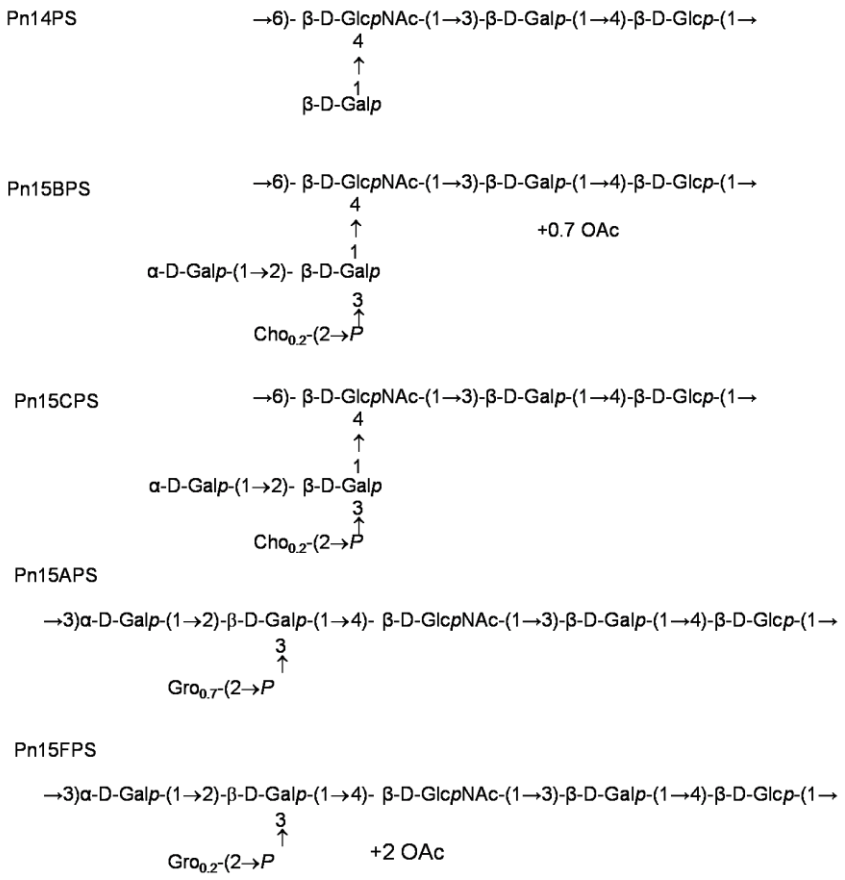

Figure 2. Comparison of structure of repeating unit of *S. pneumoniae* polysaccharides between serotype 14; Pn14PS and serogroup 15, i.e. Pn14PS: Pn15BPS, Pn15CPS, Pn15APS, and Pn15FPS (4,7).

The absence of cross reactivity is in accordance with the fact that polysaccharides of serotype 3, 4, 6B, 9V, 14, 18, 19F and 23 do not have an overlap in their primary structures with Pn14PS. The absence of the potential cross reactivity with Pn15PS might be due to a different presentation of the linear epitope, fixed in Pn15PS and flexible in the synthetic conjugate vaccine (CRM). The antibodies directed against serotype 14 which are induced by heptavalent pneumococcal conjugate vaccination apparently also do not display significant cross-reactivity because serotype 15 is one of the carriage replacement serotypes (2).

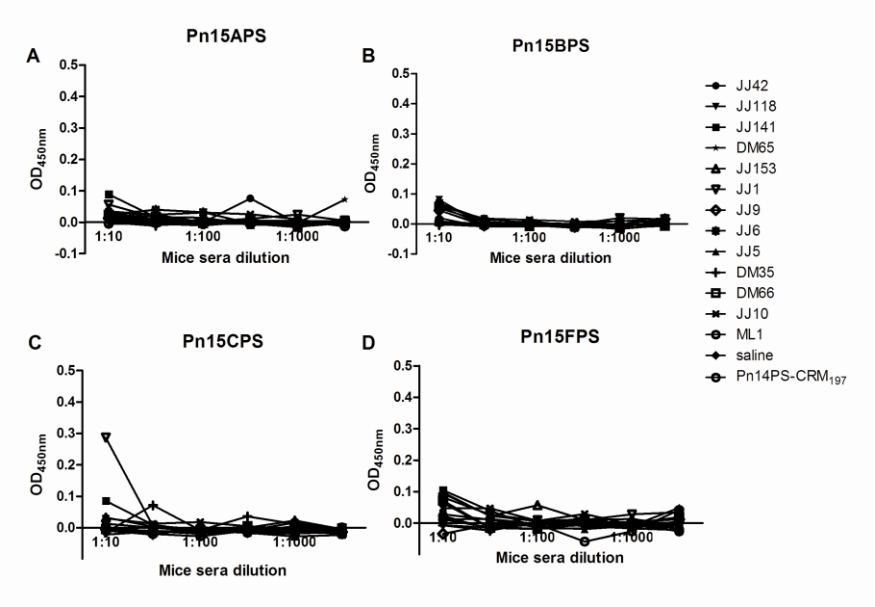

Figure 3. Binding of antibodies induced in mice by immunization with various neoglycoconjugates of Pn14PS to the capsular polysaccharides of serogroup 15 *S. pneumoniae*. Serial dilutions of mouse serum (ranging from 1:10 to 1:1000) were incubated on ELISA plates coated with serotype 15A polysaccharide Pn15APS (panel A), Pn15BPS (panel B), Pn15CPS (panel C) or Pn15FPS (panel D).

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Chapter 3

Antibody- and cell-mediated immune responses to a synthetic oligosaccharide conjugate vaccine after booster immunization

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Submitted

Abstract

Memory formation to synthetic polysaccharide conjugates was studied by immunizing mice with a synthetic branched tetrasaccharide conjugated to CRM_{197} protein (CRM-neoglycoconjugate) related to *Streptococcus pneumoniae* type 14 polysaccharide (Pn14PS). Animals were boosted with either the same neoglycoconjugate or a native polysaccharide in order to investigate the effect of booster immunization on the outcome of sustained immunity to *S. pneumoniae* type 14. Boosting with the CRM-neoglycoconjugate resulted in increased levels of interleukin 5 (IL-5) in serum on day 1, which was followed by the appearance of high levels of specific anti-Pn14PS IgG antibodies at day 7. Boosting with native polysaccharide of Pn14PS resulted in neither IL-5 induction nor evoking of anti Pn14PS IgG antibodies. *In vitro* (re)stimulation of spleen cells after booster injection with the same neoglycoconjugate revealed the presence of IL-4 and IL-5, indicative for a T helper cell type 2 (Th2) response. Spleen cells obtained from mice boosted with polysaccharide were unable to do so, neither IL-4 or IL-5 were induced *in vitro*. Stimulation of the latter mice with heat inactivated bacteria resulted in higher levels of IFNγ (Th1 marker) and lower levels of IL-17 (Th17 marker) when compared with mice boosted with the CRM-neoglycoconjugate or mock immunized mice. In conclusion, a neoglycoconjugate boosting is responsible for the activation of memory cells and the establishment of sustained immunity. A boost with native polysaccharide is not only ineffective to evoke opsonic antibodies, it also interferes with several immune regulatory mechanisms. The first sign of effective action, i.e. a successful immunization, is the appearance of IL-5 in the serum after a booster injection.

Introduction

Native polysaccharides are well-known thymus-independent type-2 (TI-2) antigens that mainly induce IgM antibodies, and to a lesser degree IgG, and lack the capability of memory T- and B-cell induction (12). Although T cells play a role in TI-2 response, the exact T-cell involvement in antibody responses to polysaccharide remains an immunological enigma (17). However, conjugation of poly- or oligo-saccharides to a protein carrier results in a switch of the anti-polysaccharide antibody response into one with characteristics of a T-cell-dependent response. This is reflected by the generation of memory B and T cells and the induction of high titers of anti-polysaccharide IgG antibodies after booster immunization (1).

The immunogenicity of synthetic carbohydrate based vaccines has been improved with conjugation of synthetic oligosaccharide fragment to a carrier protein (neoglycoconjugate). A neoglycoconjugate corresponding to *Haemofilus influenzae* (*H. influenza*) type b-conjugated to different carrier proteins, i.e. tetanus toxoid (TT), *Neisseria meningitidis* outer membrane protein complex, human serum albumin and bovine serum albumin (BSA) was reported that elicited antibodies which are reactive with the native bacterial capsular polysaccharide of *H. influenzae* type b in an animal model (10). The synthetic pentadecasaccharide representing of *O*-specific polysaccharide of *Shigella flexneria* 2a-conjugated to TT protein was also reported to induce anti-lipopolysaccharide antibody, dominated by IgG1 production (Th2-type response) in mice (24). Meanwhile we and other groups have been working on improving the immunogenicity of neoglycoconjugates against different *Streptococcus pneumoniae* (*S. pneumoniae*) serotypes in animal models: Di-, tri-, and tetrasaccharides related to polysaccharide type 17F conjugated to keyhole limpet hemocyanin (KLH) protein (7,15); and tri- and tetrasaccharides related to type 23 conjugated to KLH protein (6); Di-, tri-, and tetrasaccharides related to type 6B conjugated to KLH protein (13); Di-, tri-, and tetrasaccharide related to type 3 conjugated to the cross-reactive material of diphteria toxin (CRM_{197}) protein (2) and most recently overlapping oligosaccharide varying from tri- to dodecasaccharides related to polysaccharide type 14 conjugated to CRM_{197} protein (22,28).

The polysaccharide vaccine (without protein carrier) as a boosting dose following a priming series of a polysaccharide conjugated vaccine has been assessed in clinical pneumococcal vaccine trials and seems controversial (23). Revaccination with a pneumococcal polysaccharide vaccine after conjugate vaccination increased IgG2

anti-polysaccharide antibody titer in children with recurrent acute otitis media (AOM) (3), but did not prevent AOM in children older than 1 year who had had recurrent episodes of AOM before vaccination (30). Booster with the 23-valent pneumococcal polysaccharide vaccine (PPV-23) following the immunization with different dose of the 7-valent pneumococcal conjugate (PCV-7) was well tolerated and induced excellent responses for all serotypes which were greatest in the single PCV-7 group (27). Hyporesponsiveness has been reported in some meningococcal vaccine trials after primary immunization or booster with a native polysaccharide (18,20,33). Recently, it was reported that secondary immunization with non conjugated meningococcal serogroup C (MenC) polysaccharide following primary immunization with MenC conjugate vaccine seemed to induce a higher IgG2 response compared to MenC conjugate immunization in adults (8).

In the present study, a mouse model was used to investigate immune response to a synthetic branched tetrasaccharide fragment conjugated to CRM_{197} protein (CRM-neoglycoconjugate) related to *S. pneumoniae* type 14 polysaccharide (Pn14PS). Special attention was given to booster injections with either CRM-neoglycoconjugate or native polysaccharide. Cytokine levels and anti-Pn14PS antibodies were measured in the sera after booster immunization. In addition *in-vitro* spleen lymphocytes cells stimulations were performed with series of antigens seven days after booster immunization. The data show that the first sign of effective activation of the cellular immune system was the appearance of IL-5 in the serum after a booster injection with a neoglycoconjugate indicating a successful booster immunization.

Materials and methods

Neoglycoconjugate and polysaccharide. A neoglycoconjugate corresponding to the branched tetrasaccharides fragment of one repeating unit of Pn14PS conjugated to CRM_{197} protein (CRM-neoglycoconjugate) was used in this study as described previously (28). The native polysaccharide was isolated and purified from *S. pneumoniae* type 14 (ATCC 6314) bacteria (31).

Table 1. Immunization schedule

Group	Primary injection	Booster injection		Code
		Week 5	Week 10	
1	GC[1]	-	-	GC
2	GC	GC	-	GC-GC
3	GC	PS[2]	-	GC-PS
4	GC	GC	GC	GC-GC-GC
5	GC	GC	PS	GC-GC-PS
6	GC	PS	PS	GC-PS-PS
7	Saline	-	-	Saline

[1]GC = CRM-neoglycoconjugate; [2]PS = native polysaccharide

Immunization. The mouse vaccination studies were approved by the Ethics Committee on Animal Experiments of Utrecht University, Utrecht, The Netherlands. Inbred 6-weeks-old female BALB/c mice were maintained at the Central Animal Facility of Utrecht University. Groups of mice (n=5) were immunized intracutaneously with the CRM-neoglycoconjugate of Pn14PS (in short GC; 2.5 µg of carbohydrate per mouse) at four different sites, i.e. in the vicinity of the lymph nodes of the axillae and the groins. The booster injections were performed with either the same GC (2.5 µg) or the native polysaccharide type 14 (in short PS; 2.5 µg) at week 5 and 10 (Table 1). Control mice were injected with phosphate-buffered saline (PBS). Blood was collected at several time points after primary and booster injection and the sera were stored at −70°C.

Measurement of Pn14PS-specific antibodies. Enzyme-linked immunosorbent assay (ELISA) was performed to measure the anti-Pn14PS antibodies as previously described (28). Briefly, diluted sera were incubated for 1 h at 37°C in flat-bottom 96-well plates (Corning Inc., Corning, NY, USA) coated with Pn14PS (0.3 µg/well). Horseradish peroxidase-conjugated goat anti-mouse IgG (Nordic Immunology Laboratories, Tilburg, The Netherlands) was then applied and visualized with a mixture of 3,3',5,5'-tetramethylbenzidine (Sigma Chemical Co., St. Louis, MO, USA) and H_2O_2 (Sigma Chemical Co.). Antibody titers were expressed as the $\log_{10}$ of the dilution giving twice the optical density (OD) obtained for control mice (immunized with saline), with a cutoff value of 0.2.

Measurement of avidity index. The antibody avidity of mice sera that recognized Pn14PS as the coating material was measured by ELISA using chaotropic sodium thiocyanate (NaSCN; Sigma Chemical Co), as previously described (25,28). Briefly Pn14PS-coated plates were incubated with diluted sera.

Then series of NaSCN concentrations (0 to 3.0 M) were incubated on the plates at 37°C for 15 min. The amount of antibodies that remained bound to the plate was detected by ELISA method as described above. The avidity index was expressed as the concentration of NaSCN that was needed to reduce the OD at 450nm by 50% (25). The absorbance value of sera without NaSCN had to be at least 0.5.

Measurement of phagocytosis titer. The phagocytosis assay procedure was performed as described previously (28). Two fold dilution of heat-inactivated pooled sera with 2% complement (guinea-pig serum) in Hank's balanced salt solution (HBSS)/1% BSA were mixed with J774A.1 cells (ATCC TIB67; $3x10^6$ cells/ml) and FITC-labeled heat-inactivated *S. pneumoniae* type 14 (ATTC 634; $3x10^7$ cells/ml) in round-bottom plates (Greiner bio-one, Frickenhausen, Germany), followed by incubation at 37°C under vigorous shaking for 50 min. The mixtures were then analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA). The phagocytosis titers were expressed as the $\log_{10}$ of the serum dilution that resulted in 25% of the J774A.1 cells being positive for FITC (14).

***In-vitro* spleen lymphocytes cells stimulations**. In parallel to the immunization experiments described above, an independent immunization was performed to investigate the cell-mediated immune response after the booster immunization. Spleens of immunized mice with CRM-neoglycoconjugate of Pn14PS dose and boosted either with the same neoglycoconjugate (GC-GC and GC-GC-GC) or native polysaccharide (GC-PS and GC-PS-PS) and saline buffer (Table 1) were removed aseptically into HBSS medium one week after the booster immunization. Spleens were squeezed through a stainless steel filter in order to prepare a single-cell suspension, and then centrifuged at 1200 rpm for 10 min at 4°C. Ammonium chloride (0.2 M) was added, and the cell suspensions were kept on ice for 10 min in order to lyse the red blood cells. After washing with HBSS medium, the splenocytes were re-suspended with Roswell Park Memorial Institute (RPMI)1640 medium complemented with 10% fetal calf serum (FCS) and gentamycin, and filtered through a nylon filter mesh. The cells were then counted by Coulter Z-1 automatic counter (Beckman Coulter Corporation, Fullerton, CA, USA).
In-vitro spleen-cells stimulation was performed as previously described (5,29). Briefly, the spleen cells suspensions (10^7 cells/ml) in medium were stimulated with different antigens, i.e., CRM-neoglycoconjugate (10 µg/ml) and BSA-neoglycoconjugate (the same synthetic oligosaccharide of Pn14PS conjugated to BSA protein, 10 µg/ml), heat-inactivated *S. pneumoniae* type 14 (Pn14) and type 9V (Pn9V) bacteria (10^7 bacteria cells/ml), native polysaccharide (Pn14PS; 10

µg/ml). The cells were then incubated at 37°C, in 100% relative humidity, and with 5 % CO_2 in air. Finally, the supernatants were collected at 72 h after initiation of the cultures, and stored at –70°C until use.

Cytokine assays. We selected six different cytokines to screen the mice sera and the supernatants after the primary, booster immunization and spleen stimulations: as Th1 cytokines: IL-2 (171-G5003M), TNF-α (171-G5023M), and IFN-γ (171-G5017M); as Th2 cytokines: IL-4 (171-G5005M) and IL-5 (171-G5006); for Th17 marker: IL-17 (171-G50013M) using the luminex-multiplex cytokine assay, following the manufacturer's instructions (Bio-Rad). The lower limits of detection were 0.31 pg/ml (IL-2), 0.71 pg/ml (IL-4), 0.24 pg/ml (IL-5), 0.20 pg/ml (IL-17), 5.2 pg/ml (TNF-α), and 0.48 pg/ml (IFN-γ).

Statistical methods. All antibody titers are shown as the $\log_{10}$ of the highest dilution of serum giving a reading twofold higher than the background. Data are presented as geometric means ±SEM. Cytokine data are given as mean (±SEM) of two independent measurement.

Results

Pn14PS-specific antibody levels in mice receiving booster immunizations with either neoglycoconjugate or polysaccharide. Except for saline group, all mice received a primary immunization with the CRM-neoglycoconjugate related to Pn14PS at day zero (Table 1). Booster immunizations were given at weeks 5 and 10, respectively, using the same neoglycoconjugate or native polysaccharide of Pn14PS. Four weeks after the primary immunization, no specific anti-Pn14PS IgG antibodies were detectable in the sera of these mice (Figure 1, Group GC). A booster with the CRM-neoglycoconjugate at week 5 (Figure 1, GC-GC) evoked one week later (week 6) high levels of IgG antibodies in the sera of these mice, while the booster with native polysaccharide at week 5 (GC-PS) and week 10 (GC-PS-PS) failed to do so. A second booster at week 10 with either the CRM-neoglycoconjugate (GC-GC-GC) or polysaccharide of Pn14PS (GC-GC-PS) hardly enhanced the levels of specific anti-Pn14PS antibodies already present after the first booster with CRM-neoglycoconjugate immunization at week 5 (Figure 1). Mock-immunized mice (Saline) did not reveal specific antibodies against Pn14PS at any time point.

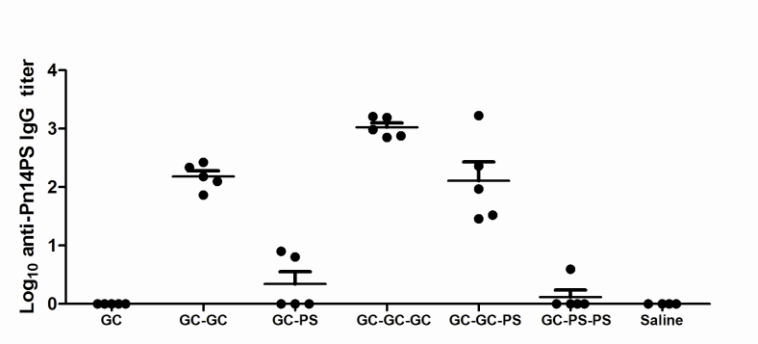

Figure 1. Level of anti-Pn14PS IgG antibodies. Groups of mice (n=5) were immunized with a CRM-neoglycoconjugate (GC) related to *S. pneumoniae* type 14 polysaccharide (Pn14PS). Booster doses containing either a GC (GC-GC and GC-GC-GC) or a native polysaccharide of Pn14PS (PS) (GC-PS, GC-GC-PS, and GC-PS-PS) were injected at Week 5 and 10 (Table 1). Sera were obtained one week later. The sera of GC group was obtained at day 28 after primary injection. Phosphate buffered saline served as a negative control. ELISA was employed to measure specific anti-Pn14PS IgG antibodies, which is expressed as the $\log_{10}$ of the sera dilution.

Pn14PS antibody avidities and phagocytic capacity of sera from mice receiving booster immunization. Using the thiocyanate elution ELISA, the avidity index (AI) for Pn14PS was measured in sera obtained one week after the booster immunizations. The threshold for the determination of an AI is a Log_{10} titer of 0.5 for anti-Pn14PS. This threshold is not reached for groups of GC-PS, GC-PS-PS, and saline (Figure 1). The second booster immunization with the CRM-neoglycoconjugate at week 10 (GC-GC-GC) resulted in an AI value higher than the first booster immunization with the same neoglycoconjugate at week 5 (GC-GC) (Figure 2). Boosting with native polysaccharide at week 5 (GC-PS) or at week 10 (GC-PS-PS) did not evoke anti-Pn14PS IgG antibodies one week later, so no AI could be determined. A boost with native polysaccharide following the primary and the first booster with CRM-neoglycoconjugate (GC-GC-PS) resulted in a decrease of the avidity index (Figure 2).

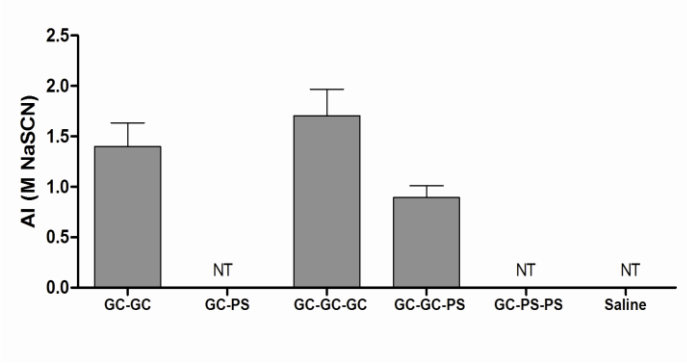

Figure 2. Antibody avidity after the booster injection. The avidity index (AI) is expressed as the concentration of NaSCN needed to reduce the OD450nm by 50%. NT= not tested (the absorbance value of sera without NaSCN was at below 0.5.

The phagocytosis titer for the uptake of heat-inactivated FITC-labelled *S. pneumoniae* type 14 by the mouse macrophage cell line J774A.1 was determined in sera obtained after the booster immunizations. The capacity to promote phagocytosis of *S. pneumoniae* type 14 by mouse cell line was absent (very low titer) in the GC-PS and GC-PS-PS groups (Fig 3). The phagocytosis titer in the GC-GC-PS group was observed to be about the same as that in the GC-GC group. This result revealed that a polysaccharide booster immunization did not enhance specific anti-Pn14PS antibodies to promote the phagocytosis of *S. pneumoniae* type 14 bacteria.

Cytokine levels in sera from mice receiving booster immunizations. The levels of six different cytokines, i.e., IL-2, IFNγ, and TNFα (as markers for Th1 activation), IL-4 and IL-5 (as Th2 activation markers) and IL-17 (Th17 marker) were measured in sera obtained at day 1 after booster immunization by multiplex immunoassay (16). In general, the cytokine levels were below the limit of detection, except for cytokine IL-5. Boosting with the CRM-neoglycoconjugate (GC-GC) at week 5 resulted in high levels of IL-5 (around 1000 pg/ml) while boosting with native polysaccharide of Pn14PS (GC-PS) failed to do so (Figure 4). It should be noted that at day 1 after a primary immunization with the CRM-neoglycoconjugate (GC group), IL-5 as well as all other tested cytokines are absent in the sera of these mice (Figure 4).

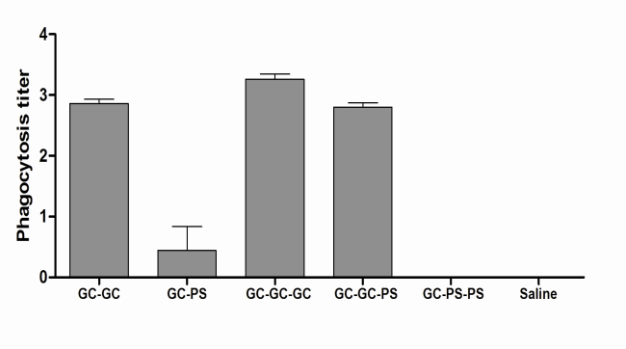

Figure 3. Phagocytosis titer from groups of sera obtained after the booster injection either with a neoglycoconjugate or polysaccharide dose. Sera were collected one week after the booster injection (Table 1) and incubate at 56ºC for 30 min. The opsonophagocytosis assay was performed with heat-inactivated FITC-labelled *S. pneumoniae* type 14 and J774A.1 cells by flow cytometry. The phagocytosis titers were expressed as the $\log_{10}$ of the serum dilution during phagocytosis that resulted in 25% of the J774A.1 cells being positive for FITC.

Cytokine levels in spleen lymphocytes cell cultures after stimulation with different antigens. Spleens were isolated at day 7 after the first booster immunization either with CRM-neoglycoconjugate (GC-GC) or native polysaccharide related to Pn14PS (GC-PS). Spleen cells isolated from mock immunized mice (saline group) served as a control (Figure 5). Single-lymphocyte cell suspensions were cultured and stimulated with the CRM-neoglycoconjugate, BSA-neoglycoconjugate, heat-inactivated *S. pneumoniae* type 14 (Pn14) bacteria, native polysaccharide type 14 or medium. After 72 hours, the levels of six different cytokines were measured in the supernatants of these cultures.

Spleen cells isolated from the GC-GC group produced high levels of cytokine IL-5, IL-4, and IL-2 upon stimulation with CRM-neoglycoconjugate, while the BSA-neoglycoconjugate, native polysaccharide and Pn14 bacteria were unable to do so (Figure 5). The IL-5 response to CRM-neoglycoconjugate is absent in spleen cells isolated from mice boosted with native polysaccharide (GC-PS). The IL-2, IL-4 and IL-5 response to native polysaccharide Pn14PS was very low in mice boosted with the CRM-neoglycoconjugate (GC-GC) or boosted with Pn14PS (GC-PS). Except for Pn14 bacteria, none of the tested antigens evoked a cytokine response in the spleen cells of the control mice (Figure5). On the other hand Pn14 bacteria induced significant levels of IFNγ, IL-17 and TNFα in the supernatant of all cell cultures: in cells derived either from unimmunized control mice (saline), from mice boosted with

CRM-neoglycoconjugate (GC-GC) or boosted with Pn14PS (GC-PS). In neither of the cell cultures, Pn14 bacteria induced IL-4 or IL-5, but minute amounts of IL-2 were detected (Figure 5).

Interestingly, we observed that the spleens obtained from the polysaccharide booster group (GC-PS) and stimulated with Pn14 bacteria produced higher amounts of IFNγ higher than spleens from CRM-neoglycoconjugate (GC-GC) or mock immunized mice (saline) (Figure 5). For IL-17 the reverse effect of previous vaccination was found: low level of IL-17 production in the GC-PS group and high levels in the GC-GC and saline group, all measured 72 h after stimulation by Pn14 bacteria (Figure 5). We observed similar response when spleens were re-stimulated with different *S. pneumoniae* serotype (data not shown). In addition, induction of IL-17 *in vitro* can only obtained with intact bacteria and not, or with a much lower efficacy, by native PS or GC. This *in vitro* induction of IL-17 appears to be independent of prior immunization with GC and/or PS vaccines (Figure 5).

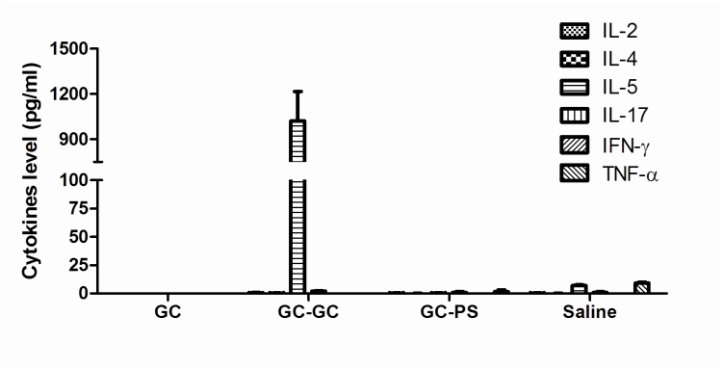

Figure 4. Cytokine levels in sera from mice receiving booster injection. Sera were collected on day 1 after the primary (GC) and the first booster immunization either with GC (GC-GC) or PS (GC-PS). Mice sera were also collected from Saline group a negative control (Table 1). Cytokine production of IL-2; IL-4; IL-5; IL-17; IFNγ; and TNFα were measured using the luminex-multiplex cytokine assay.

