Reprinted from

MOLECULAR IMMUNOLOGY

Vol. 22, *No.* 8, *pp.* 907–919

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PERGAMON PRESS

OXFORD · NEW YORK · TORONTO · SYDNEY · FRANKFURT

1985

Molecular Immunology, Vol. 22, No. 8, pp. 907–919, 1985
Printed in Great Britain

0161-5890/85 $3.00 + 0.00

SPECIFIC ANTIBODIES TO DIPHTHERIA TOXIN AND TYPE 6A PNEUMOCOCCAL CAPSULAR POLYSACCHARIDE INDUCED BY A MODEL OF SEMI-SYNTHETIC GLYCOCONJUGATE ANTIGEN*

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(*First received* 17 *September* 1984; *accepted in revised form* 23 *January* 1985)

Abstract—A molecular model of a carbohydrate–protein conjugate is described, involving the non-toxic mutant protein CRM197, serologically related to the diphtheria toxin, covalently bound to a characterized oligosaccharide derived from the molecular structure of type 6A pneumococcal capsular polysaccharide. Physicochemical and immunochemical characteristics of this oligosaccharide–protein conjugate were consistent with a molecule showing a molar carbohydrate/protein ratio of 8, an av. mol. wt of 75,000, and retention of complete immunochemical identity when tested towards the homologous antisera. The immunological characteristics obtained after immunization of 2 animal models showed a high immunogenicity of the glycoconjugate specifically directed towards diphtheria toxin and the type 6A pneumococcal capsular polysaccharide.

INTRODUCTION

Carbohydrate–protein conjugate molecules represent a promising approach in improving the immunologic response to carbohydrate antigens. Avery and Goebel (1929) and Goebel (1938) described the use of similar "hybrid" molecules more than 50 years ago, by demonstrating that immunization of animals with a synthetic pneumococcal oligosaccharide conjugated to a carrier protein could provide protection against pneumonia. The development of new meningococcal and pneumococcal capsular polysaccharide vaccines in the last decade has again demonstrated the poor immunogenicity of many carbohydrate antigens. This is a particularly serious problem in the infant population, which is at higher risk of infection by encapsulated bacteria (Austrian *et al.*, 1977; Gray *et al.*, 1980). Several studies have recently been published in the field of carbohydrate–protein conjugates and promising results have been obtained with *Haemophilus influenzae* type b and meningococcal group A and C polysaccharide antigens (Schneerson *et al.*, 1980; Jennings and Lugowski, 1981). Improved immunogenicity of polysaccharides linked to protein carriers has also been shown to be related to a thymus-dependent immunologic response, since a significant increase of serum IgG antibodies was observed following the administration of a "booster" dose of conjugate (Beuvery *et al.*, 1982). In addition, antibodies obtained towards *H. influenzae* type b and meningococcal polysaccharide conjugates showed significant bactericidal activity against the homologous bacterial strains (Schneerson *et al.*, 1980; Jennings and Lugowski, 1981), a fundamental requisite for an efficient immunological defense against invasive diseases caused by these microorganisms in man (Makela *et al.*, 1977; Gotschlich *et al.*, 1969).

In this paper we describe the immunochemistry of a semi-synthetic glycoconjugate involving the non-toxic mutant protein CRM197 as carrier protein for a type 6A pneumococcal oligosaccharide, derived from the structure of the homologous bacterial capsular polysaccharide, which retained the identified immunodominant sugar.

MATERIALS AND METHODS

Preparation of oligosaccharides

Type 6A pneumococcal capsular polysaccharide, with characteristics as reported previously (Porro *et al.*, 1983*a*), was hydrolyzed in different ways in order to obtain oligomers deriving from hydrolysis of phosphodiester or *O*-glycosidic bonds. Phosphodiester-hydrolyzed oligosaccharides were obtained by mild alkaline hydrolysis under the following conditions: polysaccharide was solubilized in 1 m*M* NaOH at a concn of 2 mg/ml, pH = 10.8. Hydrolysis was performed in sealed vials, in an oil bath at 100°C, for a time ranging between 96 and 216 hr in order to obtain several molecular sizes of the oligomers. *O*-glycosidic hydrolyzed oligosaccharides were obtained by acid hydrolysis under the following conditions: polysaccharide was solubilized in 10 m*M* acetic acid at a

*This work is dedicated to the memory of Prof. Mario Saletti, microbiologist.

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concn of 2 mg/ml, pH = 3.4. Hydrolysis was conducted in an oil bath at 100°C in sealed vials, for a time ranging between 6 and 21 hr, in order to obtain several molecular sizes of the oligomers. At various times during both alkaline and acid hydrolysis, the oligomers were purified by gel chromatography on Sephadex G-15 (Pharmacia, Uppsala, Sweden) equilibrated in 200 m*M* NaCl, pH = 7.0. The chromatographic effluents were chemically analyzed for methyl pentose, phosphorus and reducing groups, according to the reported procedures (Kabat, 1964; Chen *et al.*, 1956; Porro *et al.*, 1981). Pooling of the chromatographic effluents showing these specific chemical activities then occurred.

High-performance liquid chromatography (HPLC) analysis of oligosaccharides

Oligosaccharides obtained after 216 hr in mild alkaline hydrolysis or after 21 hr by acid hydrolysis, showing an av. mol. wt corresponding to 1200 on gel chromatography, were compared by HPLC using a Perkin–Elmer series 3B instrument and a model TSK 3000 SW column operating at a pressure of 55 atm.

NMR spectroscopy

^{13}C NMR spectra of oligosaccharides were obtained by examining their solutions in D_2O (50 mg/ml) in 5-mm probes at 35°C using a spectrometer (model CFT 20, Varian Associates, Palo Alto, CA) operating at 20 MHz in the pulsed transformed mode. Internal reference was methyl alcohol, $\delta = 39.5$ ppm.

Immunochemical properties of oligosaccharides

Low mol. wt haptens do not give immunoprecipitation reaction when tested towards homologous antibodies. For this reason, analysis of antibody specificity for oligosaccharides was performed by a method developed in our laboratory which combines the techniques of affinity electrophoresis (Horejsi, 1981) and "rocket" immunoelectrophoresis (Weeke, 1973). We termed this method "differential immunoelectrophoresis". The basic principle is constituted by the inhibiting capacity of a hapten in the homologous antigen–antibody immunoprecipitation reaction.

Technically, the method was performed as follows: a plastic plate support for immunoelectrophoresis contained three 1% (w/v) agarose compartments (Agarose M-LKB, Bromma, Sweden). Compartment A contained 0.05% (v/v) of reference group 6 pneumococcal rabbit antiserum (Statens Seruminstitut, Copenhagen, Denmark). Compartment B contained 0.05% (v/v) of reference group 6 antiserum previously incubated at 37°C for 15 min with a known amount of reference type 6A pneumococcal capsular polysaccharide. Compartment C contained 0.05% (v/v) of reference group 6 antiserum previously incubated at 37°C for 15 min with a known amount of oligosaccharide hapten. An electrophoretic run of the reference type 6A polysaccharide in 4 serial 2-fold dilutions then occurred. After electrophoresis, the plates were dried and the "rocket" immunoprecipitates were detected and quantified (Fig. 1). The sensitivity level of this method was 20 ng/ml in the detection of type 6A polysaccharide.

Inhibition by the hapten molecule was evidenced when higher "rocket" immunoprecipitates appeared in the compartment of agarose containing the reference antiserum preincubated with the hapten (C) then in the compartment containing the reference antiserum preincubated with the reference antigen (B). The heights of the 4 "rocket" immunoprecipitates detected in each agarose compartment were plotted against their respective concns of type 6A polysaccharide, in order to obtain a straight line characterized by a given intercept on the height axis. Thus, the minimal inhibitory concn (MIC) of a hapten was calculated as:

$$\mathrm{MIC}_{\mathrm{Ha}} = C_{\mathrm{Ha}} \cdot \frac{h_{\mathrm{Ag}}}{h_{\mathrm{Ha}}},$$

where

C_{Ha} = concn (w/v) of the hapten examined in the gel,

h_{Ag} = intercept of the straight line as determined by the height of the "rocket" immunoprecipitates obtained when the reference antigen was in the gel, and

h_{Ha} = intercept of the straight line as determined by the height of the "rocket" immunoprecipitates obtained when the hapten examined was in the gel.

