Conference paper

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Oligosaccharide ligand tuning in design of third generation carbohydrate pneumococcal vaccines

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Abstract: *Streptococcus pneumoniae* can cause many types of dangerous infectious diseases such as otitis media, pneumonia, meningitis and others that are more common in the very young and very old age. Available to date commercial vaccines based on capsular polysaccharides of *S. pneumoniae* of clinically important strains (first generation carbohydrate vaccines) and conjugated vaccines based on these polysaccharides (second generation carbohydrate vaccines) have certain limitations in protective efficiency. However, the efficiency of vaccines can be increased by the use of third generation vaccines based on synthetic oligosaccharide ligands representing in their structures the protective epitopes of capsular polysaccharides. The proper choice of an optimal oligosaccharide ligand is the most important step in the design of third generation carbohydrate vaccines. Herein we overview our works on the synthesis of three oligosaccharides corresponding to one, "one and a half" and two repeating units of *S. pneumoniae* type 14 capsular polysaccharide, immunogenic conjugates thereof and comparative immunological study of their conjugates with bovine serum albumin, which was used as a model protein carrier. The ability of obtained products to raise antibodies specific to capsular polysaccharide and homologous oligosaccharides, the induction of phagocytosis by immune antisera and active protection of immunized animals from *S. pneumoniae* type 14 infection were evaluated. On the basis of the results obtained tetrasaccharide comprising the repeating unit of *S. pneumoniae* type 14 capsular polysaccharide is an optimal carbohydrate ligand to be used as a part of the third generation carbohydrate pneumococcal vaccine.

Keywords: antibody; biotinylated oligosaccharide; Mendeleev XX; opsonophagocytosis; pneumococcal vaccine; protective activity; *Streptococcus pneumoniae* type 14.

Introduction

Prevention of pneumococcal diseases with polysaccharide and conjugate vaccines based on capsular polysaccharides (CPs) of *Streptococcus pneumoniae* has led to a significant reduction of the morbidity rate of otitis media, pneumonia, meningitis and mortality in all age groups [1–5]. The efficacy of polysaccharide-based

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vaccines containing CPs of clinically significant serotypes of *S. pneumoniae* is due to the fact that the polysaccharide capsule surrounding the bacterial cell is one of the major protective antigens of pneumococci [6].

The currently used first generation polysaccharide-based vaccines consist of a mixture of CPs. Their main disadvantage is a lack of efficiency in children of younger age groups because purified CPs are unable to induce T-dependent immune response with the following production of CP-specific IgG antibodies and memory cells [7, 8]. For the induction of the T-dependent immune response, the second generation of pneumococcal vaccines has been developed in which CPs are covalently bound to a protein carrier (diphtheria or tetanus toxoids or protein D of *Haemophilus influenzae* type b) [3, 9, 10].

The main disadvantages of the first and second generation carbohydrate vaccines are expensive and time-consuming processes of production and purification of CPs, the inevitable presence of bacterial impurities in the final product, insufficient immunological activity of some CPs of *S. pneumoniae* [11], the necessity to work with living bacteria cells and, additionally for conjugate vaccines, not always successful conjugation of CPs to the protein carrier [12]. Despite the relative efficacy of the first and second generation carbohydrate vaccines, their disadvantages determine the necessity of further improvement of the design of pneumococcal vaccines.

Currently, third generation carbohydrate pneumococcal vaccines [13] based on comprising protective epitopes synthetic analogues of fragments of the CPs of *S. pneumoniae* are under development. These studies include several technological approaches: development of neoglycoconjugate vaccines with adjuvant [14]; peptide-free, liposome-based oligosaccharide vaccine adjuvanted with a natural killer T cell antigen [15]; lipid-carbohydrate conjugate vaccine without protein carrier which stimulates invariant natural killer T (iNKT) cells that possess the ability to stimulate the production of high-affinity IgG antibodies specific for pneumococcal polysaccharides and long-lived memory B cells [16]; the use of gold nanoparticles as carriers for synthetic oligosaccharides [17]. Advantages of neoglycoconjugate vaccines include the well-defined structure of the protective epitope, the possibility to control the binding of ligands to the protein carrier and appropriate conformational structure of the ligands.