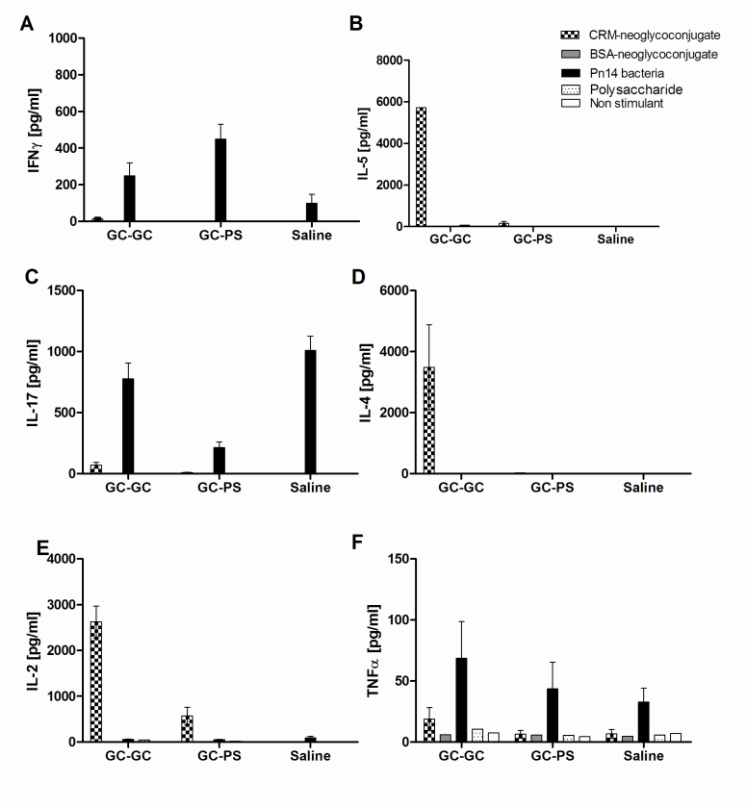

Figure 5. Level of cytokines production after spleen cells stimulation. Splenocytes were isolated from mice (n=2) seven days after the first booster injection with either CRM-neoglycoconjugate (GC-GC) or native polysaccharide Pn14PS (GC-PS) and saline group. Spleen cells (10^6 cells per well) were cultured *in vitro* and stimulated with CRM-neoglycoconjugate (black-white blocked bar), BSA-neoglycoconjugate (gray bars), heat-inactivated *S. pneumoniae* type 14 (Pn14 bacteria, black bar), native polysaccharide Pn14PS (small black dot bars) and only medium (Non stimulant, white bars). Spleen supernatants were collected 72 hours after culture initiation. The cytokine levels of INFγ (A), IL-5 (B), IL-17 (C), IL-4 (D), IL-2 (E) and TNF-α (F) were measured using the luminex-multiplex cytokine assay.

Discussion

In this study, we investigated the effect of booster immunization with either CRM-neoglycoconjugate (synthetic branched tetrasaccharides of Pn14PS conjugated to CRM_{197} protein) or native polysaccharide of Pn14PS following the primary immunization with CRM-neoglycoconjugate on the outcome of sustained immunity to *S. pneumoniae* type 14 in a mouse model. We found, as expected, that the amount of specific IgG antibodies against Pn14PS increased substantially when a CRM-neoglycoconjugate booster was given as a booster in mice previously primed with the same CRM-neoglycoconjugate. The induced antibodies were capable to opsonise *S. pneumonia* type 14. Boosting with native polysaccharide following a primary conjugate vaccine injection did not result in IgG antibody formation to Pn14PS. In addition, boosting with polysaccharide of mice already displaying high levels of circulating Pn14PS antibodies was ineffective. No further raise in antibody titers occurred, while the avidity of the Pn14PS antibodies decreased.

In order to obtain more insight into the cellular basis for these differences between a booster immunization with a neoglycoconjugate or native polysaccharide, the *in vivo* and *in vitro* cytokine production profiles were determined. In a first experiment, we measured cytokine profiles in serum 1 day after booster injection. We observed high levels of IL-5 in serum after a booster injection with CRM-neoglycoconjugate. All other tested cytokines (Il-2, IL-4, IL-17, IFN-γ and TNF-α) were not detectable. Boosting with native polysaccharide Pn14PS did not result in the induction of IL-5 nor any of the other tested cytokines. We suggest that induction of of IL-5 in serum is an early sign of a successful booster immunization and is a prerequisite for the production of specific anti-polysaccharide IgG antibodies. To further investigate the effect of a booster immunization and re-stimulation on activation of (memory) T cells, *in vitro* spleen cell cultures were set up. The cytokines IL-4 and IL-5, both well known Th2 markers, were evoked by the CRM-neoglycoconjugate in spleen cell cultures of mice previously primed and boosted with the same CRM-neoglycoconjugate. Native polysaccharide Pn14PS, a neoglycoconjugate with a different protein carrier or heat inactivated bacteria were unable to do so. These data are in line with Wuorimaa, *et al.* who also reported that the pneumococcal conjugate vaccine was associated with a Th2-type activation, as indicated by an enhanced IL-5 secretion in response to the carrier protein (tetanus or diphtheria protein) in human vaccination trials (32). Part of the increased immunogenicity of (neo)glycoconjugates therefore is the ability of the carrier protein to induce a Th2 dominated cytokine profile.

Spleen cells derived from immunized as well as unimmunized mice responded upon stimulation by heat-inactivated *S. pneumoniae* (Pn14) bacteria with the induction of the cytokines TNFα (regulatory cytokine), IFNγ (marker for Th1) and IL-17 (marker for Th17 and regulatory cytokine). These cytokines were reported to play a key role in several biological activities, influencing migration and pathogenic behavior during inflammatory disease (4). Especially IL-17 has received ample attention recently because of its role in protection against invasive pneumococcal infections in both experimental animal models as well as humans (9,19,34). We found a remarkable difference in activity for the two cytokines IFNγ and IL-17 which depended on the source of spleen cells used. Spleen cells derived from polysaccharide boosted mice displayed higher levels of IFNγ and lower levels of IL-17 in comparison to cells derived either from unimmunized mice or mice boosted with the neoglycoconjugate. Further analyses revealed that the reduced IL-17 response to heat inactivated bacteria in polysaccharide boosted mice was not serotype specific. This is in line with previous data indicating that cell wall polysaccharide (21) and pneumococcal surface protein A (11,26) (both non-serotype specific components of the pneumococcal surface) are the major activators of IL-17 production by CD4 T cells. Our data suggest that prior immunization with native polysaccharides promotes the IFNγ production when stimulated by heat inactivated non-specific *S. pneumoniae* bacteria, shifting the balance away from IL-17 production.

In conclusion, the inability of polysaccharides to boost CRM-neoglycoconjugate primed mice might be due to the incapability to induce sufficient IL-2, IL-4 and IL-5. In addition boosting with polysaccharide affects the key regulatory cytokine IL-17 which might have consequences for the overall immune defense to *S. pneumoniae*.

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Chapter 4

Codelivery of adjuvants at the primary immunization site is essential for evoking a robust immune response to neoglycoconjugates

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Vaccine (In press)

Abstract

A series of nonformulated adjuvants, i.e. Quil-A, MPL, DDA, CpG and Alum were codelivered with a synthetic branched tetrasaccharide fragment corresponding to *Streptococcus pneumoniae* type 14 polysaccharide (Pn14PS) conjugated to CRM_{197} (neoglycoconjugate) in order to investigate antibody- and cell-mediated immune responses in a mouse model. The first immunization was performed intracutaneously in the presence of adjuvants. Booster injections which were given without adjuvant dramatically enhanced and expanded the immune response from IgM to IgG1 and other IgG subclasses. Codelivery of the neoglycoconjugate with CpG or Alum had no additional effect over vaccine in saline and elicited mainly IgG1 antibodies against Pn14PS. Codelivery of the neoglycoconjugate with Quil-A, MPL, DDA alone or in combination resulted in both IgG1, IgG2a, IgG2b and IgG3 antibodies. Quil-A alone or in combination with MPL induced systemic IL-5 and IL-6 six hours after primary immunization. This adjuvant combination also increased CD4/CD8 T cells ratio in lymph nodes and peripheral blood on Day 1 after immunization. Seven day later, the ratio's in blood and lymph nodes were returned to normal. In conclusion, codelivery of Quil-A alone or in combination with MPL had the most dramatic effect on antibody- and cell-mediated immune response to neoglycoconjugate of Pn14PS.

Introduction

Adjuvants are used to create and improve the inflammatory context required for induction of an optimal innate and adaptive immune responses to vaccines as well as to ensure long-lived immunological memory. Adjuvants also allow to decrease the dose of antigen to be applied and thereby reduce the vaccine costs (19). Alum (Aluminum hydroxide; $Al(OH)_3$) is the only adjuvant used widely with routine human vaccines due to their good track record of safety and low cost but still has its limitations e.g. local reactions, augmentation of IgE antibody responses, ineffectiveness for some antigens and inability to augment cell-mediated immune responses, especially cytotoxic T-cell responses (7).

Next to alum, many other adjuvants have been developed which can be divided in 2 main categories. Alum itself and incomplete Freunds adjuvant promote the formation of depots of antigen at the site of immunization. The second category are the dispersible adjuvants, most of which engage Toll like receptors (TLRs). Monophosphoryl lipid A (MPL) suspended in saline or given as an oil-in-water emulsion stimulate TLR4 (19, 20), while oligodeoxynucleotides which contain unmethylated CpG motifs bind to TLR9 (12). For the saponin derivative Quil-A (the extract of *Quillaja saponari*) and for dimethyldioctadecyl-ammonium bromide (DDA, a lipophilic quaternary ammonium salt) it not known whether they engage TLRs. These adjuvants have been investigated intensively in experimental animals and have been shown to enhance the antibody response as well as the T cell response to a variable degree. Most of the studies have been performed with protein based vaccines (3,8,11,24) and the potential of adjuvants to improve the response to polysaccharide based vaccines has been studied less extensively. Quil-A has been shown to enhance the antibody response to a synthetic oligosaccharide fragment conjugated to protein carrier (neoglycoconjugate) related to *S. pneumoniae* polysaccharide (4,25) as well as a synthetic carbohydrate-based anticancer vaccine in animal models (13). The Quil-A administration results in increased IL-4, IL-5, and IL-13 gene expression (26). MPL enhanced the IgG antibody responses to the polysaccharide part of the glycoconjugate vaccine (18) and increased the number of IgG subclasses involved (23). CpG based adjuvants improve the response to pneumococcal polysaccharide conjugate vaccines (2).

Several adjuvants e.g. Alum, Quil-A, MPL, CpG, DDA, and combinations thereof have been studied for their capacity to improve the immunogenicity and phagocytic capacity of a neoglycoconjugate vaccine corresponding to *S. pneumoniae* type 3

polysaccharide (14). We have previously reported that the immunization of neoglycoconjugates related to *S. pneumoniae* type 14 polysaccharide (Pn14PS) conjugated to cross-reactive material of diptheria toxin (CRM_{197}) protein and co-administered with MPL and Quil-A elicited anti-Pn14PS IgG antibodies were higher as compared to mice injected with the corresponding conjugates without adjuvant in mouse model (17). In the present study, a series of different adjuvants was tested in order to investigate antibody- and cell-mediated immune responses to a neoglycoconjugate of Pn14PS in more detail in a mouse model. The antibody response was measured after primary and booster immunization. In order to obtain more insight in the mechanism of action of these adjuvants in this model system, cytokine profiles as well as CD4/CD8 T lymphocyte ratios in blood and draining lymph nodes were determined. The results showed that Quil-A alone or in combination with MPL resulted in increased CD4/CD8 ratio and cytokine production (IL-5, IL-6) which might explain the superior antibody production after booster injection.

Materials and methods

Neoglycoconjugate and adjuvants. A synthetic branched tetrasaccharides fragment β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)-]β-D-Glc*p*NAc corresponding to one repeating unit of Pn14PS conjugated to CRM_{197} protein was used as neoglycoconjugate (17). The adjuvants used in this study were described previously (14). The Quil-A was a gift from Dr. Erik B. Lindblad, Brenntag Biosector , Vedbaek, Denmark. MPL was obtained from InvivoGen (San Diego, USA), dimethyldioctadecylammonium bromide (DDA) was purchased from Eastman Kodak (Rochester, N.Y. USA), the oligodeoxynucleotides ODN1826 (CpG, TCCATGACGTTCCTGACGTT) was obtained from Eurogentec (Seraing, Belgium) and Alum was a gift from the National Institute of Public Health and the Environment (Bilthoven, The Netherlands).

Immunization. Mice immunization studies were approved by the Ethics Committee on Animal Experiments of Utrecht University, Utrecht, Netherlands. Inbred 6-week-old female BALB/c mice (obtained from Harlan, Horst, The Netherlands) were housed at the Central Animal Facility of the Utrecht University. Five mice per group were immunized intracutaneously with a neoglycoconjugate of Pn14PS (0.5 µg carbohydrate per animal) and coadministrated with adjuvant (Table 1). The vaccines and adjuvant were administered at four different sites in the proximity of the lymph nodes of the axillae and the groins. Booster immunizations

were performed intracutaneously with the same neoglycoconjugate (0.5 µg carbohydrate per animal) without adjuvant coadministration on weeks 5 and 10. Blood samples were taken at several time points before and after booster immunization.

Measurement of Pn14PS-specific antibodies by ELISA. Enzyme-Linked Immunosorbent Assay (ELISA) was performed to measure the anti-Pn14PS antibodies as previously described (17). Briefly, diluted sera were incubated for 1 h at 37ºC in flat-bottom plates (Corning Inc., Corning, NY, USA) which were coated with native polysaccharide of Pn14PS (0.3 µg/well) and blocked with 3% gelatin. After a washing step, horseradish peroxidase-conjugated goat anti-mouse IgG (1:10000) or IgG subclass specific antibodies IgG1 (1:10000); IgG2a (1:6000); IgG2b (1:2500); and IgG3 (1:5000) (Nordic Immunology Laboratories, Tilburg, Netherlands) were incubated for 1 h at 37ºC. A mixture of 3,3',5,5'-tetramethylbenzidine (Sigma Chemical Co, St. Louis, MO, USA) and H_2O_2 (Sigma Chemical Co) was then added to visualize and quantify the amount of bound peroxidase. The reaction was stopped by the addition of 0.5 M H_2SO4. Optical density (OD) values were obtained with a microtiter plate spectrophotometer at 450nm (Bio-Rad, model 3550 UV; Bio-Rad Laboratories, Hercules, CA).

Measurement of avidity. The antibody avidity of mouse sera that recognized polysaccharide of Pn14PS was measured by ELISA using chaotropic sodium thiocyanate (NaSCN; Sigma Chemical Co), as previously described (17). Briefly Pn14PS-coated plates were incubated with diluted sera (1:25) for 1 h at 37ºC. After the washing step, series of NaSCN concentrations (0-3.0 M) were incubated on the plates at 37ºC for 15 min. After five washes, horseradisch peroxidase-conjugated goat anti-mouse IgG (1:10.000) in PBS was added, and the solution was incubated for 1 h at 37ºC. The amount of residual antibodies bound to the plate was detected by ELISA as described above. The avidity index (AI) was calculated and expressed as the concentration of NaSCN needed to reduce the OD450nm by 50% (15). AI could only be determined when the OD value of a 1:25 diluted serum was at least 0.5.

Table 1. A series of adjuvants was used in this study

Group	Adjuvant	Dose[1] (µg)
1	MPL	10
2	Quil-A	20
3	MPL+Quil-A	10+20
4	DDA	100
5	CpG	50
6	DDA+CpG	100+50
7	Alum	50
8	No adjuvant	-
9	Saline	-

[1]Adjuvant doses were described previously (14).

Opsonophagocytosis. The opsonic activity of mouse sera was determined as the uptake of *S. pneumoniae* type 14 by the mouse macrophage cell line J774A.1 (ATCC TIB67). Details of the phagocytosis assay procedure have been published previously (17). Briefly, two-fold dilutions of heat-inactivated pooled mouse sera in Hanks balanced salt solution-1% BSA were added with 2% (v/v) fresh frozen guinea-pig serum (as complement source) in round-bottom 96-well tissue culture clusters (Greiner bio-one, Frickenhausen, Germany). Next, 20 µl of J774A.1 cells (3.10^6 cells/ml) and 20 µl of FITC-labeled *S. pneumonie* type 14 (3.10^7 cells/ml) were added to the wells (17), followed by incubation at 37°C under vigorous shaking for 50 mins. The mixtures were then fixed with cold 2% paraformaldehyde and analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA, USA). The percentage of FITC-positive J774A.1 cells (i.e. the cells which have taken up the fluorescent pneumococci) was used as a measure of the phagocytic activity and corrected by using the values found in the mixtures without sera. The phagocytosis titer is expressed as the log10 of the serum dilution used in the assay that resulted in 25% of the J774A.1 cells being positive for FITC (9).

Measurement of T lymphocyte subsets. Single cell suspensions were prepared from blood and lymph node as described in Chapter 3. Blood samples were collected in EDTA coated tubes at 6, 24, 72, and 168 hrs after the primary immunization with neoglycoconjugate of Pn14PS with adjuvants coadministration (Table 1). Plasma was separated by centrifugation and stored at -70°C for cytokine analysis. Red blood cells were lysed with ammonium chloride (0.16 M) and incubated for 10 min on ice. The lymph nodes (LNs) were obtained from mice (n=3) on day 1 and 7 after primary immunization. They were removed aseptically into Hanks balanced salt solution, squeezed with a cell strainer of 70 µm (BD Falcon) to prepare a single cell suspension. After washing, cells were resuspended in PBS and counted by a

Coulter Z-1 automatic counter (Beckman Coulter Corporation, Fullerton, CA, USA).

Staining of $CD4^+$ and $CD8^+$ T cells was described previously (1). Cell suspensions (10^6 cells per well) were incubated with an Fc receptor blocking antiserum (Rat Anti-Mouse CD16/32, Southern Biotech) for 5 minutes, then subsequently incubated with rat anti-mouse CD8a/Ly-2-FITC (Southern Biotech), rat anti-mouse CD4/L3T4-PE (Southern Biotech) and rat anti-mouse CD3e-APC (Southern Biotech) for 30 mins on ice. Cells were then washed and resuspended with PBS and analyzed by flow cytometry. From every sample 20,000 cells were analyzed. Lymphocytes were gated based on forward and side-scatter characteristics. The CD4/CD8 ratio was calculated from the percentage of $CD4^+$ and $CD8^+$ in the lymphocyte cell population.

Measurement of cytokine levels. The mouse sera were screened for cytokine levels of TNF-α, IFN-γ, IL-1β, IL-5, IL-6, IL-10, and IL-12(p20) using the Luminex-multiplex cytokine assay, following the manufacturer's instructions (Bio-Rad). The lower limits of detection were 1.1 pg/ml (TNF-α), 0.49 pg/ml (IFN-γ), 1.27 pg/ml (IL-1β), 0.19 pg/ml (IL-5), 0.07 pg/ml (IL-6), 0.21 pg/ml (IL-10), and 0.43 pg/ml (IL-12(p20)).

Statistical methods. Data are presented as geometric means ±SEM. Unpaired t test was used to determine differences in antibody titer or the CD4/CD8 ratio with P value of ≤ 0.05 to be considered statistically significant. In all analyses of antibody responses, the $\log_{10}$ of dilutions was used.

Results

Effect of adjuvants on anti-Pn14PS IgG antibody response, IgG subclass distribution, avidity and phagocytosis titer. Groups of five mice were immunized intracutaneously with neoglycoconjugate of Pn14PS in combination with different adjuvants or without adjuvant (Table 1). Booster immunizations were given on week 5 and 10 with the same dose of neoglycoconjugate without adjuvant. The level of anti-Pn14PS antibodies before and after booster immunization was determined by ELISA. Immunization of mice with the neoglycoconjugate of Pn14PS without adjuvant induces no or very little IgG antibodies in a primary response (less than 0.2 log unit). All tested adjuvants, except CpG, improved the primary IgG response

by 1 – 1.5 log units at week 4 after immunization (Figure 1). Except for the CpG group, the anti-Pn14PS total IgG titers increased dramatically after the booster immunization. CpG as an adjuvant was not very effective in this respect; in fact the IgG antibody levels after the booster immunization were even lower than that in the no-adjuvant group (p *value* = 0.0242; Figure 1). The second booster immunization at week 10 did not significantly enhanced these titers further, e.g. the titer of IgG anti-Pn14PS antibodies obtained from the DDA group between week 6 and week 11 (p-*value* =0.9036; Figure 1)

The functional activity of the anti-Pn14PS IgG antibodies was determined by antibody avidity and phagocytosis titer in sera obtained 1 week after the second booster immunization. The avidity index (AI) of the sera increased in those groups who had received at the primary immunization neoglycoconjugate of Pn14PS in combination with a single dose of Quil-A or DDA. Combinations of Quil A and MPL or DDA and CpG resulted in an AI equal to those groups in which no adjuvant was used (Figure3). Quil-A was the only tested adjuvant which caused a higher rate of phagocytosis of *S. pneumoniae* type 14 bacteria than serum of mice vaccinated without adjuvant (p *value* = 0.0063; Figure 4). Remarkably, CpG as an adjuvant at the moment of primary immunization resulted in a low AI (p-*value* = 0.2525) and a lower phagocytosis capacity (p *value* = 0.002) than non-adjuvant group after the booster immunization.

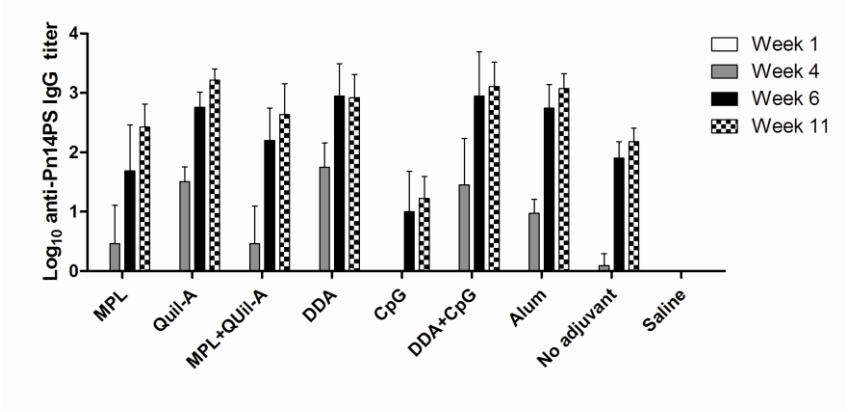

Figure 1. Level of anti-Pn14PS IgG antibodies. Group of mice (n=5) were immunized intracutaneously with neoglycoconjugate of Pn14PS and codelivered with series of adjuvants (Table 1). Mice sera were collected after the primary and booster immunization. ELISA was performed to measure the anti-Pn14PS antibodies.

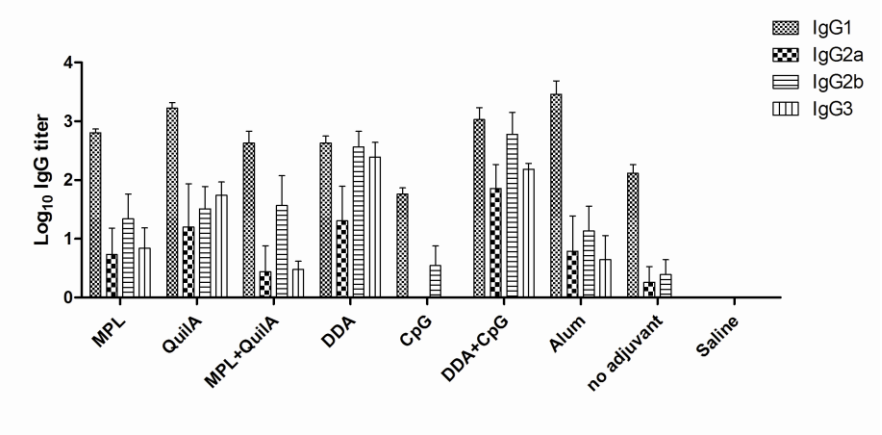

Figure 2. Anti-Pn14PS IgG antibodies subclass distribution. Mice sera were collected after the The first booster immunization. ELISA was performed to measure the anti-Pn14PS IgG antibodies subclass distribution: IgG1, IgG2a , IgG2b, and IgG3.

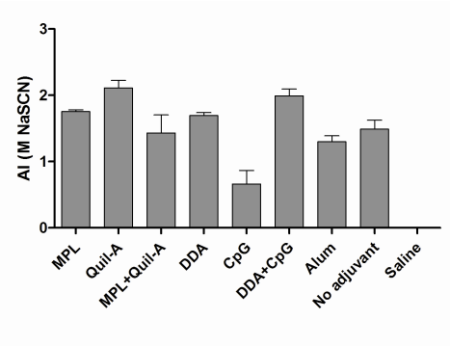

Figure 3. Antibody avidity. Mice sera (n=5) were collected on Week 11. The avidity index (AI) is expressed as the concentration of NaSCN needed to reduce the OD450nm by 50% (15). The absorbance value of sera without NaSCN should be at least 0.5.

Without adjuvants, the neoglycoconjugate of Pn14PS mainly induces, after booster immunization, an IgG antibody response which is dominated by the IgG1 subclass (Figure 2). The diversification of anti-Pn14PS antibodies to other IgG subclasses was investigated after the booster immunization given at week 5 (Figure 2). Primary immunization with neoglycoconjugate and Quil-A or DDA as a single adjuvant or in combination with MPL and CpG, respectively evoked after the first booster anti-Pn14PS IgG antibodies of the IgG1, IgG2a, IgG2b and IgG3 subclasses (Figure 2). CpG and Alum as a single adjuvant mainly induces anti-Pn14PS IgG1 antibodies subclass. The highest levels of IgG3 (1.96±0.56 log unit) were reached in mice which received DDA as an adjuvant.

Cytokine production after the primary immunization. Pooled sera from mice immunized with neoglycoconjugate of Pn14PS and the various adjuvants were used to measure the cytokine profiles. Cytokines were measured in serum obtained at six hours and 72 hours after primary immunization by luminex-multiplex cytokine assay. We found that most adjuvants cause increased serum levels of IL-6 and IL-1β as compared to the mice vaccinated without adjuvants (Figure 5). Quil-A, either as a single adjuvant or in combination with MPL induced the highest levels of the Th2 cytokine IL-5 at six hours after primary immunization which declined three day later (Figure 5). MPL and DDA, as a single adjuvant or in combination, caused detectable levels of IL-12 p70 (a Th1 inducing cytokine). Serum levels of IL-10 and TNF-α did not discriminate between the various adjuvants. No increase in systemic IFNγ was detectable after primary immunization in any of the groups (data not shown).

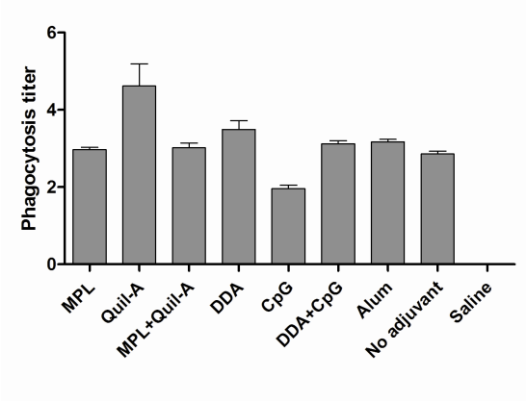

Figure 4. Phagocytosis titer. The phagocytosis titer was established using flow cytometry with heat-inactivated FITC-labelled *S. pneumoniae* type 14 and J774A.1 cells. The phagocytosis titers are expressed as the $\log_{10}$ of the serum dilution during phagocytosis that resulted in 25% of the J774A.1 cells being positive for FITC (9).

Changes in CD4/CD8 T cells ratio. The dynamics of the T cell response after primary immunization was studied by measuring CD4/CD8 T cells ratios in peripheral blood and draining lymph nodes (LNs). We found that a single dose of Quil-A led to higher CD4/CD8 T cells ratios than the other adjuvant groups and the no adjuvant group as well as saline group in peripheral blood at 6 (p *value* = 0.0147) and 24 hrs (p *value* = 0.0407; Figure 6) as well as in combination with MPL. The CD4/CD8 T cells ratio in LNs was observed to increase in the group with single dose of Quil-A (p *value* = 0.1410) or in combination with MPL adjuvant (p *value* = 0.0093) at 24 hrs with the no adjuvant group (Figure 6). The ratio's in blood and LNs were restored after 168 hrs (day 7) after primary immunization.

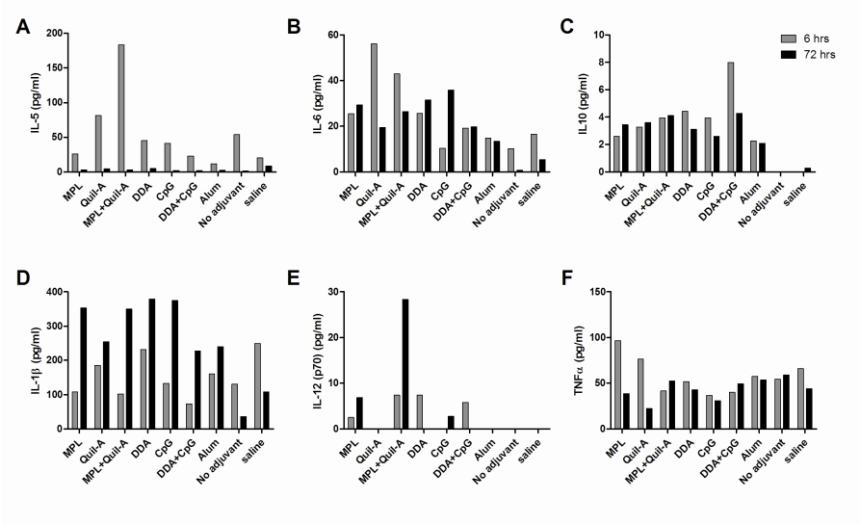

Figure 5. *In-vivo* cytokine production after primary immunization. Mice sera were collected at 6 and 72 hours Day 3 after primary immunization. Level of cytokines, i.e. IL-5 (A); IL-10 (B); IL-6 (C); IL-1β (D); IL-12 (E); and TNFα (F), was measured using the luminex-multiplex cytokine assay.

Discussion

In the present study, a neoglycoconjugate to Pn14PS was codelivered with a series of adjuvants to investigate the antibody- and cell-mediated immune responses in a mouse model. The adjuvant effect was shown in an increase of the magnitude of the IgG antibody response and by a diversification of anti-Pn14PS IgG antibodies (other subclasses were evoked), and an enhanced avidity and enhanced opsonic activity of these antibodies. Previously it was reported that especially the Quil-A adjuvant enhanced and promoted the diversification of the antibody response against different antigens (4,10,14,23). That was confirmed in case Quil-A was administered either alone as a single adjuvant or in combination with MPL. We found that next to Quil-A also DDA as a single dose or in combination had a similar effect on the diversification elicited a broader anti-Pn14PS IgG antibody subclasses. CpG or alum as adjuvants lead to an immune response dominated by IgG1 antibodies after booster immunization comparable to the response in mice immunizaed without adjuvants.

