STRATEGIES FOR TYPE-SPECIFIC GLYCOCONJUGATE VACCINES OF STREPTOCOCCUS PNEUMONIAE

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INTRODUCTION

Prevention of bacterial infections in infants by Gram-positive and Gram-negative encapsulated microrganisms is still a world-wide concern. According to reliable estimates (US National Institute of Allergy and Infectious Diseases, 1986) the infant mortality by Streptococcus pneumoniae approaches to seven million cases annually and the mortality by encapsulated bacteria (Streptococcus pneumoniae, Haemophilus influenzae type B and Neisseria meningitidis) accounts for about 38% of the total burden by infectious diseases.

The highly purified polysaccharide vaccines introduced in the seventies (Gotschlich et al., 1969; Austrian, 1979) have been demonstrated to be efficacious in adults and older children but not significantly immunogenic in young infants (Teele et al., 1981; Makela et al., 1981; Douglas and Miles, 1984). The main reason for the poor immunogenicity of purified capsular polysaccharides in infants seems related to the immunological properties of the polysaccharides (helper T-cell independent antigens or TI-2 antigens) as well as to the immaturity of the host's immune system.

In this respect, data collected from immunological experiments in animals and humans lead to some insight in the activation mechanism of the host's immune system by polysaccharides. Specifically, capsular polysaccharides of Streptococcus pneumoniae have been reported to bind and activate different lymphocyte populations. Activation of B cells mainly results in the secretion of antibodies of IgM and IgG2 isotype in man and IgM and IgG3 in mice (Rijkers and Mosier, 1985); activation of regulatory T-cells (suppressor and contrasuppressor) results in the suppression of amplifier and helper T-cells leading, as ultimate result, to the inhibition or very low induction of IgG isotype antibodies (Paul et al., 1971; Braley Mullen, 1986a; 1986b; Baker, 1990); activation of Natural Killer (NK) cells, in infant mice, results in age-related down-regulation of IgM isotype (Khater et al., 1986).

Taken together, the data suggest that a polysaccharide antigen induces modest levels of IgM antibodies due to the

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concomitant activation of B and NK cells and low levels of IgG antibodies, which do not show a secondary immune response following a booster dose of the same antigen, because the activity of suppressor T-cells results in the inhibition of amplifier and helper T-cells.

A strategy to overcome the problems associated with the polysaccharide vaccines involves the use of glycoconjugates, that is covalently linked carbohydrate antigens and haptens to protein carriers working as T-helper dependent antigens. This strategy goes back to the pioneering and elegant studies of immunochemistry performed by Avery and Goebel in the thirties (Avery and Goebel,1929; Goebel, 1938) and it is now taking advantage of the newer technologies developed in recent years.

An oligosaccharide-based conjugate vaccine (Anderson et al, 1986) and a polysaccharide conjugate vaccine (Lepow et al, 1987) for prophylaxis of Haemophilus influenzae type b infections in infants have been recently licensed by the U.S. Food and Drug Administration. A comparison of immunological activity for the two basically different strategies used in the synthesis of a glycoconjugate vaccine (the use of short-chain oligosaccharides versus long-chain polysaccharides) will be determined on the basis of efficacy in field trials and this will require some time.

It is the purpose of this work to investigate the immunogenic activity in animal models, of glycoconjugates synthesized according to the same protocol, with the same carrier protein but using carbohydrate haptens of two different chain lenght: short-chain versus long chain oligosaccharides. In an attempt to get significant informations from similar work, we have considered four polysaccharide structures of Streptococcus pneumoniae with monosaccharide sequences completely unrelated to each other (type 6A,14,19F and 23F). The choice of these four serotypes has been done on the basis of the observation that they were found to be poor immunogens in respect to other polysaccharides of Streptococcus pneumoniae in the pediatric population (Douglas et al, 1983) and that at least one of them (type 6A) has been reported as the poorest antigen among the serotypes present in the combined polysaccharide-based pneumococcal vaccine (Makela et al,1981). The carrier protein CRM197 has been chosen because of its high immunogenicity as a carrier which is also able to induce protective levels of neutralizing antibodies to diphtheria toxin (Porro et al, 1985; Porro et al, 1986; Porro,1987).

MATERIALS AND METHODS

Preparation of oligosaccharides

Purified capsular polysaccharides of Streptococcus pneumoniae type 6A, 14, 19F and 23F had immunochemical characteristics comparable to those previously reported (Porro et al, 1983). Oligosaccharides of different sizes were generated by controlled acid hydrolysis in the following conditions: polysaccharides were solubilized at 2-5 mg/ml in 10^{-2} M acetic acid (type 6A), $5x10^{-1}$ M trifluoroacetic acid (type 14), $1.5x10^{-2}$ M phosphoric acid (type 19F) and 10^{-1} M acetic

acid (type 23F). Hydrolysis proceeded in sealed vials at 100°C (type 6A and 23F), T=70°C (type 14) and T=50°C (type 19F) for various times in order to get oligosaccharides of desired size according to Fig.1. The rate of hydrolysis of the capsular polysaccharides were investigated by the molar ratios: end-reducing group/total phosphorus (type 6A and 23F), end-reducing group/N-Acetyl glucosamine (type 14) and ester-bound phosphate group/total phosphorus (type 19F), according to the structures of the capsular polysaccharides type 6A (Rebers and Heidelberger, 1961), type 14 (Lindberg et al, 1977), type 19F (Jennings et al, 1980; Ohno et al, 1980) and type 23F (Richards and Perry, 1988). Chemical methods for assays of phosphorus (Chen et al, 1956), end-reducing groups (Porro et al, 1981), hexosamine (Ashwell, 1957) and ester-bound phosphate groups by alkaline phosphatase hydrolysis (Eby R.,personal communication), were used. The sites of hydrolysis in each polymer have been investigated by ^{13}C and ^{1}H-Nuclear Magnetic Resonance (NMR) as previously reported (Porro et al, 1985) and by Gas-chromatography (Dick W.E., personal communication). The homogeneity of the oligosaccharide preparations were tested by gel chromatography on Sephadex G-15 and G-50 (Pharmacia, Uppsala, Sweden) by chemical analysis of the eluates, using the above reported methods.