The immunochemical specificity as the ponderal amount of hapten vs antigen recognized by reference rabbit antibodies was then expressed in the ratio:

$$\text{specificity} = \frac{\mathrm{MIC}_{\mathrm{Ag}}}{\mathrm{MIC}_{\mathrm{Ha}}}.$$

The MIC for type 6A polysaccharide was experimentally found to be 5 ng/gel vol, using the method and the conditions reported. Several "nonsense" experiments, performed with heterologous haptens or antigens with respect to the group 6 antiserum, confirmed the reliability of the test.

A comparative analysis of specificity for oligomer haptens obtained from type 6A polysaccharide by both mild alkaline or acid hydrolysis followed, using this procedure. In particular, a scale of antibody specificity was established for the high mol. wt polysaccharide and derived oligomers, until the lowest value of specificity identified the immunodominant sugar present in the structure of the carbohydrate.

Selection of the type 6A oligosaccharide hapten

On the basis of the physico-chemical, spectroscopic and immunochemical properties showed by oligomers obtained by hydrolysis of phosphodiester or *O*-glycosidic bonds, a selection of the molecules

followed. The hapten obtained by acid hydrolysis with dimensions of an octasaccharide (about 2 basic repeating units), which exhibited an end-reducing group and a more restricted molecular polydispersion with respect to a similar hapten obtained by mild alkaline hydrolysis, was selected for conjugation to the protein carrier. ^{13}C NMR spectroscopic data showed that the discovered end-reducing group did not significantly involve the galactose residues.

The selected dimensions for a similar hapten were chosen on the basis of the maximal determinant size reported for polypeptide and polysaccharide homopolymers, which range between 6 and 8 monomeric units, as determined by several authors using immunochemical methods (Kabat, 1966; Van Vunakis *et al.*, 1966). However, the exact dimensions of the type 6A pneumococcal immunodeterminant group could only be determined by measuring the affinity constant value using specific monoclonal antibodies (which at present are not available in our laboratory), since anti-polysaccharide immunoglobulins raised in animals against whole bacterial cells are heterogeneous for specificity with respect to different sizes of a polydispersion (Porro *et al.*, 1983*b*; Bishop and Jennings, 1982).

Preparation of CRM197

CRM197 was produced by *Corynebacterium diphtheriae* strain C7 ($\beta^{tox-197}$) as previously described (Porro *et al.*, 1980). The protein was purified by molecular filtration using a Millipore membrane XM-50 (NMWL $\leq 5 \times 10^4$), then precipitated from the solution by the addition of ammonium sulfate to 65% saturation. The precipitate was sedimented by centrifugation and solubilized in 0.01 *M* phosphate buffer, pH = 7.2. Following dialysis against the phosphate buffer, CRM197 was then adsorbed on a DEAE-Sepharose 6B/CL (Pharmacia) column which was equilibrated in 0.01 *M* phosphate buffer, pH = 7.2. Elution of CRM197 from the column was obtained by increasing the ionic strength of the buffer to 0.09 *M* NaCl. CRM197 purified in this manner was *ca* 80% in the unnicked form, as determined by SDS–PAGE under reducing conditions. The level of final purity obtained was *ca* 400 Lf/mg protein.

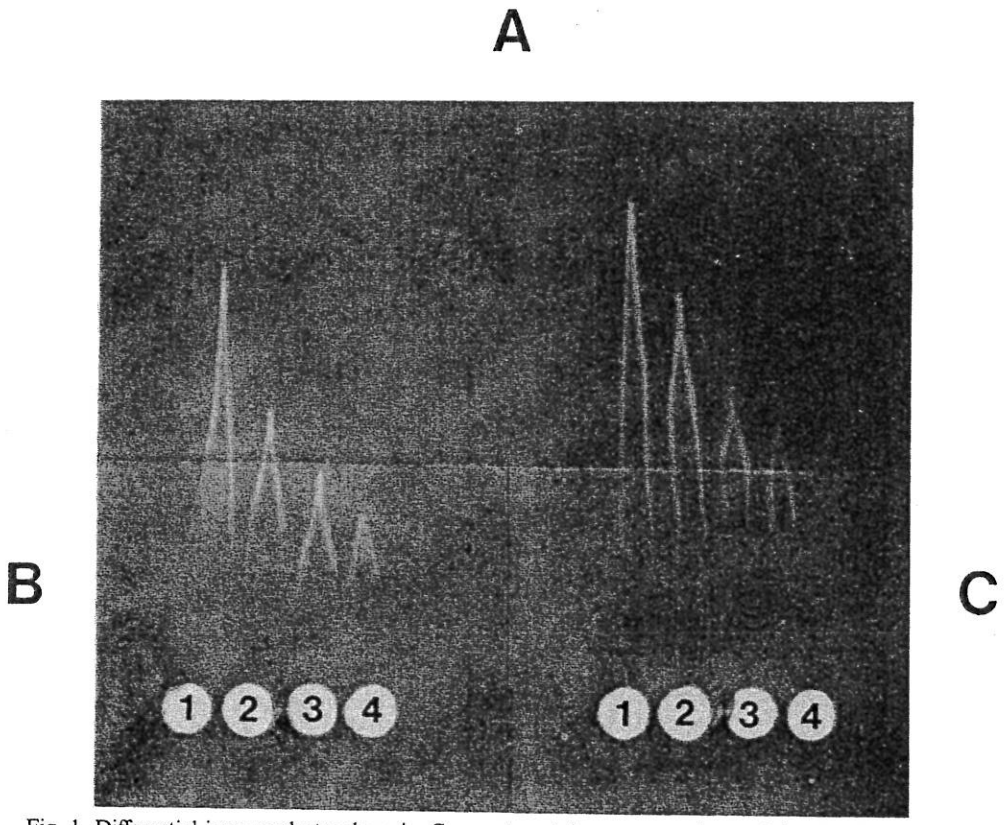


Fig. 1. Differential immunoelectrophoresis. Compartment A—agarose gel containing 0.05% (v/v) reference group 6 pneumococcal rabbit antiserum. Compartment B—agarose gel containing 0.05% (v/v) reference group 6 pneumococcal rabbit antiserum, previously incubated at 37°C for 15 min with the MIC (5 ng) of reference type 6A pneumococcal capsular polysaccharide. Compartment C—agarose gel containing the same amount of reference antiserum as in b, previously incubated under the same conditions, with 50 μg of oligo 6A hapten characterized by $R = 0.34$ in gel chromatography (see legend of Fig. 4 for definition of R). Wells 1, 2, 3 and 4 ($V = 10\ \mu$l) contained reference type 6A polysaccharide in amounts of 10, 5, 2.5 and 1 ng, respectively. Electrophoretic run was performed at 70 V/cm $\times$ 90 min with 20 m*M* Tris–barbiturate buffer, pH = 8.8. The gel was silver stained. For explanations, see text.

Synthesis of oligo 6A–CRM197 conjugate

A direct coupling of 6A oligomers to the protein carrier CRM197, via their end-reducing groups, yielded a very low amount of conjugate product, even after several days of reaction. For this reason we studied a new method of synthesis for this kind of glycoconjugate, as recently reported (Porro *et al.*, 1983*a*). For a typical preparation, the procedure involved the following steps:

Introduction of primary amino groups in the end-reducing group of the oligomer. The oligosaccharide was solubilized in distilled water at a concn of 6 mg/ml. Ammonium chloride (20 mg/ml) and $NaBH_3CN$ (7.5 mg/ml) were then added and the pH adjusted to 9 using 1 *N* NaOH.

Sealed vials containing the solution were kept at 50°C under gentle magnetic stirring for 2 weeks. After this period of reaction, the amino group derivatized oligosaccharide was purified by gel chromatography on Sephadex G-15 column, equilibrated in 10 m*M* NaCl, pH = 7.0. The chromatographic effluents showing $K_d \leq 0.1$ and chemical activity by methyl pentose, phosphorus and amino groups were pooled. Chemical analysis was performed using the methods reported by Kabat (1964), Chen *et al.* (1956) and Habeeb (1966).