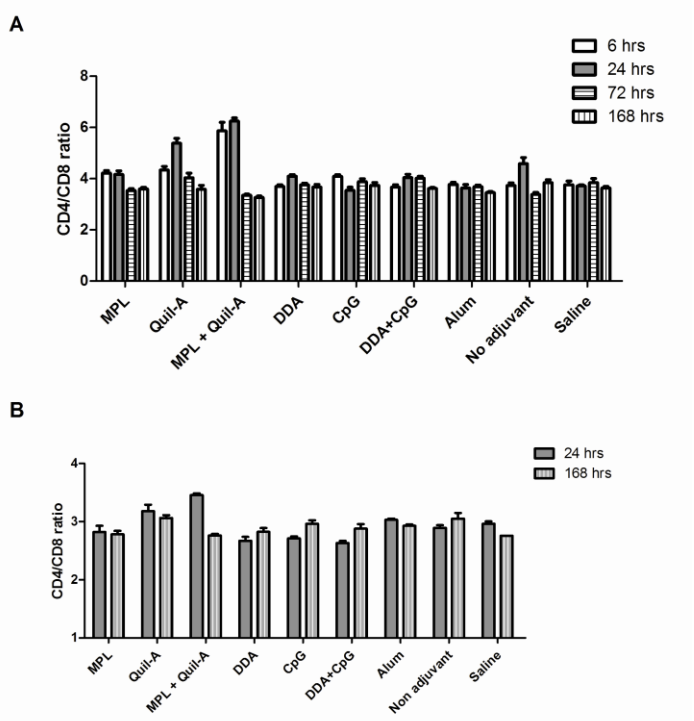

Figure 6. CD4/CD8 T cells ratio after the primary immunization. Lymphocyte cells were isolated from peripheral blood (A) and lymph nodes (B). Blood were collected at 6 hours (white bars), 24 hours (grey bars), 72 hours (black bars), and 168 hours (black and white bars) after the primary immunization. Mice (n=2) were killed by cervical dislocation on 24 hours and 168 hours. CD4/CD8 T cells ratio was calculated from percentage of $CD4^+$ and $CD8^+$ T cells population in lymphocytic cells.

We also observed that the neoglycoconjugate of Pn14PS immunization with codelivered of CpG showed low anti-Pn14PS IgG antibodies, antibody avidity and phagocytosis titer compared with other adjuvants. Lefeber et al. also observed that neoglycoconjugate related to *S. pneumoniae* type 3 polysaccharide (Pn3PS) immunized and codelivered with CpG as an adjuvant did not lead to higher anti-Pn3PS IgG antibodies, the response was even lower than that in mice vaccinated without adjuvants (14). However other studies showed that polysaccharide-protein conjugate vaccine immunization with codelivered of CpG significantly enhanced anti-polysaccharide IgG responses in a mouse model (2,27).

Cytokines are important mediators of a number of critical steps during the immune response, including differentiation of effector cells (20,21). Victoratos et al. suggested that adjuvants may direct the isotype switching process via induction of certain cytokines (26). Here we observed that cytokine profiles in blood after primary immunization could not be related directly to switch to other subclasses evoked after the booster immunization. Lefeber et al. reported that the use of Quil-A or a combination of the adjuvants CpG and DDA resulted in the highest phagocytic capacities and the highest levels of T helper 1 (Th1)-related IgG subclasses (14). In the present study we found that the Quil-A or in combination with MPL induced a rather high level of IL-5 after the primary immunization. IL-5 produced by Th2 cells acts as a B-cell differentiation factor by stimulating activated B cells to secrete antibody (16). The adjuvant of Quil-A was also observed to induce more IL-6 than the other adjuvants. Both IL-6 and IL-β are markers of inflammation. One of the effects of IL-6 is to inhibit Th1 differentiation and promote Th2 differentiation (5,6). Here we observed that the adjuvant of Quil-A induced higher IL-6 than other adjuvants after primary immunization. Overall our data suggest that Quil-A induces a Th2 response rather than Th1 response. Codelivery of adjuvants of DDA or MPL as a single dose induces anti-polysaccharide IgG antibodies of various subclass and detectable IL-12 p70 after primary immunization. IL-12 is a potent inducer of Th1 cells differentiation and inducer of IFN-γ production by T and natural-killer cells (22). The data thus suggested that DDA and MPL are Th1 directing adjuvant. Overall the data indicate that both Th1 and Th2 inducing adjuvants can enable an anti-polysaccharide antibody response with participation of various IgG subclasses.

The dynamics of T cells after primary immunization were followed by measuring CD4/CD8 T cells ratio in peripheral blood and lymph nodes (LNs). In this study, we found that a single dose of Quil-A or in combination with MPL displayed higher CD4/CD8 T cells ratio than other adjuvant groups in peripheral blood and LNs after primary immunization. This result confirmed that codelivered of Quil-A enhanced the cell-mediated immune response to neoglycoconjugates. It is unknown what constitutes the rate limiting step for reaching sufficiently high anti-polysaccharide antibodies with neoglycoconjugates: antigen presentation, T cell activation by the carrier protein or the B cell activation by the oligosaccharide. Quil-A adjuvant given during primary immunization determines the outcome of the antibody response to neoglycoconjugate by promoting an inflammatory response and a temporary increase of CD4 T cells in LNs and blood. From previous studies in Chapter 6, we know that splenic T cells can be activated by carrier peptide to

produce Th1 and Th2 cytokines. Further studies will be required to determine whether Quil-A, and other adjuvants for that matter, recruits peptide specific Th cells to the local LNs which can interact with the oligosaccharide specific B cells to generate an optimal anti-polysaccharide antibody response.

Acknowledgement

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Chapter 5

The immune response to group B streptococcus type III capsular polysaccharide is directed to the -Glc-GlcNAc-Gal- backbone epitope

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Submitted

Abstract

The structures of the branched capsular polysaccharides of *Streptococcus pneumoniae* type 14 (Pn14PS) and group B streptococcus type III (GBSIIIPS) are identical apart from the (α2→3)-linked sialic acid in the side chains of GBSIIIPS. The present study tries to determine the minimal epitope in GBSIIIPS, using both a panel of anti-Pn14PS mouse sera and sera of humans vaccinated with either Pn14PS or GBSIIIPS. Type-specific Pn14PS antibodies that recognize the branched structure of Pn14PS have a low affinity for the native GBSIIIPS and do not promote opsonophagocytosis of GBSIII. Desialylation of GBSIIIPS results in dramatically higher affinity of anti-Pn14PS antibodies and subsequent opsonophagocytosis. Epitope specific anti-Pn14PS mouse antibodies and human sera of PCV7 vaccinees only recognized structures with the branching element -Glc-(Gal-)GlcNAc-, in particular -Gal-Glc-(Gal-)GlcNAc- in Pn14PS. On the other hand anti-GBSIIIPS human antibodies recognize predominantly the linear structure in the backbone of Pn14PS or GBSIIIPS, i.e., -Glc-GlcNAc-Gal-. This difference in antigenicity of Pn14PS and GBSIIIPS could be explained by differences in flexibility of the two polysaccharides caused by the presence or absence of sialic acid.

Introduction

Infections by group B streptococcus (GBS) or by *Streptococcus pneumoniae* constitute a major health risk in neonates and young children worldwide (6,18,30). The key to prevention of infection by capsulated bacteria such as *S. pneumoniae* (5,24,25), and GBS (2,3,21) is the generation of antibodies against their capsular polysaccharide antigens. GBS isolates derived from patients bear any one of nine antigenically distinct polysaccharides which determine the GBS type and are thought to play a key role in virulence (7). All CPSs are high molecular weight and type Ia, Ib, II, III and V are composed of repeating units of glucose, galactose, *N*-acetylglucosamine, and *N*-acetylneuraminic acid (sialic acid) (1). Isolates from newborns are most commonly GBS type III (GBSIII) whereas isolates from adults are most commonly GBS type V (4,9,12,14,23). More than ninety serotypes of *S. pneumoniae* have been identified on the basis of their capsular polysaccharides, whereby *S. pneumoniae* type 14 (Pn14) is one of the major infection sources in newborns and adults (16).

The relation between GBSIII and Pn14 bacteria, in particular the similarity of their capsular polysaccharides, has long been investigated (8,13,15,19). The structures of the branched capsular polysaccharides of Pn14 (Pn14PS) and GBSIII (GBSIIIPS) differ only in the absence (in Pn14PS) or presence (in GBSIIIPS) of the (α2→3)-linked sialic acid in their side chains (Figure 1) (19). In general, sialic acid on bacteria mimics mammalian cell surface sialic acid residues and thus can subvert immune clearance mechanisms (28).

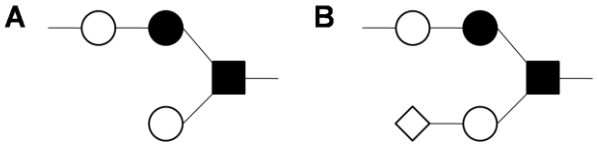

Figure 1. Schematic polysaccharide structures of Pn14 and GBSIII. The primary structures of the branched capsular polysaccharides of *S. pneumoniae* type 14, Pn14PS (A), and group B streptococcus type III, GBSIIIPS (B), differ only in that their side chains do not contain (Pn14PS) or do contain (GBSIIIPS) (α2→3)-linked sialic acid, *N*-acetylneuraminic acid (Neu5Ac): {→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[±α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-]β-D-Glc*p*NAc-(1→}$_n$. Open circle = galactose (Gal); filled circle = glucose (Glc); filled square = *N*-acetylglucosamine (GlcNAc); and open diamond = sialic acid, *N*-acetylneuraminic acid (Neu5Ac).

The majority of healthy adults responding to GBSIII vaccines with a fourfold or greater increase in GBSIII-specific IgG antibodies developed antibodies cross-reacting with Pn14PS (i.e. desialylated GBSIIIPS) (15). In previous reports, we have shown in a mouse model that the branched tetrasaccharide structure Gal-Glc-(Gal-)GlcNAc, which represents one repeating unit of Pn14PS, is essential and sufficient for inducing Pn14PS-specific antibodies that promote opsonophagocytosis of *S. pneumonia* (22,26). In fact, the branching element Glc-(Gal-)GlcNAc was fundamental for induction of an antibody response (26).

The present study was aimed to elucidate the antigenic determinant in GBSIIIPS. Two approaches were followed. First, heat-inactivated GBSIII bacteria, which were treated or not treated with sialidase (desialylated GBSIII), were tested for their capacity to be recognized by specific anti-Pn14PS antibodies. The type-specific Pn14PS antibodies were prepared in mice using Pn14PS and a series of synthetic overlapping oligosaccharide fragments of Pn14PS, all conjugated to the cross-reactive material of diphtheria toxin, CRM_{197} (CRM-neoglycoconjugates) (26). Second, sera of adult humans vaccinated with a pneumococcal conjugate vaccine containing a.o. the Pn14PS-CRM_{197} conjugate (PCV7, Prevnar, Wyeth) and one pool of sera obtained from five humans vaccinated with an experimental GBSIIIPS-tetanus toxoid conjugate vaccine were tested for their binding capacity to immobilized Pn14PS, GBSIIIPS and the synthetic Pn14PS oligosaccharide fragments, mentioned above. The combined data point towards -Glc-GlcNAc-Gal- as the dominant epitope in GBSIIIPS.

Materials and Methods

Group B streptococcus. GBS of different serotypes were obtained from the Reference Laboratory for Bacterial Meningitis, Department of Medical Microbiology, Academic Medical Center, Amsterdam, Netherlands. The GBS strains were plated on blood agar and incubated at 37°C overnight, after which they were inoculated into yeast extract broth containing 0.2% glucose and incubated at 37°C overnight. The culture was then centrifuged and washed with phosphate buffered saline (PBS) and the pellet was resuspended in PBS to an optical density (OD) value of 1.0 at 660 nm. The bacteria were inactivated by heating at 56°C for 1 h and stored at –20°C. Purified polysaccharide of GBSIII (GBSIIIPS) was a kind gift from Dr. Dennis Kasper (Channing laboratory, Harvard Medical School, Boston, MA, USA).

Table 1. Summary of CRM-neoglycoconjugates and anti-Pn14PS antibody levels in mice, immunized with these conjugates.

Code	Oligosaccharide fragment	Log_{10} IgG titer[1]
JJ42	Gal-Glc-GlcNAc-spacer	ND[2]
JJ141	Glc-GlcNAc-Gal-spacer	ND
JJ118	GlcNAc-Gal-Glc-spacer	ND
DM65	Gal-Glc-GlcNAc-Gal-spacer	ND
JJ153	Glc-GlcNAc-Gal-Glc-spacer	ND
JJ5	Glc-(Gal-)GlcNAc-Gal-spacer	1.1±0.2
JJ1	Gal-Glc-(Gal-)GlcNAc-spacer	3.0±0.3
JJ9	Gal-GlcNAc-Gal-Glc-spacer	ND
JJ6	Glc-(Gal-)GlcNAc-Gal-Glc-spacer	ND
DM35	Gal-Glc-GlcNAc-Gal-Glc-spacer	ND
DM66	Gal-Glc-(Gal-)GlcNAc-Gal-spacer	2.8±0.5
JJ10	Gal-GlcNAc-Gal-Glc-(Gal-)GlcNAc-spacer	0.5±0.4
DM36	Gal-Glc-(Gal-)GlcNAc-Gal-Glc-spacer	2.4±0.7
JJ4	Gal-Glc-(Gal-)GlcNAc-Gal-Glc-(Gal-)GlcNAc-spacer	2.3±0.4
ML2	Gal-Glc-(Gal-)GlcNAc-Gal-Glc-(Gal-)GlcNAc-Gal-Glc-(Gal-)GlcNAc-spacer	2.6±0.4
Pn14PS	Native polysaccharide type 14	2.6±0.5

[1] Levels of anti-Pn14PS antibodies obtained from mice immunized with CRM-neoglycoconjugates of synthetic fragments of Pn14PS and Pn14PS itself (Data from Safari et al (26)).
[2] ND = not detectable (The limits of detection level is <0.1 (log_{10} IgG titer))

Mouse and human sera. A panel of sera was obtained from mice immunized with 16 different neoglycoconjugates, consisting of the natural Pn14PS itself and synthetic overlapping oligosaccharide fragments of Pn14PS conjugated to the protein CRM_{197} (Table 1), as reported previously (26). Seven human sera (Code: AH-1 to AH-7) were available from healthy individuals vaccinated with the 7-valent pneumococcal conjugate vaccine (PCV7; Prevnar, Wyeth, Madison, NJ, USA) containing Pn14PS-CRM_{197} as one of the constituents. The paired sera (pre- and post-vaccination) were kindly provided by the Department of Medical Microbiology and Immunology, St Antonius Hospital, Nieuwegein, Netherlands. Standard Human Reference Serum III (SHRSIII) is a pool of five sera of healthy volunteers vaccinated with an experimental GBSIII-tetanus toxoid conjugate vaccine as described previously (21). The SHRSIII as well as a mouse monoclonal antibody against GBSIIIPS were kindly provided by Dr. Dennis Kasper.

Direct-binding assay by flow cytometry. Heat inactivated GBS bacteria (10^8 cells/ml) were treated without or with *Arthrobacter ureafaciens* sialidase (0.2 U/ml; Roche Diagnostic Corp., Mannheim, Germany) at 37°C for 1 h with shaking at 600 rpm. After washing with PBS, 50 µl of a series of diluted mouse sera (see Table 1) were added, and the mixture was incubated at 4°C for 30 min with shaking at 600 rpm, then washed with PBS. Goat-anti-mouse IgG labelled with fluorescein

isothiocyanate (FITC) (Dako, Glostrup, Denmark; diluted 1:100) was added and the mixture incubated again under the same conditions. After washing, the bacterial pellets were resuspended in PBS and analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA).

Opsonophagocytosis assay. The opsonophagocytosis assay was performed by using human polymorphonuclear leukocytes (PMN cells), isolated from peripheral blood of healthy donors, as previously described (10). Heat-inactivated GBSIII bacteria (10^9 cells/ml) were treated with or without *Arthrobacter ureafaciens* sialidase (0.2 U/ml) at 37ºC for 1 h with shaking at 600 rpm. After washing, the bacterial pellet was resuspended in PBS and incubated with 0.5 mg/ml of FITC (Sigma Chemical Co.) at room temperature for 2 h. The pellet was washed and resuspended in PBS (OD_{660nm} = 1.0) and stored at –20ºC until use. The assay was performed by mixing 20 µl of PMN cells ($3x10^6$ cells/ml), 20 µl of FITC-labeled heat-inactivated GBS treated or not treated with sialidase ($3x10^7$ cells/ml), and 20 µl diluted heat-inactivated (56°C, 30 min) diluted sera in PBS, followed by incubation at 37°C under vigorous shaking for 50 min. The mixtures were then fixed with cold 2% paraformaldehyde in PBS and analyzed by flow cytometry. For each sample, the FITC intensity was measured of 10,000 PMNs, gated on forward and side-scatter characteristics.

ELISA. Enzyme-linked immunosorbent assay (ELISA) was performed as previously described (26). Briefly, purified GBSIIIPS (3 µg/ml), Pn14PS (3 µg/ml) or bovine serum albumi (BSA)-neoglycoconjugates of Pn14PS (1 µg/ml) was coated on flat-bottom 96-well plates (Corning, Inc., Corning, NY, USA) and incubated at 37ºC overnight. The carbohydrate parts of the BSA-neoglycoconjugates comprise the synthetic oligosaccharide fragments JJ118, JJ141, JJ42, JJ9, JJ153, JJ5, DM65, DM35, JJ6, DM66, and JJ10, related to Pn14PS (Table 1), and mannose (BSA-mannose). After washing, the coated plates were treated with 3% (w/v) gelatin to block non-specific binding, then diluted human sera were added and the plates incubated at 37°C for 1 h. This step was followed by incubation with horseradish-peroxidase-conjugated goat-anti-human IgG (Dako, Glostrup, Denmark) for 1 h at 37ºC. Color development was initiated by incubation with a mixture of 3,3',5,5'-tetramethylbenzidine (Sigma Chemical Co, St. Louis, MO, USA) and H_2O_2 (Sigma Chemical Co), and the reaction stopped by addition of 0.5 M of H_2SO_4. Absorbance was measured at 450 nm.

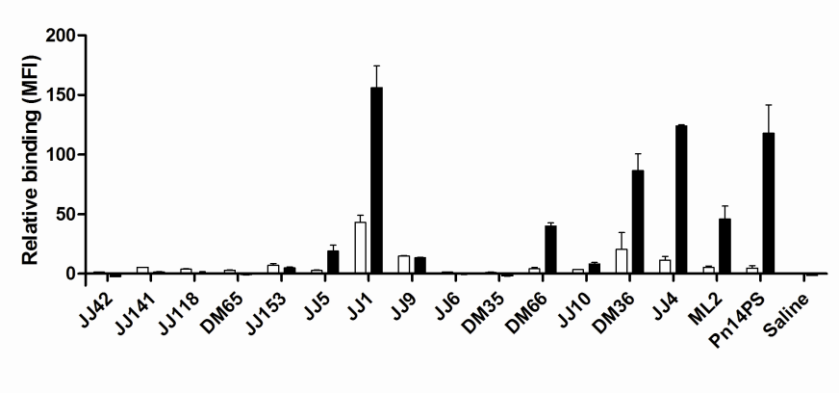

Figure 2. Direct binding between positive and negative mice anti-Pn14PS sera and GBSIII bacteria. Heat-inactivated native GBSIII (open bars) or desialylated GBSIII (filled bars) were incubated with pooled mice sera (1:50) obtained from a series of CRM-neoglycoconjugates of Pn14PS immunization (Table 1) and mice sera obtained from Pn14PS-CRM_{197} (native polysaccharide type 14 conjugated to CRM_{197} protein; code: Pn14PS) immunization. Sera from Immunization with saline buffer was used as the negative control. The relative binding between mice sera and GBS III bacteria was measured by flow cytometry and was expressed as mean fluorescence intensity (MFI).

Results

Recognition of native and desialylated GBSIII bacteria by mouse type-specific Pn14PS sera. Flow cytometry was used to detect interaction between antibodies induced in mice with a series of CRM-neoglycoconjugates of Pn14PS (Table 1) and heat-inactivated GBSIII in native or desialylated form. The sera which were positive in a Pn14PS ELISA, obtained from mice immunized with the branched tetrasaccharide fragment of Pn14PS: JJ1 (one repeating unit), DM36 (one repeating unit with two extra monosaccharides), DM66 (one repeating unit with one extra monosaccharide), JJ4 (two repeating), and ML2 (three repeating), as well as polysaccharide conjugate (Pn14PS) itself displayed significant binding (mean fluorescence intensity) to the desialylated GBSIII (Figure 2). Sera with low titers of anti-Pn14PS antibodies (Table 1; JJ5 and JJ10) showed also low binding to desialylated GBSIII. Sera in which anti-Pn14PS IgG antibodies were not detectable (Table 1) did not show any binding to desialylated GBSIII (Figure 2). Native GBSIII bacteria were only bound by antibodies from mice immunized with neoglycoconjugates of JJ1, JJ4, and DM36, and even then to a much lower degree

as desialylated GBSIII bacteria. Sera from mice immunized with the other CRM-neoglycoconjugates, showed virtually no binding to native GBSIII.

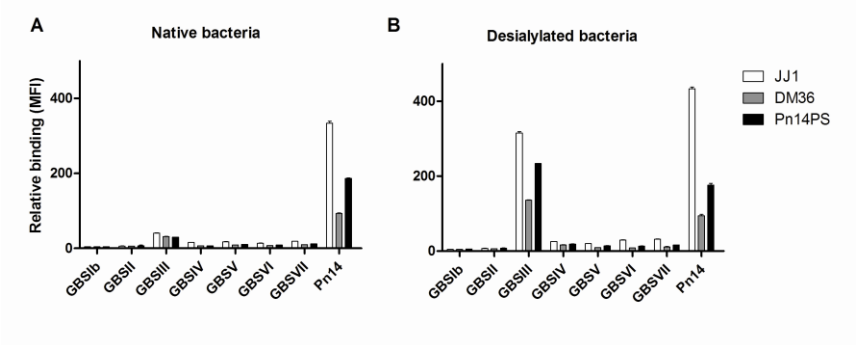

Figure 3. Cross reactivity between mice anti-Pn14PS sera and different GBS serotypes. Seven GBS different serotypes and *S. pneumoniae* type 14 (Pn14), native bacteria (A) or desialylated bacteria (B), were incubated with three mouse sera (diluted 1:50) obtained from neoglycoconjugate of JJ1 and DM36, and the polysaccharide conjugate (Pn14PS) immunization. The relative binding between mice sera and the bacteria was measured by flow cytometry and was expressed as mean fluorescence intensity (MFI).

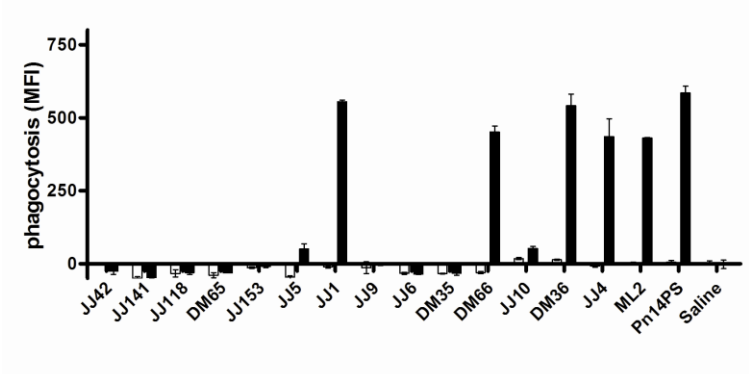

Figure 4. Opsonophagocytic capacity of PMN cells in the presence of sera from mice immunized with Pn14PS-related CRM-neoglycoconjugates (Table 1). The opsonophagocytosis assay was performed by using PMN cells, FITC-labeled heat-inactivated native (open bars) or desialylated (filled bars) GBSIII and heat-inactivated sera (diluted 1:20). The opsonophagocytosis promoted by different sera are expressed as mean fluorescence intensity (MFI).

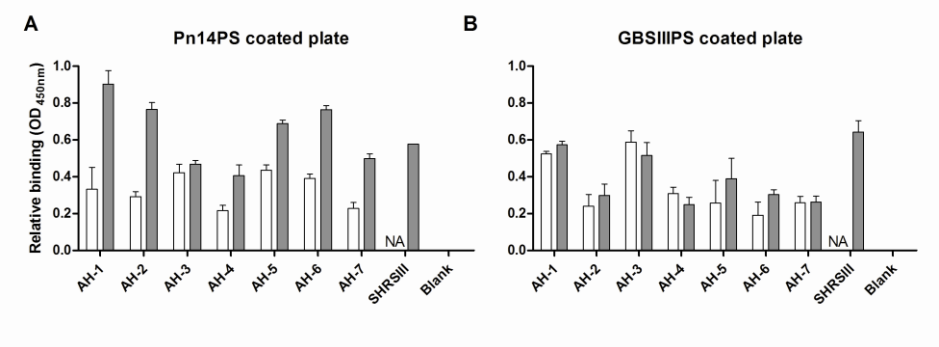

Figure 5. Level of human serum IgG antibodies against the polysaccharides Pn14PS (A) and GBSIIIPS (B) as coated materials. ELISA was performed to measure the level of antibodies against Pn14PS (A) and GBSIIIPS (B) from a series of human sera (diluted 1:50) before (open bars) and after (filled bars) immunization with PCV7, containing CRM_{197}-Pn14PS as one of its constituents (AH-1 to AH-7). SHRSIII is a pool of 5 human sera obtained after vaccination with an experimental GBSIII-tetanus toxoid conjugate vaccine. The data are expressed as the optical density (OD) value at 450 nm. NA = Not available.

Interaction of mouse anti-Pn14PS antibodies with other GBS serotypes than GBSIII. Although the various serotypes of GBS are covered by different polysaccharide capsules, they all contain a terminal ($\alpha 2 \rightarrow 3$)-linked sialic acid residue. In a subsequent series of experiments, the reactivity of type-specific Pn14PS mouse sera with the six GBS serotypes Ib, II, IV, V, VI, and VII were investigated. To this end, three mouse sera containing high levels of anti-Pn14PS IgG (Table 1; JJ1, DM36, and Pn14PS) were selected. None of these sera showed binding to the different native GBS serotypes nor to their desialylated forms (Figure 3). Sera with low or undetectable levels of anti-Pn14PS IgG antibodies also were negative for binding to native or desialylated GBS bacteria of any serotype (data not shown).

Anti-Pn14PS antibodies promote opsonophagocytosis of desialylated GBSIII but not native GBSIII bacteria. Human PMN cells were used to determine whether type-specific Pn14PS mouse sera (Table 1; CRM-neoglycoconjugates) promote the opsonophagocytosis of native and desialylated GBSIII. As is evident from Figure 4, none of the sera promoted the opsonophagocytosis of the native GBSIII bacteria. However, opsonophagocytosis of the desialylated GBSIII bacteria was promoted by all type-specific Pn14PS sera containing a high level (log IgG titer of 2.3 or more) of anti-Pn14PS antibodies: JJ1, DM66, DM36, JJ4, ML2, and native polysaccharide of Pn14PS conjugate (Figure 4,

Table 1). Sera with low anti-Pn14PS titers (JJ10 and JJ5) did not promote phagocytosis of the native or desialylated GBSIII bacteria by the PMN cells (Figure 4, Table 1). Mouse sera, which contained no detectable anti-Pn14PS IgG antibodies (Figure4, Table 1), also did not promote phagocytosis of either the native or the desialylated GBSIII bacteria.

Recognition of Pn14PS and GBSIIIPS by human sera. An ELISA was performed to measure the level of antibodies in humans, vaccinated against Pn14 and GBSIII bacteria. The paired human sera AH-1 to AH-7 were derived from individuals pre- and post-vaccination with the pneumococcal conjugate vaccine PCV7, containing Pn14PS-CRM_{197} as one of its constituents. For GBSIII, a pool of five sera of healthy volunteers vaccinated with an experimental GBSIII-tetanus toxoid conjugate vaccine was used (SHRSIII). The relative binding of the human sera AH-1 to AH-7 to coated Pn14PS increased after vaccination, except for AH-3 where there is no significant difference between pre- and post-vaccination (Figure 5A). When GBSIIIPS was used as a coat in the ELISA plate, significant binding was detected in AH1-AH7 but this did not increase after vaccination (Figure 5B). The human anti-GBSIII (SHRSIII) did interact strongly with GBSIIIPS as expected, but also with Pn14PS (Figure 5). Whether or not this is due to vaccination with GBSIII cannot be concluded from this experiment.

In subsequent studies the epitope specificity of human antibodies induced by vaccination with Pn14PS or GBSIII conjugate vaccines was investigated. To that end, BSA-neoglycoconjugates of linear JJ42, JJ141, JJ118, DM65, JJ153, JJ9, and DM35 (which do not induce detectable anti-Pn14PS titers when used as CRM-neoglycoconjugates), BSA-neoglyconjugates of branched JJ5, JJ10, and JJ6 (which induce low anti-Pn14PS IgG titers) and a BSA-neoglycoconjugate of branched DM66 (inducing a high anti-Pn14PS IgG titer) (Table 1) were used on coated plates. Vaccination with PCV7 resulted in the induction of antibodies which bind to the branched tetrasaccharide DM66 (Gal-Glc-(Gal-)GlcNAc-Gal) by sera of the individuals AH-1, AH-2 and AH-3 (Figure 6). Individuals AH-4, AH-5, and AH-6, while showing a comparable anti-Pn14PS antibody response after conjugate vaccination, did not recognize any of the linear or branched oligosaccharides tested (Figure 6). The pooled human serum SHRSIII (anti-GBSIIIPS) interacts strongly with the BSA-neoglycoconjugates JJ141 (Glc-GlcNAc-Gal), JJ42 (Gal-Glc-GlcNAc), JJ153 (Glc-GlcNAc-Gal-Glc) but displayed lower binding to the other linear or the branched oligosaccharides, including DM66 (Figure 6). It was noted that the pre- and post-PCV7 human sera of individual AH-3 bound equally well to JJ141, JJ42,

and JJ153 (Figure 6) in a pattern similar to that observed for the SHRSIII antiserum. Human sera AH-1 and AH-3 also interacted with BSA-JJ5 (Glc-(Gal-)GlcNAc-Gal) and BSA-JJ6 (Glc-(Gal-)GlcNAc-Gal-Glc); in fact JJ5 and JJ6 miss the galactosylated Glc residue at the non-reducing site, present in DM-66 (Gal-Glc-(Gal-)GlcNAc-Gal).