Immunochemical characterization of the oligosaccharides

Negatively-charged oligosaccharides (type 6A, 19F and 23F) were tested for immunochemical reactivity against their specific pneumococcal reference rabbit antisera (Statens Seruminstitut, Copenhagen, Denmark) by inhibition of immunoprecipitation in differential immunoelectrophoresis (Porro et al, 1985) using the homologous capsular polysaccharides as reference antigens. The neutral type 14 oligosaccharide and the homologous reference polysaccharide were analyzed by inhibition of immunoprecipitation in radial immunodiffusion. The immunochemical specificity for each purified oligosaccharide, with respect to that detected for the reference homologous capsular polysaccharide, was calculated on the basis of the ratio between the minimal inhibitory concentration found for the polysaccharide antigen (MIC Ag) and that found for the selected oligosaccharide hapten (MIC Hp), as formerly described (Porro et al, 1985).

Synthesis of glycoconjugates

The purified oligosaccharides (5 mg/ml) of the selected sizes for the four serotypes, were reacted at pH=9.0 (0.2M KH_2PO_4) T=100°C for 15 min, with diaminoethane in a molar excess 10:1 with the estimated amount of end-reducing groups. The temperature was then reduced at T=50°C and pyridine borane at a molar excess 25:1 with respect to the amount of end-reducing groups in the oligosaccharide was used in reaction. Reduction followed for 48hs. Purification of the amino-activated oligosaccharides followed on Sephadex G-15 superfine equilibrated in $1.5x10^{-2}$ M sodium chloride. The purified oligosaccharides were freeze-dried and then transformed to monosuccinimidyl ester of adipic acid as previously reported (Porro et al, 1985). Conjugation of the activated oligosaccharides to the protein CRM197 (purified and characterized as described by Porro et al, 1985) occurred

overnight a T=4°C in a 50% v/v solution dimethylsulfoxide (DMSO)/10^{-1} M sodium bicarbonate buffer (pH=8.0), using a molar ratio activated oligosaccharide/amino groups of CRM197 (titered according to Habeeb, 1966) 1:2.

Finally, each glycoconjugate was purified on Sephadex G-100 superfine or Sephacryl S-200 according to the size of the glycoconjugate synthesized and estimated by SDS-PAGE analysis in the conditions published (Porro et al, 1985).

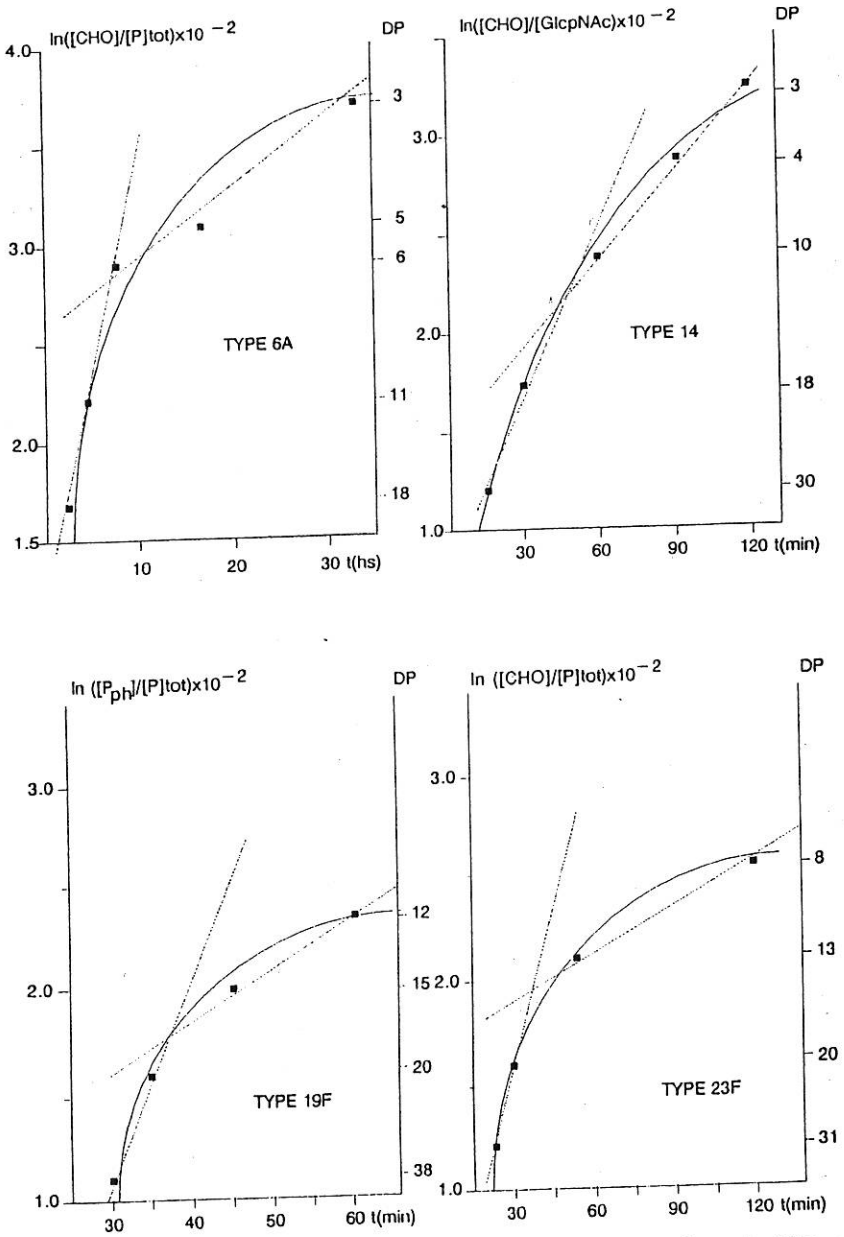


Figure 1. Rate of hydrolysis of S.pneumoniae type-specific polysaccharides. (For explanation see text, Methods section)

Physical-Chemical and Immunochemical characterization of the glycoconjugates

The analytical procedures used for characterization of the four type-specific glycoconjugates, were basically the same previously reported (Porro et al, 1985).