Conversion of the amino group derivatized oligomer to its corresponding active ester. The purified amino-derivatized oligosaccharide was diluted with dimethyl sulfoxide to obtain a concn of organic solvent in water of 80% (v/v). This solution was dropwise added to a solution of dimethyl sulfoxide containing the disuccinimidyl ester of adipic acid, prepared according to the reported methods (Anderson *et al.*, 1964; Hill *et al.*, 1979) in the same molar amount of the amino groups introduced in the oligosaccharide. Analysis for disappearance of amino groups was performed after this step.

Conjugation of the activated oligomer to CRM197. The solution containing the ester-activated oligomer was added to an aq. solution of CRM197 (2 mg/ml) to obtain a final concn of dimethyl sulfoxide in water of 20% (v/v). The solution was gently stirred for 15 hr at room temp. Recovery of the glycoconjugate molecule was performed by gel chromatography on Sephadex G-100 column, equilibrated in 0.2 *M* NaCl, pH = 7.0. The chromatographic effluents showing chemical reactivity by both protein and phosphorus reactions were pooled, sterile filtered, and stored at 4°C. This glycoconjugate will be referred to in the text as oligo 6A-AD–CRM197.

Physico-chemical analysis of oligo 6A-AD–CRM197

The glycoconjugate was analyzed by gel filtration on Sephadex G-100; SDS–PAGE according to the Laëmmli (1970) procedure, using an acrylamide gradient of 3–9% (w/v) and silver staining of the gel (Porro *et al.*, 1982); chemical analysis for content in protein and phosphorus (Lowry *et al.*, 1951; Chen *et al.*, 1956); estimation of the amount of residual amino groups (Habeeb, 1966) in CRM197 after the coupling procedure. All these analyses were performed in direct comparison with native CRM197. The stoichiometry of the glycoconjugate, defined as the substitution degree (sd), represented the value of the ratio oligosaccharide/protein (moles/mole) and was calculated as follows: since the ratio methyl pentose/phosphorus in the type 6A pneumococcal polysaccharide was identical to that found in the derived oligosaccharide used for the glycoconjugate preparation, the amount of phosphorus estimated in the glycoconjugate analysis permitted calculation of the ponderal ratio between carbohydrate and protein. By considering the av. mol. wt of the oligomer as estimated by ^{13}C NMR spectroscopy (about 2 basic repeating units = 1400), the ratio carbohydrate/protein was transferred on a molar basis (sd). This value was compared with the number of amino groups lost for each mole of CRM197, after the coupling reaction. Based on the SD value, the calc. mol. wt of the glycoconjugate was compared to that obtained by SDS–PAGE and to the K_d shift shown on Sephadex G-100 gel chromatography with respect to native CRM197.

Immunochemical properties of oligo 6A-AD–CRM197

Exposure of the type 6A oligomer haptens on the surface of the protein carrier was tested using differential immunoelectrophoresis, by evaluating the specificity shown by the rabbit reference group 6 pneumococcal antiserum. Immunochemical properties of the protein carrier CRM197 in conjugate form were tested by tandem-crossed immunoelectrophoresis (Weeke, 1973).

Doses and preparation of the vaccine solutions

The synthesized glycoconjugate was prepared in two different vaccine forms: as solution and as $AlPO_4$-adsorbed form, both in a vol of 0.5 ml. In the case of the adsorbed form of the antigen, the preparation involved 1.5 mg $AlPO_4$/0.5 ml and adsorption was performed at 35°C in saline, pH = 6.8, by stirring in an orbital shaker at 150 rpm for 15 hr. The amount of adsorbed glycoconjugate was then tested by rocket immunoelectrophoresis analysis by determining the residue amount of CRM197 in conjugate form still present in the supernatant obtained after centrifugation (2000 rpm) of the suspension. Adsorption occurred for more than 80% of the amount of glycoconjugate employed.

0.5 ml of both the vaccine forms contained 37.5 μg of glycoconjugate, equivalent to 12.5 Lf of CRM197 and 6.8 μg of type 6A oligosaccharide. The amount of CRM197 was chosen on the basis of the consideration reported previously (Porro *et al.*, 1980). The amount of type 6A oligosaccharide was derived from the selected dose of CRM197, in consideration of the stoichiometry of the glycoconjugate.

Immunization of animal models

Two groups of 8 albino rabbits, 7–8 weeks old, and 2 groups of 10 guinea pigs, weighing about 350 g, were immunized subcutaneously with the 2 different vaccine forms, as follows: 1 group of both albino rabbits and guinea pigs received 0.5 ml of 1 of the 2 vaccine forms. Twenty-eight days after the basal immunization, a booster dose of the homologous vaccine form was given to all the animals. A second booster dose was given 11 days later to 3 rabbits and to 5 guinea pigs of each group. The time at which the second booster dose was given corresponded to half the half-life period known for the IgG immunoglobulin class. The animals were bled a few minutes before they received the first and second booster doses and 11 days after the second booster dose. The other groups of both rabbits and guinea pigs were immunized with the other vaccine form, using the same schedule described.

Pooled sera from 5 albino rabbits and 5 guinea pigs, obtained before vaccination, were tested for the absence of antibodies to type 6A pneumococcal polysaccharide and diphtheria toxin. Control immunizations with type 6A pneumococcal polysaccharide or CRM197 alone were omitted, based upon previous experiences (Porro *et al.*, 1980, 1983*a*) that neither type 6A polysaccharide nor CRM197, when injected into the animals reported and in the dosage range adopted, were able to raise detectable antibodies.

During the period of immunization, the animals were observed for wt gain and adverse reactions at the site of injection.

Antibody titrations

Antibodies to diphtheria toxin in the pooled sera of each group of guinea pigs were titered according to the required test of the US Pharmacopea for the human vaccine. Antibodies to type 6A pneumococcal polysaccharide, in the serum of each rabbit, were determined by indirect ELISA as follows: polystyrene micro plates were coated overnight at 4°C with 100 μl of a 1 mg/ml type 6A pneumococcal polysaccharide solution in 0.5 *M* carbonate buffer, pH = 8.5. After 3 washes with 100 μl PBS solution containing 0.01% (v/v) Tween 20, 4 serial 2-fold dilutions of rabbit sera in the range 1/100–1/800 were distributed in the wells. Incubation was at 37°C for 1.5 hr. Three washes followed, and the plates were incubated at 37°C for 1.5 hr with 100 μl of a solution of goat anti-rabbit IgG conjugated to peroxidase (Miles-Yeda, Israel). After 3 more washes, 100 μl of the chromogen solution [2.9 ml of 0.075% (w/v) ABTS (Boehringer, Mannheim, F.R.G.) in 0.1 *M* citrate buffer, pH = 5.6, containing 0.1 ml of a 0.043% (w/v) H_2O_2 solution] were added, and the colorimetric reactions recorded after 20 min by absorbance (ABS) level at 405 nm. Parallel analysis for non-specific reactions was performed with plates not coated with the type 6A polysaccharide. The titers of rabbit IgG anti-6A pneumococcal polysaccharide were determined as follows: when the 4 serial 2-fold dilutions of a serum were plotted against their respective logarithmic value of ABS levels, a straight line was obtained for each rabbit serum. The intercept of this straight line with the ABS axis was taken as the maximum value of ABS exhibited in ELISA by that serum (ABS_{max}). Comparison of this value followed after each bleeding, according to the immunization schedule, for each rabbit immunized by the homologous vaccine form. The geometric mean (X_g) of the ABS value obtained in each group of rabbits after each bleeding was then calculated, and the increase of these values was estimated (R_{X_g}).

Qualitative analysis of rabbit antisera

Rocket immunoelectrophoresis of type 6A pneumococcal capsular polysaccharide towards both reference and vaccine-raised antisera was performed as follows: 2 adjacent layers of agarose [1% (w/v)] contained the vaccine-raised or reference rabbit antiserum, respectively. Electrophoresis of the type 6A polysaccharide was performed using 0.02 *M* Tris–barbiturate buffer, pH = 8.8. The gel plate was dried and silver stained as reported by Porro *et al.* (1982).