Discussion

The present study investigated the presence of common and unique epitopes in GBSIIIPS and Pn14PS by using type-specific mouse and human sera. The capsular polysaccharide in both GBSIII and Pn14 share a common polysaccharide backbone and differ only in their side chains: Pn14PS has β-D-Gal*p*-(1→4)- attached to the GlcNAc residue and GBSIIIPS α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-. The structure of Pn14PS (which is thus equivalent to desialylated GBSIIIPS) has been reported to be more flexible and disordered than the structure of GBSIIIPS. The rigidity of GBSIIIPS could have an impact on its immunogenicity (13,19), and based on the GBSIIIPS model it has been suggested that anti-GBSIIII antibodies recognize the core and not the surface of the helix (13). The conformation of the backbone is stabilized by interactions (hydrogen bonding) with the side chains, in particular with sialic acid.

Earlier, one structural repeating unit of Pn14PS, i.e. Gal-Glc-(Gal-)GlcNAc, has been reported to induce a specific antibody response to Pn14PS (22). Recently, we confirmed that the branched trisaccharide element Glc-(Gal-)GlcNAc is essential in inducing Pn14PS-specific antibodies and that the extra galactose unit at the glucose residue contributes clearly to the immunogenicity of the epitope (26). It has been shown in direct binding studies of GBSIIIPS with a panel of GBSIII specific monoclonal antibodies that 2 repeating backbone units form the minimum binding epitope (31). The present study has shown that type-specific Pn14PS antibodies that recognize the branched structure of Pn14PS have a low affinity for the native GBSIIIPS and do not promote opsonophagocytosis of GBSIII. Desialylation of GBSIIIPS, however, resulted in dramatically higher affinity of anti-Pn14PS antibodies in mice. These antibodies also promoted opsonophagocytosis of the desialylated GBSIII. These results demonstrate that GBSIII bacteria are protected from binding of antibodies against Pn14PS by a residue of (α2→3)-linked sialic acid, as described previously (17,20).

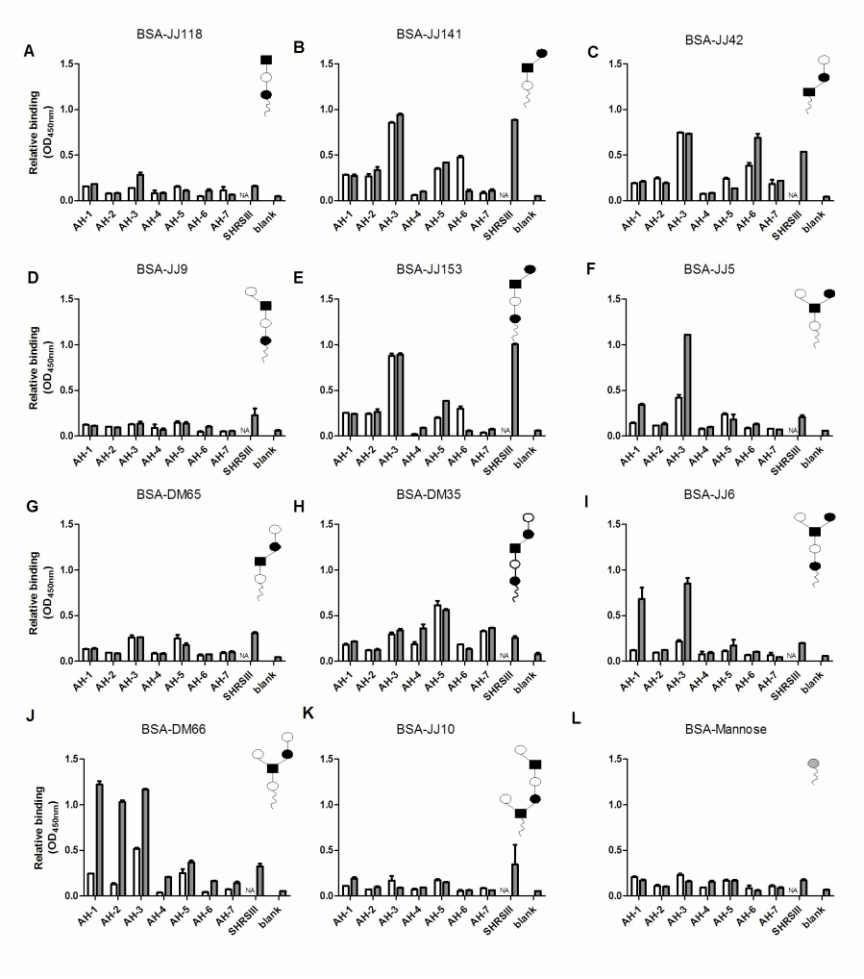

Figure 6. Direct binding between human serum and BSA-neoglycoconjugates. The same sera as presented in Figure 5 were tested in ELISA (dilution of 1:100) for their capacity to bind to a series of synthetic oligosaccharide fragments. The following BSA-neoglycoconjugates were used as coating material, i.e. JJ118 (A; GlcNAc-Gal-Glc), JJ141 (B; Glc-GlcNAc-Gal), JJ42 (C; Gal-Glc-GlcNAc), JJ9 (D; Gal-GlcNAc-Gal-Glc), JJ153 (E; Glc-GlcNAc-Gal-Glc), JJ5 (F; Glc-(Gal-)GlcNAc-Gal), DM65 (G; Gal-Glc-GlcNAc-Gal), DM35 (H; Gal-Glc-GlcNAc-Gal-Glc), JJ6 (I; Glc-(Gal-)GlcNAc-Gal-Glc), DM66 (J; Gal-Glc-(Gal-)GlcNAc-Gal), JJ10 (K; Gal-GlcNAc-Gal-Glc-(Gal-)GlcNAc), and mannose (L; man). Schematic structure of oligosaccharide fragments: filled circle = glucose; open circle = galactose, and filled square = *N*-acetylglucosamine. The data are expressed as the optical density (OD) value at 450 nm. NA = Not available.

Bacterial pathogens such as GBS are coated with terminal sialic acids which provides resistance to components of the host's innate immune response system (7,27). In a neonatal rat model of otherwise lethal GBS infection, loss of capsular sialic acid in a mutant strain was associated with a loss of virulence (11). Although GBS of all serotypes possess a terminal (α2→3)-linked sialic acid (7), we found no cross-reaction between type-specific Pn14PS sera and GBS of several serotypes, whether native or desialylated.

The linear Gal-Glc fragment in the backbone of the rigid GBSIIIPS has been reported to be part of the immunodominant epitope of GBSIII (29), and cross-reactions between some anti-GBSIII antibodies and Pn14PS (15) likely arise from recognition of a small linear epitope that is associated with the shared immunodominant region (19). In rabbits, immunized with GBSIII it was reported that two distinct populations of antibodies were induced: the major population of antibodies is dependent of the presence of sialic acid residues (the branched structure) and the other population of antibodies is not sialic acid dependent (located in the backbone of GBSIIIPS) (17). In the present study, we showed that human anti-Pn14PS antibodies only recognized the branched structure of Pn14PS with -Glc-(Gal-)GlcNAc-Gal- as the minimum epitope while human anti-GBSIIIPS antibodies recognized the linear structure of the backbone of Pn14PS and GBSIIIPS: -Glc-GlcNAc-Gal-.

Mice immunized with CRM-neoglycoconjugates of linear oligosaccharide fragments of Pn14PS and GBSIIIPS, e.g. Gal-Glc-GlcNAc, Glc-GlcNAc-Gal, and GlcNAc-Gal-Glc, do evoke specific oligosaccharide antibodies (26) but these antibodies neither bind native nor desialylated GBSIII. These linear structures are either too small or too flexible to evoke antibodies to the linear structure of the backbone of Pn14PS and GBSIIIPS. The difference in antigenicity of Pn14PS and GBSIIIPS might be due to a difference in flexibility of the two polysaccharides caused to the presence or absence of sialic acid (19).

In conclusion, the sialic acid of GBSIIIPS effectively shields the inner core of the polysaccharide and prevents recognition by type-specific Pn14PS antibodies. Pn14PS immunization elicits antibodies that only recognize the branched structure of Pn14PS while GBSIIIPS immunization elicits antibodies to the backbone structure of GBSIIIPS and Pn14PS. The antigenic determinant for GBSIII is the –Glc-GlcNAc-Gal- epitope located at the linear structure of the backbone.

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Chapter 6

Hybrid gold nanoparticles coated with a synthetic tetrasaccharide of *Streptococcus pneumoniae* type 14 capsular polysaccharide and ovalbumin peptide $OVA_{323\text{-}339}$ induce a carbohydrate-specific humoral immune response

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In preparation

Abstract

Gold nanoclusters coated with different amounts of the synthetic repeating unit branched tetrasaccharide β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)-]β-D-Glc*p*NAc-(1→spacer of *Streptococcus pneumoniae* type 14 polysaccharide (Pn14PS), the $OVA_{323-339}$-peptide fragment, and the monosaccharide D-glucose were prepared and their immunogenicity studied in BALB/c mice. Using well-defined thiol-terminated conjugates of the three components and a gold salt ($HAuCl_4$), it was possible to prepare the hybrid gold nanoparticles by *in situ* reduction with $NaBH_4$ in one step. The presence of the T-cell stimulating peptide $OVA_{323-339}$ in the hybrid gold nanoparticles was a prerequisite for the induction of specific anti-Pn14PS IgG antibodies. The molar ratio between tetrasaccharide, glucose and peptide in the hybrid gold nanoparticles turned out to be critical for optimal immunogenicity: Gold nanoparticles containing 45%of tetrasaccharide and 5% of peptide, supplemented with inert glucoconjugate, were able to trigger anti-Pn14PS antibodies. These data show that well designed hybrid gold nanoparticles display a promising platform towards the development of synthetic carbohydrate-based vaccines.

Introduction

Carbohydrates are usually poorly immunogenic and a strategy to improve carbohydrate-based vaccines is based on coupling capsular polysaccharides of pathogens to suitable immunogenic protein carriers (conjugate vaccines) (1). Current advances in identification and synthesis of carbohydrate epitopes has opened new ways to vaccine design. The group of Vérez-Bencomo developed the first approved humane vaccine against *Haemophilus influenzae* type b based on synthetic oligosaccharide fragments of the capsular polysaccharide antigen of this bacterium (31). Synthetic oligosaccharide fragments conjugated to protein carriers (neoglycoconjugates) have been investigated for a number of different *S. pneumoniae* capsular polysaccharides (29). The synthetic branched tetrasaccharide β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)-]β-D-Glc*p*NAc-(1→)-$(CH_2)_3NH_2$ [Gal-Glc-(Gal-)GlcNAc),corresponding to a single repeating unit of the *S. pneumoniae* type 14 capsular polysaccharide {→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)-]β-D-Glc*p*NAc-(1→}$_n$ (Pn14PS) was reported to be able to induce anti-Pn14PS specific antibodies when conjugated to cross reactive material of diphtheria toxin (CRM_{197}) protein (19,25).

Nanoparticulate systems, especially liposomes, have been proposed as carriers for vaccines delivery (28). Generally, the insertion of mannose derivatives into vaccine-loaded liposomes allow targeting of antigen presenting cells (APCs) to enhance the antigenic effect (33). Recently, a potential carbohydrate-based vaccine against the Gram-negative bacteria *Shigella flexneri* was developed by functionalization of liposomes with synthetic mono-, di-, and tri-meric oligosaccharides corresponding to the antigenic pentasaccharide repeating unit of its lipopolysaccharide and the "universal" T-helper peptide from the influenza virus hemagglutinin (26).

The possibility of bioconjugation of metal and semiconductor nanomaterials has opened new opportunities in design and synthesis of multifunctional and multimodal assembled systems for biomedical applications (9). Metallic nanoparticles display unique magnetic, electrical, optical, mechanical and chemical properties, which can be tuned by controlling size, shape and dispersity (approximately 1-100 nm), and by varying the core materials (11). Among the many possibilities in the choice of the core material, gold nanoparticles have been explored for their relative inertness, low toxicity, easy manipulation and ability to control the chemistry on the metal surface (4).

Our group has developed a simple and versatile method for obtaining sugar-functionalised gold nanoclusters (glyconanoparticles [GNPs]) that have 3D polyvalent

carbohydrate display and globular shapes. GNPs are water dispersible and stable under physiological conditions (3) and it is possible to insert different ligands in the same gold nanoplatform in a controlled way (3,21). Metallic glyconanoparticles have been widely used to study and evaluate carbohydrate-based interactions (16). Gold GNPs have been successfully used as multivalent chemical tools to demonstrate Ca^{2+}-dependent carbohydrate-carbohydrate interactions of the antigen determinant Le^X trisaccharide (8), the carbohydrate epitopes in the marine sponge *Microciona prolifera* (6), and lactose (24). GNPs have also been employed in selective aggregation studies where carbohydrate–protein interactions are involved by means of bioassays, in the detection, labelling and control of microorganisms through protein targeting, and in other biomedical applications (10).

In the present study, we explore the potential application of GNPs as vaccine carrier with synthetic oligosaccharides derived from bacterial capsular polysaccharides in a mouse model. A series of GNPs were prepared with different molar ratios between the above mentioned synthetic branched tetrasaccharide fragment of Pn14PS (25), and the immunodominant ovalbumin T-cell epitope ($OVA_{323-339}$-peptide) (5,23), and the monosaccharide glucose as inert component. Glucose was used as an inner and inert component to assist water dispersibility and biocompatibility and to allow the tetrasaccharide fragment moiety, armed with a long amphiphilic linker, protruding above the organic shell of GNPs (15,18). Subsequent studies on the immunogenicity of these hybrid gold nanoparticles, GNPs showed that they do induce a specific immune response against Pn14PS after intracutaneous administration to BALB/c mice. Furthermore, it has been proven by measuring cytokine levels *in vitro* that T cells recognizing the $OVA_{323-339}$–peptide play a crucial role in the induction of antibodies against native Pn14PS.

Materials and methods

General procedures. Except for chloroauric acid (Strem Chemicals), all chemicals were purchased as reagent grade from Sigma-Aldrich and used without further purification. Purified water was obtained from a Simplicity Ultrapure Water System (Millipore). NMR spectra were recorded on a Bruker instrument at 500 MHz for ^1H or at 125 MHz for ^{13}C; chemical shifts (δ) are given in ppm relative to the residual solvent signal. Infrared spectra (IR) using KBr pellets were recorded from 4000 to 400 cm^{-1} with a Nicolet 6700 FT-IR spectrometer (Thermo Spectra-Tech). UV/Vis spectra were measured with a Beckman Coulter DU 800 spectrophotometer. For transmission electron microscopy (TEM) examinations, a single drop (10 µl) of the aqueous solution (*ca.* 0.1

mg ml^{-1} in milliQ water) of the GNPs was placed onto a copper grid coated with a carbon film (Electron Microscopy Sciences). The grid was left to dry in air for several hours at room temperature. TEM analyses were carried out in a Philips JEOL JEM-2100F microscope working at 200 kV. Additional information is provided in the supplementary section.

Synthesis of the branched tetrasaccharide conjugate 6. Following a reported protocol, the known protected thioethyl tetrasaccharide 1 (Figure S-1 supplementary data) was treated with 3-azidopropanol in presence on NIS/AgOTf as a promoter system, leading to the corresponding 3-azidopropyl glycoside (90% yield) (30). Concomitant removal of the benzoyl groups and conversion of the phthalimido group into the *N*-acetylamido group provided 2 (74% yield over three steps). Subsequent cleavage of the bromoisopropylidene acetal in 2 with 90% CF_3COOH (96% yield) and reduction of the azido group under Pd-catalysed hydrogenation conditions provided β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→6)-[β-D-Gal*p*-(1→4)-]β-D-Glc*p*NAc-(1→$(CH_2)_3NH_2$ 3 (89% yield) as hydrochloric acid salt. Following a reported procedure, coupling of aminopropyl tetrasaccharide 3 with the isothiocyanate linker 4 in a water/isopropanol/acetonitrile mixture and triethylamine (18) gave thioacetyl-protected neoglycoconjugate 5 (71% yield). After deprotection neoglycoconjugate 6 was obtained as a mixture of disulfide and thiol (~2.5:1, 81% yield), which could be used without further separation for the preparation of the GNPs under reductive conditions (Figure S-2 and Figure 1). Experimental details are provided in the supplementary section.

Synthesis of glucose neoglycoconjugate 7. Glucose conjugate 7 (Figure 1) was prepared according to the literature (18). Experimental details are provided in the supplementary section.

$OVA_{323-339}$-peptide fragment. The $OVA_{323-339}$-peptide fragment 8, consisting of ISQAVHAAHAEINEAGR with an additional glycine and mercaptopropionic acid (MPA) linker at the *N*-terminal end (Figure 1) was ordered from GenScript Corp. (New Jersey, USA).

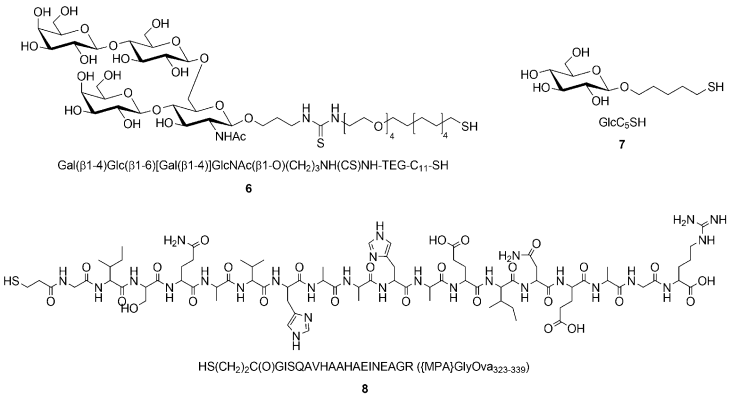

Figure 1. Synthetic branched tetrasaccharide conjugate 6, the monosaccharide glucose conjugate 7, and $OVA_{323-339}$ peptide conjugate 8. For clarity reasons, all conjugates are depicted as thiols.

Gold Glyconanoparticles. A 0.012 M (3 equiv.) methanolic solution of the appropriate mixture of thiol-armed compounds 6, 7 and 8 in different ratios was added to a solution of tetrachloroauric acid (0.025 M, 1 equiv.) in water (Figure 2). An excess of 1 M aqueous solution of $NaBH_4$ was then added in four portions, with vigorous shaking. The black suspension formed was shaken for an additional 2 h at 25 ^{o}C after which the supernatant was removed and analysed. The residue was dissolved in a minimal volume of Nanopure water and purified by dialysis or by centrifugal filtering. For the ligand analysis, ^{1}H NMR spectra of the glycoconjugate mixtures used for the GNPs synthesis and of the products recovered from the supernatants after GNPs formation were recorded. The ratio of the ligands in the GNPs was evaluated through integration of the signals of the anomeric protons of tetrasaccharide 6 with respect to the anomeric proton of glucoside 7 and the shielded methyl groups of isoleucine (Ile) and valine (Val) of OVA peptide conjugate 8 (Table 1). The particle size distribution (average gold diameter) of the gold nanoparticles was evaluated from TEM micrographs (see supplementary data). The average number of gold atoms and molecular formulas of the GNPs were calculated on the basis of the average diameter obtained by TEM according to previous works (Table 1) (12,18).

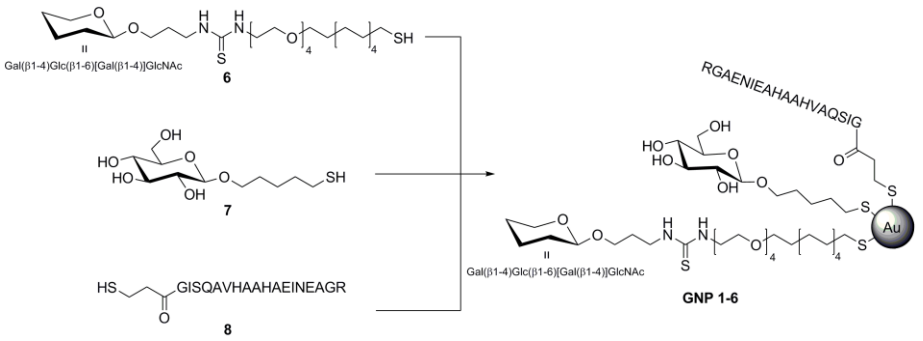

Figure 2. One-step synthesis of gold glyconanoparticles (GNPs) incorporating synthetic conjugates of branched tetrasaccharide (compound 6), glucose (compound 7), and Ova peptide (compound 8) in a controlled way. Reagents and conditions: $HAuCl_4$, $NaBH_4$, H_2O/MeOH, 2 h, 25 ^oC. For clarity reasons, all conjugates are depicted as thiols and the dimension of the gold nanocluster is not in scale with respect to the ligands.

GNP-1a and GNP-1b. OVA peptide derivative 8 (0.34 mg, 0.18 µmol) was dissolved in CF_3COOD (50 µl) and dried under air stream until formation of an oil. Tetrasaccharide conjugate 6 (1.89 mg, 1.6 µmol) and glucose conjugate 7 (0.5 mg, 1.8 µmol) were added to the oil, and the mixture was dissolved in CD_3OD-D_2O (1:1) (600 µl). ^1H NMR analysis of the mixture showed a compound 6:7:8 ratio of ~9:10:1 (Figure S-4, supplementary data). After evaporation of the solvent, MeOH was added up to a 0.012 M concentration of organic material, and the pH was adjusted to 1 by addition of CF_3COOH. Then, an aqueous solution of $HAuCl_4$ (28.3 µl, 0.025 M) was added followed by an aqueous $NaBH_4$ solution (15 µl, 1 M) under rapid shaking. The black suspension was shaken for 2 h, then the supernatant was separated by decantation. The black solid was washed with EtOH (4 x 1 ml) and MeOH (3 x 1 ml), dissolved in Nanopure water (0.5 ml) and purified by centrifugal filtering (AMICON MWCO 10000, 50 min, 10000 rpm). The residue in the AMICON filter was dissolved in 0.5 ml of Nanopure water and lyophilised to afford 0.7 mg of GNP-1. UV/Vis (H_2O, 0.1 mg ml^{-1}): surface plasmon band not observed. TEM: average gold diameter 1.8 ± 0.5 nm (Figure S-11; see supplementary data). Average molecular formula: $Au_{201}(C_{49}H_{90}N_3O_{25}S_2)_{32}(C_{11}H_{21}O_6S)_{35}(C_{79}H_{126}N_{27}O_{27}S)_4$ (MW 95 KDa). IR (KBr) and ^1H NMR (500 MHz, D_2O): see supplementary data.

GNP-2. Tetrasaccharide conjugate 6 (4.6 mg, 3.9 µmol) and glucose conjugate 7 (1.1 mg, 3.9 µmol) were dissolved in D_2O (656 µl). ^1H NMR analysis of the mixture showed a compound 6:7:8 ratio of ~1:1 (Figure S-5, see supplementary data). After evaporation of the solvent, MeOH was added up to a 0.012 M concentration of organic material. Then

an aqueous solution of $HAuCl_4$ (62.8 µl, 0.025 M) was added followed by an aqueous $NaBH_4$ solution (33 µl, 1 M) under rapid shaking. The black suspension was shaken for 2 h, then the supernatant was separated by decantation. The black solid was washed with EtOH (4 x 1 ml) and MeOH (3 x 1 ml), dissolved in Nanopure water (0.5 ml) and purified by centrifugal filtering (AMICON MWCO 10000, 50 min, 10000 rpm). The residue in the AMICON filter was dissolved in 0.5 ml of Nanopure water and lyophilised to afford 0.86 mg of GNP-2. UV/Vis (H_2O, 0.1 mg ml^{-1}): surface plasmon band not observed. TEM: average gold diameter 1.9 ± 0.3 nm (Figure S-11, see supplementary data). Average molecular formula: $Au_{225}(C_{49}H_{90}N_3O_{25}S_2)_{36}(C_{11}H_{21}O_6S)_{35}$ (97 KDa). ^1H NMR (500 MHz, D_2O): see supplementary data.

GNP-3. OVA peptide derivative 8 (1.68 mg, 0.87 µmol) was dissolved in CF_3COOD (100 µl) and dried under air stream until formation of an oil. Glucose conjugate 7 (2.2 mg, 7.8 µmol) was added to the oil, and the mixture was dissolved in CD_3OD-D_2O (1:1) (600 µl). ^1H NMR analysis of the mixture showed a compound 7:8 ratio of ~9:1 (Figure S-6, supplementary data). After evaporation of the solvent, MeOH was added up to a 0.012 M concentration of organic material. Then, an aqueous solution of $HAuCl_4$ (70 µl, 0.025M) was added followed by an aqueous $NaBH_4$ solution (37 µl, 1 M) under rapid shaking. The black suspension was shaken for 2 h, then the supernatant was separated by decantation. The black solid was washed with EtOH (4 x 1 ml) and MeOH (3 x 1 ml), dissolved in Nanopure water (3 ml) and purified by dialysis (Slide-A-Lyzer Dialysis cassette, Pierce, 10000 MWCO) against a 3 L of distilled water. The contents of the beaker was stirred slowly, recharging with fresh distilled water every 3–4 h over the course of 72 h. The solution in the membrane was then lyophilised to regain 0.60 mg of GNP-3. UV/Vis (H_2O, 0.1 mg ml^{-1}): surface plasmon band not observed. TEM: average gold diameter 1.9 ± 0.5 nm (Figure S-11 see supplementary data). Average molecular formula: $Au_{225}(C_{11}H_{21}O_6S)_{64}(C_{79}H_{126}N_{27}O_{27}S)_7$ (76 KDa). IR (KBr) and ^1H NMR (500 MHz, D_2O): see supplementary data.

GNP-4. OVA peptide derivative 8 (0.16 mg, 0.08 µmol) was dissolved in CF_3COOD (50 µl) and dried under air stream until formation of an oil. Tetrasaccharide conjugate 6 (0.37 mg, 0.31 µmol) and glucose conjugate 7 (0.105 mg, 0.375 µmol) were added to the oil, and the mixture was dissolved in CD_3OD-D_2O (1:1) (600 µl). ^1H NMR analysis of the mixture showed a compound 6:7:8 ratio of ~4:5:1 (Figure S-7, see supplementary data). After evaporation of the solvent, MeOH was added up to a 0.012 M concentration of organic material and the pH was adjusted to 1 by addition of CF_3COOH. An aqueous solution of $HAuCl_4$ (6.0 µl, 0.025 M) was added followed by an aqueous $NaBH_4$ solution (3.2 µl, 1 M) under rapid shaking. The black suspension was shaken for 2 h and the

supernatant was separated by decantation. The black solid was washed with EtOH (4 x 1 ml) and MeOH (3 x 1 ml), dissolved in Nanopure water (0.5 ml) and purified by centrifugal filtering (AMICON MWCO 10000, 50 min, 10000 rpm). The residue in the AMICON filter was dissolved in 0.5 ml of Nanopure water and lyophilised to afford 0.3 mg of GNP-4. UV/Vis (H_2O, 0.1 mg ml^{-1}): surface plasmon band not observed. TEM: average gold diameter 2.1 ± 0.4 nm (Figure S-11, see supplementary data). Average molecular formula: $Au_{225}(C_{49}H_{90}N_3O_{25}S_2)_{28}(C_{11}H_{21}O_6S)_{35}(C_{79}H_{126}N_{27}O_{27}S)_8$ (103 KDa). ^1H NMR (500 MHz, D_2O): see supplementary data.

GNP-5. OVA peptide derivative 8 (0.38 mg, 0.20 µmol) was dissolved in CF_3COOD (50 µl) and dried under air stream until formation of an oil. Tetrasaccharide conjugate 6 (0.48 mg, 0.40 µmol) and glucose conjugate 7 (0.395 mg, 1.4 µmol) were added to the oil, and the mixture was dissolved in CD_3OD (600 µl). ^1H NMR analysis of the mixture showed a compound 6:7:8 ratio of ~2:7:1 (Figure S-8, supplementary data). After evaporation of the solvent, MeOH was added up to a 0.012 M concentration of organic material, and the pH was adjusted to 1 by addition of CF_3COOH. Then, an aqueous solution of $HAuCl_4$ (16 µl, 0.025 M) was added followed by an aqueous $NaBH_4$ solution (9 µl, 1 M) under rapid shaking. The black suspension was shaken for 2 h, then the supernatant was separated by decantation. The black solid was washed with EtOH (4 x 1 ml) and MeOH (3 x 1 ml), dissolved in Nanopure water (0.5 ml) and purified by centrifugal filtering (AMICON MWCO 10000, 50 min., 10000 rpm). The residue in the AMICON filter was dissolved in 0.5 ml of water and lyophilised to afford 0.8 mg of GNP-5. UV/Vis (H_2O, 0.1 mg ml^{-1}): surface plasmon band not observed. TEM: average gold diameter 1.7 ± 0.6 nm (Figure S-11 supplementary data). Average molecular formula: $Au_{201}(C_{49}H_{90}N_3O_{25}S_2)_{14}(C_{11}H_{21}O_6S)_{50}(C_{79}H_{126}N_{27}O_{27}S)_7$ (84 KDa). ^1H NMR (500 MHz, D_2O): see supplementary data.

Mice immunization studies. All immunization studies were approved by the Ethics Committee on Animal Experiments of Utrecht University, Utrecht, The Netherlands. Inbred 6-week-old female BALB/c mice were immunized intracutaneously with series of GNPs (6 µg) which contain of tetrasaccharide fragment (approximately 3 µg per dose) and/or $OVA_{323-339}$-peptide (approximately 3 µg per dose) with adjuvant coadministration (Table S-1 supplementary data). The GNPs were injected at four different sites in the proximity of the lymphnodes of the axillae and the groins. The adjuvants of 10 µg monophosphoryl lipid-A [MPL derived from *S. minnesota* R595 LPS; Ribi ImmunoChem Research Inc., Hamilton, MT, USA] and 20 µg Quil-A [Quil-A was a gift from Dr. Erik B. Lindblad, Brenntag Biosector , Vedbaek, Denmark] were used as described previously (25). The following antigens served as positive controls: *S. pneumoniae* type 14

polysaccharide conjugated to CRM_{197} (Pn14PS-CRM_{197}; Wyeth Research, Pearl River, NY, USA, 2.5 µg of carbohydrate), free $OVA_{323-339}$-peptide (2.5 µg) and $OVA_{323-339}$-peptide conjugated to CRM_{197} protein ($OVA_{323-339}$-peptide-CRM_{197}, 50 µg). The $OVA_{323-339}$-peptide-CRM_{197} was constructed by coupling of the $OVA_{323-339}$-peptide fragment to CRM_{197} protein as described previously (25). All control antigens were injected into mice in combination with the adjuvants mentioned above (Table 1S see supplementary data). All booster immunizations were given without adjuvant and were performed on weeks 5 and 10, respectively. Blood samples were taken before and after the booster and the sera were stored at −20 °C.