The main problem in the development of such vaccines is the absence of convenient rules for correct choice of efficient oligosaccharide ligands to be used in vaccines. The search for them includes the synthesis of oligosaccharides corresponding to different fragments of the polysaccharide chain, their conjugation to a protein carrier for induction of T-dependent immune response, evaluation of their immunogenicity by measuring the level of specific IgG antibodies in sera of immunized animals and the ability of sera to promote opsonophagocytosis, as well as the study of the protective activity of the obtained neoglycoconjugates by challenging animals with the relevant serotype of *S. pneumoniae*. In the 2000s, such an approach was applied to the search for an optimal oligosaccharide ligand for *S. pneumoniae* type 14 [18]; however, one of the most important characteristics of a vaccine candidate, namely, the protective activity in vivo was not determined.

In this review, we summarize the results of our studies on the determination of the most appropriate oligosaccharide ligand related to *S. pneumoniae* type 14 for the design of candidate pneumococcal vaccine with the use of synthetic oligosaccharides representing one (**1a**), "one and a half" (**2a**) and two (**3a**) repeating units of the CP (Fig. 1); their conjugates **1b**, **2b** and **3b** with the model protein carrier BSA; as well as biotinylated oligosaccharides **1c**, **2c** and **3c** used as coating antigens in ELISA assays. The choice of *S. pneumoniae* type 14 is explained by its high prevalence in the population, a high degree of invasiveness and the ability to cause severe pneumococcal diseases in children [19, 20].

Synthesis of oligosaccharides related to the capsular polysaccharide of *S. pneumoniae* type 14

The capsular polysaccharide of *S. pneumoniae* type 14 is built of tetrasaccharide repeating units **4** (Fig. 2), which in turn consist of lactose and N-acetyllactosamine blocks connected through a β-(1→3′)-linkage. In the late 1980s, Kochetkov and coworkers synthesized polysaccharide **4** with an average degree of polymerization of 10 (n = 10) by polycondensation of a tetrasaccharide monomer bearing a donor 1,2-O-(1-cyanoethylidene)