Immunization procedures and immunological analysis

For each of the four type-specific capsular antigens of Streptococcus pneumoniae , two kinds of glycoconjugate vaccines were prepared, different only in the length of the oligosaccharide chain (DP=3-6 and DP=10-14, see the Results section). Each kind of glycoconjugate vaccine was formulated as mixture of the four glycoprotein antigens in either soluble or aluminium hydroxide-adsorbed form. Each immunizing dose contained 2.5 μg of conjugated type-specific oligosaccharide in 0.5 ml volume of PBS pH=7.2. The adsorbed formulation contained 1 mg/dose of aluminium hydroxide and all the conjugate material was adsorbed under these conditions.

Groups of five New Zealand rabbits (Av.wt.= 2 kg) and five 8 week-old Swiss-Webster mice were immunized s.c. with each vaccine formulation respectively at week 0,4 and 8 (bleeding at week 0,4,6 and 10) and at week 0 and 4 (bleeding at week 0,4 and 6).Three groups of five guinea pigs each, were injected with the purified protein CRM197 according to the schedule previously reported (Porro et al, 1980) with the following amount: priming dose of 30,60 and 120 μg followed by a booster dose, 6 weeks later, respectively of 60,120 and 240 μg.The "range" of the CRM197 doses injected included 0.5,1 and 2 times the maximum dose (50 μg) of conjugated CRM197 present in the mixture of the four type-specific glycoconjugates.

IgG antibodies to type specific capsular polysaccharides of Streptococcus pneumoniae have been titered for each rabbit and for the pool of mice sera by ELISA assay, basically in the conditions published (Porro et al, 1985) coating the PVC plates with type-specific polysaccharides at 5 μg/ml. Titers were detected as the highest dilution yielding an optical density (OD) value at 405 nm twice of the background level and were expressed as geometric mean of the titers determined in each rabbit. Inhibition-ELISA was performed by pre-incubating (2hs at r.t.) rabbit sera (at constant dilution 1:5,000 v/v) with serial concentrations of competitors (homologous or heterologous type-specific polysaccharides and oligosaccharides). The molar concentration of each competitor was calculated on the basis of the molecular weight of the basic repeating unit present in its structure, in order to avoid inaccurate evaluation of the molecular weight of pneumococcal polysaccharide structures which appear as size-distributed systems by gel-chromatography and by antibody recognition of the eluates (Porro et al, 1983). Neutralizing antibodies to diphtheria toxin raised in rabbits by the conjugates, have been titered on sera pool using Vero cells (Strain P 142) culture in COSTAR plates, basically according to Zucher and Murphy 1984, and using a diphtheria toxin (LIST BIOLOGICS lot #06 1/28/82) concentration of 5×10^{-13} M with a

reference anti DT antiserum (U.S.Standard #47 09/04/88,Office of Biologics, Food and Drug Administration). Neutralizing antibodies to diphtheria toxin in guinea pigs have been titered as previously published (Porro et al,1980). Statistical analysis was performed by the Student's t test .

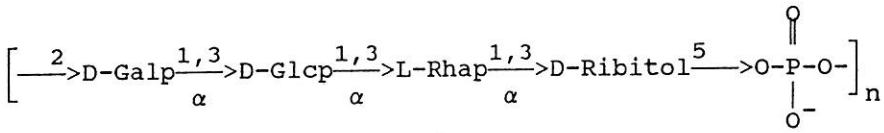
RESULTS

Physical-chemical and immunochemical characteristics of oligosaccharide haptens type 6A,14,19F and 23F

The capsular polysaccharides of Streptococcus pneumoniae type 6A,14,19F and 23F are complex polymers of basic repeating units whose structures have been already investigated and clarified.

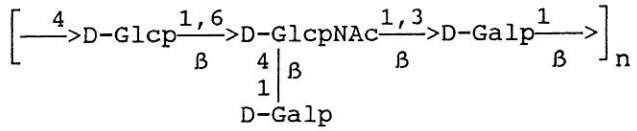
Depolymerization of the four type-specific polysaccharides by chemical hydrolysis in mild conditions,as reported in the METHODS section, was investigated by the rate of hydrolysis.

Type 6A is a polymer with the linear structure:



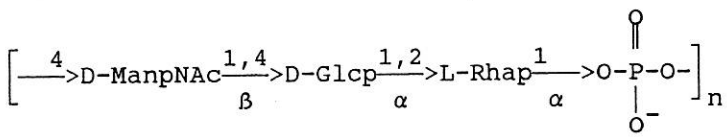
(Rebers and Heidelberger,1961)

Type 14 is a polymer with branched structure:



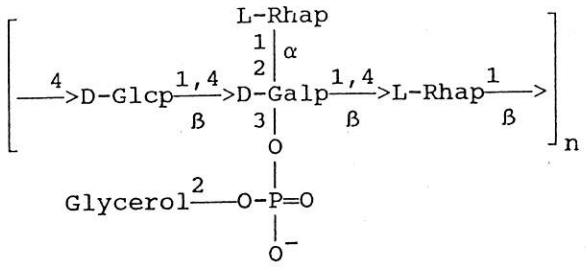
(Lindberg et al, 1977)

Type 19F is a polymer with linear structure:



(Jennings et al, 1980; Ohno et al, 1980)

Type 23F is a polymer with branched structure:



(Richards and Perry, 1988).