Table 1. Properties of the charged gold nanoparticles prepared in this study

GNPs	Mean metal core diameter [nm][a]	Average number of gold atoms[b]	Average MW (KDa)	Average molecular formula	Molar ratio of ligands Tetra : Glc : OVA[c]
GNP 1a	1.8 ± 0.5	201	95	$Au_{201}(Tetra)_{32}(Glc)_{35}(OVA)_4$	45 : 50 : 5
GNP 2	1.9 ± 0.3	225	97	$Au_{225}(Tetra)_{36}(Glc)_{35}$	50 : 50 : 0
GNP 3	1.9 ± 0.5	225	76	$Au_{225}(Glc)_{64}(OVA)_7$	0 : 90 : 10
GNP 4	2.1 ± 0.4	225	103	$Au_{225}(Tetra)_{28}(Glc)_{35}(OVA)_8$	40 : 50 : 10
GNP 5	1.7 ± 0.6	201	84	$Au_{201}(Tetra)_{14}(Glc)_{50}(OVA)_7$	20 : 70 : 10
GNP 1b	1.8 ± 0.5	201	95	$Au_{201}(Tetra)_{32}(Glc)_{35}(OVA)_4$	45 : 50 : 5

[a] Diameter of the gold nanocluster (as measured by TEM). [b] Average number of gold atoms per nanoparticle were calculated from the size of the gold cluster obtained by TEM as reported previously (12). [c] Molar ratio of ligands per nanoparticle was determined by analysing the mixture of the ligands by NMR before and after the nanoparticles formation (Tetrasaccharide, Glc, and OVA stand for branched tetrasaccharide conjugate 6, glucoside conjugate 7, and Ova peptide conjugate 8, respectively).

Detection of type-specific antibodies by ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed to measure the antibodies to native Pn14PS, to the synthetic branched tetrasaccharide structure Gal-Glc-(Gal-)GlcNAc-Gal (one repeating unit of Pn14PS with extra one galactose residue), and to ovalbumin, as described previously (25). Briefly, serially diluted sera were incubated for 1 h at 37 °C in flat-bottom plates (Corning, Inc., Corning, NY), coated with native Pn14PS (0.3 µg/ml), Gal-Glc-(Gal-)GlcNAc-Gal conjugated to bovine serum albumin (BSA) (1 µg/ml) or ovalbumin protein (1µg/ml). After coating the plates were blocked with 3% gelatin, then washed, and horseradish peroxidase-conjugated goat anti-mouse IgG (Nordic Immunology Laboratories, Tilburg, The Netherlands) was added and incubated for 1 h at 37°C. Then, a mixture of 3,3′,5,5′-tetramethylbenzidine (Sigma Chemical Co., St. Louis, MO) and H_2O_2 (Sigma Chemical Co.) was added to visualize the amount of bound

peroxidase. The reaction was stopped by the addition of 0.5 M H_2SO_4. Optical density (OD) values were obtained with a microtiter plate spectrophotometer at 450 nm (Bio-Rad, model 3550 UV; Bio-Rad Laboratories, Hercules, CA). Antibody titers were expressed as the $\log_{10}$ of the dilution giving twice the OD obtained for control mice (immunized with saline) with a cut-off value of 0.2.

Measurement of phagocytosis titer. The opsonophagocytosis assay was performed by using human polymorphonuclear leukocytes (PMN cells) isolated from peripheral blood of healthy donors, as previously described (13,25). Twofold dilutions of heat-inactivated pooled sera in Hank's balanced salt solution-1% BSA were mixed with 2% complement (guinea-pig serum) in round-bottom plates (Greiner Bio-One, Frickenhausen, Germany). The assay was performed by mixing 20 µl of PMN cells (3×10^6 cells/ml) and 20 µl of heat-killed fluorescein isothiocyanate (FITC)-labeled *S. pneumoniae* type 14 (ATTC 634; 3×10^7 cells/ml), followed by incubation at 37 °C under vigorous shaking at 900 rpm for 50 min. The mixtures were then fixed with cold 2% paraformaldehyde and analyzed in a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA). The percentage of FITC-positive PMN cells was used as a measure of the phagocytic activity and corrected by using the values found in the mixtures without sera. The phagocytosis titers are expressed as the $\log_{10}$ of the serum dilution during phagocytosis that resulted in 25% of the PMN cells being positive for FITC.

***In-vitro* spleen cell stimulation**. *In-vitro* spleen-cell stimulation was performed as previously described (7,27). Briefly, mouse spleens (n=2) were isolated three weeks after the second booster immunization. The spleen cells suspensions (10^7 cells/ml) were stimulated with free $OVA_{323-339}$-peptide (10 µg). The cells were then incubated at 37 °C, in 100% relative humidity, and with 5% CO_2 in air. Finally, the supernatants were collected at 72 h after initiation of the cultures, and stored at −70°C until use. Six different cytokines were selected to screen the supernatants: as Th1 cytokines: IL-2 (171-G5003M), TNF-α (171-G5023M), and IFN-γ (171-G5017M); as Th2 cytokines: IL-4 (171-G5005M) and IL-5 (171-G5006); and IL-17 as Th17 marker (171-G50013M) using the luminex-multiplex cytokine assay, following the manufacturer's instructions (Bio-Rad). The lower limits of detection were 0.31 pg/ml (IL-2), 0.71 pg/ml (IL-4), 0.24 pg/ml (IL-5), 0.20 pg/ml (IL-17), 5.2 pg/ml (TNF-α), and 0.48 pg/ml (IFN-γ).

Statistical methods. Data are presented as geometric means ±SEM. Unpaired *t* test was used to determine the differences in antibody titers and a *P* value of ≤ 0.05 was considered statistically significant.

Results

Preparation of hybrid GNPs. Gold glyconanoparticles (GNPs) used in this study were prepared from thiol-armed conjugates 6, 7, and 8 (Figure 1) which are constituted by the antigenic synthetic branched tetrasaccharide Gal-Glc-(Gal-)GlcNAc, inert D-glucose, and the immunodominant ovalbumin (OVA) T-cell epitope $OVA_{323-339}$ peptide, respectively. The aim was to use gold nanoclusters as novel scaffolds for multimerization of Gal-Glc-(Gal-)GlcNAc, i.e. the establish carbohydrate-epitope of Pn14PS. GNPs with variable density of Gal-Glc-(Gal-)GlcNAc were obtained by a slight modification of our earlier reported single-phase procedure (21) which consists of reducing a gold salt ($HAuCl_4$) with sodium borohydride in the presence of a mixture of thiol-functionalized conjugates 6, 7 and 8 in the desired molar proportions and in a one-step reaction (Figure 2). The use of thiol-armed ligands that strongly bind the *in situ* nascent gold due to the soft character of both gold and sulphur is well documented and allows the protection of the metallic core by a covalent Au-S bond. The ratio of neoglycoconjugate 6 with respect to stealth component 7 and OVA peptide conjugate 8 in the initial solution can be controlled by NMR analysis (see supplementary data).

GNP-1a and GNP-1b, GNP-4, and GNP-5 were prepared using 45:50:5, 40:50:10 and 20:70:10 molar ratios of 6, 7, and 8, respectively. GNP-2 containing only tetrasaccharide conjugate 6 and glucoside 7 in 50:50 proportions was prepared to study the response in the absence of the immunodominant OVA peptide conjugate 8. GNP-3, not functionalized with the tetrasaccharide conjugate 6 and containing ~10% of OVA peptide and ~90% of glucose conjugate 7, was prepared as control system. The ratio of the different ligands on the gold nanocluster surface was determined by comparison of the ^{1}H NMR spectra of the initial mixtures, the formed GNPs and the recovered supernatants after the self-assembly process. The ^{1}H NMR spectra of the bound ligands in the GNPs featured broader signals compared to those of the corresponding free ligands, preventing an accurate integration. However, a qualitative assessment of the presence of the ligands could be achieved by individuation of selected signals (see supplementary data). The resulting GNPs showed exceptionally small mean gold core diameters, ranging from 1.7 to 2.1 nm, as demonstrated by TEM analysis. TEM micrographs showed uniform dispersion of the GNPs and no aggregation was observed. The average molecular formulas and weights are reported in Table 1 and estimated according to the literature (12). The UV/Vis spectra were characterized by the lack of a gold surface plasmon band at around 520 nm, confirming the small core-size of the GNPs.

The obtained GNPs were water soluble and stable for months in solution. They could survive freeze-drying processes without losing their properties or aggregating. Glucoside 7 and OVA peptide derivative 8 were armed with short aliphatic linkers. By contrast, 3-aminopropyl tetrasaccharide 3 was conjugated to a long amphiphilic linker in order to make the antigenic sugar moiety tetrasaccharide more accessible. The eleven carbon-atoms aliphatic part of the linker allows good SAMs packaging and confers rigidity to the inner organic shell to protect the gold core, while the external polyether moiety, due to its flexibility upon solvation in water, ensures accessibility to ligands and assists water solubility. Furthermore, SAMs that present poly(ethyleneglycol) units are known to resist the adsorption of proteins (14,20).

GNPs immunization elicited specific IgG antibodies recognizing native polysaccharide of Pn14PS. Six preparations of GNPs (Table 1), different in their molar ratio of tetrasaccharide conjugate 6, glucose conjugate 7, and $OVA_{323-339}$-peptide conjugate 8 were injected intracutaneously into BALB/c mice with or without adjuvant coadministration (supplementary data Table 1S). Specific IgG antibodies against native Pn14PS were determined by ELISA. The four GNP preparations, GNP-1a, GNP-1b, GNP-4 and GNP-5 elicited different levels of specific IgG antibodies against native Pn14PS (Figure 3). In this study, the GNP-1a and GNP-1b which have the same molar composition (molar ratio 6:7:8 = 40:50:5) were constructed using different batches of tetrasaccharide. Immunization and boosting with 6 µg GNP-1b resulted in a clear positive anti-Pn14PS antibody response, which however was one $\log_{10}$ lower than the response to the positive control antigen Pn14PS-CRM_{197} (a polysaccharide-protein conjugate vaccine) (Figure 3). When GNP-1a was boosted in a fivefold higher dose (30 µg) than primary injection, we observed that the levels of the evoked IgG antibodies against Pn14PS increased slightly (*p value* = 0.3402). Immunization with GNP-2 which contains only tetrasaccharide and glucose (molar ratio 6:7:8 = 50:0:50) only or immunization with GNP-3 which contains only glucose and $OVA_{323-339}$-peptide (molar ratio 6:7:8 = 0:90:15) did not elicit any specific IgG antibodies against Pn14PS. Also when mice were immunized and boosted twice with a mixed suspension of 6 µg GNP-2 and 6 µg GNP-3, no (or very low) specific anti-Pn14PS IgG antibodies were detected (Figure 3). Immunization with GNP-4 (molar ratio 6:7:8 = 40:50:10) or GNP-5 (molar ratio 6:7:8 = 20:70:10) elicited lower levels of specific IgG antibodies against Pn14PS in comparison to immunization with GNP-1b although mice were boosted with a higher dose than the primary injection (Table 1S supplementary data, Figure 3).

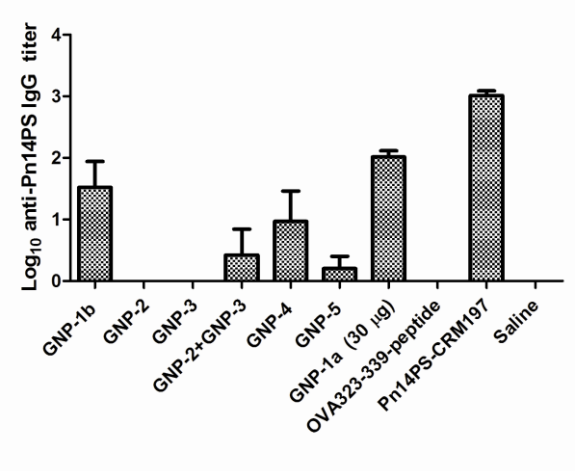

Figure 3. Specific anti-*S. pneumoniae* type 14 polysaccharide (Pn14PS) IgG antibodies. Groups of mice were immunized with series of GNPs with adjuvant coadministration at the primary injection. Sera were collected one week after the second booster injection which was given without adjuvant. The GNPs differed in their molar ratio for the tetrasaccharide: glucose: OVA-peptide (Table 1). Pn14PS-CRM_{197} and saline buffer immunization served as positive and negative control respectively. ELISA was performed to measure specific anti-Pn14PS IgG antibodies with native polysaccharide of Pn14PS as a coating material.

Low level of antibodies against ovalbumin. The used $OVA_{323\text{-}339}$-peptide is a well-known immunodominant T-cell epitope of ovalbumin in BALB/c mice. This however does not exclude that also antibodies to this peptide might be raised. Therefore, the sera obtained one week after the second booster immunization were tested by ELISA for specific antibodies (IgG) against ovalbumin. In addition to the mice immunized with the six above-mentioned GNP preparations and Pn14PS-CRM_{197}, two extra groups of mice were immunized intracutaneously with the $OVA_{323\text{-}339}$ peptide (2.5 μg) and an $OVA_{323\text{-}339}$ peptide-CRM_{197} conjugate (50 μg). In both cases specific IgG antibodies to ovalbumin were detected, in contrast to the GNP preparations which elicited no or very low levels of IgG antibodies against ovalbumin (Figure 4). Only mice sera boosted twice with the GNP preparation GNP-1a elicited an anti-ovalbumin response above the detection level by ELISA (Figure 4). Because the epitopes in the 323-339 peptide may be hidden in the complete OVA protein, sera from the immunized mice were also tested for antibodies on ELISA plates coated with the $OVA_{323\text{-}339}$-peptide conjugated to BSA. Similar results (data not shown) as obtained with OVA coated ELISA plates were obtained, indicating that immunization with $OVA_{323\text{-}339}$-peptide containing GNP does hardly induce anti-OVA antibodies.

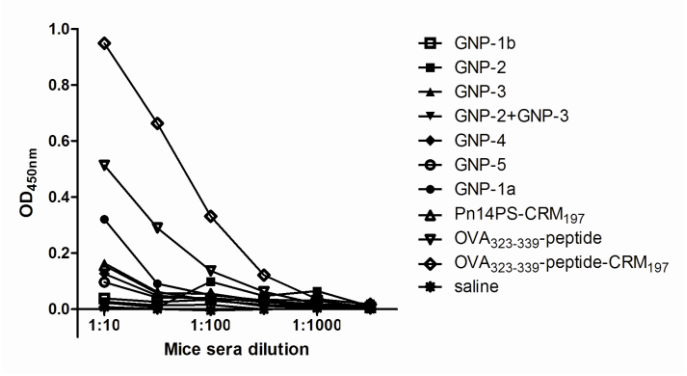

Figure 4. Level of antibodies against ovalbumin protein. Pooled mice sera were tested by ELISA for the presence of IgG antibodies against ovalbumin protein. The sera were obtained from mice previously immunized with series of GNPs and control sera were obtained from mice immunized with CRM_{197}-$OVA_{323-339}$-peptide and free $OVA_{323-339}$-peptide. Level of antibodies are expressed as optical density (OD) at 450nm.

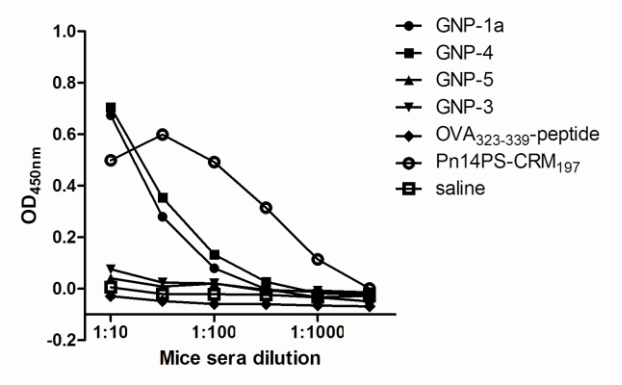

Figure 5. Level of antibodies against branched oligosaccharides fragment Gal-Glc-(Gal-)GlcNAc-Gal structure of Pn14PS. Pooled sera were obtained from mice previously immunized with GNPs. Specific IgG antibodies against the branched oligosaccharides structure of Pn14PS one week after the second booster immunization were measured by ELISA using the synthetic branched oligosaccharides fragment Gal-Glc-(Gal-)GlcNAc-Gal related to Pn14PS conjugated to BSA as coating material. Level of antibodies were expressed as optical density (OD) at 450nm.

Antibodies against the branched tetrasaccharide structure of Pn14PS. Mice sera were tested by ELISA for antibodies against the branched Gal-Glc-(Gal-)GlcNAc structural fragment of Pn14PS. For this assay the neoglycoconjugate built up from Gal-Glc-(Gal-)GlcNAc-Gal (one extra monosaccharide at the reducing end) and BSA was used as coating material. As shown in Figure 5, sera obtained from the immunizations with GNP-1a or GNP-4 recognized the branched structure of Pn14PS. Serum of mice immunized with Pn14PS-CRM_{197} also bind to BSA-neoglycoconjugate. However, sera of mice immunized with GNP-3 or GNP-5 did not recognize the BSA-neoglycoconjugate (Figure 3).

Antibodies against the branched structure of polysaccharide of Pn14PS. Mice sera were tested by ELISA for antibodies against the branched structure of Pn14PS. In this assay, we used the BSA-neoglycoconjugate of Pn14PS which contain the branched tetrasaccharide Gal-Glc-(Gal-)GlcNAc-Gal with one extra monosaccharide at the reducing end conjugated to BSA protein as coating material in this experiment. Sera obtained from GNP-1a or GNP-4 immunization recognized the branched structure of polysaccharide of Pn14PS as shown in Figure 3. Serum of mice immunized with Pn14PS-CRM_{197} also bind to BSA-neoglycoconjugate of Pn14PS. Sera of mice immunized with GNP-3 or GNP-5 did not recognize BSA-neoglycoconjugate (Figure 5).

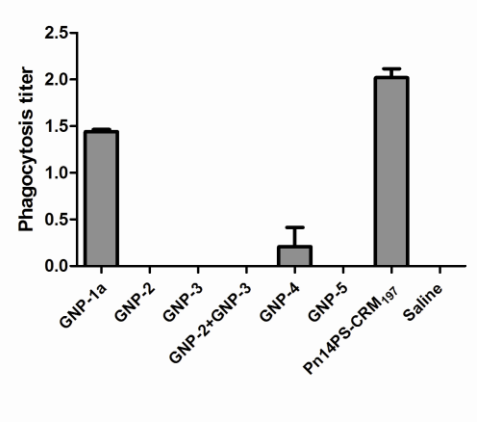

Figure 6. Phagocytosis titer. Groups of mice were immunized with series of GNPs with adjuvant coadministration. Sera were collected one week after the second booster injection. Pn14PS-CRM_{197} and saline buffer immunization served a positive and negative control respectively. The sera were heat inactivated and supplemented with 2% complement. The assay was performed with heat-inactivated FITC labelled *S. pneumoniae* type 14 and PMN cells by flow cytometry. The titers are expressed as the $\log_{10}$ of the serum dilution during phagocytosis resulting in 25% of PMN cells being positive for FITC.

Phagocytic capacity in sera obtained from the GNPs immunization. Mice sera obtained one week after the second booster injection (GNP-1a, GNP-4, and GNP-5) were tested for their capacity to promote the phagocytosis of heat inactivated *S. pneumoniae* type 14 bacteria by human PMN cells. It was found that sera obtained from mice immunized with GNP-1a were capable to opsonize *S. pneumoniae* type 14 bacteria, although in a lower fashion if compared with sera of mice immunized with Pn14PS-CRM_{197} (Figure 6). A low phagocytosis titer was measured for sera obtained from mice immunized with GNP-4. Sera obtained from mice immunized with GNP-5 were not capable to opsonize *S. pneumoniae* type 14 bacteria (Figure 6).

Cytokine levels after spleen cell stimulation. To investigate whether immunization with $OVA_{323-339}$-peptide containing GNPs actually leads to activation of memory T-cells , spleen cells were isolated from mice treated with GNP-1b, GNP-2, GNP-3, and a mixture of GNP-2+GNP-3 three weeks after the second booster immunization. Spleen cells were (re)stimulated *in vitro* with 10 μg $OVA_{323-339}$-peptide or medium for 72 h. The cytokine levels of IL-2, IL-4, IL-5, IL-17, IFNγ, and TNFα were measured in the supernatants using the luminex-multiplex cytokine assay. Except for TNFα, spleen cells obtained from mice immunized previously with GNP-2 (not containing the $OVA_{323-339}$ –peptide) did not respond to *in vitro* stimulation with $OVA_{323-339}$ –peptide with production of significant amounts of IL-2, IL-4, IL-5, IL-17 or IFNγ (Figure 7). Spleen cells of mice obtained from mice previously immunized with GNP-1b, GNP-3 or a combination of GNP-2 and GNP-3 did respond to the stimuli. Induction of IL-5 occurred only in those cells previously immunized with GNP-1b which correlates with the production of specific IgG antibodies to Pn14PS *in vivo* (Figure 7). Spleen cells were also cultured with ovalbumin, which lead to a lower but similar cytokine production profile (data not shown). The data indicate that immunization with GNP-1b, GNP-3 and a combination of GNP-2 and GNP-3 all resulted in the activation of T-cells , but that the specific antibody production to the branched structure fragment or native Pn14PS only occurred if both tetrasaccharide and $OVA_{323-339}$ –peptide were presented at the same gold glyconanoparticle, i.e. GNP-1b (Figure 7).

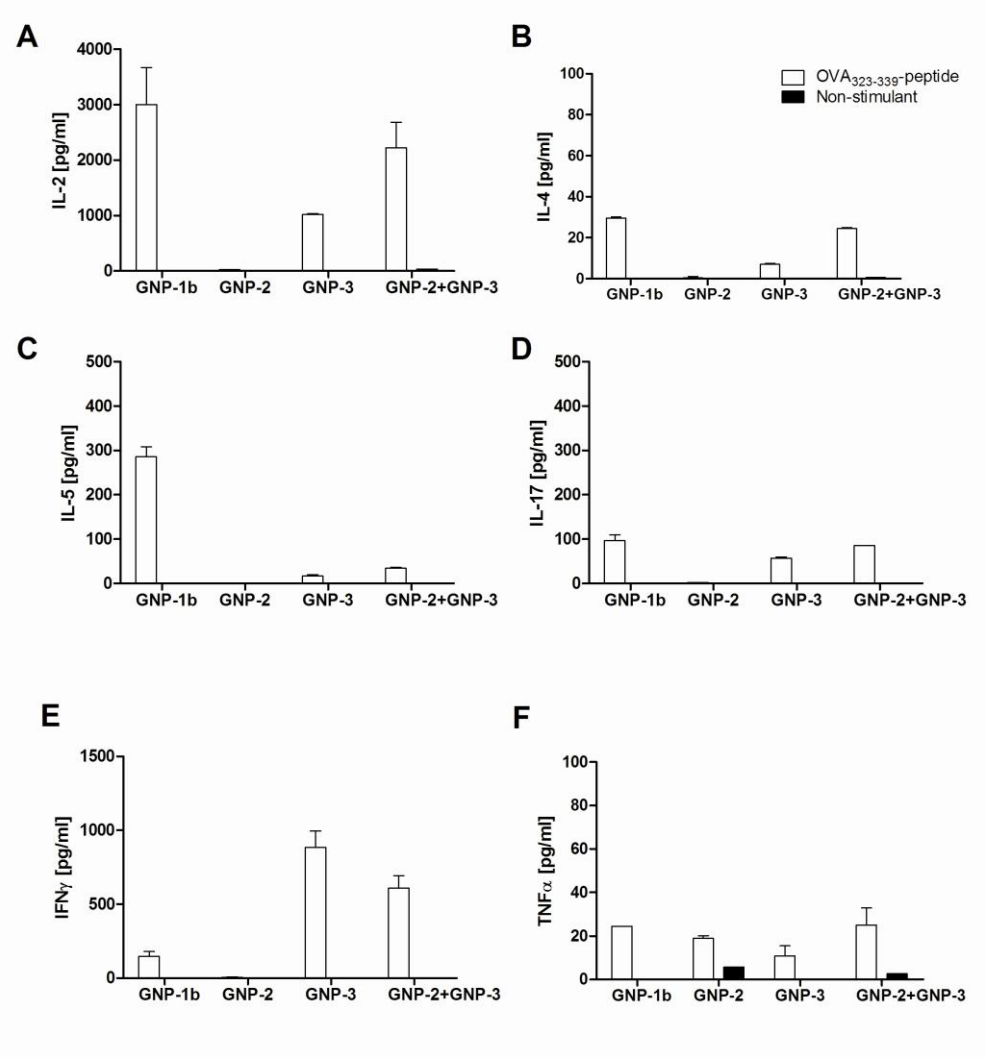

Figure 7. Level of cytokines production after spleen cells stimulation. Splenocytes were isolated from mice immunized with GNP-1b, GNP-2, GNP-3, and GNP-2+GNP-3 and adjuvants coadministration two weeks after the second booster injection. Spleen cells (10^7 cells/ml) were cultured *in vitro* and stimulated with 10 µg $OVA_{323-339}$ peptide (white bars), and no stimulant (black bars). Spleen supernatants were collected 72 hours after culture initiation. The cytokine levels of IL-2 (A), IL-4 (B), IL-5 (C), IL-17 (D), IFNγ (E) and TNFα (F) were measured using the luminex-multiplex cytokine assay.

Discussion

Metal-based glyconanoparticles (GNPs) are biofunctional nanomaterials that combine the unique physical, chemical and optical properties of the metallic nucleus with the characteristics of the carbohydrate coating (10). In the present study, we propose gold nanoclusters as a versatile platform to construct hybrid gold glyconanoparticles (GNPs) with different thiol-end linkers of synthetic branched tetrasaccharide related to *S. pneumoniae* type 14 polysaccharide (Pn14PS), $OVA_{323-339}$-peptide and glucose. Glucose was used as an inner and inert component to assist water dispersibility and biocompatibility and to allow the tetrasaccharide fragment moiety, armed with a long amphiphilic linker, to protrude above the organic shell of GNPs (15,18).

We found that GNPs coated with the tetrasaccharide and OVA-peptide induce specific IgG antibodies that recognize the branched tetrasaccharide homologue Gal-Glc-(Gal-)-GlcNAc-Gal and the native polysaccharide of Pn14PS. A first interesting observation is that the synthetic carbohydrate epitope Gal-Glc-(Gal-)GlcNAc does not lose its biofunctionality after conjugation to the gold nanoplatform. In general, three main factors are of importance to reach a detectable carbohydrate-directed immune response: (i) the density of the antigenic tetrasaccharide on the gold nanoplatform; (ii) the presence of T-cell stimulating peptide $OVA_{323-339}$; and (iii) the combination of both T-helper and B-cell epitopes in the same GNP.

The different molar ratio of tetrasaccharide conjugate 6, glucose conjugate7 and $OVA_{323-339}$ peptide conjugate 8 has a key effect on the immunogenic response. The GNP with a molar ratio 6:7:8 = 20:70:10 induced a very low titer of anti-Pn14PS IgG antibodies compared to the GNPs which have molar ratios 6:7:8 = 45:50:5 or 40:50:10. These experimental data indicate that epitope density on gold surface is of crucial importance to obtain significant levels of IgG antibodies. The ligand density and the nature of spacers used to separate a selected ligand from the gold cluster are important factors for proper receptor targeting of GNPs, as demonstrated with different types of lactose-coated gold GNPs in aggregation experiments with *Ricinus communis* agglutinin (22) or in enzymatic studies with *Escherichia coli* β-galactosidase (2), and with (oligo)mannose-coated GNPs in cellular models of inhibition of HIV trans-infection of T cells (17).

Another major finding is that the presence of the T-cell stimulating peptide $OVA_{323-339}$ in the hybrid GNPs was crucial for the induction of specific carbohydrate-directed IgG antibodies. In this study, we revealed that GNPs which do not contain $OVA_{323-339}$ peptide

are not able to elicit anti-Pn14PS IgG antibodies. *In vitro* spleen (re)stimulation revealed that spleen cells derived from mice immunized with GNPs containing the $OVA_{323-339}$-peptide produced cytokine IL4 when stimulated by $OVA_{323-339}$-peptide. This indicates a Th2 response to the peptide. Induction of IL-5 occurred only in those cells from animals previously immunized with GNPs which contain tetrasaccharide and peptide correlates with the production of specific anti-Pn14PS IgG antibodies *in vivo*. These results are in line with other immunization studies in which the same synthetic tetrasaccharide was conjugated to the CRM_{197}. In a mouse *in vitro* stimulation model, the cytokines IL-4 and IL-5 were only produced in response to the carrier protein (Chapter 3). In human vaccination trials with the pneumococcal conjugate vaccine an enhanced IL-5 secretion was observed in response to the carrier protein (32). Our experiments indicate that T cells recognizing the $OVA_{323-339}$–peptide play a crucial role in the induction of antibodies to the native polysaccharide of Pn14PS. We suggest that the direct covalent linkage of a T-helper epitope to a B-cell epitope is not a strict condition for the induction of IgG-mediated immune response against Pn14PS. The presentation of both the antigenic carbohydrate and the T-helper peptide in the same gold nanoplatform seems to be crucial for eliciting an antibody response.