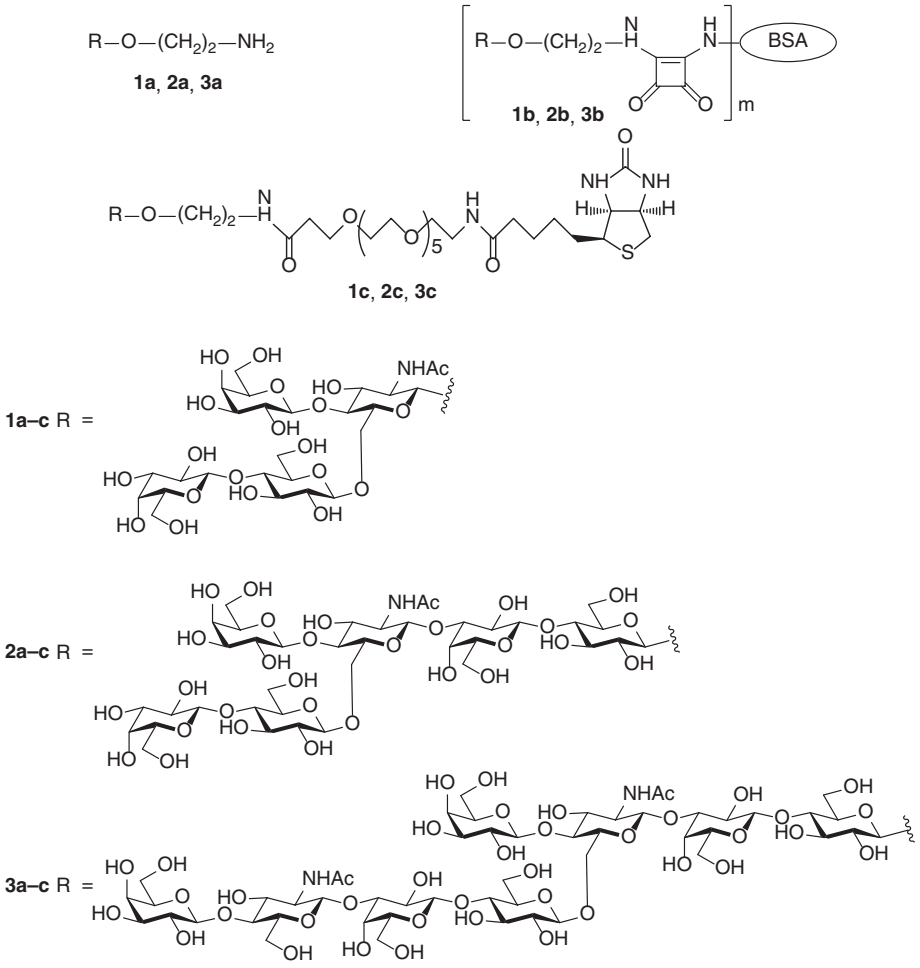

Fig. 1: Structures of synthetic oligosaccharides and their BSA and biotin conjugates used to determine an optimal ligand for a synthetic glycoconjugate vaccine.

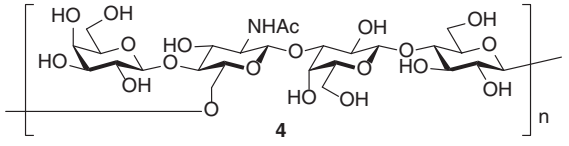

Fig. 2: Repeating unit of capsular polysaccharide of *S. pneumoniae* type 14.

function in the glucose residue and an acceptor 6-trityloxy group in the glucosamine unit [21]. A large set of oligosaccharides related to the capsular polysaccharide of *S. pneumoniae* type 14 was synthesized in the late 1990s – early 2000s [22, 23]. This set included oligosaccharides representing different regions of the polysaccharide chain from trisaccharides up to the dodecasaccharide comprising three repeating units.

According to the structure of the polysaccharide, disaccharide synthetic blocks derived from lactose (**5**, **8**) and lactosamine (**6**, **7**, **9**) were employed for the assembling of the target oligosaccharides (Fig. 3) [24].

The synthesis of the oligosaccharides has been carried out in a straightforward manner using regioselective glycosylation of 3-OH in galactose and 6-OH in glucosamine. The protected precursor of tetrasaccharide **1a** was obtained in 87% yield by TMSOTf-catalyzed glycosylation of lactosamine acceptor **9** with lactose trichloroacetimidate **8** (Fig. 4). Deprotection of tetrasaccharide **10** included acidic removal of the isopropylidene group, removal of all O- and N-acyl groups by treatment with hydrazine hydrate, exhaustive N,O-acetylation

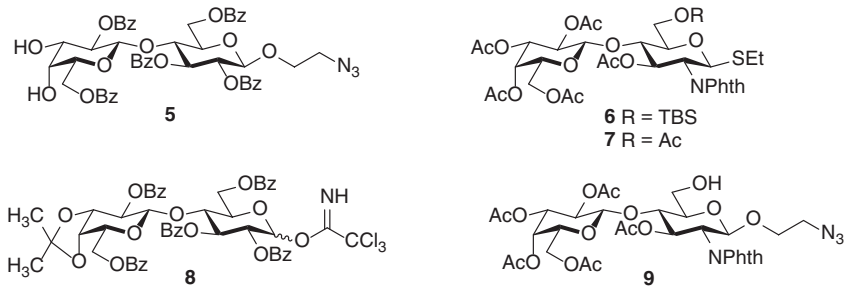

Fig. 3: Lactose and lactosamine building blocks for assembling target oligosaccharides.