The following molar ratios were calculated for each purified oligosaccharide in order to estimate the degree of polymerization (DP) attained:end-reducing groups (CHO)/total phosphorus $(P)_{tot}$ (type 6A); end-reducing groups (CHO) / N-Acetyl Glucosamine (Glcp-NAc) (type 14); alkaline phosphatase-hydrolyzed phosphates (Pph)/total phosphorus $(P)_{tot}$ (type 19F); end-reducing groups (CHO)/total phosphorus $(P)_{tot}$ (type 23F). In Fig.1 the relationship between time of hydrolysis and DP is reported. For type 19F the ratio of alkaline phosphatase-hydrolyzed phosphate/total phosphorus was used in the place of end-reducing groups/total phosphorus since the rhamnose residue (Rhap) (where hydrolysis occurs) is α-linked via the C-2 to the D-Glcp residue and it shows a lower reducing activity than a Rhap residue not substituted at the near carbon (C-2) of the end-reducing anomeric carbon (C-1). In Fig.2, the same relationship is referred to the amount of end-reducing groups estimated per mg of carbohydrate material: this allowed a reliable and fast method for selection of type-specific oligosaccharide of a desired DP.

The sites of hydrolysis for the type-specific polymers have been detected in the following sequences:

L - Rhap $\xrightarrow[\alpha]{1,3}$ D - Ribitol (type 6A)

D - Glcp $\xrightarrow[ß]{1,6}$ D - GlcpNAc
D - Galp $\xrightarrow[ß]{1,4}$ D - Glcp (type 14)

L-Rhap $\xrightarrow[\alpha]{1}$ O-P-O- (type 19F)
O⁻

L-Rhap $\xrightarrow[ß]{1,4}$ D-Glcp (type 23F)

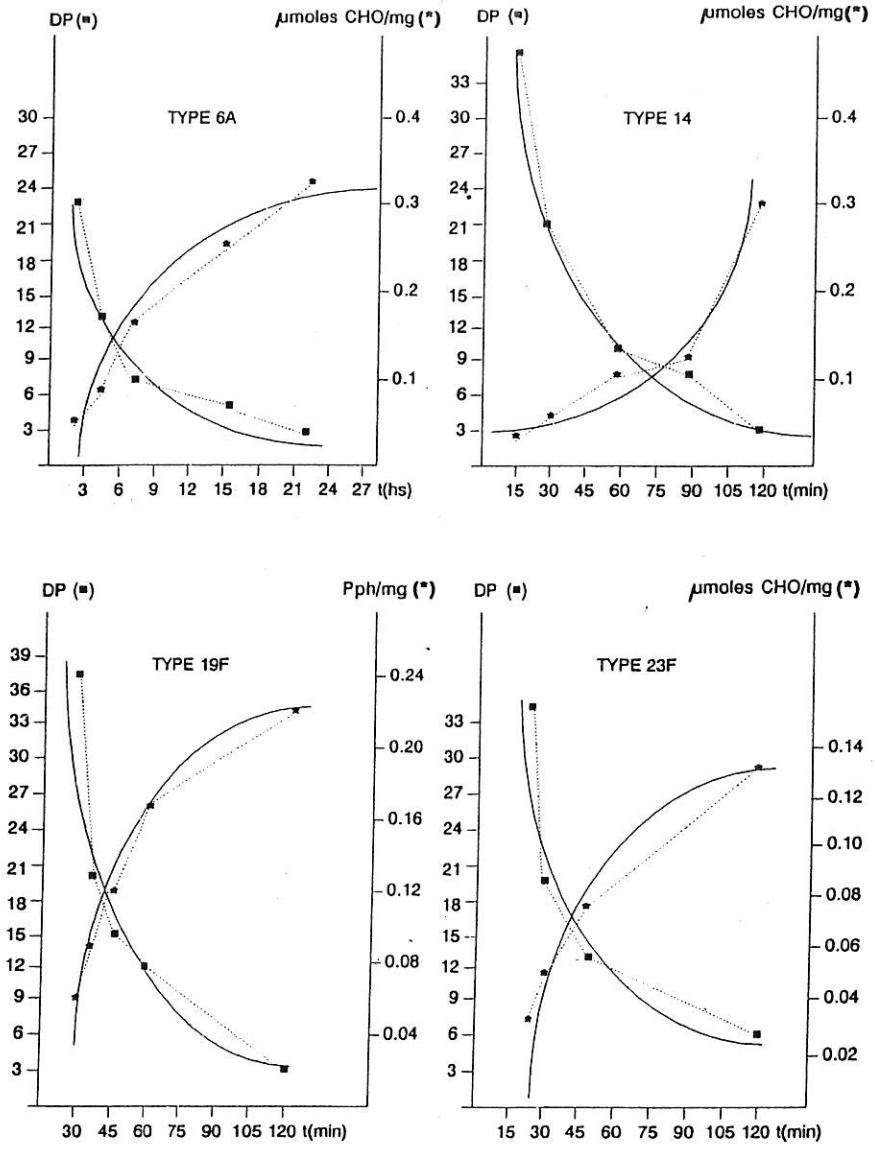


Figure 2. Rate of hydrolysis of S.pneumoniae type-specific polysaccharides.
(For explanation see text, Methods section)

With the exception of type 14, which shows two equally sensitive linkages to mild acid conditions, the other three type-specific polymers have shown a preferred site of hydrolysis. This homogeneity is mirrored by the comparable behaviour of the type-specific polysaccharides in the rate of hydrolysis reported in Fig.1. The size-distribution of the oligosaccharides generated by acid hydrolysis tends toward the exponential form as the random degradation theory (random hydrolysis) for a polysaccharide would predict (Mazur, 1984). Thus, the theory and the data reported suggest that the oligosaccharides generated by mild acid hydrolysis in controlled conditions are the result of random degradation of size-distributed carbohydrate systems (type specific polysac-

charides) through cleavage of preferred sites of hydrolysis. Accordingly, the obtained oligosaccharide structures behaved on gel-chromatography with a size-distribution similar in shape to the one characteristic for the homologous polysaccharide from which they were derived (data not shown). Thus, our definition of degree of polymerization (DP) for a given purified oligosaccharide is the expression of its average DP, that is the mean of the size-value within the oligosaccharide size-distribution (mean of a bell-shaped normal distribution).