The classical Quellung reaction with living type 6A and 6B *Streptococcus pneumoniae* strains was performed according to Austrian (1976), using both reference group-specific rabbit antiserum and the vaccine-raised rabbit antiserum.

RESULTS

Physico-chemical and immunochemical characteristics of oligosaccharides

When oligosaccharides obtained from both mild alkaline or acid hydrolysis showed similar molecular sizes ($\overline{MW} = 1200$) on gel chromatography, a comparative HPLC analysis was performed in order to investigate the heterogeneity of their mol. wts. Figure 2 shows a qualitative comparison of oligomers with the native type 6A pneumococcal polysaccharide (Ps6A); whereas Ps6A appeared to have a restricted polydispersion, since the large carbohydrate was eluted with the V_o of the column [retention time (Rt) = 6.3 min and $MW \geq 3 \times 10^5$], the oligosaccharide obtained by acid hydrolysis actually had a restricted mol. wt polydispersion when compared with a similar oligosaccharide obtained by mild alkaline hydrolysis. In fact, although the model values of the polydispersion of the two oligomers showed a similar Rt (= 13.8 and 13.7 min, respectively), the polydispersion referred to the oligomer obtained by mild alkaline hydrolysis showed other chromatographic peaks to higher and lower molecular sizes, evidencing a more heterogeneous polydispersion.

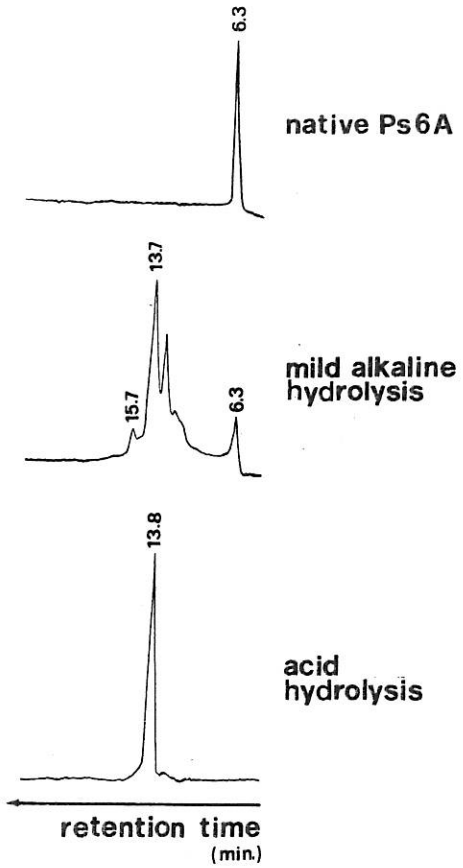
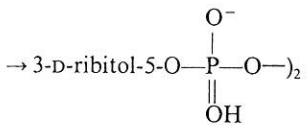


Fig. 2. Qualitative comparison by HPLC of mol. wt heterogeneity in oligomers obtained by 2 different hydrolytic procedures (see text). Oligomers were characterized by a similar value of R in gel chromatography on Sephadex G-50.

Chemical analysis for both oligomers showed a phosphorus/rhamnose ratio of 1 (moles/mole) as in the native Ps6A, but in the oligomer obtained by acid hydrolysis, end-reducing groups in a rhamnose/reducing group ratio of 3 (moles/mole) were found.

^{13}C NMR spectroscopy data (Fig. 3) evidenced two different kinds of hydrolytic cleavage of the type 6A polysaccharide. In the case of acid hydrolysis, the galactose residue did not appear to be significantly affected by the breaking of the O-glycosidic bond, since the area corresponding to the signal at 98.9 ppm (typical of the anomeric carbon C_1 of the galactose) was similar to that observed in higher fields (96.7 ppm). In contrast, the smaller area of the anomeric carbon evidence at 101.7 ppm, when compared to the same signals obtained with the native polysaccharide (Kenne *et al.*, 1979), suggested that this was the site(s) affected by hydrolysis. Investigations of the localization of the sugar(s) involved in the hydrolysis are in progress, since end-reducing groups were demonstrated in this oligomer. Estimation of the dimensions of a similar oligosaccharide corresponded to approx. 8 monose residues, which means about 2 basic repeating units:

(→ 2-α-D-Galp-1 → 3-α-D-Glcp-1 → 3-α-L-Rhap-1



In the case of mild alkaline hydrolysis, breakage of the phosphodiester bonds was indicated by the disappearance of the signal corresponding to the anomeric carbon C_1 of the galactose (98.9 ppm) and of the 2 signals assigned to the C_2 and C_3 of the galactose unit which is substituted in C_2 by the phosphate group. Under these conditions, no significant end-reducing groups were estimated in the oligomer. The immunochemical specificity of reference group 6 pneumococcal rabbit antibodies for oligomers was demonstrated to be a function of their molecular size. Figure 4 shows the specificity scale obtained by mild alkaline hydrolysis derived oligomers in comparison with native Ps6A. Galactose was shown to be the immunodominant sugar present in the molecular structure of the type 6A carbohydrate, since the other sugars, glucose and rhamnose, did not show inhibition towards specific group 6 rabbit antiserum. Very interestingly, similar specificity values were found when similar molecular size of acid or mild alkaline hydrolysis derived oligomers were compared. This observation appeared to be in good correlation with the results obtained by ^{13}C NMR spectroscopy showing the substantial integrity of the galactose residues in the acid hydrolysis derived oligomer, and well supported the finding for galactose as the immunodominant sugar. Specificity of reference antibodies for the selected octasaccharide used in the chemical synthesis of the glycoconjugate was 10^{-3}, when compared to the native type 6A polysaccharide.

Physico-chemical and immunochemical characteristics of oligo 6A-AD–CRM197 conjugate

Chemical analysis of the glycoconjugate indicated a carbohydrate/protein ratio of 0.18 (w/w). This value, when the dimensions of the oligomer were estimated by ^{13}C NMR spectroscopy, permitted definition of the stoichiometry of this glycoconjugate as equivalent to an sd of 8. This sd value was in excellent correlation with the number of amino groups that disappeared for each mole of the protein carrier CRM197, after the coupling procedure. The gel chromatographic profile of the glycoconjugate appeared as a restricted polydispersion with a K_d "shift" of 0.1, when compared with the native CRM197 on Sephadex G-100 (Fig. 5). The apparent mol. wt was estimated by SDS–PAGE (Fig. 6), where the polydisperse glycoprotein showed an av. mol. wt of 75,000. Furthermore, glycosylation appeared to be distributed on the 2 fragments constituting the CRM197 molecule (Uchida *et al.*, 1973). Because of the high sensitivity of the silver staining adopted, all