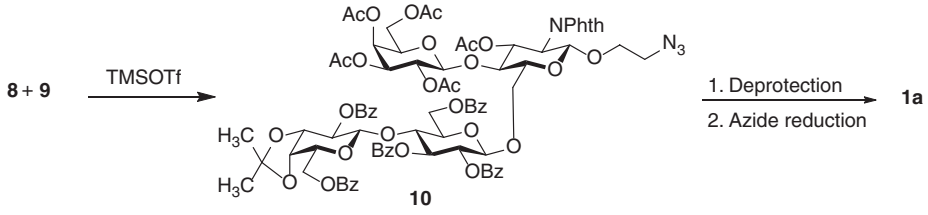

Fig. 4: Synthesis of tetrasaccharide corresponding to one repeating unit of capsular polysaccharide of *S. pneumoniae* type 14.

with Ac_2O in pyridine and O-deacetylation with sodium methoxide. In the final step, the azido group was reduced by hydrogenation over PdO/C to produce tetrasaccharide **1a**.

The synthesis of hexa- and octasaccharides is outlined in Fig 5. Lactoside acceptor **5** was subjected to NIS–TfOH-promoted regioselective 3-*O*-glycosylation with lactosamine thioglycoside **6** followed by removal of the TBS protection to afford tetrasaccharide diol **11** in 76% yield. Further glycosylation of **11** with lactose imidate **8** proceeded highly regioselectively at the primary hydroxyl group and furnished the corresponding hexasaccharide (89%) that provided triol **12** after removal of the isopropylidene group from the terminal

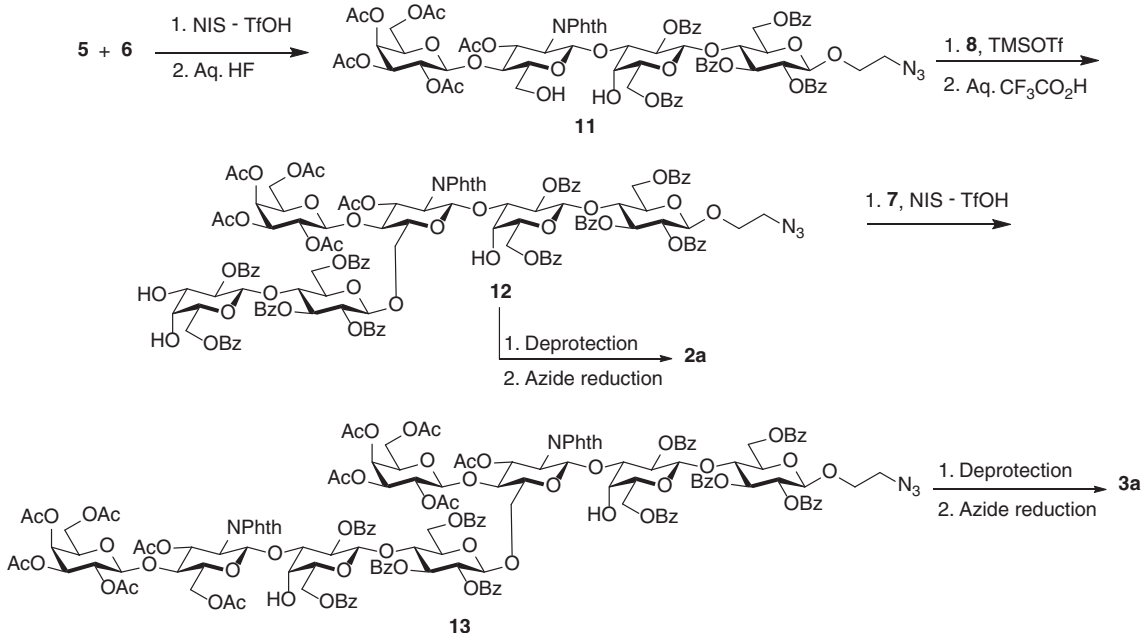

Fig. 5: Synthesis of hexa- (**2a**) and octasaccharide **3a** representing to "one and a half" and two repeating units of capsular polysaccharide of *S. pneumoniae* type 14.