When the purified type-specific oligosaccharides of different size (DP) have been analyzed by antibody recognition using the reference polyclonal rabbit antiserum, the exponential function shown in Fig.3 was obtained. The immunochemical specificity detected by the ratio MIC Ag/MIC Hp of oligosaccharides compared to their homologous type - specific polysaccharide gave evidence for a logarithmic increase (10^{-6} - 10^{-1}) of specificity beginning from some simple monosaccharide present in the basic repeating unit of the polymers (immunodominant sugar) up to a size for the oligosaccharides of DP=5. In the size-range equivalent to DP=10 and DP=25, the calculated specificity was approaching the one estimated for a type-specific polysaccharide with a DP value several orders of magnitude higher (DP≥1000). From this analysis, two significantly different sizes of type-specific oligosaccharides have been selected for conjugation to the protein carrier: oligosaccharides in the size-range DP=3-6 and DP=10-14, the former having shown a specificity value between 10 and 100 times lower than the homologous polysaccharides, while the latter were mimicking the complete antigenic repertoire of the native polymers.

Physical-chemical and immunochemical characteristics of glycoconjugates type 6A,14,19F and 23F

In Table I, the properties of the two kinds of glycoconjugates are compared. In Fig.4, the qualitative aspect of the four type-specific DP=3-6 glycoconjugates in SDS-PAGE analysis is shown. The DP=10-14 glycoconjugates did not significantly penetrate the 9% (w/w) acrylamide running gel (data not shown). In all cases tested, conjugation of the oligosaccharides resulted in their increased immunochemical specificity, an observation previously reported and explained (Porro et al, 1985;1987).

Immunology of the glycoconjugates

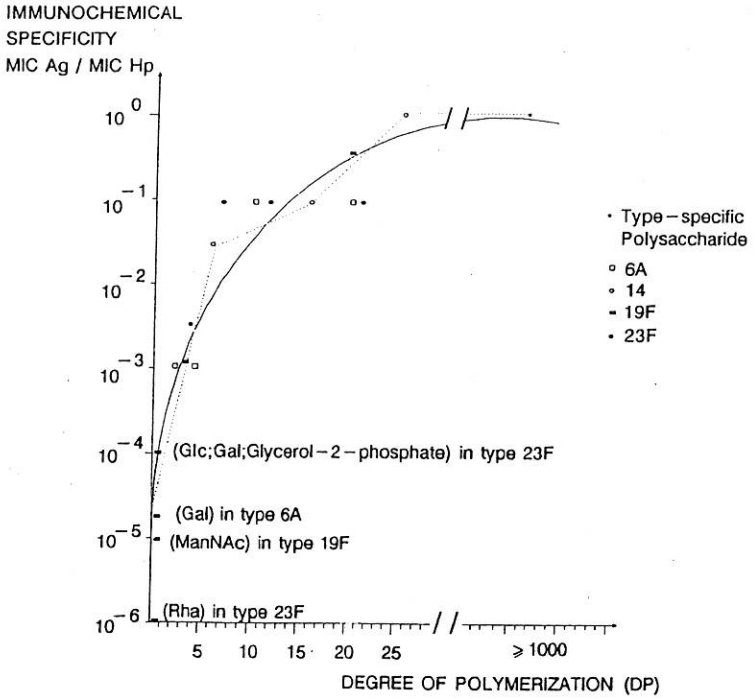
Comparison of the immunological properties found in rabbits for the two kinds of glycoconjugates, short-chain vs long-chain conjugated oligosaccharides, is shown in Fig.5. The IgG titers induced by the short-chain conjugated oligosaccharides were found at higher levels than those estimated for the long-chain conjugated oligosaccharides. For each serotype, in either the priming or booster activity, the IgG titers of the former aluminium adsorbed conjugate model exceeded the titers obtained with the latter-one at a statistically significant level $P<0.005$ (Fig.5A vs Fig.5C). For the same model of conjugate, the booster activity of each serotype was significantly higher than the priming-one at a statistically significant level $P<0.01$ (Fig.5A and Fig.5C). Titers obtained

with each of the two models, either against the type-specific capsular polysaccharides or against the carrier protein, were significantly higher in the formulation employing aluminium hydroxide, at a level $P<0.01$ (Fig.5A vs Fig.5B and Fig.5C vs Fig.5D). This result confirms the one previously reported for type 6A conjugate (Porro et al,1985).

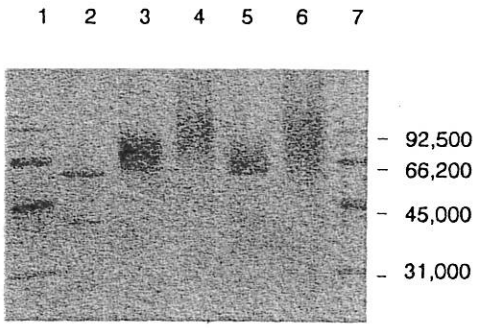
TABLE I. Characterization of the Glycoconjugates