In conclusion, hybrid gold nanoparticles coated with synthetic branched tetrasaccharide Gal-Glc-(Gal-)GlcNAc and $OVA_{323-339}$–peptide are capable of inducing IgG antibodies against native polysaccharide of *S. pneumoniae* type 14. The molar ratio between tetrasaccharide, glucose and peptide in the hybrid gold nanoparticles turned out to be critical for optimal immunogenicity: Gold nanoparticles containing 45%of tetrasaccharide and 5% of peptide, supplemented with inert glucoconjugate, were able to trigger anti-Pn14PS antibodies. For the future, the high degree of multimerization of tetrasaccharide on the nanometric gold platform and the possibility of functionalizing the same gold nanoparticles with various ligands provide a versatile system that can be tailor-made to obtain synergistic effects against lots of biological problems.

Supplementary data

Synthesis and characterization of thiol-armed tetrasaccharide conjugate 6 and corresponding intermediates, selected ^{1}H NMR and IR spectra of all compounds, TEM and size distribution of GNPs and mice immunization details are available. Supplementary data associated with this article can be found, in appendix II.

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Appendix II

Supplementary data:

Hybrid gold nanoparticles coated with a synthetic tetrasaccharide of *Streptococcus pneumoniae* type 14 capsular polysaccharide and ovalbumin peptide OVA323-339 induce a carbohydrate-specific humoral immune response

Preparation of synthetic branched tetrasaccharide fragment

General information. Chemicals were purchased as reagent grade from Sigma-Aldrich and used without further purification, unless otherwise stated. Dichloromethane (DCM) was distilled from calcium hydride before use. Dry methanol was kept over molecular sieves 3 Å. Purified water was obtained from a Simplicity Ultrapure Water System (Millipore). TLC was performed on 0.25 mm pre-coated silica gel glass plates or aluminium backed sheets (Merck silica gel 60 F_{254}) with detection by UV-light (254 nm) and/or heating at over 200 °C after staining either with 10 % sulfuric acid (aqueous solution) or p-anisaldehyde solution [p-anisaldehyde (25 mL), H_2SO_4 (25 mL), EtOH (450 mL), and CH_3COOH (1 mL)]. Organic solvents were removed by rotary evaporation under reduced pressure at approximately 40 °C (water bath). Silica gel (0.041 – 0.063 mm, Amicon and 0.063-0.200 mm, Merck) was used for flash column chromatography (FCC). Polyethylene (PE) columns equipped with PE frits (20 µm pore-size) were used for flash chromatography. Size-exclusion column chromatography was performed on Sephadex LH-20 (GE Healthcare) or P2 gel (BioRad, polyacrylamide, fine) using 1 % n-butanol in water at a flow rate recommended by the manufacturer. To remove small particles (e.g. Pd/C), a PE column was equipped with a filter sandwich made by stacking PE frits (20 µm, 10 µm, and 5 µm pore-size) on-top of each other.

NMR spectra were recorded at 500 MHz (Bruker) (^{1}H) or 125 MHz (^{13}C). If not otherwise stated, chemical shifts (δ) are given in ppm relative to the residual solvent signal. Infrared spectra (IR) were recorded from 4000 to 400 cm^{-1} with a Nicolet 6700 FT-IR spectrometer (Thermo Spectra-Tech), solids were pressed into KBr pellets. Optical rotations were determined with a Perkin-Elmer 341 polarimeter. UV/Vis spectra were measured with Beckman Coulter DU 800 spectrophotometer. Mass spectrometric data was obtained from a Waters LCT Premier XE instrument with a standard ESI source by direct injection. The instrument was operated with a capillary voltage of 1.0 kV and a cone voltage of 200 V. Cone and desolvation gas flow were set to 50 and 500 L/h, respectively; source and desolvation temperatures were 100 °C. High resolution mass was determined using glycocholic acid (Sigma) as an internal standard (2 M+Na^+, m/z = 953.6058). The masses for compound 2 and 3 were recorded on a Bruker Daltonics MicrOTOF (ESI). MALDI-TOF spectra were recorded on a Bruker Reflex IV using 2',4',6'-trihydroxy-acetophenone monohydrate (THAP) as matrix.

3-Azidopropyl 3,4-*O*-(1-bromomethylethylidene)-β -D-galactopyranosyl-(1→4)-β-D-glucopyranosyl)-(1→6)-[(β-D-glucopyranosyl)-(1→4)]-2-deoxy-2-acetamido-β-D-glucopyranoside 2. Compound 1 (150 mg, 82 µmol) was dissolved in dry dichloromethane (2 ml), then molecular sieves (4 Å, 200 mg), azidopropanol (30 µl, 0.32 mmol) and *N*-iodosuccinimide (30 mg, 0.13 mmol) were added (Figure S-2). The reaction mixture was stirred at room temperature for 15 min. After that, the mixture was cooled down to 0 °C in an ice bath, and a catalytic amount of AgOTf was added. The reaction was monitored by TLC (Toluene/ EtOAc 6:1), while stirring of the reaction mixture continued for 1 hour at room temperature. After complete conversion, the mixture was concentrated and purified by flash column chromatography (toluene → toluene/EtOAc, 15:1→10:1→7:1→5:1) to give 3-azidopropyl [2,6-di-*O*-benzoyl-3,4-*O*-(1-bromomethylethylidene)-β-D-galactopyranosyl]-(1→4)-(2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl)-(1→6)-[(2,3,4,6-tetra-*O*-benzoyl-β -D-glucopyranosyl)-(1→4)]-2-deoxy-2-phthalimido-β -D-glucopyranoside as diastereomeric mixture (138 mg, 71 µmol, 90 %). MALDI-Tof calcd. for $C_{101}H_{89}BrN_4O_{31}$ Calcd. 1933.4767 $[M + H]^+$, 1955.4586 $[M + Na]^+$. Found 1933.5150 $[M + H]^+$ ± 2.0 ppm, 1955.6625 $[M + Na]^+$ ± 1.0 ppm. NMR data was in agreement with the previously reported one (2). This material (138 mg, 71 µmol) was dissolved in dry ethanol (5 ml), and after addition of ethylenediamine (500 µl, 7.4 mmol) the reaction mixture was stirred at 60 °C overnight. After complete consumption of the starting material (TLC: 6:1, DCM/MeOH), the mixture was concentrated and the residue co-evaporated 2 times with toluene. Then dry methanol (5 ml) and acetic anhydride (250µl) was added to the residue, and the resulting mixture was stirred for 1 hour at room temperature, concentrated, co-evaporated with toluene, and again dissolved in dry methanol (10 ml). Then NaOMe (approx.1 M solution in MeOH, 10 drops) was added, and the reaction mixture was stirred at room temperature overnight.

The progress of the reaction was monitored by Maldi-Tof. After complete conversion, the mixture was neutralized using H^+-ion-exchange resin (Dowex-50WX-8). The resin was filtered off the reaction solution, and the filtrate was concentrated before the residue applied onto a column. Flash chromatography (DCM/MeOH 15:1→10:1→8:1→6:1→3:1→1:1→methanol) gave compound 2 as diastereomeric mixture (48 mg, 53 µmol, 74 %). HResMS calcd for $C_{32}H_{53}BrN_4O_{21}$ Calcd. 931.2278 $[M + Na]^+$. Found 931.1931 $[M + Na]^+$ ± 3.7 ppm; ^{13}C NMR (126 MHz, MeOD) 173.6 (N*C*(O)CH_3), 110.1 (C(CH_3)(CH_2Br)), 104.9, 104.6, 104.2, 103.1 (C-1^{I-IV}), 81.8, 81.5, 81.0, 80.5, 77.0, 76.5, 76.5, 76.0, 75.9, 75.5, 75.4, 74.9, 74.4, 74.1, 72.8, 70.5, 68.7, 67.5, 62.6, 62.5, 62.0, 56.8 (C-2^{I-IV}-6^{I-IV}, O*C*$H_2CH_2CH_2N_3$), 37.0

($C(CH_3)(CH_2Br)$), 30.2 ($OCH_2CH_2CH_2N_3$), 25.0, 23.1 ($C(CH_3)(CH_2Br)$) and ($NC(O)CH_3$). ($OCH_2CH_2CH_2N_3$) undetected (under MeOD signals).

3-Aminopropyl-(1→4)-β-D-glucopyranosyl)-(1→6)-[(β-D-glucopyranosyl)-(1→4)]-2-acetamido-β-D-glucopyranoside·HCl salt (3). Compound 2 (48 mg, 53 µmol) was dissolved in 90% trifluoroacetic acid (1.8 ml) (Figure S-1). The reaction mixture was stirred at room temperature for 3 hours and the progress of the reaction monitored by TLC (DCM/MeOH, 6:1) and Maldi-Tof. After complete consumption of the starting material, the mixture was co-evaporated with toluene (2 x 5 ml). Purification by size exclusion chromatography (P2 column, 1% n-BuOH in purified water) provided 3-azidopropyl-(1→4)-β-D-glucopyranosyl)-(1→6)-[(β-D-glucopyranosyl)-(1→4)]-2-acetamido-β-D-glucopyranoside· (40 mg, 51 µmol, 96%). (D_2O, 25 °C,): ^{1}H NMR (D_2O, 25 °C) δ 4.49-4.42 (m, 3H, 3xH-1); 4.36 (d, 1H, J = 7.8 Hz, H-1); 4.20 (d, 1H, J = 9.8 Hz); 3.94-3.82 (m, 5H); 3.81-3.54 (m, 15H), 3.54-3.49 (m, 1H); 3.49-3.42 (m, 1H); 3.33-3.24 (m, 2H), 1.96 (s, 3H, NAc); 1.79-1.72 (m, 2H, $OCH_2CH_2CH_2N_3$). ^{13}C NMR (126 MHz, D_2O) 174.5 ($NC(O)CH_3$), 103.0, 102.8, 102.4, 101.2 (C-1^{I-IV}), 78.4, 77.9, 75.4, 75.3, 74.7, 74.3, 73.5, 72.7, 72.5, 72.3, 71.0, 68.6, 67.4, 67.3, 61.1, 61.0, 60.1, 55.1 (C-2^{I-IV}-6^{I-IV}, $OCH_2CH_2CH_2N_3$), 47.8 ($OCH_2CH_2CH_2N_3$), 28.1 ($OCH_2CH_2CH_2N_3$), and 22.2 ($NC(O)CH_3$). HResMS calcd for $C_{29}H_{50}N_4O_{21}$ Calcd. 813.2860 $[M + Na]^+$. Found 813.2754 $[M + Na]^+$ ± 1.3 ppm, This material (40 mg, 51 µmol) was dissolved in water/MeOH (1:1, v/v) and 2M HCl (1eq, 30 µl) and a catalytic amount Pd/C were added.

The reaction mixture was stirred under hydrogen atmosphere at room temperature. When complete conversion had taken place, as indicated by MALDI-Tof, the catalyst was removed by filtration over a filter sandwich. Then, the solvents were removed by evaporation. The residue was dissolved in water (2 ml), and after freeze drying, compound 3 was obtained (36 mg, 45 µmol, 89%). (D_2O, 25 °C,): ^{1}H NMR (D_2O, 25 °C) δ 4.41-4.33 (m, 3H, 3xH-1); 4.29 (d, 1H, J = 7.8 Hz, H-1); 4.14 (d, 1H, J = 9.0 Hz); 3.88-3.77 (m, 3H); 3.76 (sb, 2H); 3.72-3.47 (m, 15H), 3.47-3.42 (m, 1H); 3.41-3.35 (m, 2H); 3.25-3.18 (m, 1H), 2.92 (t, 2H, J = 6.8 Hz, $OCH_2CH_2CH_2N_3$), 1.88 (s, 3H, NAc); 1.78 (dt, 2H, J = 13.0 Hz, $OCH_2CH_2CH_2N_3$). ^{13}C NMR (126 MHz, D_2O) d 174.7 ($NC(O)CH_3$), 103.0, 102.8, 102.4, 101.3 (C-1^{I-IV}), 78.4, 77.7, 75.4, 75.3, 74.7, 74.3, 73.4, 72.7, 72.5, 72.1, 71.0, 68.6, 68.1, 61.0, 61.0, 55.1 (C-2^{I-IV}-6^{I-IV}, $OCH_2CH_2CH_2NH_2$), 37.7 ($OCH_2CH_2CH_2NH_2$), 26.6 ($OCH_2CH_2CH_2NH_2$), and 22.1 ($NC(O)CH_3$). HResMS calcd for $C_{29}H_{52}N_2O_{21}$ Calcd. 787.2955 $[M + Na]^+$. Found 787.3105 $[M + Na]^+$ ± 1.9 ppm.

***S*-Protected tetrasaccharide conjugate 5 [Gal(β1-4)Glc(β1-6)[Gal(β1-4)]GlcNAc(β1-O)$(CH_2)_3$NH(CS)NH-TEG-C_{11}-SAc].** Isothiocyanate linker 4 and 5-(thio)pentyl D-glucopyranoside (7) were prepared as previously reported (1). A solution of isothiocyanate linker 4 (12.1 mg, 26.1 µmol, 1.8 equiv.) in H_2O-^{i}PrOH-CH_3CN (1:1:1 proportion, 0.6 mL) was added to a solution of 3-aminopropyl tetrasaccharide 3 (11.62 mg, 14.5 µmol, 1 equiv.) in H_2O-^{i}PrOH-CH_3CN (1:1:1 proportion, 1.8 mL) and the pH was set to basic by addition of triethylamine (18 µL, 130 µmol, 9 equiv.) (Figure S-2). The mixture was stirred at room temperature for 17 hours and then evaporated. The crude was kept in high vacuum to remove the residual triethylamine and then triturated with Et_2O (4 x 2 mL) in order to get rid of the excess of the linker. The insoluble solid was purified by Sephadex LH-20 chromatography (column: diameter = 2 cm; height = 45 cm) using as eluent MeOH/H_2O = 9/1 to afford the *S*-protected tetrasaccharide conjugate 5 as a white solid after lyophilisation (12.6 mg, 10.3 µmol, 71%).

^{1}H NMR (D_2O) δ 4.61-4.52 (m, 3H, 3xH-1); 4.48 (br d, 1H, J = 7.6 Hz, H-1); 4.31 (d, 1H, J = 10.2 Hz); 4.03-3.60 (m, 40H); 3.57 (m, 2H, 2xH-2); 3.50 (br t, 2H, J = 6.0 Hz, OCH_2CH_2CH_2); 3.41 (br t, 1H, J = 7.6 Hz, H-2); 2.88 (t, 2H, J = 7.1 Hz, CH_2SAc); 2.35 (s, 3H, SAc), 2.07 and 2.06 (s, 3H, NAc); 1.91-1.82 (m, 2H, OCH$_2$CH_2CH$_2$N); 1.65-1.53 (m, 4H, CH_2CH$_2$SAc and OCH$_2$CH_2CH$_2$); 1.44-1.26 (m, 14H, (CH_2)$_7$). ^{13}C NMR (D_2O) δ 102.9 (d, 2C, C-1); 102.4 (d, 1C, C-1); 101.1 (d, 1C, C-1); 78.4; 77.8; 75.3; 74.7; 74.2; 73.4; 72.7; 72.6 (d, 1C, C-2); 72.5; 72.3; 71.0 (t, 1C, OCH$_2$CH$_2$CH$_2$); 70.9 (d, 2C, 2xC-2); 70.3; 70.0; 69.8; 69.7; 68.6; 67.3; 67.2 (d, 1C); 61.0; 60.9; 60.2; 43.7 (br t; CH$_2$NH); 30.2 (q, 1C, SC(O)CH$_3$); 29.7-29.0 and 25.8 (t, 9C, (CH$_2$)$_9$); 28.8 (t, 1C, CH$_2$SAc); 28.3 (t, 1C, OCH$_2$CH$_2$CH$_2$N); 22.2 (q, 1C, NC(O)CH$_3$); C=S and C=O undetected. IR (KBr): ν˜ 3424 (broad), 2925, 2850, 1745, 1655, 1374, 1232, 1068 cm^{-1}. MS *m/z* Calcd. for $C_{51}H_{93}N_3NaO_{26}S_2^+$ $[M+Na]^+$ 1250.5381, Found 1250.5468; Calcd. for $C_{51}H_{94}N_3O_{26}S_2^+$ $[M+H]^+$ 1228.5561, Found 1228.5591. $[\,]^{29}_D$ = -8.1 (c = 0.4; H_2O) .

Tetrasaccharide-linker6 [Gal(β1-4)Glc(β1-6)[Gal(β1-4)]GlcNAc(β1-O)$(CH_2)_3$NH(CS)NH-TEG-C_{11}-S]. Sodium methoxide (0.5 mg, 9.2 µmol, 1 equiv.) was added to a solution of the coupled sugar 5 (11.28 mg, 9.2 µmol, 1 equiv.) in MeOH (5 mL) (Figure S-2). The mixture was stirred at room temperature for 4 hours and then evaporated. The crude was concentrated and purified by Sephadex LH-20 chromatography (column: diameter = 2 cm; height = 45 cm) using as eluent MeOH/ H_2O = 9/1 to afford the tetrasaccharide conjugate 6 (~2.5:1

mixture of disulfide and thiol) as a white solid after lyophilisation (8.8 mg, 7.4 µmol (considered as thiol), 81%).

^{1}H NMR (D_2O) δ 4.60-4.52 (m, 3H, 3xH-1); 4.48 (br d, 1H, H-1, J = 7.3 Hz); 4.30 (br d, 1H, J = 9.9 Hz); 4.03-3.59 (m, 40H); 3.57 (m, 2H, 2xH-2); 3.53-3.46 (m, 2H, $OCH_2CH_2CH_2$); 3.42 (br t, 1H, J = 7.6 Hz, H-2); 2.76-2.68 (br signal, 1.5H, CH_2SS); 2.57-2.51 (br signal, 0.6H, CH_2SH); 2.52 (bs, 2H, CH_2SH); 2.07 and 2.05 (s, 3H, NAc); 1.90-1.82 (m, 2H, $OCH_2CH_2CH_2N$); 1.77-1.68 (m, 1.5H, CH_2CH_2SS); 1.65-1.55 (m, 2.5H, CH_2CH_2SH and $OCH_2CH_2CH_2$); 1.50-1.22 (m, 14H, $(CH_2)_7$). ^{13}C NMR (D_2O) δ 103.0 (d, 1C, C-1); 102.8 (d, 2C, C-1); 101.2 (d, 1C, C-1); 78.6; 77.8; 75.3; 74.7; 74.2; 72.5 (d, 1C, C-2); 72.3; 71.1 (t, 1C, $OCH_2CH_2CH_2$); 71.0; 70.9 (d, 2C, 2xC-2); 70.0; 68.4; 67.3 (d, 1C); 67.2; 61.0; 60.1; 43.7 (br t; CH_2NH); 38.8 (t, ~0.7C, CH_2SS); 24.4 (t, ~0.3C, CH_2SH); 29.1 (t, 2C, CH_2CH_2S and $OCH_2CH_2CH_2$); 29.7-28.8 and 25.9 (t, 9C, $(CH_2)_9$); 28.2 (t, 1C, $OCH_2CH_2CH_2N$); 22.3 (q, 1C, $NC(O)CH_3$); C=S and C=O undetected. IR (KBr): ν˜ 3369 (broad), 2925, 2854, 1654, 1564, 1381, 1075, 1047. MS m/z Calcd. for $C_{49}H_{91}N_3NaO_{25}S_2^+$ $[M+Na]^+$ 1208.5275, Found 1208.5261 (Figure S-3).

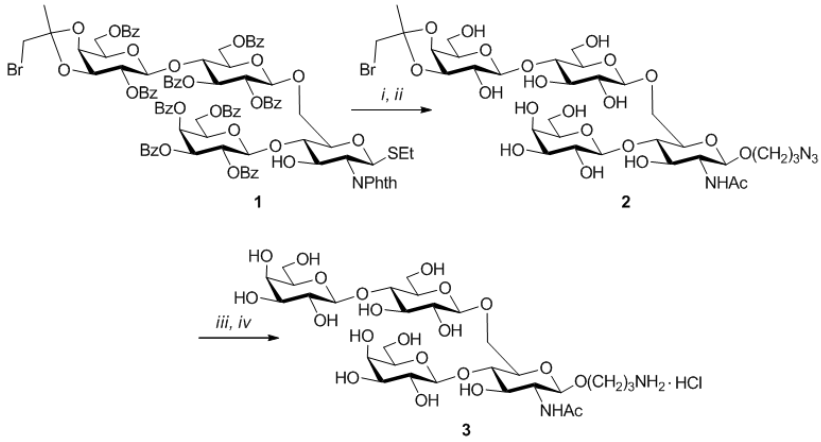

Figure S-1. *i)* 3-azidopropanol, NIS/AgOTf (cat.), DCM, 90 %; *ii)* 1. ethylenediamine, EtOH; 2. Ac_2O, MeOH; 3. NaOMe, MeOH; (74 % over three steps); *iii)* 90% TFA, 96 %; *iv)* H_2, Pd/C, MeOH/H_2O (1:1, *v/v*), 89 %.

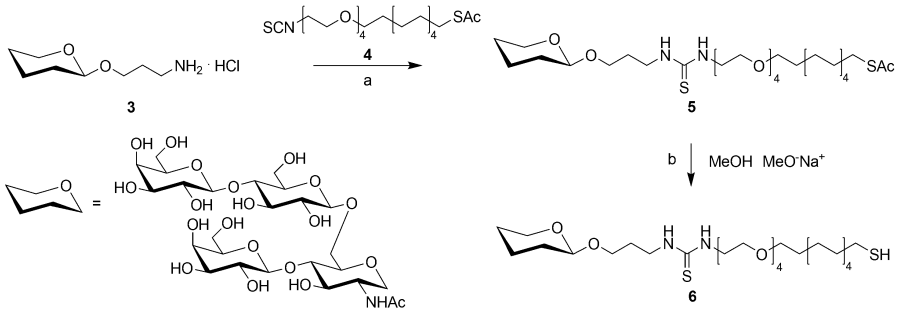

Figure S-2. Reagents and conditions: (a) NEt_3, $H_2O/^iPrOH/CH_3CN$, 17 h, 25 °C; (b) MeONa/MeOH, 4 h, 25 °C. For clarity reasons, neoglycoconjugate 6 is depicted as thiol.

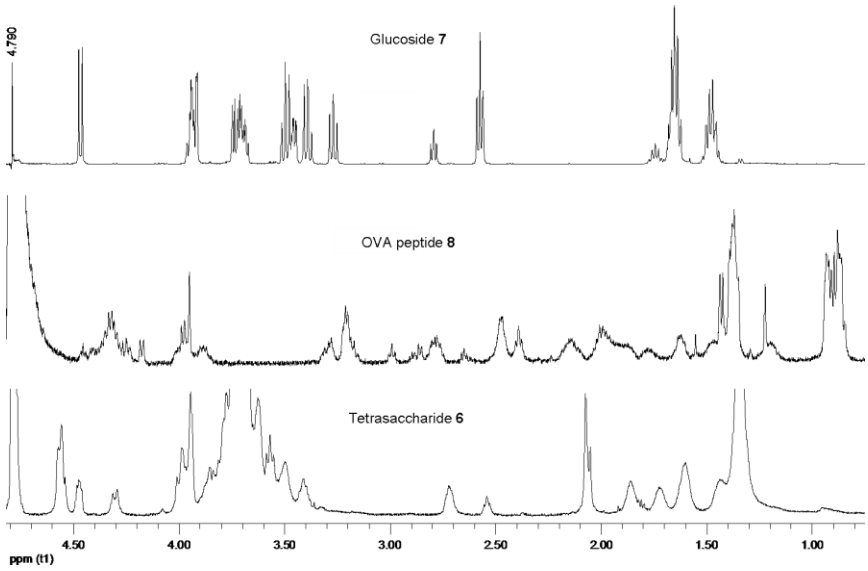

Figure S-3. NMR spectra of the starting material (6, 7, and 8). From up to bottom: ^{1}H NMR (500 MHz, D_2O) of glucose conjugate 7 (water suppression), OVA peptide derivative 8 (His signals out of scale), and tetrasaccharide 6.

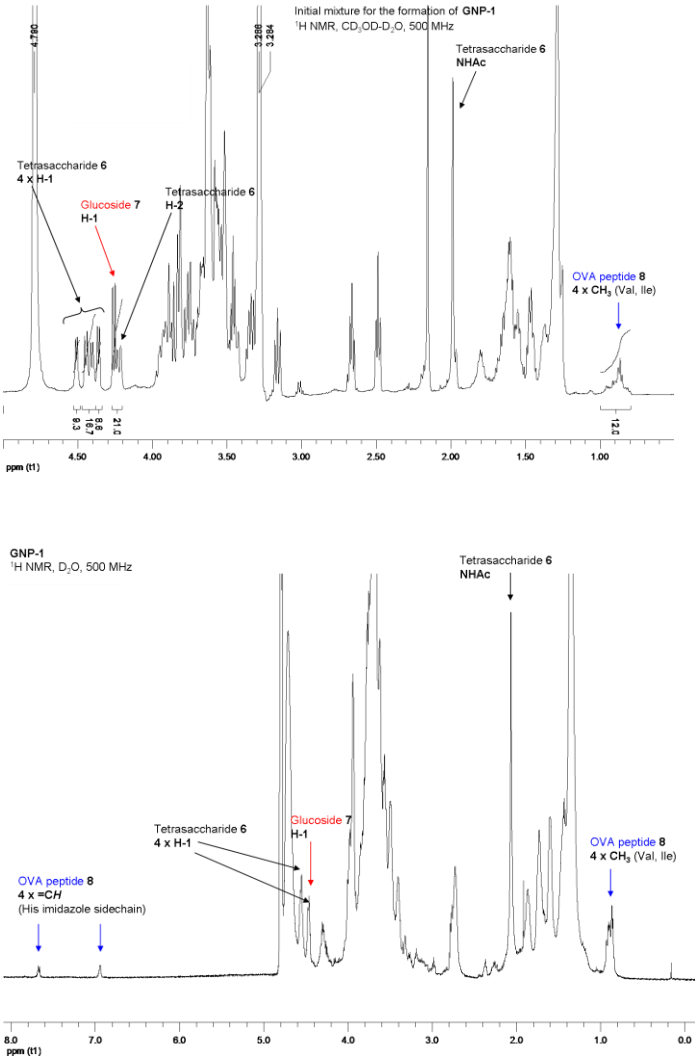

Figure S-4. *Upper panel*: ^{1}H NMR (500 MHz, CD_3OD:D_2O = 1:1) of the mixture used to prepare GNP-1. Integration of selected signals shows that the ratio between tetrasaccharide 6, glucose conjugate 7, and OVA peptide derivative 8 is about 9:10:1. *Bottom panel*: ^{1}H NMR (500 MHz, D_2O, water suppression) of GNP-1. The selected signals show the presence of all three components (tetrasaccharide 6, glucose conjugate 7, and OVA peptide derivative 8) in the same nanoparticle.

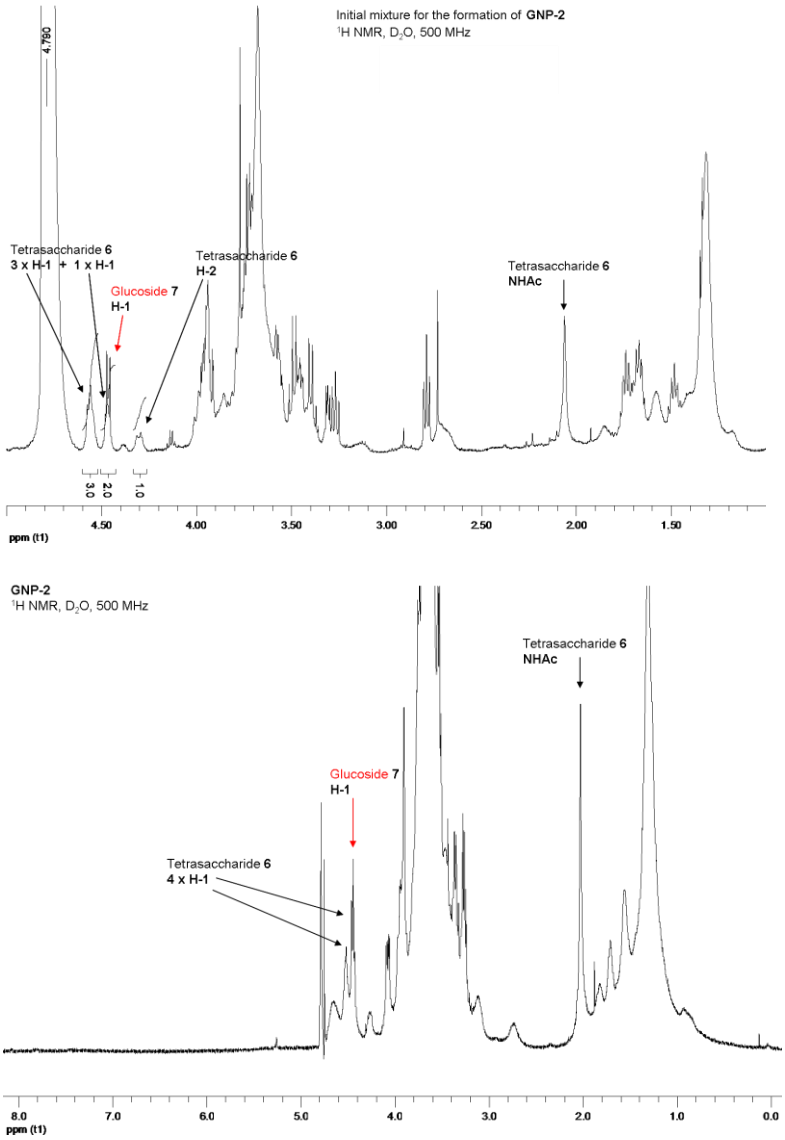

Figure S-5. *Upper panel*: ^{1}H NMR (500 MHz, D_2O) of the mixture used to prepare GNP-2. Integration of selected signals shows that the ratio between tetrasaccharide 6 and glucose conjugate 7 is about 1:1. *Bottom panel*: ^{1}H NMR (500 MHz, D_2O, water suppression) of GNP-2. The selected signals show the presence of both tetrasaccharide 6 and glucose conjugate 7 in the same nanoparticle.