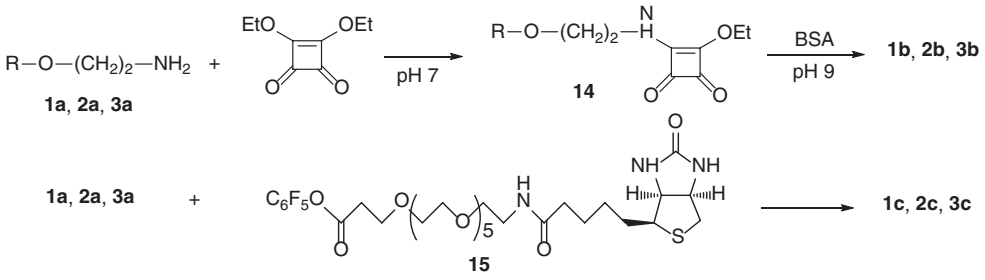

Fig. 6: Conjugation of oligosaccharides with BSA and biotin.

galactose residue. Deprotection of **12** followed by reduction of the spacer azido group as described above produced free hexasaccharide **2a**.

Final NIS–TfOH-promoted glycosylation of **12** with lactosamine thioglycoside **7** also demonstrated high regioselectivity and afforded octasaccharide **13** in 80% yield. The latter was converted into free oligosaccharide **3a** using the standard procedure described above.

The synthesized oligosaccharides were converted to immunogens **1b–3b** [24] by conjugation to the model protein carrier BSA via a squarate linker [25] (Fig. 6). Acylation of the oligosaccharides with pentafluorophenyl ester **15** [26] derived from biotin equipped with a flexible and hydrophilic hexaethylene glycol linker provided biotin conjugates **1c–3c** [27] applied as coating antigens in the ELISA assay.

Search for an optimal oligosaccharide ligand of *S. pneumoniae* type 14

For a correct determination of the structure of the ligand that could be suitable for the construction in the future of a multicomponent third generation carbohydrate pneumococcal vaccine, a series of immunological tests was conducted including the ability of conjugates to induce the production of IgG antibodies specific to homologous oligosaccharides (OSs) and capsular polysaccharides (CP), the ability of sera obtained after immunization with the conjugates to promote opsonophagocytosis of bacterial cells and the protective activity of the conjugates.

Immunogenicity of neoglycoconjugates was assessed by the formation of antibodies in mice. We determined the level of antibodies in the sera to homologous OSs **1c–3c** and CP of *S. pneumoniae* type 14. All conjugates **1b–3b** were immunogenic but to a different extent. The highest titer of antibodies specific to CP induced conjugated octasaccharide **3b** (Fig 7b), whereas production of the highest level of antibodies to the homologous OS evoked conjugated tetrasaccharide **1b** (Fig 7a). Hexasaccharide conjugate **2b** possessed the lowest immunogenicity giving only a low antibody production to CP and to homologous biotinylated OS **2c**. The titers of antibodies to OSs **1c–3c** in the same sera of mice immunized with conjugates **1b–3b** absorbed on aluminum hydroxide were 66, 32 and 16 times higher than the titers of antibodies detected by CP (Fig. 7) [28]. It was shown previously that some oligosaccharides conjugated to a protein induce the formation of antibodies to capsular polysaccharides at a higher level than traditional polysaccharide conjugate vaccines [29]. We revealed a higher titer of antibodies to the OSs compared to the level of antibodies to CP in the same immune sera probably due to the fact that antibodies to conjugated OSs do not precisely correspond to the conformational structure of the CP. For conjugated OSs **1b–3b** related to *S. pneumoniae* type 14, this fact has been observed for the first time.