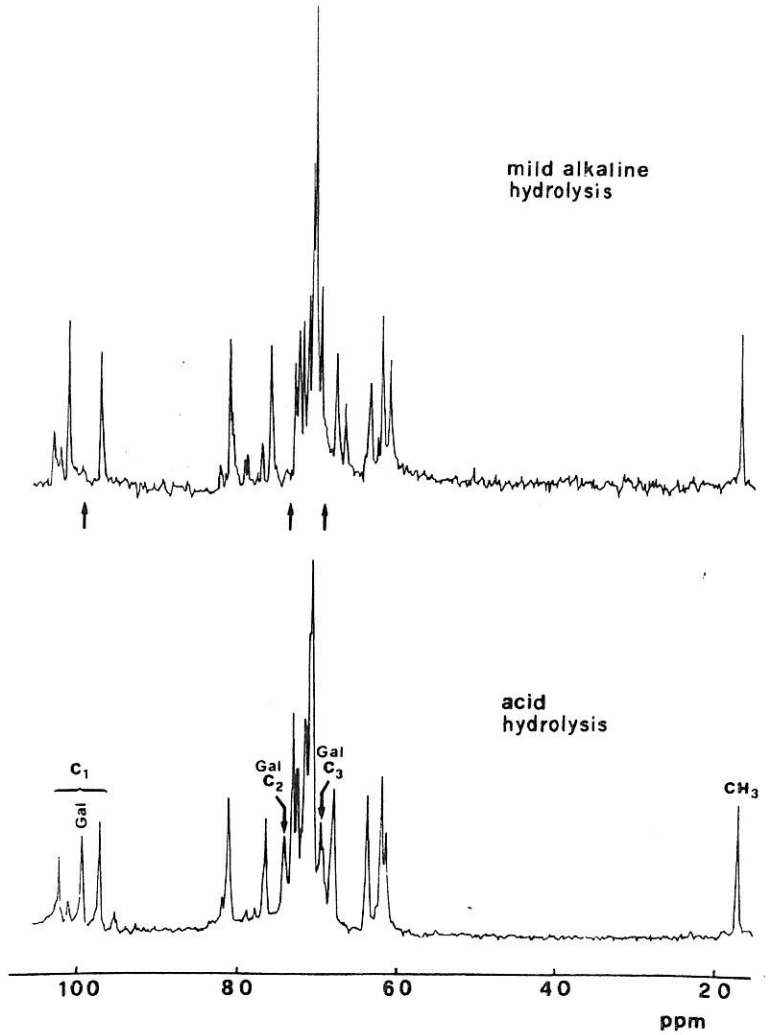


Fig. 3. Evidence for the 2 different kinds of hydrolytic cleavage obtained by mild alkaline or acid hydrolysis of type 6A pneumococcal polysaccharide, as shown by ^{13}C NMR spectroscopy. For explanations, see text.

the carrier protein was shown in glycoconjugate form since bands of free CRM197 did not appear in the gel. This result was expected, in light of the stoichiometry of the glycoconjugate and the statistical considerations which derived from an sd higher than 1. The estimated molecular size of the glycoconjugate by both gel filtration and SDS–PAGE was in the range of that theoretically calculated from the sd value.

Immunochemical analysis of this glycoconjugate, with respect to both the oligosaccharide and the protein carrier, showed that the antigenic determinants were exposed and recognized by their respective homologous antibodies. The immunochemical specificity of the reference group 6 pneumococcal rabbit antiserum for the glycoconjugate, when compared with the native 6A polysaccharide, was 10^{-1} as indicated by differential immunoelectrophoresis (data not shown). This value appeared to be 100 times higher than that obtained using the corresponding oligomer in unconjugated form. This finding could be explained by the effective increase in molecular size of the oligosaccharides in conjugate form with respect to the oligosaccharide alone, emphasizing the role of the protein carrier as a support for carbohydrate haptens. In fact, the immunochemical specificity of a homologous polyclonal antiserum for a polysaccharide is related to the molecular size of the carbohydrate as we have also demonstrated for type 6A polysaccharide. It should be emphasized, however, that the immunochemical specificity obtained by heterogeneous antibody populations, as present in a typical immune serum, is the expression of the amount of homologous antibodies recognizing the antigen and not of their affinities (Berzofsky and Schechter, 1981), which would require experiments with monoclonal antibodies. The protein carrier CRM197 showed immunochemical identity with native CRM197 when tested by diphtheria toxin antiserum (Fig. 7).

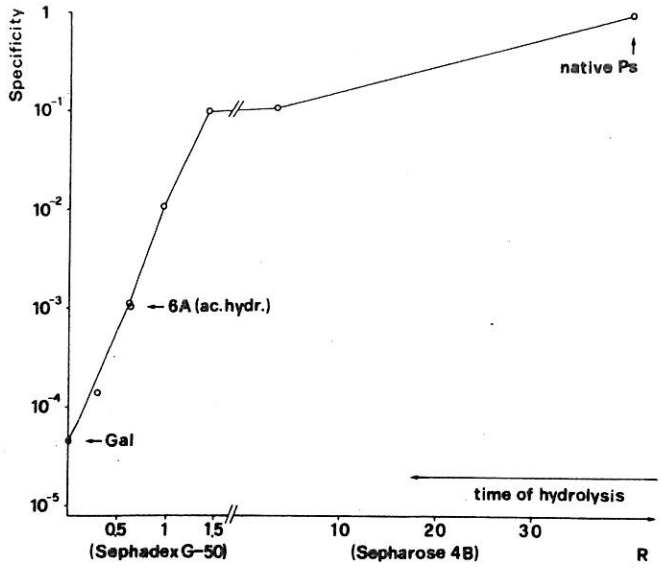


Fig. 4. Specificity of reference group 6 pneumococcal rabbit antibodies for haptens of different molecular size obtained by mild alkaline hydrolysis of type 6A pneumococcal capsular polysaccharide (see text), as detected by differential immunoelectrophoresis. R ($K_d < 0.5/K_d > 0.5$) represents the chromatographic recovery of the molecular polydispersion of the carbohydrates in respect to $K_d = 0.5$ characteristic for a chromatographic column containing the indicated gel (Sepharose 4B or Sephadex G-50). Also shown is a comparison of specificity for the native type 6A polysaccharide and for an oligomer obtained by acid hydrolysis, characterized by an R value similar to that of a hapten obtained by mild alkaline hydrolysis. Gal indicates the immunochemical specificity for galactose.

Immunological properties of oligo 6A-AD–CRM197 conjugate

Quantitative determination of serum antibodies to diphtheria toxin, induced in guinea pigs immunized with the glycoconjugate in the 2 vaccine forms described, are shown in Fig. 8.

Quantitative estimations of IgG immunoglobulins anti-6A pneumococcal capsular polysaccharide induced in rabbits receiving either 2 or 3 injections are reported in Tables 1 and 2, respectively, and summarized in Fig. 9. The ratios between the geometric means (R_{X_g}) of the IgG titers indicated that an increase of specific antipolysaccharide antibodies was obtained after each booster dose of the 2 vaccine forms. In addition, the use of $AlPO_4$-adsorbed gly-

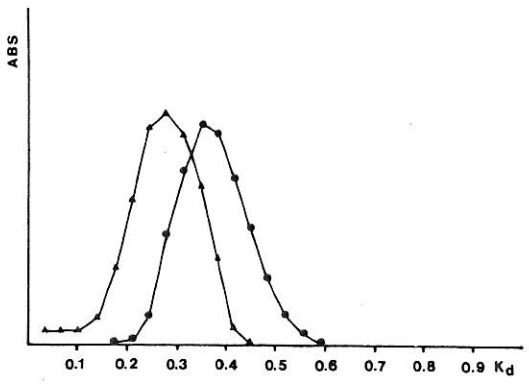


Fig. 5. Gel chromatographic profile on Sephadex G-50 of oligo 6A-AD–CRM197 (▲——▲) in comparison with CRM197 (●——●).

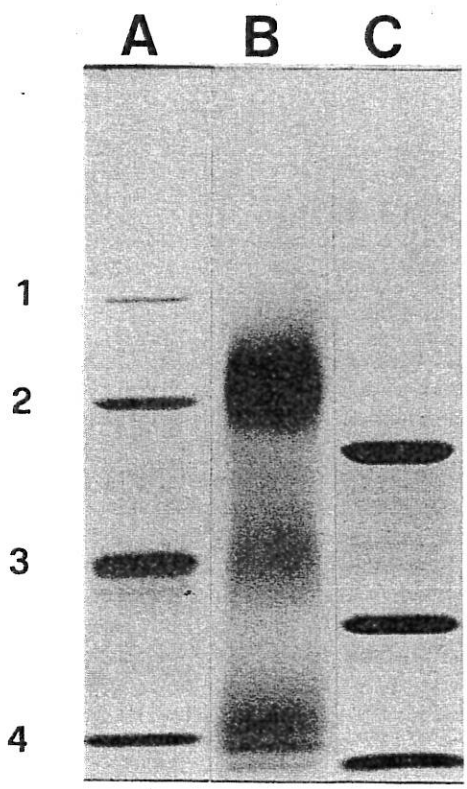


Fig. 6. SDS–PAGE, in reducing conditions, of the glycoconjugate molecule as compared to CRM197 alone. (A) Reference proteins for MW determination: (1) Phosphorylase b (94,000); (2) BSA (67,000); (3) ovalbumin (43,000); (4) carbonic anhydrase (30,000). (B) Oligo 6A-AD–CRM197. (C) CRM197 in partial "nicked" form. In both samples B and C, the amount of CRM197 was 5 μg.