TYPE	HAPTENS		MW OF GLYCO-CONJUGATES	RATIO OLIGOS/PROT	SPECIFICITY of RABBIT Ab for HAPTENS	
	DP	MW	(SDS-PAGE)	(moles/mol)	free	conj.
6A	3	2.1×10^3	77.6×10^3	7	10^{-3}	10^{-2}
14	5	3.5×10^3	85.1×10^3	6	5×10^{-2}	10^{-1}
19F	3	1.9×10^3	69.2×10^3	4	10^{-3}	10^{-2}
23F	6	4.5×10^3	85.0×10^3	5	10^{-1}	10^{0}
6A	10	7.1×10^3	$>10^5$	6	10^{-1}	10^{0}
14	12	8.3×10^3	$>10^5$	10	10^{-1}	10^{0}
19F	10	6.1×10^3	$>10^5$	5	10^{-1}	10^{0}
23F	14	11.1×10^3	$>10^5$	10	10^{-1}	10^{0}

Type 19F and 23F (DP=10-14) oligosaccharide conjugates were immunologically silent in either soluble or adsorbed for mulation (Fig.5C and Fig. 5D). In all experiments reported, higher IgG titers to type-specific polysaccharides were detected when the anti-carrier protein immune response was also higher as expected for a true T-helper dependent antigen. Significantly higher anti-carrier protein titers were observed with the short-chain conjugated oligosaccharide model of the vaccine (Fig.5A vs Fig.5C and Fig.5B vs Fig.5D). It is important to note, that the protein carrier CRM197, although "in vitro" it is antigenically cross-reactive with diphtheria toxin, is not significantly immunogenic in animals unless a specific treatment increasing its stability to proteolytic enzymes is performed (Pappenheimer et al, 1972; Porro et al, 1980). As Table II shows, even injecting into guinea pigs up to four times the amount of CRM197 present in the formulations of the combined glycoconjugate vaccines, no significant or very low anti-diphtheria toxin titers have been elicited in comparison to those seen with CRM197 in conjugated form. These data, combined with those mentioned above for CRM197, strongly suggest that chemical glycosylation of the carrier protein CRM197 results in improved stability to "in vivo" proteolysis.



gure 3. Oligosaccharides generated from controlled hydrolysis of homologous polysaccharides of S.pneumoniae type 6A, 14, 19F and 23F : immunochemical specificity of reference rabbit antisera as estimated by inhibition of immunoprecipitation in differential Immuno−electrophoresis. (For explanation see text, Methods Section)



Lane 1 and 7 = Mw Standars
Lane 2 = CRM 197
Lane 3 = Conj. oligo 6A−CRM197
Lane 4 = Conj. oligo 14−CRM197
Lane 5 = Conj. oligo 19F−CRM197
Lane 6 = Conj. oligo 23F−CRM197

ure 4. Qualitative aspect of the four (DP=3−6) type−specific glycoconjugates in SDS−PAGE. All glycoconjugates were loaded in equal amount of protein (2µg). Gel was silver stained as reported previously (Porro et al, 1985).

Immunological analysis in mice for two out of the four type specific short-chain (DP=3-6) oligosaccharide conjugates (6A and 14) has been performed in order to investigate the optimal dose-response curve in this animal model (Fig.6).

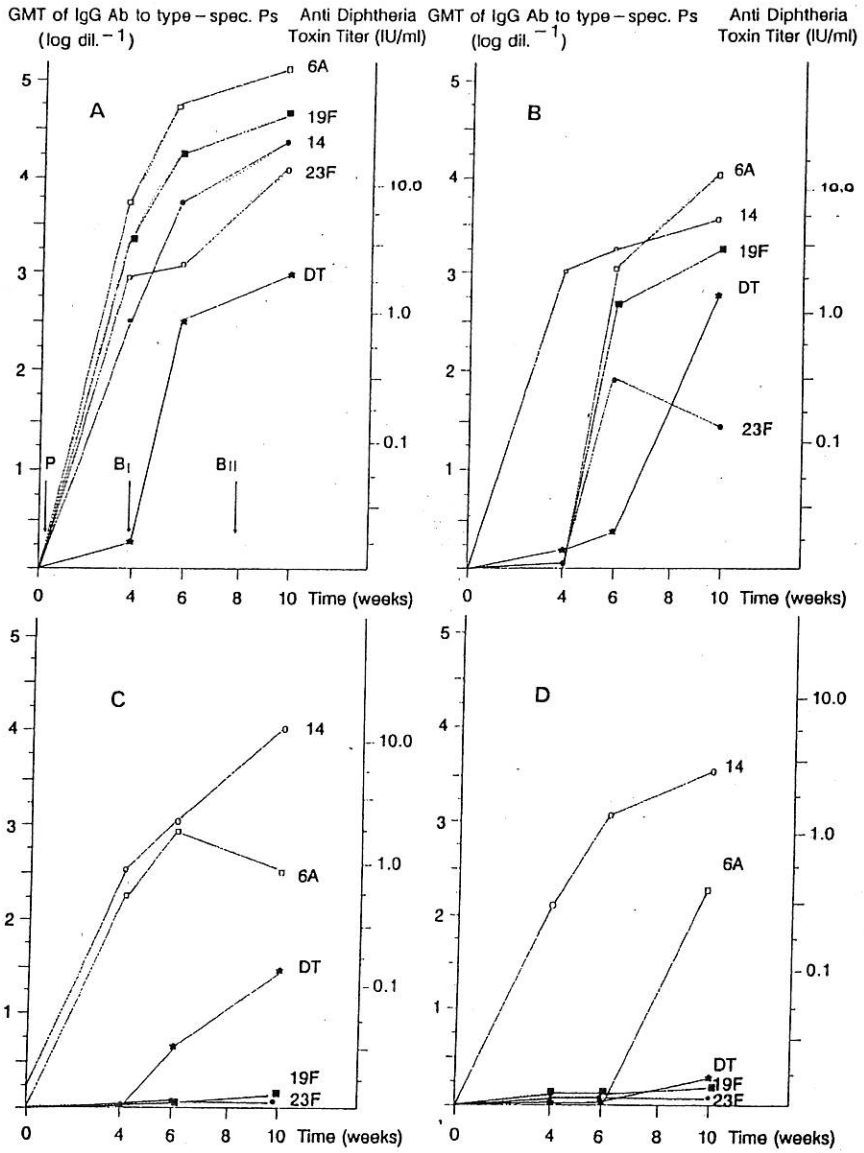


Figure 5. Comparison of the immunological properties in rabbit of the two models of glycoconjugates specific for S. pneumoniae type 6A, 14, 19F and 23F.