Thus, in the ELISA assay for the evaluation of the level of antibodies specific to biotinylated OSs **1c–3c**, conjugate **1b** based on tetrasaccharide **1a** corresponding to the repeating unit of CP of *S. pneumoniae* type 14 possessed the highest immunogenic activity. At the same time, it was revealed that antibodies to conjugate **1b** have relatively low affinity to bacterial CP.

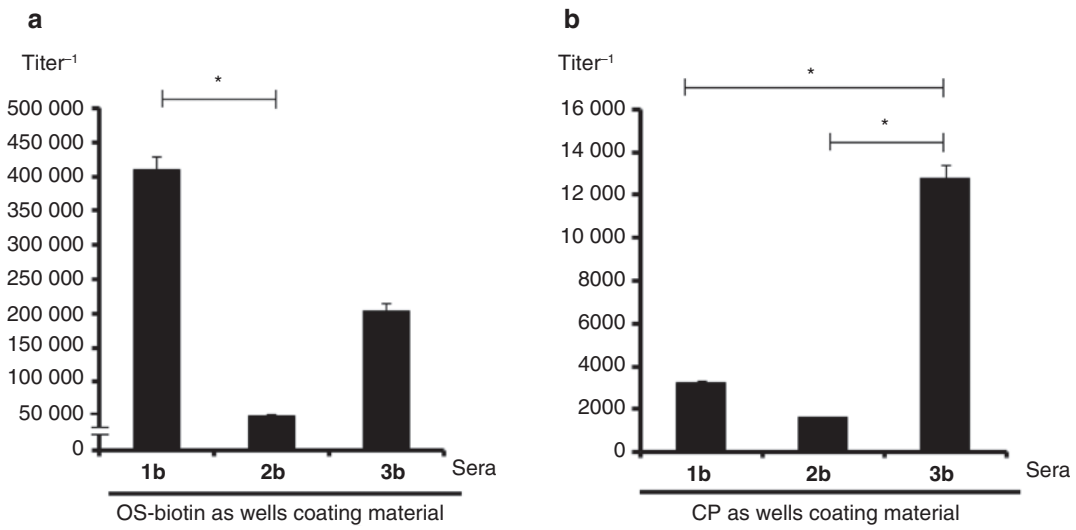

Fig. 7: Titer of IgG1 antibodies to biotinylated OSs **1c–3c** as coating antigens (a) and to CP as coating antigen (b).

The specificity of antibody binding in sera to conjugates **1b–3b** and CRM_{197}-CP with ligands **1a–3a** in ELISA inhibition assay demonstrated that tetrasaccharide **1a** possessed the higher capacity to inhibit the binding between anti-OSs antisera and biotinylated OSs or CP also used as a coating material as compared to ligands **2a** and **3a** (data are not presented) [30]. The functional activity of IgG antibodies to tetrasaccharide conjugate **1b** was confirmed in the test of slide agglutination of living bacteria *S. pneumoniae* type 14 on adding to them the immune serum (Table 1).

The data obtained confirmed efficient agglutination (++++) caused by the serum to tetrasaccharide conjugate **1b** related to the repeated unit of CP of *S. pneumoniae* type 14 and the fact that the serum to octasaccharide conjugate **3b** was less active, whereas the serum to hexasaccharide conjugate **2b** was found to be inactive in this test [28].

The ability of the serum to glycoconjugates **1b–3b** to promote phagocytosis of inactivated bacteria confirmed their high immunological activity (Table 2). The difference in opsonizing activity of the serum to glycoconjugates **1b–3b** could not be detected, because the mice were immunized with the most effective dose (10 μg/mouse of carbohydrate). Also no difference was found in the experiments on the passive protection of mice treated with immune serum to glycoconjugates **1b–3b** and challenged with *S. pneumoniae* type 14 (data are not presented) [30].