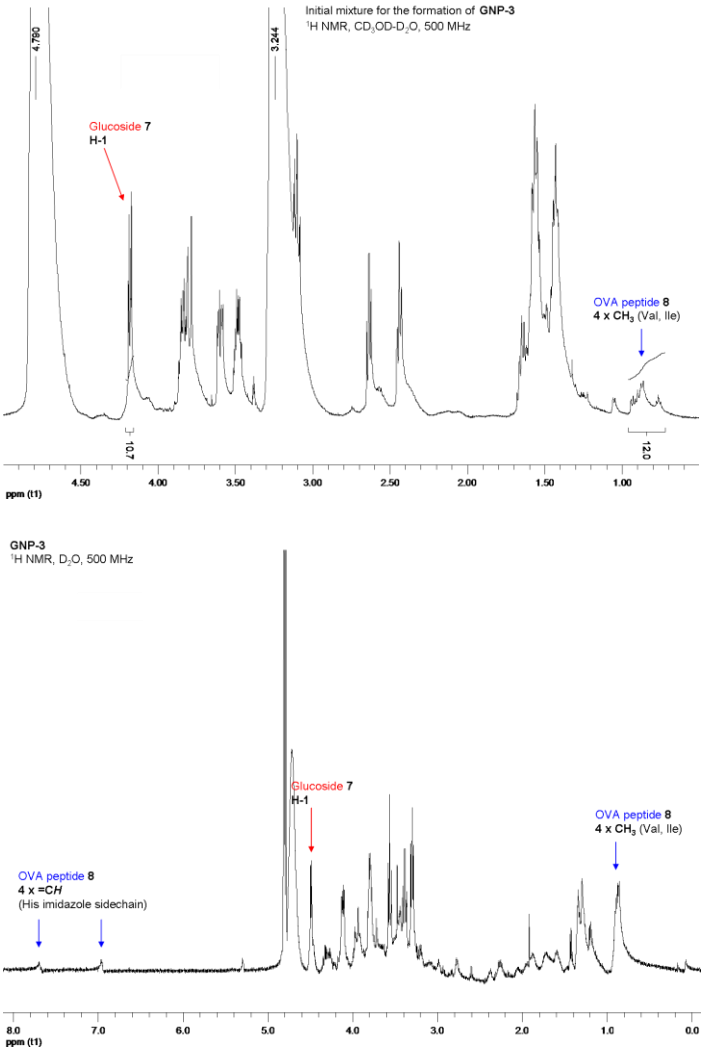

Figure S-6. *Upper panel*: ^{1}H NMR (500 MHz, CD_3OD:D_2O = 1:1) of the mixture used to prepare GNP-3. Integration of selected signals shows that the ratio between glucose conjugate 7 and OVA peptide derivative 8 is about 9:1. *Bottom panel*: ^{1}H NMR (500 MHz, D_2O, water suppression) of GNP-3. The selected signals show the presence of both glucose conjugate 7 and OVA peptide derivative 8 in the same nanoparticle.

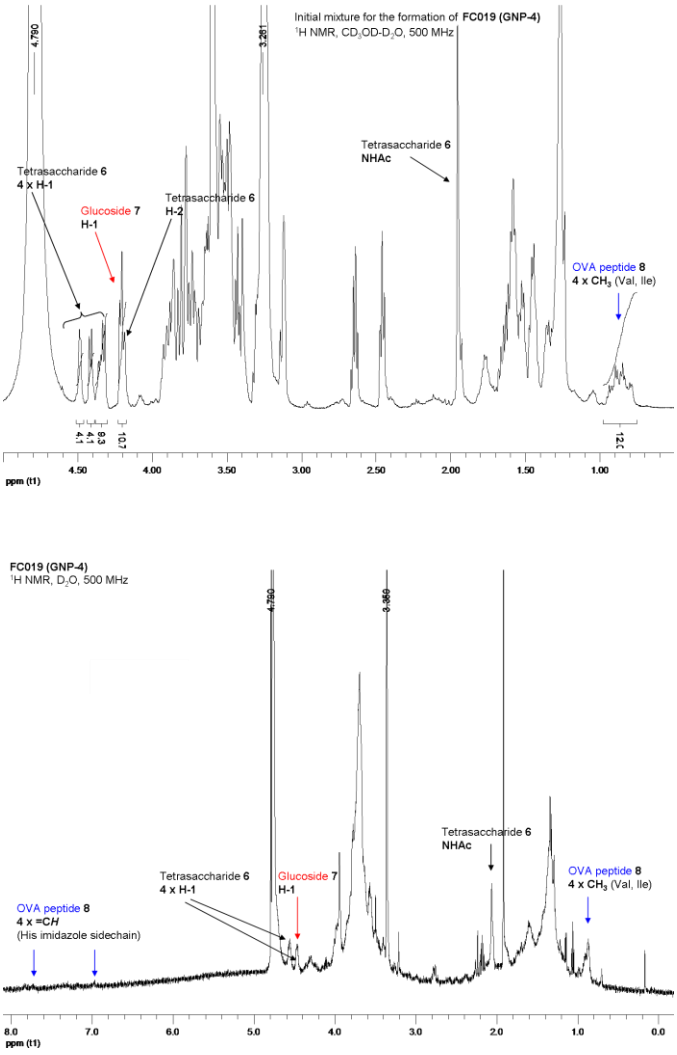

Figure S-7. *Upper panel:* ^{1}H NMR (500 MHz, CD_3OD:D_2O = 1:1) of the mixture used to prepare GNP-4. Integration of selected signals shows that the ratio between tetrasaccharide 6, glucose conjugate 7, and OVA peptide derivative 8 is about 4:5:1. *Bottom panel:* ^{1}H NMR (500 MHz, D_2O, water suppression) of GNP-4. The selected signals show the presence of all three components (tetrasaccharide 6, glucose conjugate 7, and OVA peptide derivative 8) in the same nanoparticle.

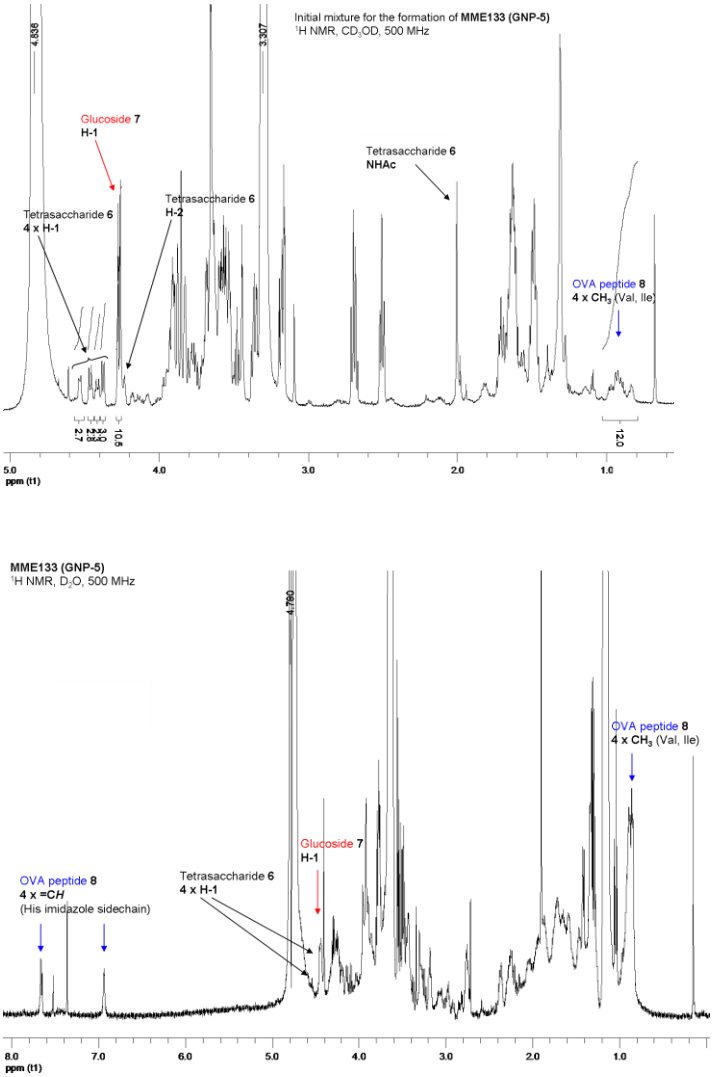

Figure S-8. *Upper panel*: ^{1}H NMR (500 MHz, CD_3OD) of the mixture used to prepare GNP-5. Integration of selected signals shows that the ratio between tetrasaccharide 6, glucose conjugate 7, and OVA peptide derivative 8 is about 2:7:1. *Bottom panel*: ^{1}H NMR (500 MHz, D_2O, water suppression) of GNP-5. The selected signals show the presence of all three components (tetrasaccharide 6, glucose conjugate 7, and OVA peptide derivative 8) in the same nanoparticle.

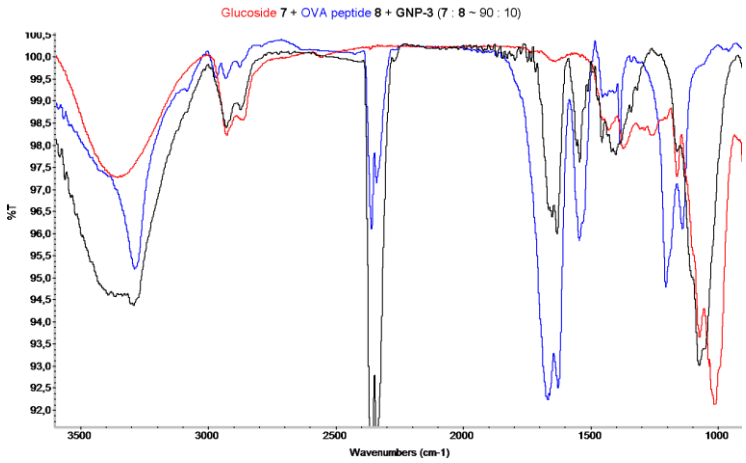

Figure S-9. Infrared spectra of gold glyconanoparticle GNP-3. Infrared (IR) spectra of GNP-3 (black) and its free components glucoside 7 (red) and OVA peptide conjugate 8 (blue), obtained after pressing each compound into KBr pellets. The bands around 1650 cm^{-1} which are present in the spectrum of GNP-3, and absent in the glucoside 7, indicate the incorporation of the OVA peptide conjugate 8 into GNP-3.

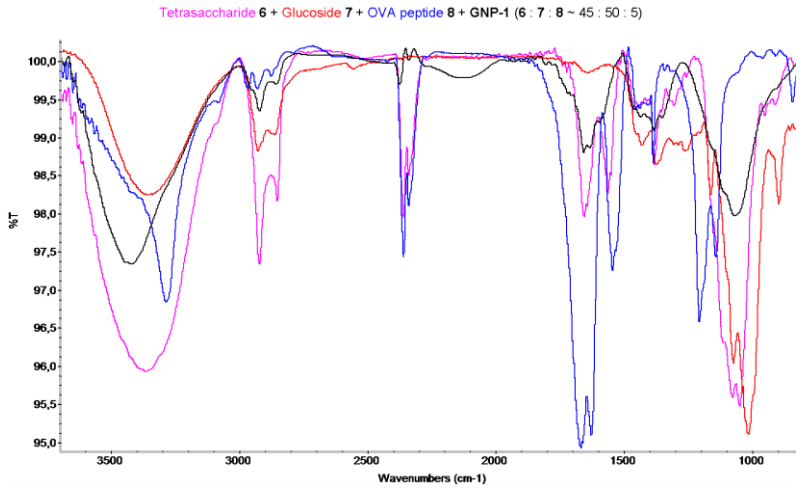

Figure S-10. Infrared spectra of gold glyconanoparticle GNP-1. Infrared (IR) spectra of GNP-1 (black) and its free components tetrasaccharide 6 (magenta), glucoside 7 (red) and OVA peptide conjugate 8 (blue), obtained after pressing each compound into KBr pellets.

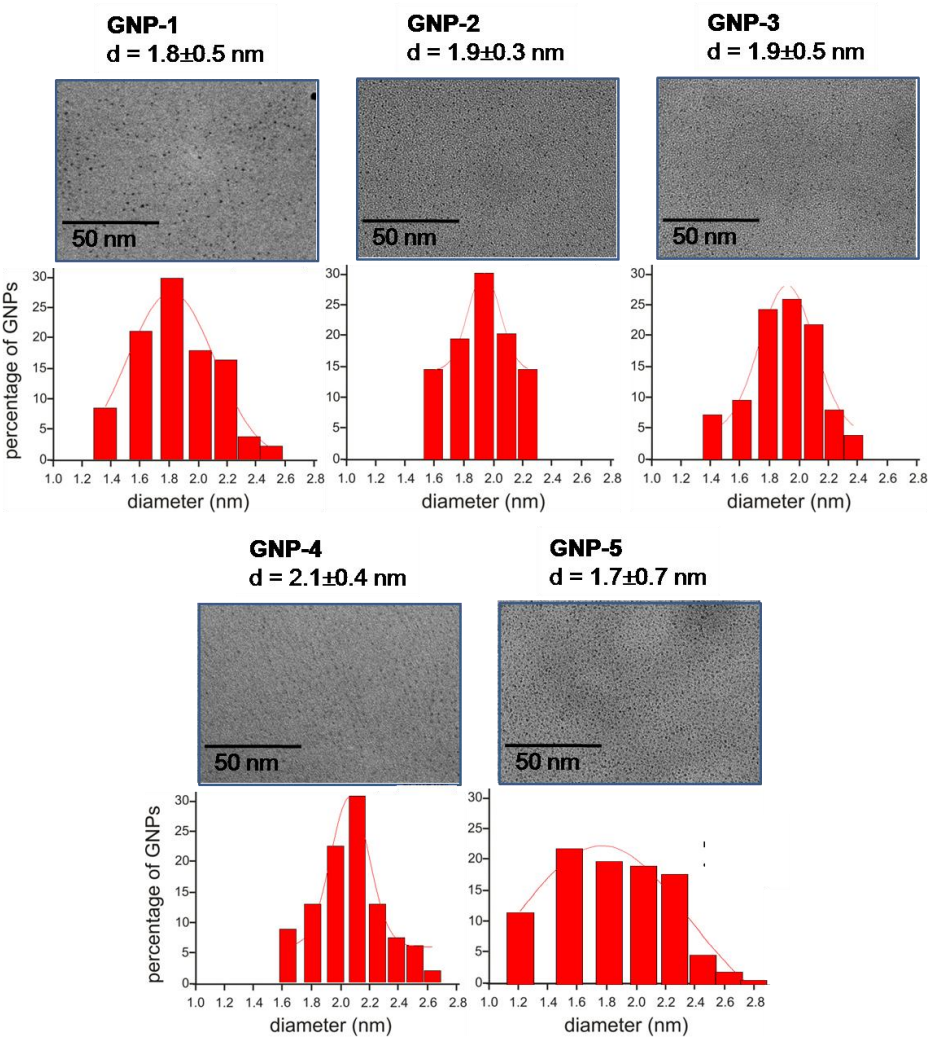

Figure S-11. TEM and size distribution of five GNPs.

Table 1S. Mice immunization studies

GNPs	n	Primary immunization		Approximately quantity[1]		Booster
		Adjuvant	GNP (µg)	Tetrasaccharide (µg)	Peptide (µg)	GNP (µg)
GNP-1b	5	MPL+QuilA	6	2.4±0.2	0.48±0.08	6
GNP-2	4	MPL+QuilA	6	2.6±0.2	-	6
GNP-3	3	MPL+QuilA	6	-	1.1±0.2	6
GNP-2 + GNP-3	5	MPL+QuilA	6+6	2.6±0.2	1.1±0.2	6+6
GNP-4	3	MPL+QuilA	6	2.0±0.2	0.88±0.15	15
GNP-5	5	MPL+QuilA	12	2.4±0.4	1.9±0.2	30
GNP-1a	5	MPL+QuilA	6	2.4±0.2	0.48±0.08	30
Pn14PS-CRM_{197}-	5	MPL+QuilA	0.5 µg conjugate[2]	-	-	2.5 µg conjugate[2]
$OVA_{323-339}$-peptide	5	MPL+QuilA	2.5 µg peptide	-	-	12.5 µg peptide
$OVA_{323-339}$-peptide-CRM_{197}-	5	MPL+QuilA	50 µg conjugate[3]	-	-	50 µg conjugate[3]
Saline	5	No	-	-	-	No

[1] Approximately quantity of GNPs are based on molar ratio of ligands of GNPs (Table 1)

[2] Dose of CRM_{197}-Pn14PS is based on carbohydrate part

[3] Dose of CRM_{197}-OVA-peptide immunization is based on complete conjugate

References

1. Martinez-Avila, O., Hijazi, K., Marradi, M., Clavel, C., Campion, C., Kelly, C., and Penades, S. (2009). Gold manno-glyconanoparticles: multivalent systems to block HIV-1 gp120 binding to the lectin DC-SIGN. Chemistry. 15**:** 9874-9888.
2. Sundgren, A., Lahmann, M., and Oscarson, S. (2005). Block synthesis of *Streptococcus pneumoniae* type 14 capsular polysaccharide structures. J. Carbohydr. Chem. 24**:** 379-391.

Chapter 7

General Discussion

General discussion

Streptococcus pneumoniae (*S. pneumoniae* or pneumococcus) infection is a leading cause of bacterial pneumonia, meningitis, and sepsis in children worldwide and it is estimated that 1.6 million people die from these infections each year, one million of whom are children (4,28). Capsular polysaccharides are well known as the major virulence factors of *S. pneumoniae*. Numerous studies have demonstrated that antibodies against polysaccharide are essential for protection against pneumococcal disease (3,19,21). Currently, two vaccine types are commercially available: a 23-valent pneumococcal polysaccharide vaccine (PPV23) and 7-valent pneumococcal conjugate vaccine (PCV7) (1).

In the search of new candidate pneumococcal conjugate vaccines investigations have been made by many researchers or industries such as covering more serotypes, reducing the effect of serotype replacement and making vaccines for a reasonable price and/or storing properties for people in the third world (Chapter 1). Our group has been working on a conjugate vaccine based on a synthetic carbohydrate (neoglycoconjugate) against *S. pneumoniae* (25). The advantages of such a vaccine are well-defined chemical structures (chain length, epitope conformation and carbohydrate/protein ratio) and absence of the impurities present in natural polysaccharides (2).

This thesis focuses on the *S. pneumoniae* type 14 capsular polysaccharide (Pn14PS) which consists of biosynthetic repeating units of the tetrasaccharide (13) {6)-[β-D-Galp-(1→4)-]β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-Glcp-(1→}n. Mawas, F. et al. reported that a synthetic branched tetrasaccharide, corresponding to a single structural repeating unit of Pn14PS conjugated to the cross-reactive material of diptheria toxin (CRM_{197}), was found to induce anti-polysaccharide type 14 antibodies (15). We are interested to investigate further how small the minimal structure in Pn14PS can be and still produce specific antibodies against native polysaccharide type 14 (Chapter 2). 16 overlapping oligosaccharide fragments of Pn14PS were synthesized as described previously (8,9,16,26) and were conjugated to the protein carrier CRM_{197}. The mice immunization studies were performed to investigate the immunogenicity of the neoglycoconjugates. We found that the fragments with a linear and/or incomplete branched structure did not elicit specific antibodies against native Pn14PS (Figure 1A). High titer of anti-Pn14PS IgG antibodies was observed when the complete branched structure fragments, conjugated to the protein carrier were used in the mouse model (Figure 1B).

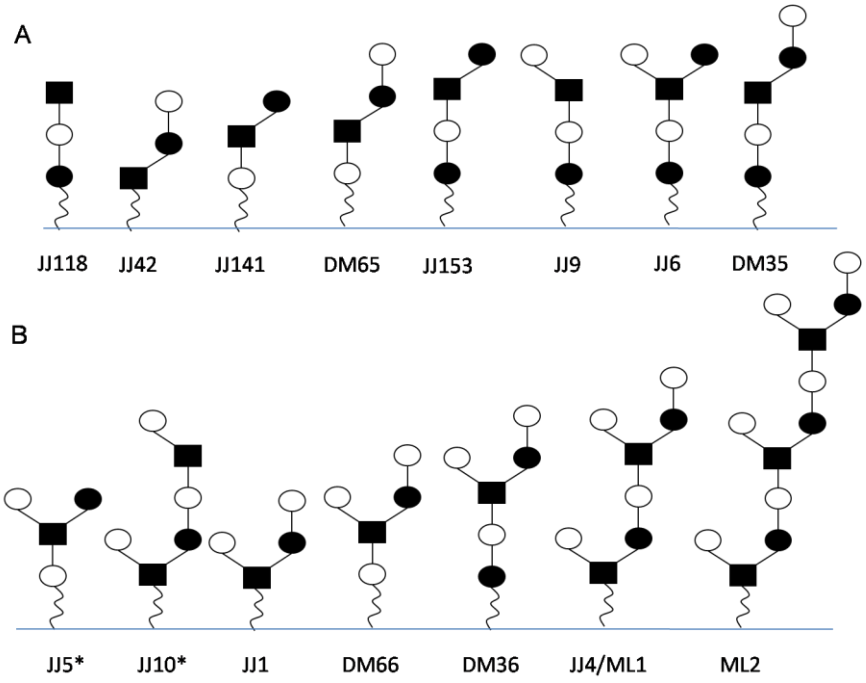

Figure 1. Schematic structure of overlapping synthetic oligosaccharide fragments of Pn14PS. The oligosaccharides were conjugated to CRM_{197} protein and the immunogenicity of those conjugates were studies in a mouse model. ELISA was employed to measure specific anti-Pn14PS IgG antibodies after the booster immunization. (A): Series of the oligosaccharide structure which did not elicit anti-Pn14PS IgG antibodies. (B): Series of the oligosaccharide structure which elicited high titer of anti-Pn14PS IgG antibodies, excepted for JJ5 and JJ10 (* low titer). Filled circle = glucose (Glc); open circle = galactose (Gal), and filled square = *N*-acetylglucosamine (GlcNAc).

All conjugates are equally potent in inducing antibodies against the spacer and against the carrier protein. Furthermore, all conjugates, also the ones belonging to group A in Figure 1, are able to induce antibodies to the homologous structure.The sera containing antibodies against Pn14PS were also capable of promoting the phagocytosis of *S. pneumoniae* type 14 by human polymorph nuclear cells and a mouse macrophage cell line. Conjugates that did not evoke specific antibodies against polysaccharide type 14 also did not display phagocytic capacity. We also confirmed that type-specific anti-Pn14PS antibodies did not cross-react with different types of polysaccharides of *S. pneumoniae* (appendix I). In conclusion, the

present study has shown that the branched trisaccharide Glc-(Gal-)GlcNAc is the core structure inducing Pn14PS-specific antibodies and that the neighboring galactose at the non-reducing end significantly contributes to the induction of phagocytosis-promoting antibodies. Our study provides evidence that the branched tetrasaccharide Gal-Glc-(Gal-)GlcNAc is a prime candidate for a synthetic oligosaccharide conjugate vaccine against infections caused by *S. pneumoniae* type 14 (23).

Native Pn14PS is structurally related to and has cross-reactivity with group B streptococcus type III polysaccharide (GBSIIIPS) (6). The branched structures of Pn14PS and GBSIIIPS differ only in the absence (in Pn14PS) or presence (in GBSIIIPS) of the (α2→3)-linked sialic acid N-acetylneuraminic acid (Neu5Ac) in their side chains: {→4)-β-D-Glcp-(1→6)-[±α-Neu5Ac-(2→3)-β-D-Galp-(1→4)-]β-D-GlcpNAc-(1→3)-β-D-Galp-(1→}n (10). Because of this we decided to also determine the minimal epitope in GBSIIIPS, using both a panel of anti-Pn14PS mouse sera and sera of humans vaccinated with either Pn14PS or GBSIIIPS (Chapter 3). The present study has shown that type-specific Pn14PS antibodies that recognize the branched structure of Pn14PS have a low affinity for the native GBSIIIPS and do not promote opsonophagocytosis of GBSIII. Desialylation of GBSIIIPS, however, resulted in dramatically higher affinity of anti-Pn14PS antibodies in mice. These results revealed that GBSIII bacteria are protected from binding of antibodies against Pn14PS by a residue of (α2→3)-linked sialic acid, as described previously (7,11). We showed that human anti-Pn14PS antibodies only recognized the branched structure of Pn14PS having -Glc-(Gal-)GlcNAc-Gal- as the absolute minimum, whereas human anti-GBSIIIPS antibodies recognized the linear structure of the backbone of both Pn14PS and GBSIIIPS: -Glc-GlcNAc-Gal-. In conclusion, the sialic acid in GBSIIIPS effectively shields their inner core of the polysaccharide and prevents recognition by type-specific Pn14PS antibodies. Pn14PS immunization elicits antibodies that only recognize the branched structure of Pn14PS while GBSIIIPS immunization elicits antibodies to the backbone structure of GBSIIIPS and Pn14PS. What is the importance of these findings? For the human host this means that immunity to serotype 14 pneumococci does not confer protection against GBSIII or vice versa, even though the capsular polysaccharides are antigenic similar. For the bacteria an important question remains to be solved. Is linkage of sialic acid to the capsular polysaccharide an effective way to escape from attack by the immune system? If so, are there more examples in nature? And is GBSIII a more successful invasive bacterium than type 14 pneumococci? What does it mean for our understanding of the immune system: Has sialidation of proteins a similar major

impact on the tertiary structure of proteins? Do specific antibodies to the proteins still bind after sialidation?

We investigated further the immune response to a neoglycoconjugate of Pn14PS on the outcome of sustained immunity to *S. pneumoniae* type 14 in a mouse model. Special attention was given to booster injections with either neoglycoconjugate or native Pn14PS (Chapter 4). Administration of pneumococcal polysaccharide vaccine in a boosting dose following a priming series of a pneumococcal conjugated vaccine has been assessed in clinical pneumococcal vaccine trials and seems controversial (18). In a number of studies, mostly in infants and young children, booster vaccination with native polysaccharide vaccines leads to an enhanced IgG anti-polysaccharide response (24,27). On the other hand, studies in adults and elderly have been performed which fail to demonstrate this effect (5,17). A study by Selma Wiertsema (unpublished) revealed that the avidity of the evoked antibodies still increased, which is a sign of ongoing memory formation. Others argue that booster vaccination with native polysaccharides may activate, but at the same time exhausts the memory B cell pool.

We found, as we expected, that the amount of specific IgG antibodies against Pn14PS increased substantially when a neoglycoconjugate booster was given to mice previously primed with the same neoglycoconjugate. The induced antibodies were capable to opsonise *S. pneumonia* type 14. Boosting with native polysaccharide following a primary conjugate vaccine injection did not result in IgG antibody formation to Pn14PS. In order to explain these phenomena we investigated how a booster immunization with a neoglycoconjugate or native polysaccharide affects the cell-mediated immune response by measuring the production profile of a panel of cytokines. We observed a high level of IL-5 in serum after a booster injection with neoglycoconjugate. Boosting with native polysaccharide Pn14PS did not result in the induction of IL-5 nor of any of the other tested cytokines. We conclude that induction of the cytokine IL-5 in serum is an early sign of a successful booster immunization and is a prerequisite for the production of specific anti-polysaccharide IgG antibodies. *In-vitro* spleen cell cultures were also used to investigate the effect of a booster injection on activation of memory T cells. IL-4 and IL-5, well known Th2 cytokines, were evoked by the neoglycoconjugate in spleen cell cultures of mice previously primed and boosted with the same neoglycoconjugate. Native polysaccharide Pn14PS, a neo-glycoconjugate with a different protein carrier and heat inactivated Pn14 bacteria all were unable to do so. Next to that we found a remarkable difference in activity for the two cytokines IFNγ and IL-17 which appeared to be related to the source of spleen cells used. Spleen cells derived from

polysaccharide boosted mice displayed higher levels of IFNγ and lower levels of IL-17 in comparison to cells derived either from unimmunized mice or mice boosted with the neoglycoconjugate. Further analyses revealed that the lowered IL-17 response to heat inactivated bacteria in polysaccharide boosted mice was not serotype specific. In conclusion, the inability of polysaccharide to boost primed mice might be due to the incapability to induce the cytokines IL-2, IL-4 and IL-5. In addition boosting with polysaccharide affects the key regulatory cytokine IL-17 which might have consequences for the overall immune defense to *S. pneumoniae* infections. Our experiments did not solve the questions raised above about the immunological consequences of a booster vaccination. They stressed the important role of cytokines, in particular IL-5 in the activation and stimulation of a B cell response and extended the role of cytokines in the involvement and activation of innate and cell mediated immune responses in the defense mechanism for *S. pneumoniae* infections.

The immunogenicity of synthetic carbohydrate vaccine was increased with adjuvant coadministration (12,23). We set out to investigate in a mouse model the effect of adjuvant coadministration on both the antibody- and cell-mediated immune response against a neoglycoconjugate of Pn14PS (Chapter 5). In the absence of adjuvant, immunization with neoglycoconjugate leads after a booster merely to IgG1 antibodies against PnP14PS. Coadministration of adjuvant had multituple effects: a diversified anti-Pn14PS IgG antibody response (also other IgG subclasses than IgG1 were evoked), an enhanced avidity and increased opsonic activity of these antibodies. We found that next to Quil-A also DDA as a single dose or in combination with CpG had similar effects on the diversification of eliciting a broader variety of anti-Pn14PS IgG antibody subclasses. Meanwhile, CpG or alum on their own showed in majority IgG1 antibodies after booster immunization in a same pattern as in non adjuvant groups. Compared to other adjuvants, codelivered Quil-A strongly improved the antibody avidity and enhanced the phagocytosis of *S. pneumoniae* type 14. In this study codelivering CpG showed to a lesser extent the induction of anti-Pn14PS IgG antibodies, antibody avidity and phagocytosis titer compared with other adjuvants. Quil-A only or in combination with MPL induced a rather high cytokine level of IL-5 and IL-6 just within six hours after primary immunization whereas other adjuvants did not. IL-5 and IL-6 both act as B-cell differentiation factors for polysaccharide specific cells by stimulating activated B cells to secrete antibodies (20). IL-5 is produced by T lymphocytes; IL-6 can be produced by a variety of cells, including T lymphocytes. The changes in systemic cytokine patterns are the consequence of the impact of the adjuvant on the local innate and acquired immune response. We have analyzed overall changes in CD4

and CD8 T cells in blood and draining lymph nodes and found a temporary increase in the number of CD4 T cells. This is compatible with studies of Malherbe et al who demonstrated that adjuvants like MPL increase TCR selection thresholds and enhance antigen-specific clonal expansion. In their view, vaccine adjuvants control the local accumulation of Th cells expressing TCR with the highest peptide MHC class II binding (14). Whether in our model also the changes in plasma cytokines are caused by the activation of CRM_{197}-specific T lymphocytes cannot be concluded yet. At any rate, our data show that codelivery of Quil-A enhanced antibody- and cell-mediated immune response to neoglycoconjugates. Quil-A as adjuvant given at the primary immunization site determines the outcome of immune response to neoglycoconjugate through. As stated above, the cellular and molecular processes by which these effects are achieved, are still largely unknown. Therefore, still a number of questions remain to be solved. Is targeting of the antigen by Quil-A to the dendritic cell the crucial step for the initiation of a successful immunization? Is it possible to replace Quil-A by incorporating specific ligands in the antigen/vaccine in such a way that targeting to dendritic cells could take place in a more physiological way? Does Quil-A initiate direct or indirect certain immune response genes and thereby determine the outcome of the booster immunization: evocation of other immunoglobulin isotypes and increase of their avidity to the antigen.