A : DP 3 – 6 oligo – CRM197 conjugates $Al(OH)_3$ – adsorbed
B : DP 3 – 6 oligo – CRM197 conjugates soluble
C : DP 10 – 14 oligo – CRM197 conjugates $Al(OH)_3$ – adsorbed
D : DP 10 – 14 oligo – CRM197 conjugates soluble

TABLE II. Neutralizing antibodies to Diphtheria Toxin (DT) in sera pool of guinea pigs immunized with CRM197

DOSE OF ANTIGEN (μg)		NEUTRALIZING Ab TO DT (IU/ml SERUM)
PRIMING	BOOSTER	AFTER BOOSTER DOSE
30	60	< 0.01
60	120	< 0.01
120	240	0.01

NOTE: pre-immunization sera contained titers of less than 0.01 IU/ml. The estimated protective level in man is 0.01 IU/ml (Wilson and Miles, 1975).

In the case of type 14 oligosaccharide conjugate, priming and boosting activity for IgG antibodies has been detected in the range 2.5-30 μg of conjugated oligosaccharide. In the case of type 6A oligosaccharide conjugate, the priming activity was seen only at the highest dose of oligosaccharide (30 μg). In both cases, increasing the dose of conjugate resulted in the increase of the IgG titers, either after the first or the second dose injected.No plateauing of the IgG titers was seen in the dose-range adopted for the two type-specific conjugates.

Estimation of affinity constant value of the rabbit IgG induced by one (type 6A) out of the four (DP=3-6) oligosaccharide conjugates injected, is reported in Fig.7. Inhibition of the ELISA reaction specifically occurred by type 6A purified capsular polysaccharide and by type 6A (DP=10) oligosaccharide but no inhibition occured by either a heterologous type 14 (DP=12) oligosaccharide activated by the molecular spacer adipic acid or conjugated to the carrier protein CRM197.These data confirmed the specificity of the immune response induced and the absence of neodeterminants introduced in the oligosaccharide structures via the chemical activation procedure. Such a comparable result has been previously reported for other conjugates using oligosaccharides activated by the succinimidyl ester of adipic acid (Porro, 1987).

Inhibition by 50% of the ELISA-detected IgG titers to type 6A capsular polysaccharide occurred at the same molar value for either the homologous polymer or the type 6A (DP=10) oligosaccharide: $Ka=4.0 \times 10^9 M^{-1}$, when the carbohydrate concentration was referred to the basic repeating unit of the structure. The estimated value falls in the range of affinity expected for a highly specific IgG antibody population.

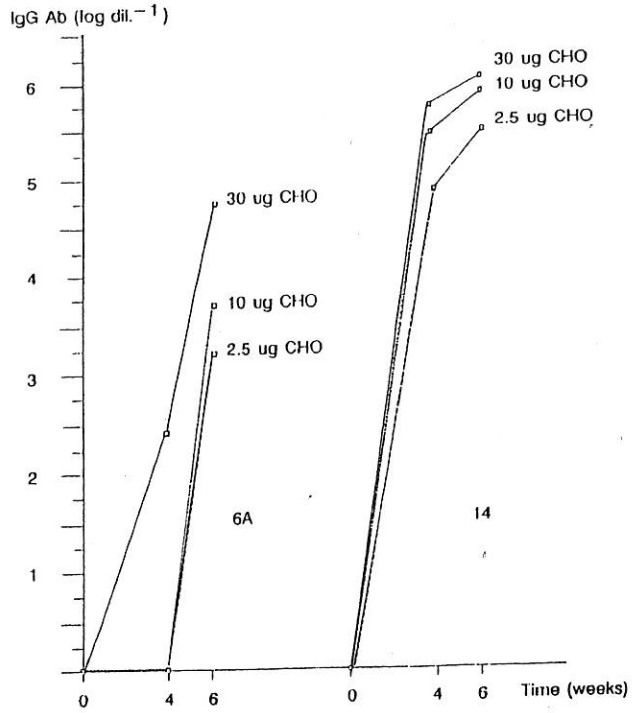


Figure 6. IgG antibody response in sera pool of mice to (DP = 3 – 6) oligosaccharide – CRM197 conjugates for serotype 6A and 14: dose – response analysis.

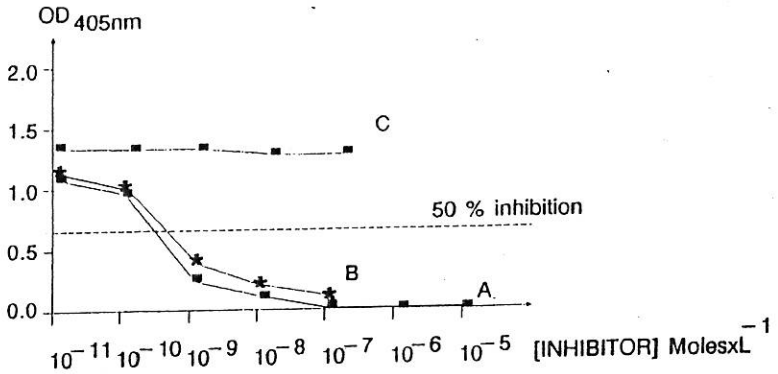


Figure 7. Rabbit IgG immune response to type 6A (DP = 3) oligosaccharide – CRM197 conjugate: affinity value of IgG isotype induced to the capsular polysaccharide as estimated by inhibition – ELISA.