The selection of protective oligosaccharide related to CP of *S. pneumoniae* type 14 is initially based on the protocols of Safari et al. [18]. The authors presented the data for the comparative evaluation of the antibody response and the avidity and opsonizing activity of antibodies to conjugated synthetic oligosaccharides (from tri – to dodecasaccharides) and made the conclusion that tetrasaccharide is a serious candidate for a synthetic oligosaccharide conjugate vaccine against infections caused by *S. pneumoniae* type 14 [18]. However, the studies have not been confirmed by experiments on active protection of immunized animals against infection caused by *S. pneumoniae* type 14.

Table 1: Agglutination of living bacteria of *S. pneumoniae* type 14 in the presence of immune serum.

Serum to conjugated OSs	Value
Tetrasaccharide-BSA **1b**	++++
Hexasaccharide-BSA **2b**	+
Octasaccharide-BSA **3b**	+++
Positive control – standard serum to CP	++++
Negative control – native serum	–

Table 2: Opsonophagocytosis of inactivated bacterial cells of *S. pneumonia* type 14.

Blood cells	Number of blood cells (%) that phagocytized bacteria in the presence of sera, M ± SD				
	Without sera	Native	Tetrasaccharide-BSA (1b)	Hexasaccharide-BSA (2b)	Octasaccharide-BSA (3b)
Neutrophils	50.4 ± 6.3	55.5 ± 9.8	85.3 ± 9.3[a]	84.7 ± 9.4[a]	93.5 ± 3[a]
Monocytes	51.7 ± 6.4	58.8 ± 6.2	71.8 ± 5.2[a]	64.8 ± 5.8[a]	73.1 ± 3.6[a]

[a]Significance of the difference between immune sera and native sera, $P < 0.05$.

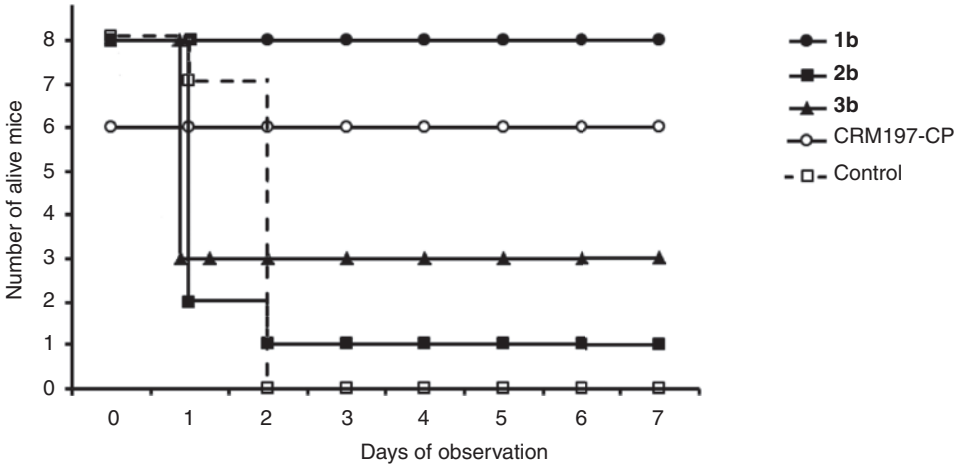

Fig. 8: Protective activity of glycoconjugates absorbed on aluminum hydroxide.

We studied the protective activity of the conjugates in experiments on active immunization of mice [30]. The highest protective activity after immunization of mice with glycoconjugates **1b–3b** in a single dose of 2.5 μg of carbohydrate was revealed for tetrasaccharide conjugate **1b** and CRM_{197}-CP of *S. pneumoniae* type 14 conjugate (positive control) that is a part of the commercial pneumococcal conjugate vaccine Prevenar-13 (Fig. 8). Protective activity of octasaccharide conjugate **3b** and, in particular, hexasaccharide conjugate **2b** was significantly lower. Notably, immunization of mice with glycoconjugates **1b–3b** without adjuvant did not protect animals against infection caused by *S. pneumoniae* type 14.