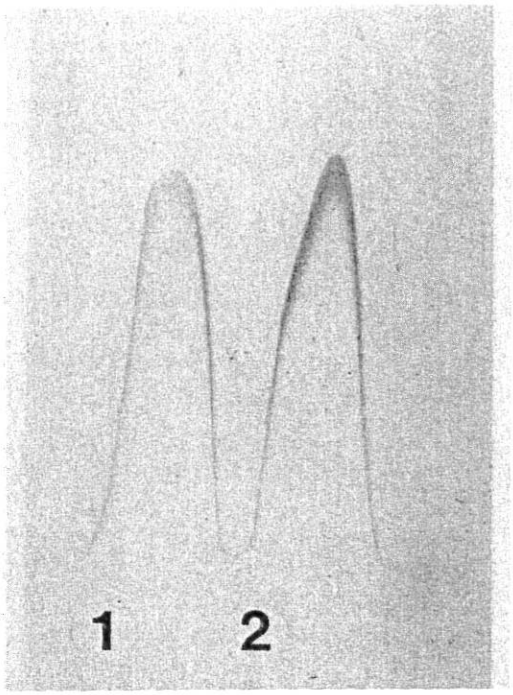


Fig. 7. Immunochemical identity of CRM197 in conjugate form as analyzed by tandem-crossed immunoelectrophoresis: (1) CRM197 (0.5 μg), and (2) oligo 6A-AD–CRM197 (0.5 μg in protein). Diffusion time: 30 min. First electrophoretic run: 10 V/cm for 30 min. Second electrophoretic run: 70 V/cm for 60 min.

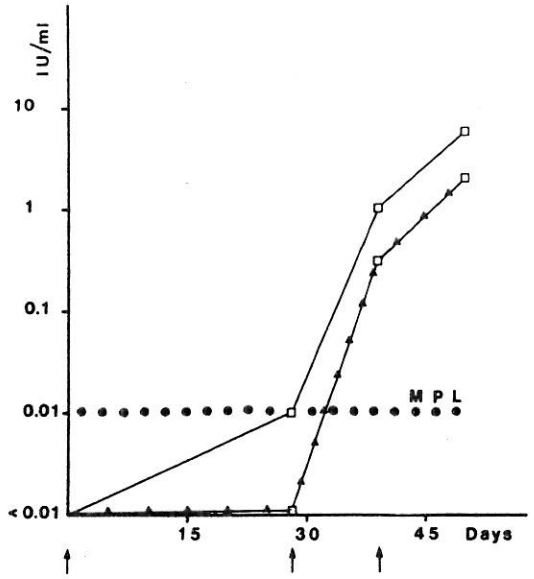


Fig. 8. Diphtheria antitoxin titers obtained by immunization of guinea pigs using 2 vaccine forms of the glycoconjugate: (□——▲——▲——□) oligo 6A-AD–CRM197 in solution form, and (□——□) oligo 6A-AD–CRM197 in $AlPO_4$-adsorbed form. Each point represents the obtained titer of the sera pool of each group of guinea pigs as described in the text. Arrows indicated the time in which injections of the vaccine were given. MPL indicates the value of the minimum protective level of diphtheria antitoxin estimated in man (Wilson and Miles, 1975).

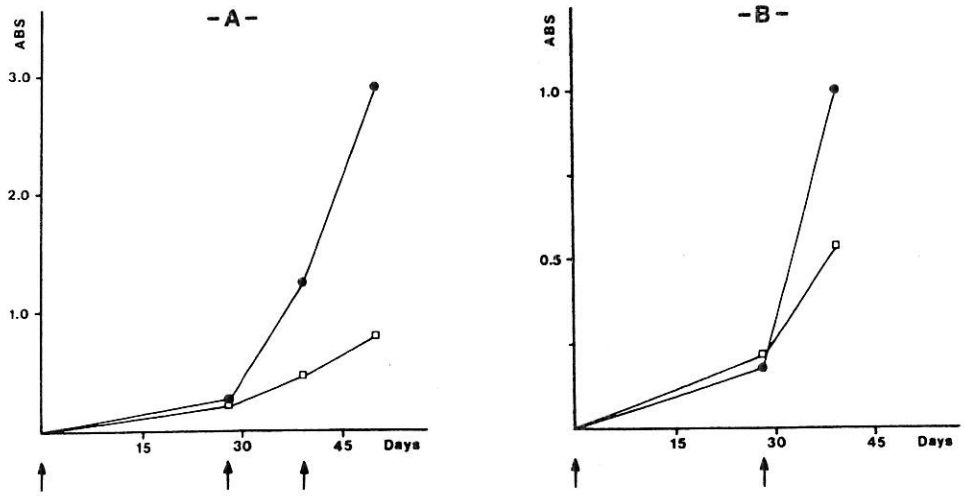


Fig. 9. ELISA titers of IgG antibodies to type 6A pneumococcal capsular polysaccharide, obtained by immunization of albino rabbits, using 3 (A) or 2 (B) injections of 2 vaccine forms of the glycoconjugate. (□——□) oligo 6A-AD–CRM197 in solution form, and (●——●) oligo 6A-AD–CRM197 in $AlPO_4$-adsorbed form. Each point represents the geometric mean (X_g) of ABS obtained in each group of rabbits as described in the text. Arrows indicate the time at which injections of the vaccine were given.

Table 1. ELISA titers of rabbit IgG anti-type 6A pneumococcal capsular polysaccharide

Rabbit No.	Antigen administered	1st injection ABS	Booster dose ABS
1	Oligo 6A-AD–CRM197	0.446	0.891
3		0.281	0.446
4		0.251	0.354
5		0.125	0.398
7		0.199	0.281
8		0.141	1.584
9		0.158	0.501
10		0.251	0.630
		$X_g = 0.213$	0.537
			$R_{X_g} = 2.5$
11	$AlPO_4$-adsorbed oligo 6A-AD–CRM197	0.251	0.630
13		0.063	1.778
14		0.079	0.251
15		0.630	1.258
16		0.199	1.000
17		0.199	1.000
18		0.199	2.511
20		0.158	1.584
		$X_g = 0.178$	1.000
			$R_{X_g} = 5.6$

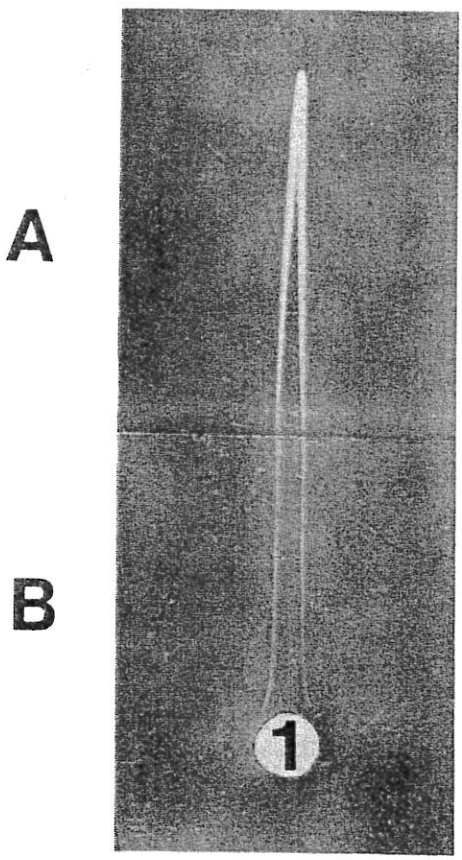


Fig. 10. Qualitative aspect, detected by rocket immunoelectrophoresis, of rabbit antiserum (B) raised by oligo 6A-AD–CRM197 conjugate, as compared to the reference group 6 pneumococcal rabbit antiserum (A): (1) type 6A pneumococcal polysaccharide (50 ng). The agarose gels (A and B) contained 0.5 and 1% (v/v) of the respective rabbit antisera. Electrophoresis was performed at 70 V/cm for 90 min.

coconjugate vaccine showed an increase in the IgG antibody titers when compared to the soluble form of the vaccine. During the period of immunization, rabbits and guinea pigs had a normal wt gain, and no significant reactions appeared at the sites of injection. Qualitative aspects of the vaccine-raised antibodies to type 6A capsular polysaccharide are shown in Figs 10 and 11.