The synthetic branched tetrasaccharide (Gal-Glc-(Gal-)GlcNAc) of Pn14PS was applied for the synthesis of gold glyconanoparticles [GNPs]. The advantages of GNPs as a potential vaccine are numerous and include multivalency, control over variation in ligand number, size of the nanoparticles, water solubility, nontoxicity, high storage stability and also resistance to enzyme degradation (22). In the present study, we prepared a series of GNPs with different molar ratios of tetrasaccharide, inert D-glucose, and $OVA_{323-339}$-peptide in a one-step reaction. The immunization studies were performed to investigate the immunogenicity of GNPs in a mouse model (Chapter 6). We observed that in the hybrid GNP the presence of the T-cell stimulating peptide $OVA_{323-339}$-peptide was a prerequisite for the induction of specific IgG antibodies against native polysaccharide of Pn14PS or its own branched structure fragment. GNPs containing 45% of tetrasaccharide and 5% of OVA peptide, supplemented with inert D-glucose, were able to induce anti-Pn14PS antibodies. These experimental data indicate that epitope density on the gold surface is crucial to obtain significant levels of IgG antibodies. We could hardly detect specific antibodies against ovalbumin after immunization with these GNPs, which as such was not so surprising because the OVA peptide encompasses a T cell epitope. Indeed, *in-vitro* spleen stimulation demonstrated that memory T cells against $OVA_{323-339}$-peptide and ovalbumin protein were evoked. Spleen cells derived

from mice immunized with these GNPs were observed to produce cytokine IL-4 and IL-5 (Th2 markers, necessary to promote antibody production) when stimulated by OVA323-339-peptide. Such a response was not found in spleen cells derived from mice immunized with GNPs lacking the $OVA_{323-339}$-peptide. In conclusion, gold GNPs bearing the combination of tetrasaccharide and OVA323-339–peptide are capable to induce IgG antibodies specifically directed to the tetrasaccharide itself and to the capsular polysaccharide of *S. pneumoniae* type 14. Gold GNPs displaying at their surface the synthetic tetrasaccharide mimicking the polysaccharide B-cell epitope of *S. pneumoniae* type 14 are useful systems towards synthetic oligosaccharide-conjugate vaccines. Our findings that a synthetic oligosaccharide maintains its antigenic function and yet can be suitably presented to the immune system by gold nanoplatforms should encourage the use of gold GNPs as new systems in the development of a synthetic pneumococcal vaccine.

Future researches

As discussed in the introduction section of this thesis, synthetic oligosaccharide-protein conjugates are proven to be effective vaccines in mice. A logical next step would be a feasibility and immunogenicity study in human volunteers. Before that, a study should be started with synthetic oligosaccharide-protein conjugates for at least the pneumococcal serotypes 1, 4, 5, 9V and 18C and should even have been completed, because the minimal epitopes for these polysaccharides are still unknown.

To improve the immunogenicity of oligosaccharide-protein conjugates co-delivery of adjuvants are required. As an alternative to the addition of adjuvants, studies should be initiated to direct oligosaccharide-protein conjugates to dendritic cells by incorporation of specific ligands. Targeting to and activation of dendritic cells by TLR5 is a possibility to be explored.

The immunogenicity of the hybrid GNPs described in Chapter 6 is a major breakthrough. Previously a number of immunization studies with GNP was performed by our group, but failed for several different reasons: incorrect oligosaccharide exposure, crowding (by) and length of spacer, lack of a proper stimulating T-cell epitope etc. A next step is to extend these GNP studies by incorporation of target molecules to dendritic cells and to incorporate build-in adjuvants. So far we have proven the effectiveness of GNP for just one serotype. How to make a multivalent GNP vaccine? Should that be done by presentation of

all those oligosaccharides on one or on multiple different nanoparticles? Another line of research could focus on the fate of these GNPs in the body. As the GNP have by nature a metallic core, tracing should be possible *in vivo* and *in vitro*. Other than a complete new vaccine concept, GNPs might be an ideal antigen to solve basic immunologic questions.

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Summary

Streptococcus pneumoniae (pneumococcus) is a leading cause of bacterial pneumonia, meningitis, and sepsis in children worldwide. Incidence of invasive pneumococcus disease varies substantially by age, genetic background, socioeconomic status, immune status, and geographical location. Nowadays, the antibiotic resistance of S. pneumoniae bacteria has increased worldwide. This makes treatment of S. pneumoniae infections more difficult and stresses the importance of the development of effective vaccines as a strategy to reduce morbidity and mortality caused by S. pneumoniae infection.

Capsular polysaccharides are the major virulence factors of S. pneumoniae. Many studies have demonstrated that antibodies against polysaccharide are essential for protection against pneumococcal disease. A total of 92 different S. pneumoniae capsular polysaccharides exist, organized into serotypes and serogroups. Serotype-specific antibodies do not cross-react and for the immune system the 92 different polysaccharides correspond to 92 different bacteria.

Polysaccharides are well-known thymus-independent type-2 (TI-2) antigens that lack T-helper epitopes and therefore mainly induce IgM antibodies, and to a lesser degree IgG antibodies. The downside of TI-2 antigens is that they do not generate an antibody response in young children and that they are unable to induce immunological memory. Repeated immunization therefore does not lead to higher antibody titers.

The immunobiological characteristics of polysaccharide can be altered by conjugation of polysaccharide to a protein carrier (glycoconjugate) resulting in a switch to an anti-polysaccharide antibody response with characteristics of a T-cell-dependent response. This is reflected by the generation of memory B and T cells and the induction of high titers of anti-polysaccharide IgG antibodies after booster immunization. Furthermore, glycoconjugate vaccines are able to induce an antibody response in infants and young children. Currently, two vaccine types are commercially available: a 23-valent pneumococcal polysaccharide vaccine (PPV23) and 7-valent pneumococcal conjugate vaccine (PCV7). The PPV23 (23 purified capsular polysaccharides) vaccine was

shown to be moderately effective in young adults but not in young children and elderly and also not in immunocompromised patients. Large scale introduction of PCV7 (seven polysaccharides are conjugated to the non-toxic cross reactive material from diphtheria toxin [CRM197]) has resulted in an overall decline in invasive pneumococcal disease (IPD). However IPD caused by the non-vaccine serotypes has increased (replacement disease), highlighting the need for inclusion of additional serotypes in improved vaccine formulations to come.

In the search of new candidate pneumococcal conjugate vaccines investigations have been made by many researchers (both in academies as well as vaccine industries) on issues such as covering more serotypes, reducing the effect of serotype replacement and making vaccines for a reasonable price and/or more reliable storing properties for use in the third world (Chapter 1). Our group has been working on a conjugate vaccine based on a synthetic carbohydrate (neoglycoconjugate) against S. pneumoniae bacteria. The advantages of such a vaccine are well-defined chemical structures (chain length, epitope conformation and carbohydrate/protein ratio) and absence of the impurities often present in natural polysaccharides. Neoglycoconjugates have been prepared for saccharides of different microorganisms, i.e., Haemophilus influenza, Vibrio cholera O1, Shigella flexneri, and also for a synthetic carbohydrate-based antitumor candidate vaccine.

In this thesis, we focus on the immunogenicity of a series of synthetic overlapping oligosaccharide fragments corresponding to S. pneumoniae type 14 polysaccharide (Pn14PS) which were conjugated to CRM197 protein and evaluated in a mouse model (Chapter 2). We demonstrated that the branched trisaccharide element Glc-(Gal-)GlcNAc is essential in inducing Pn14PS-specific antibodies and that the neighboring galactose unit at the non-reducing end contributes clearly to the immunogenicity of this epitope. The effectivety of the antibodies was measured in a functional assay (opsonophagocytosis). The antibodies in sera obtained from mice immunized with neoglycoconjugates of Pn14PS did not cross-react with other S. pneumoniae polysaccharide serotypes (Appendix 1) and displayed very low binding activity with group B streptococcus type III polysaccharide (Chapter 5). We showed that a neoglycoconjugate booster vaccination is required for the activation of memory cells and the establishment of sustained immunity. A booster with native polysaccharide is ineffective to evoke functional antibodies (Chapter 3). We observed that codelivery of Quil-A alone or in combination with MPL had the most dramatic effect on antibody- and cell-mediated immune response to neoglycoconjugate of Pn14PS (Chapter 4). From these studies, we concluded that the branched tetrasaccharide Gal-Glc-(Gal-

)GlcNAc is a serious candidate for a synthetic oligosaccharide conjugate vaccine against infections caused by S. pneumoniae type 14.
The above mentioned synthetic branched tetrasaccharide was used for the synthesis of hybrid gold nanoparticles [GNPs] (Chapter 6). The advantages of GNPs as a potential vaccine platform are numerous and include multivalency, control over variation in ligand number, size of the nanoparticles, water solubility, nontoxicity, high storage stability and also resistance to enzyme degradation. In this thesis, we prepared a series of GNPs with different molar ratios of tetrasaccharide, inert D-glucose, and OVA323-339-peptide in a one-step reaction. Their immunogenicity was evaluated in the mouse model mentioned above.We found that the presence of the T-cell stimulating peptide OVA323-339 in the hybrid gold nanoparticles was a prerequisite for the induction of specific anti-Pn14PS IgG antibodies. We showed that well designed hybrid gold nanoparticles display a promising starting point towards the development of synthetic oligosaccharide-based vaccines.

The synthetic oligosaccharide-protein conjugates have proven to be effective vaccines in mice. A logical next step would be a feasibility and immunogenicity study in human volunteers. But before that, a study should be started with synthetic oligosaccharide-protein conjugates for other pneumococcal serotypes. As an alternative to the addition of adjuvants, experiments should be initiated directing oligosaccharide-protein conjugates to dendritic cells by incorporation of specific ligands. Dendritic cells are present in the skin and present foreign material to the immune system. Targeting to and activation of dendritic cells by TLR5 is well worth to be explored. The immunogenicity of the hybrid GNPs described in Chapter 6 is a major breakthrough. A next step is to extend these GNP studies by incorporation of target molecules to dendritic cells or to incorporate build-in adjuvants. So far we have proven the effectiveness of GNP for just one serotype. How to make a multivalent GNP vaccine? Should one nanoparticle contain all known variants or a multitude of particles each loaded with just one serotype? Another line of research could focus on the fate of these GNPs in vivo. As the GNP has by nature a metallic core, tracing should be possible in vivo and in vitro. Next to being a complete new vaccine concept, GNPs might be an ideal antigen to solve basic immunological questions.

Samenvatting

Streptococcus pneumoniae (de pneumococ) is wereldwijd de belangrijkste bacteriële verwekker van longontsteking, nekkramp en bloedvergiftiging bij kinderen. Het ziekmakend vermogen van de pneumococ varieert met de leeftijd, de genetische opmaak en de algehele gezondheid van het kind, de sociaal-economische status en geografische locatie. Antibiotica resistente pneumococcen komen tegenwoordig over de gehele wereld voor. Dit maakt het steeds moeilijker om pneumococcen infecties goed te behandelen en geeft aan dat het heel belangrijk is om vaccins te ontwikkelen teneinde de ziekte en sterfte door *S. pneumoniae* effectief te bestrijden.

Het kapselpolysacharide is de belangrijkste virulentie factor van S. pneumoniae. Veel studies hebben aangetoond dat afweerstoffen (antilichamen) die gericht zijn tegen het polysacharide essentieel zijn in de bescherming tegen pneumococcen infecties. Er bestaan 92 verschillende vormen van het kapsel die onder te verdelen zijn in serotypen en serogroepen. De 92 verschillende polysachariden komen overeen met 92 verschillende bacteriën. Antilichamen, specifiek voor het een bepaald serotype reageren niet kruislings met een ander serotype.

De afweerreactie tegen polysachariden verloopt zonder tussenkomst van T-cellen en daarom worden zij thymus onafhankelijke antigenen type 2 (TI-2) genoemd. Deze antigenen wekken in volwassenen voornamelijk IgM antistoffen op en in mindere mate IgG antistoffen. De keerzijde is dat jonge kinderen in het geheel niet reageren op TI-2 antigenen en zij zijn ook nog niet in staat een immunologisch geheugen op te bouwen.

Door koppeling van het polysacharide aan een eiwitdrager (glycoconjugaat) verandert het immunobiologisch karakter van polysachariden. Na immunizatie worden er nu anti-polysacharide antilichamen opgewekt met eigenschappen die ook voorkomen op T-cel-afhankelijke antigenen. Dit uit zich in de vorming van een immunologisch geheugen (zowel B- als T-cellen) en de vorming van grote hoeveelheden anti-polysacharide IgG antilichamen na een tweede of derde injectie. Bovendien zijn deze glycoconjugaat vaccins in staat antilichamen op te wekken in baby's en jonge kinderen.

Momenteel zijn er twee typen pneumococcen vaccins commercieel verkrijgbaar: het 23-valente pneumococcen polysacharide vaccin (PPV23) en het 7-valente pneumococcen conjugaat vaccin (PCV7). Het PPV23 vaccin (dat 23 gezuiverde kapselpolysachariden bevat) was matig effectief in jong-volwassenen maar in jonge kinderen, ouderen en patiënten met een verminderde afweer was het in het geheel niet werkzaam. De grootschalige introductie van PCV7 (dat zeven polysachariden bevat die gekoppeld zijn aan het niet toxisch eiwit CRM197) heeft geresulteerd in een afname van infecties veroorzaakt door de pneumococ bij jonge kinderen.

De zoektocht naar nieuwe kandidaat-vaccins voor de pneumococ wordt voortgezet door wetenschappers aan universiteiten en in de industrie. Er wordt onderzocht of het glycoconjugaat vaccin PCV7 verbeterd kan worden door meer serotypes hierin op te nemen. Anderen richten zich op het maken van een goed houdbaar vaccin voor een redelijke prijs voor de derde wereld (Hoofdstuk 1). Onze groep richt zich op de ontwikkeling van een conjugaat vaccin voor de pneumococ waarbij gebruikt gemaakt wordt van synthetische koolhydraten (neoglycoconjugaten). Het voordeel van een dergelijk vaccin is dat het om goed gedefinieerde structuren gaat (ketenlengte en koolhydraat/eiwit ratio) die vrij zijn van onzuiverheden die voorkomen in natuurlijke polysachariden. Door andere onderzoeksgroepen zijn ook neoglycoconjugaten gemaakt voor verschillende andere microorganismen, b.v. *Haemophilus influenzae*, *Vibrio cholera* O1 en *Shigella flexneri*. Zelfs een antitumor-kandidaat-vaccin op basis van synthetische koolhydraten wordt onderzocht.

In dit proefschrift richten wij ons op de immunogeniciteit (het vermogen om antilichamen op te wekken) van een reeks synthetische, deels overlappende oligosacharide structuren die overeenkomen met het kapselpolysacharide van S. pneumoniae type 14 (Pn14PS). De structuren werden gekoppeld aan het CRM197 eiwit en geëvalueerd in een muis model (Hoofdstuk 2). Wij stelden vast dat Gal-Glc-(Gal-)GlcNAc (een vertakte koolhydraat structuur bestaande uit 4 suikers) in staat was Pn14PS-specifieke antilichamen op te wekken. De opgewekte antilichamen die verkregen werden na immunizatie van muizen met de neoglycoconjugaten van Pn14PS gaven geen kruisreactie met andere S. pneumoniae polysacharide serotypen (Appendix 1) en vertoonden een zeer lage binding met groep B streptococcus type III polysacharide (Hoofdstuk 5). Wij toonden aan dat een tweede of derde injectie met het neoglycoconjugaat noodzakelijk was om geheugencellen te activeren en langdurige beschermende immuniteit op te bouwen. Polysachariden alleen waren niet in staat om functionele antilichamen op te wekken (Hoofdstuk 3). Gelijktijdige toediening van Quil-A, alleen of in combinatie met MPL met een neoglycoconjugaat had een dramatisch effect op de antilichaamproductie en op alle tussengelegen immunologische

reacties (Hoofdstuk 4). Uit al deze studies blijkt dat het vertakte koolhydraat Gal-Glc-(Gal-)GlcNac een serieuze kandidaat voor de ontwikkeling van een synthetisch koolhydraat-conjugaat vaccin is ter voorkoming van infecties veroorzaakt door S. pneumoniae type 14.

Het bovengenoemde koolhydraat is vervolgens gebruikt voor de synthese van samengestelde goud-nanopartikels [GNPs]. De voordelen van GNPs als potentieel vaccin zijn talrijk. De grootte, de samenstelling van de verschillende componenten (koolhydraat, peptide etc.) in de GNPs is volledig te variëren en te controleren. GNPs zijn in water oplosbaar, niet toxisch en langdurig houdbaar. In dit proefschrift hebben wij een reeks van GNPs gemaakt met verschillende hoeveelheden van het koolhydraat, glucose en het peptide OVA323-339. De bereiding van GNPs vond plaats in een één staps reactie. De werkzaamheid als vaccin werd geëvalueerd in het eerder genoemde muismodel. Wij vonden dat de aanwezigheid van het peptide OVA323-339 in de samengestelde GNPs noodzakelijk was om specifieke anti-Pn14PS antilichamen op te wekken in muizen. Wij toonden aan dat goed ontworpen hybride GNPs veelbelovend zijn als toekomstige volledig synthetische vaccins.

De synthetische koolhydraat-eiwitconjugaat vaccins zijn effectief in een muis model. Een logische volgende stap is deze vaccins uit te testen in menselijke vrijwilligers. Maar voordat een dergelijke studie uitgevoerd kan worden moeten er eerst synthetische conjugaat vaccins gemaakt worden voor andere serotypen van de pneumococ. Als alternatief voor het gebruik van adjuvantia, moeten er studies gestart worden om de koolhydraat-eiwitconjugaten direct te kunnen sturen naar dendritische cellen. Dendritische cellen komen in de huid voor en presenteren lichaamsvreemde stoffen aan het afweersysteem. Sturing naar en activatie van dendritische cellen door inbouw van eiwitten die kunnen binden aan TLR5 in het koolhydraat-eiwitconjugaat en GNP moet onderzocht worden. De toepassing van GNP als vaccin is een echte doorbraak. De werkzaamheid nu nog is slechts aangetoond voor één serotype. Hoe moet het vaccin eruit zien om effectief te zijn voor meerdere serotypen? Moet dat door alle verschillende koolhydraten tegelijk te presenteren op het oppervlak van één nanopartikel of moeten er meerdere verschillende GNPs gemaakt worden? Daarnaast kan er een onderzoekslijn opgestart worden over het gedrag van GNPs in het lichaam. Waar verblijft het na injectie. Omdat GNPs van nature een kern van goud bevatten, moet het mogelijk zijn deze partikels zowel in het lichaam alswel in een individuele cel te vervolgen. Naast het geschetste nieuwe vaccinconcept, zijn GNPs ideale structuren om basale immunologische vragen op te lossen.

Ringkasan

Streptococcus pneumoniae (pneumokokus) adalah penyebab utama pneumonia, meningitis, dan sepsis pada anak-anak di seluruh dunia. Kejadian penyakit invasi pneumokokus bervariasi secara substansial oleh umur, latar belakang genetik, status sosial ekonomi, status kekebalan, dan lokasi geografis. Saat ini, resistensi antibiotik terhadap bakteri *S. pneumoniae* telah meningkat di seluruh dunia. Hal ini membuat pengobatan infeksi ini lebih sulit yang menekankan betapa pentingnya pengembangan vaksin yang efektif sebagai strategi untuk mengurangi kesakitan dan kematian yang disebabkan oleh infeksi bakteri pneumokokus.

Kapsul polisakarida adalah faktor virulensi yang utama dari pneumokokus. Banyak penelitian telah menunjukkan bahwa antibodi terhadap polisakarida sangat penting untuk melindungi terhadap penyakit pneumokokus. Terdapat sebanyak 92 jenis polisakarida dari pneumokokus yang dikelompokan kedalam serotipe and serogroup. Antibody specific serotipe tertentu tidak terjadi reaksi silang dan untuk sistem immun, 92 jenis polisakarida korespondensi dengean 92 bakteri yang berbeda.

Polisakarida terkenal sebagai *timus-independent tipe-2* (TI-2) antigen memiliki kekurangan epitop *T-helper* sehingga hanya menginduksi antibodi IgM, dan dengan kadar lebih rendah menginduksi antibodi IgG. Kelemahan dari TI-2 antigen adalah tidak menghasilkan respon antibodi pada anak-anak dan tidak dapat menginduksi memori imunologi. Imunisasi berulang tidak menyebabkan kadar antibodi lebih tinggi.

Karakteristik immunobiologi dari polisakarida dapat diubah dengan konjugasi polisakarida ke pembawa protein (glikokonjugat) sehingga karakteristik dari anti-polosakarida antibodi berubah menjadi *T-cell-dependent*. Hal ini tercermin dengan adanya memori sel B dan T yang menginduksi antibodi IgG dengan kadar yang tinggi setelah immunisasi berulang. Selanjutnya, vaksin glikokonjugat dapat merangsang respon antibodi pada bayi dan anak-anak muda. Saat ini, tedapat dua jenis vaksin yang tersedia secara komersial: vaksin polisakarida pneumokokus 23-valent (PPV23) dan 7-valent pneumokokal konjugat (PCV7). Vaksin PPV23 (23 polisakarida kapsul murni) terbukti cukup efektif dalam dewasa muda tetapi tidak pada anak-anak dan orang tua dan juga tidak pada pasien dengan kondisi sistem immun tertekan.

pengenalan skala besar PCV7 (tujuh polisakarida yang terkonjugasi dengan *the non-toxic cross reactive material* dari *diphtheria toxin* [CRM197]) telah menghasilkan penurunan secara keseluruhan pada penyakit invasi dari pneumokokus (IPD: *invasive pneumococcal disease*). Namun IPD yang disebabkan oleh serotipe non-vaksin telah meningkatkan (*replacement disease*), sehingga dibutuhkan polisakarida serotipe lain sebagai tambahan dalam formulasi vaksin yang baru.

Pencarian vaksin pneumokokus yang baru telah banyak dilakukan oleh para peneliti (baik di institusi pendidikan serta perusahaan vaksin) dengan focus pada peningkatan jumlah serotipe dari vaksin untuk mengurangi efek *replacement disease* dan membuat vaksin dengan harga yang wajar yang dapat aplikasikan di negara-negara berkembang (Bab 1). Group kami bekerja dengan vaksin konjugat berdasarkan sintetik karbohidrat (neoglikokonjugat) untuk melawan bakteri *S. pneumoniae*. Keuntungan dari vaksin ini adalah struktur kimiawi yang terdefinisi dengan baik (panjang rantai, konfirmasi epitop dan rasio karbohidrat/protein) dan tidak adanya kontaminsai yang sering terdapat dalam polisakarida. Neoglikokonjugat telah diteliti untuk sakarida dari mikroorganisme yang berbeda, yaitu *Haemophilus influenza*, *Vibrio cholera* O1, *Shigella flexneri*, dan juga untuk kandidat vaksin antitumor berdasarkan karbohidrat sintetik.

Dalam tesis ini, kami memfokuskan pada imunogenisitas dari serangkaian fragmen oligosakarida sintetik yang saling tumpang tindih berdasarkan struktur dari polisakarida dari *S. pneumoniae* tipe 14 (Pn14PS) yang terkonjugasi dengan CRM197 protein dan dievaluasi dengan tikus sebagai modelnya (Bab 2). Kami menunjukkan bahwa elemen trisakarida bercabang Glc-(Gal-)GlcNAc sangat penting dalam menginduksi speficik antibodi untuk Pn14PS dan unit galaktosa yang berdekatan dengan ujung non-reduksi sangat jelas berkontribusi pada imunogenisitas dari epitop tersebut. Keefektifan dari antibodi diukur dengan uji fungsional (*opsonophagocytosis*). Antibodi di serum yang diperoleh dari tikus yang telah diimunisasi dengan neoglikokonjugat dari Pn14PS tidak bereaksi silang dengan polisakarida dari serotipe *S. pneumoniae* yang lain (Lampiran 1) dan menunjukan interaksi yang sangat rendah dengan polisakarida dari bakteri group B streptococcus tipe III (Bab 5). Kami menyakinkan bahwa vaksinasi berulang dengan neoglikokonjugat diperlukan untuk mengaktivasi sel memori dan pembentukan kekebalan yang berkesinambungan. Immunisai berulang dengan polisakarida asli tidak efektif untuk membangkitkan antibodi fungsional (Bab 3). Kami mengamati bahwa ko-administrai dari *adjuvant* Quil-A sendiri atau dalam kombinasi dengan MPL memiliki pengaruh yang paling dramatis terhadap respon immun via antibodi and sel-mediasi terhadap neoglikokonjugat dari Pn14PS (Bab 4). Dari penelitian ini, kami menyimpulkan

bahwa tetrasakarida bercabang Gal-Glc-(Gal-)GlcNAc merupakan kandidat serius untuk vaksin konjugat oligosakarida sintetik terhadap infeksi yang disebabkan oleh *S. pneumoniae* tipe 14.

Sintetik bercabang tetrasakarida di atas diaplikasikan untuk sintesis dari hibrida nanopartikel emas [GNPs] (Bab 6). Keuntungan dari GNPs sebagai platform vaksin potensial dengan multivalensi adalah dapat mengkontrol variasi dalam jumlah ligan, ukuran nanopartikel, kelarutan, tidak beracun, stabilitas penyimpanan yang tinggi dan juga tahan terhadap enzim degradasi. Dalam tesis ini, kami meneliti serangkaian GNPs dengan rasio molar berbeda antara tetrasakarida, D-glukosa, dan OVA323-339-peptida dalam satu langkah reaksi. Imunogenitas dari GNPs dievaluasi di tikus. Kami menemukan bahwa kehadiran peptida OVA323-339 yang merangsang sel T dalam hibrida nanopartikel emas merupakan prasyarat untuk menginduksi spesifik antibodi terhadap Pn14PS IgG. Kami menunjukkan bahwa nanopartikel emas dirancang dengan hibrida merupakan sebuah awal yang menjanjikan terhadap pengembangan vaksin berbasis oligosakarida sintetik.

Konjugasi oligosakarida sintetik dengan protein telah terbukti sebagai vaksin yang efektif pada tikus percobaan. Langkah logis berikutnya adalah studi kelayakan dan immunogenesitas pada sukarelawan manusia. Tapi sebelum itu, studi harus dimulai dengan konjugasi oligosakarida sintetik-protein untuk pneumokokus serotipe lainnya. Sebagai alternatif dengan penambahan *ajduvant*, eksperimen harus dimulai dengan target terhadapa sel dendritik dengan penggabungan ligan tertentu. Sel dendritik hadir di dalam kulit dan bahan asing untuk sistem kekebalan tubuh. Target dan aktivasi sel dendritik oleh TLR5 sangat layak untuk di ujicoba. Imunogenisitas dari hibrida GNPs yang dijelaskan dalam Bab 6 adalah sebuah terobosan besar. Langkah selanjutnya adalah memperluas studi GNP ini dengan penggabungan target molekul untuk sel dendritik atau dengan *adjuvant*. Sejauh ini kami telah membuktikan efektivitas GNP hanya satu serotipe. Bagaimana cara membuat vaksin GNP yang multivalent? Haruskah satu nanopartikel berisi semua varian yang diketahui atau banyak partikel masing-masing sarat dengan hanya satu serotipe? Penelitian lain bisa difokuskan untuk studi GNP secara *in vivo*. Memungkinkannya penelusuran GNP yang mempunyai inti metalik secara *in vivo* and *in vitro*. Sehingga GNP akan menjadi sebuah konsep vaksin baru yang lengkap, GNPs mungkin merupakan antigen yang ideal untuk memecahkan pertanyaan imunologi dasar.

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Curriculum vitae

Dodi Safari was born on March 5th 1977 in Cianjur, West Java, Indonesia. He grew up in Nagrak - Cipanas, a small village closed by the Puncak tea plantation. After senior high school, the author moved to Bogor – West Java and started to study chemistry in 1996, at the Department of Chemistry, Bogor Agricultural University. He was awarded the degree of bachelor of science with *cum laude*. His final year project was on "the evolution study from three subspecies of *Oryza sativa*: Indica, Javanica, and Japonica, by apocytochrome b sequence analysis", under the supervision of Dr. H. Sudoyo and Dr. Sulistiyani.

In 2001, he joined the mitochondrial group at Eijkman Institute for Molecular Biology, Jakarta, as a research asisstant, supervised by Dr. H. Sudoyo. He was actively involved in the study of human genome diversity in Indonesia based on the mitochondrial DNA sequence and provided support for the Sequencing and DNA forensics units of the Institute.

In 2006, he left for Utrecht, the Netherlands as a PhD student in the Department of Medical Microbiology, University Medical Center Utrecht. He joined and was supported in his endeavor by the European network program (Glycogold), a Marie Curie research training network program. As described in this thesis, his PhD project is on the development of new synthetic oligosaccharide vaccines, under the supervision of Dr. H. Snippe, Dr. G.T. Rijkers, Prof. Dr. J. Verhoef, and Prof. Dr. J.P. Kamerling.