A – Type 6A capsular polysaccharide

B – Type 6A oligosaccharide (DP = 10) in free form or conjugated to the carrier CRM197

C – Type 14 oligosaccharide (DP = 12) activated by the molecular spacer or conjugated to CRM197

DISCUSSION

Although the idea to improve the immunogenicity of saccharide haptens through covalent coupling to a carrier protein was first proposed and realized about half-century ago, only recently glycoconjugate vaccines have been introduced in clinical medicine as new prophylactic agents to overcome the problems associated with the use of polysaccharide vaccines. Neverthless, basic questions about the immunological mechanism by which a carbohydrate structure does acquire the immunological characteristics peculiar of protein antigens, still remain to be answered.

A significant immunological feature of protein antigens is their capability in activating helper and amplifier T-cells showing priming and amplification of the immune response serologically detectable in mammalians as anamnestic induction of IgG isotype antibodies. Carbohydrates do not share such a property neither in animals nor in humans (Kabat, 1961;Paul et al, 1971; Gotschlich, 1975).

From the chemical point of view, a given glycoconjugate can be synthesized using different methods of coupling and carbohydrates of significantly different molecular size, so that an obvious question to be raised is whether any model of glycoconjugate synthesized can exhibit the desired immunological properties. In particular, it would be important to understand the immunological role of the chain length of a saccharide hapten in a given glycoconjugate as related to the acquired T-cell dependency and consequently to the anamnestic induction of specific IgG antibodies.

With this specific desire in mind, we have undertaken the present work and the results obtained with two models of glycoconjugates comparable in terms of chemistry and carrier protein adopted but different in the length of the carbohydrate chain coupled, have given some significant answers at least in animal models. The model synthesized by short-chain (DP=3-6) type specific oligosaccharide haptens has shown a significantly higher activity either in the primary or in the secondary immune response when compared to the model synthesized by long-chain (DP=10-14) oligosaccharides. It is noteworthy to observe that the immunochemical specificity estimated "in vitro" for the latter model of glycoconjugate by type-specific reference antisera, was significantly higher than the one estimated for the former model. Thus, since the "in vivo" results were obtained with each of the four different saccharide structures, it seems evident that the size of the oligosaccharides is responsible for the different immunological activity of the glycoconjugates found in animal immunizations.

The oligosaccharide size seems to directly affect the expression of immunogenicity of the carrier protein, since any enhancement of the anti-carrier immune response was paralleled by the enhancement of the type-specific IgG response to the carried oligosaccharide. In the case of the vaccine model synthesized by short-chain oligosaccharides

the anti-carrier immune response was significantly higher than the one detected using the long-chain conjugated oligosaccharides. This modulating activity related to the carbohydrate moiety of the glycoconjugates, may be interpreted by the following hypothesis. Since pneumococcal polysaccharides do not significantly activate helper T-cells and do not amplify the immune system response because the activation of suppressor T-cells, a longer carbohydrate chain in a given glycoconjugate could limit the extent of the immune response induced by the protein carrying the carbohydrate. In other words, several degrees of T-cell dependency might exist for a glycoconjugate antigen, resulting from the balance of the specific immunological features of the two structurally different components. Although other hypothesis may also be considered, like a "covering up" phenomenon occurring by a long-chain oligosaccharide on specific epitopes of the carrier protein responsible for activation of helper and amplifier T-cells, on the basis of the antibody specificity detected for the anti-carrier immune response we believe that the former hypotesis better fits with the experimental results.

About the specificity and affinity of the anti-carbohydrate IgG induced in rabbit by the type 6A (DP=3) conjugate, the comparable (sigmoidal) shape of the binding curves obtained when either the homologous capsular polysaccharide or oligosaccharide were competing for the same specific antigen-antibody complex, is demonstrating the identical chemical nature of the antigenic site present in the two saccharide structures. Thus, the antibody response to a conjugated short-chain oligosaccharide hapten generated from the homologous native polymer, results in IgG antibodies having complete immunochemical specificity for the native antigen. Furthermore, the same affinity constant value of the IgG antibody population found for the oligosaccharide and the homologous polysaccharide, is demonstrating identity between the antigenic sites recognized and not cross-reactivity. Since both specificity and affinity of the induced IgG antibody were identical for the carbohydrate structures, one can assume the IgG antibody population to be homogeneous (Berzofsky and Schechter,1981), that is all the IgG antibodies present in the rabbit sera are directed against the same determinant.

The size of the recognized determinant was not estimated because an unambiguous measure of it would require structurally defined saccharides, like synthetic oligomers of the basic repeating unit for each type-specific carbohydrate. However, since in two out of the four type-specific glyccconjugates the IgG antibodies have been induced by DP=3 oligosaccharides (ten to twelve monosaccharide residues),the minimal structure for such oligosaccharide haptens able to induce a consistent and specific secondary immune response in mammalians after conjugation to a carrier protein would most likely encompass two basic repeating unit (DP=2) or six to eight monosaccharide residues, which is roughly the size of the maximal determinant reported in literature for repeating polypeptide and polysaccharide homopolymers (Schlossman et al, 1965; Kabat,1960;1966; Van Vunakis et al, 1966) and proteins (Atassi,1975).

In conclusion, the data obtained by comparison of "in vitro" versus "in vivo" immunochemical properties of two models of glycoconjugate synthesized by making use of oligosaccharides with different chain length, have indicated that short-chain oligosaccharides are significantly better immunogens than long-chain homologous oligosaccharides, after coupling to a carrier protein.In addition, they have shown the property to induce a primary and secondary IgG immune response which is homogeneous and specifically directed to the same determinants of the native polysaccharides. On the basis of the size of the smallest oligosaccharides used in this work, the data also strongly suggest that the IgG immune response specific for the carbohydrate chain of a glycoconjugate is restricted to the linking area of the glycoconjugate and most likely confined to few monosaccharide residues of the carbohydrate structure.

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