DISCUSSION

Development of new biologicals as synthetic polypeptide vaccine (Sela, 1983) and carbohydrate–protein conjugates (Schneerson *et al.*, 1980; Jennings and Lugowski, 1981) constitutes a promising approach to overcome a series of problems coupled to the classic vaccines and to the modern polysaccharide vaccines for human use. These problems include some aspects of undesirable reactogenicity of toxoids such as diphtheria and tetanus (Matuhasi and Ikegami, 1982) and basic aspects related to the poor immunogenicity of the polysaccharide vaccines in infants (Sell *et al.*, 1981; Gotschlich, 1975).

In the present work we used the type 6A pneumococcal capsular polysaccharide which is present in the polyvalent pneumococcal vaccine for human use, because in the clinical use of this vaccine several investigators reported the failure of the type 6A antigen to protect children between 2 and 6 years of age (Sell *et al.*, 1981; Makela *et al.*, 1981). In addition, pneumococcal serotype 6A seems to have a marked statistical incidence in childhood infection (Gray *et al.*, 1980). The carrier protein CRM197, a non-toxic mutant protein serologically cross-reactive with diphtheria toxin, was considered because we described this protein as a potential antidiphtheria antigen (Porro *et al.*, 1980) after a formalin treatment of this

Table 2. ELISA titers of rabbit IgG anti-type 6A pneumococcal capsular polysaccharide

Rabbit No.	Antigen administered	1st injection ABS	1st booster dose ABS	2nd booster dose ABS
1		0.446	0.891	1.584
5	Oligo 6A-AD–CRM197	0.125	0.398	0.794
7		0.199	0.281	0.398
		$X_g = 0.233$	0.464	0.794
		$R_{X_g} = 2.1$	$R_{X_g} = 1.7$	
15	$AlPO_4$-adsorbed	0.630	1.258	2.511
16	oligo 6A-AD–CRM197	0.199	1.000	3.981
20		0.158	1.584	2.511
		$X_g = 0.271$	1.258	2.928
		$R_{X_g} = 4.6$	$R_{X_g} = 2.3$	

protein was performed. This treatment increased the stability of CRM197 to trypsin-like enzymes "*in vitro*" and this appeared to be related to its acquired immunogenic properties (Porro *et al.*, 1980; Pappenheimer *et al.*, 1972).

Using the molecular model reported, the synthesized glycoconjugate recalled the properties of a thymus-dependent antigen for both the carbohydrate and the protein moiety, since immunologic memory was stimulated when booster doses were administered

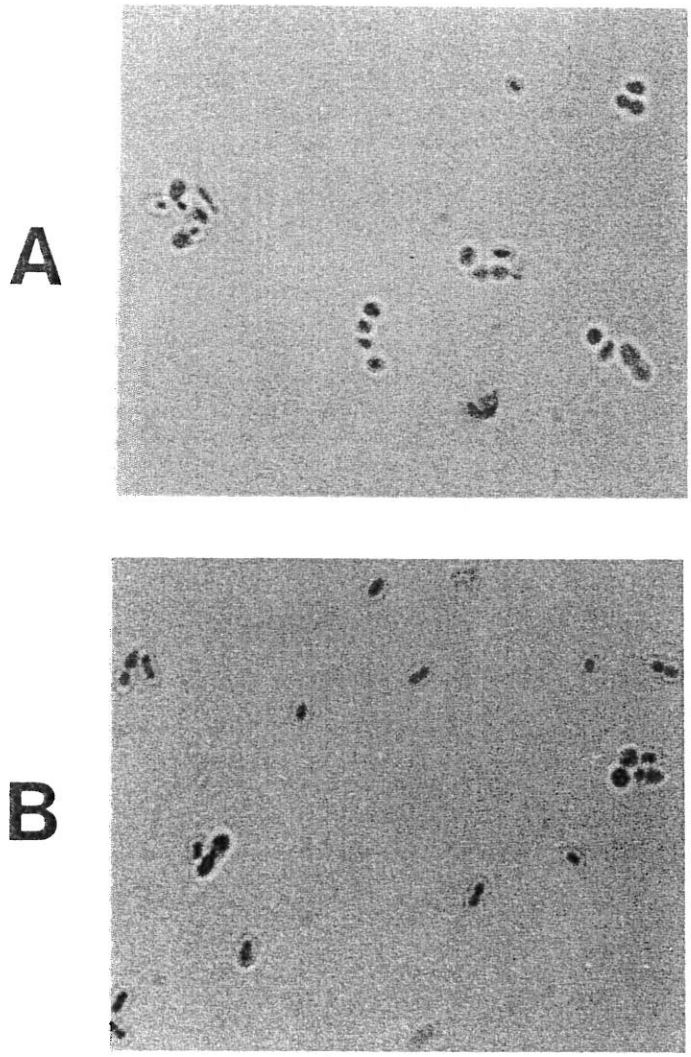


Fig. 11. Quellung reaction on type 6A *S. pneumoniae* strain: (A) rabbit antiserum raised by oligo 6A-AD–CRM197 conjugate, and (B) reference group 6 rabbit antiserum. Magnification ×585, in an Orthoplan optical microscope (Leitz, Germany).

after basal immunization. In particular, the rabbit humoral response to the type 6A pneumococcal capsular polysaccharide was evidenced as a significant increase in antibody of IgG class. The use of the mineral adjuvant $AlPO_4$, largley used in vaccines for human use, amplified the immunological response in both antitoxic and antipolysaccharide antibodies. This observation appeared significant since over 80% of the glycoconjugate administered was shown to be adsorbed to the mineral adjuvant.

The antitoxic level in guinea pigs obtained by 2 booster doses of CRM197 in the conjugate form adsorbed to $AlPO_4$, increased 100 and 1000 times with respect to the estimated minimum protective level in man. This amount of antibodies doubled that reached when the glycoconjugate was administered in soluble form.

Rabbit IgG antibodies to type 6A pneumococcal capsular polysaccharide were shown at high levels of concn by an ELISA technique. The absence of statistical correlation between ELISA and RIA in the pneumococcal antibody titration (Callahen *et al.*, 1979) did not permit us to translate the ELISA titers into ng antibody nitrogen/ml of serum, which is the usual expression to evaluate an antipneumococcal titer (Schiffman *et al.*, 1980). Nevertheless, human antisera to pneumococcal type 6A polysaccharide with an antibody level ranging from 250 to 500 ng antibody nitrogen/ml showed an IgG content of not more than 0.28 ABS using the same ELISA test performed for quantitative analysis of rabbit IgG anti-type 6A polysaccharide. The qualitative aspect of the rabbit immunological response supported the quantitative data since the glycoconjugate-elicited rabbit antisera were specific for the type 6A pneumococcal polysaccharide as a reference group 6 rabbit antiserum. In addition, these antisera were able to evidence a clear Quellung reaction on living type 6A and 6B *S. pneumoniae* strains, in accordance with the known structural similarity of these capsular carbohydrates (Kenne *et al.*, 1979; Rebers and Heidelberger, 1961). This finding appeared very significant, because it evidenced the specificity of these antibodies in the recognition of the natural polysaccharide capsule, an essential requisite for antipneumococcal immunity (Austrian, 1979).

Although preliminary tests gave evidence for the absence of general toxicity of these glycoconjugates, both in rabbits and guinea pigs, further studies on these hybrid molecules will involve pharmacotoxicological investigations before clinical trials in man begin.

REFERENCES

Anderson G. W., Zimmerman J. E. and Callahan F. M. (1964) The use of *N*-hydroxysuccinimide in peptide synthesis. *J. Am. chem. Soc.* **86,** 1839–1842.

Austrian R. (1976) The Quellung reaction, a neglected microbiologic technique. *Mt Sinae J. Med.* **43,** 699–709.

Austrian R. (1979) Pneumococcal vaccine: development and prospects. *Am. J. Med.* **67,** 547–549.