Some authors indicate that conjugated OSs with different chemical structures related to CP of *S. pneumoniae* type 14, including hexasaccharide conjugate, may be immunologically active [18, 31]. Synthetic hexasaccharide conjugate **2b** was characterized by serotype specificity in the ELISA assay, was used as coating antigen recognizing the antibodies only to the antimicrobial serum to *S. pneumoniae* type 14 and did not interact with sera to serotypes 19A and 19F [32]. We showed that hexasaccharide conjugate **2b** also possessed immunological activity but less pronounced than that of conjugates **1b** and **3b**. The presence of two extra monosaccharides in addition to the tetrasaccharide repeating unit of the CP in its structure imparts it greater similarity to CP of *S. pneumoniae* type 14 compared to OSs with a shorter chain length. This was the basis for the in-depth immunological study of conjugated hexasaccharide **2b** to evaluate its action on the stimulation of innate immunity with the following development of cell-mediated and antibody immune response.

A single immunization of mice with hexasaccharide conjugate **2b** resulted in an increase in bactericidal activity of the peripheral blood leukocytes against the heterologous pathogen, *Staphylococcus aureus* [33]. When administered to mice, hexasaccharide conjugate **2b**, non-absorbed or absorbed on the aluminum hydroxide, increased the number of cells expressing Toll-like receptor 2 (TLR2) on mononuclear leukocytes in the spleen of mice. It was proved that the activation of TLR2 was not the result of a direct ligand-receptor interaction. The addition of conjugated hexasaccharide **2b** to the culture of cells generated from the mice bone marrow led to maturation of dendritic cells ($CD11c^+$, $CD80^+$ and $MHCII^+$), which produced cytokines IL-1β, IL-6, and TNFα into the culture medium [34].

After a single administration of hexasaccharide conjugate **2b** to mice, the levels of cytokines IL-1β, IL-6, IL-10, IFNγ and TNF α increased in the interval from 2 to 24 h. Immunization with hexasaccharide conjugate **2b** absorbed on aluminum hydroxide stimulated the production of a broader spectrum of cytokines GM-CSF, IL-1β, IL-5, IL-6, IL-10, IL-17, IFNγ and TNFα [34].

The number of $CD3^+$ T lymphocytes increased after the second immunization with conjugated hexasaccharide **2b** absorbed on aluminum hydroxide, whereas that of $CD4^+$ T lymphocytes remained within normal values and the number of $CD8^+$ T lymphocytes decreased. The amount of B cells ($CD5^+$, $CD19^+$), NK^+ cells and molecules of the antigen presentation $MHCII^+$ increased during this period, thus indicating the influence of hexasaccharide conjugate **2b** on the activation of cell-mediated immune response [34]. After two-fold immunization of mice with conjugated hexasaccharide **2b** absorbed on aluminum hydroxide, the level of antibodies to CP diminished on the 92nd day. Booster immunization with hexasaccharide conjugate **2b** with the adjuvant stimulated the production of IgG memory antibodies, which were determined within 97 days. Immunization with conjugated hexasaccharide **2b** absorbed on aluminum hydroxide elicited the formation of predominantly IgG1 antibodies [34]. The obtained data characterizing the immunological activity of the synthetic analog of CP (**2b**) of *S. pneumoniae* type 14 on the stimulation of innate and adaptive immunity can be used to improve the quality of the second and third generation carbohydrate pneumococcal vaccines.

Thus, the set of the obtained results indicated the advisability of the use of tetrasaccharide **1a** as a carbohydrate ligand specific to CP of *S. pneumoniae* type 14 for the development of a synthetic third generation carbohydrate pneumococcal vaccine. Moreover, tetrasaccharide **1a** can be applied for the development of diagnostic assays and for obtaining pneumococcal antisera for serotyping *S. pneumoniae* type 14.

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