Austrian R., Howie V. M. and Ploussard J. H. (1977) The bacteriology of pneumococcal otitis media. *Johns Hopkins med. J.* **141,** 104–111.

Avery O. T. and Goebel W. F. (1929) Chemo-immunological studies on conjugated carbohydrate proteins. II. Immunological specificity synthetic of sugar–protein antigens. *J. exp. Med.* **50,** 533–550.

Berzofsky J. A. and Schechter A. N. (1981) The concepts of cross-reactivity and specificity in immunology. *Molec. Immun.* **18,** 751–763.

Beuvery E. C., Rossum F. V. and Nagel J. (1982) Comparison of the induction of immunoglobulin M and G antibodies in mice with purified pneumococcal type 3 and meningococcal Group C polysaccharides and their protein conjugates. *Infect. Immun.* **37,** 15–22.

Bishop C. T. and Jennings H. J. (1982) Immunology of polysaccharides. In *The Polysaccharides* (Edited by Aspinal G. O.), Vol. 1, pp. 292–330. Academic Press, New York.

Callahen L. T., Woodhour A. F., Meeker J. B. and Hilleman M. R. (1979) Enzyme-linked immunosorbent assay for measurement of antibodies against pneumococcal polysaccharide antigens: comparison with radioimmunoassay. *J. clin. Microbiol.* **10,** 459–463.

Chen P. S., Toribara T. Y. and Warner H. (1956) Microdetermination of phosphorus. *Analyt. Chem.* **28,** 1756–1758.

Goebel W. F. (1938) Chemo-immunological studies on conjugated carbohydrate proteins. IV. The immunological properties of an artificial antigen containing cellobiuronic acid. *J. exp. Med.* **68,** 469–484.

Gotschlich E. C. (1975) Development of polysaccharide vaccine for the prevention of meningococcal diseases. *Allergy* **9,** 245–258.

Gotschlich E. C., Goldschneider I. and Artenstein M. S. (1969) Human immunity to the meningococcus. IV. Immunogenicity of Group A and Group C meningococcal polysaccharides in human volunteers. *J. exp. Med.* **129,** 1367–1384.

Gray B. M., Converse G. M. and Dillon H. C. (1980) Epidemiologic studies of *S. pneumoniae* in infants; acquisition, carriage and infection during the first 24 months of life. *J. infect. Dis.* **143,** 923–933.

Habeeb A. F. S. A. (1966) Determination of free amino groups in proteins by trinitrobenzensulfonic acid. *Analyt. Biochem.* **14,** 328–338.

Hill M., Bechet J. J. and D'Albis A. (1979) Disuccinimidyl esters as bifunctional cross-linking reagents for proteins. *FEBS Lett.* **102,** 282–286.

Horejsi V. (1981) Affinity electrophoresis. *Analyt. Biochem.* **112,** 1–8.

Jennings H. J. and Lugowski C. (1981) Immunochemistry of Groups A, B and C meningococcal polysaccharide–tetanus toxoid conjugates. *J. Immun.* **127,** 104–108.

Kabat E. A. (1964) Methylpentose determination. In *Experimental Immunochemistry* (Edited by Kabat E. A. and Mayer M.), pp. 538–541. Thomas, Springfield, IL.

Kabat E. A. (1966) The nature of an antigenic determinant. *J. Immun.* **97,** 1–11.

Kenne L., Lindberg B. and Madden J. K. (1979) Structural studies of the capsular antigen from *Streptococcus pneumoniae* type 26. *Carbohydr. Res.* **73,** 175–182.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, Lond.* **227,** 680–685.

Lowry O. H., Rosebrough N. J., Farr L. and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265–275.

Makela P. H., Leinonen M., Pukander J. and Karma P. (1981) A study of the pneumococcal vaccine in prevention of clinical acute attacks of recurrent otitis media. *Rev. infect. Dis.* **3,** S124–S130.

Makela P. H., Peltola H., Kayhty H., Jousimies H., Ruos-

alhti E., Sivonen A. and Renkonen O. V. (1977) Polysaccharide vaccines of Group A *Neisseria meningitidis* and *Haemophilus influenzae* type b: a field trial in Finland. *J. infect. Dis.* **136,** S43–S50.

Matuhasi T. and Ikegami H. (1982) Elevation of levels of IgE antibody to tetanus toxin in individuals vaccinated with diphtheria–pertussis–tetanus vaccine. *J. infect. Dis.* **136,** 290.

Pappenheimer A. M., Jr, Uchida T. and Harper A. A. (1972) An immunological study of the diphtheria toxin molecule. *Immunochemistry* **9,** 891–906.

Porro M., Costantino P., Viti S. and Saletti M. (1983*a*) Immunochemistry of glycoconjugate molecules obtained by chemical synthesis of two bacterial antigens. In *Proceedings of the 7th International Symposium on Glycoconjugates* (Edited by Chester M. A., Heinegard D., Lundblad A. and Svenson S.) pp. 247–248. Lund–Ronneby, Sweden.

Porro M., Fabbiani S., Marsili I., Viti S. and Saletti M. (1983*b*) Immunoelectrophoretic characterization of the molecular weight polydispersion of polysaccharides in multivalent bacterial capsular polysaccharide vaccines. *J. biol. Stand.* **11,** 65–74.

Porro M., Saletti M., Nencioni L., Tagliaferri L. and Marsili I. (1980) Immunogenic correlation between cross-reacting material (CRM197) produced by a mutant of *C. diphtheriae* and diphtheria toxoid. *J. infect. Dis.* **143,** 716–724.

Porro M., Viti S., Antoni G. and Neri P. (1981) Modifications of the Park–Johnson ferricyanide submicromethod for the assay of reducing groups in carbohydrates. *Analyt. Biochem.* **118,** 301–306.

Porro M., Viti S., Antoni G. and Saletti M. (1982) Ultrasensitive silver-stain method for the detection of proteins in polyacrylamide gels and immunoprecipitates on agarose gels. *Analyt. Biochem.* **127,** 316–321.

Rebers P. A. and Heidelberger M. (1961) The specific polysaccharide of type VI pneumococcus. II. The repeating unit. *J. Am. chem. Soc.* **83,** 3056–3059.

Schiffman G., Douglas R. M., Bonner J. M., Robbins M. and Austrian R. (1980) A radioimmunoassay for immunological phenomena in pneumococcal diseases and for the antibody response to pneumococcal vaccine. I. Method for the radioimmunoassay of anticapsular antibodies and comparison with other techniques. *J. immun. Meth.* **33,** 130–144.

Schneerson R., Barrera O., Sutton A. and Robbins J. B. (1980) Preparation, characterization and immunogenicity of *Haemophilus influenzae* type b polysaccharide–protein conjugates. *J. exp. Med.* **152,** 361–376.

Sela M. (1983) From synthetic antigens to synthetic vaccines. *Biopolymers* **22,** 415–424.

Sell S. H., Wright P. F., Vaughan W. K., Thompson J. and Schiffman G. (1981) Clinical studies of pneumococcal vaccine in infants. I. Reactogenicity and immunogenicity of two polyvalent pneumococcal vaccines. *Rev. infect. Dis.* **3,** S97–S107.

Uchida T., Pappenheimer A. M., Jr and Greany R. (1973) Diphtheria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. *J. biol. Chem.* **248,** 3838–3844.

Van Vunakis H., Kaplan H. and Levine L. (1966) Immunogenicity of polylisine and polyornithine when complexed to phosphorylated bovine serum albumin. *Immunochemistry* **3,** 393–402.

Weeke B. (1973) Rocket immunoelectrophoresis. In *A Manual for Quantitative Immunoelectrophoresis* (Edited by Axelson N. H., Kroll J. and Weeke B.), *Scand. J. Immun.* Vol. 2, pp. S37–S46.

Wilson G. S. and Miles A. (1975) Diphtheria and other diseases due to corynebacteriae. In *Topley and Wilson's Principles of Bacteriology, Virology and Immunity* (Edited by Arnold E.), pp. 1800–1842. Arnold, London.

NOTE ADDED IN PROOF

^{13}C and ^{1}H NMR spectra recently obtained with the carried type 6A oligosaccharide hapten, identified the hydrolysis of *O*-glycosidic bonds at the level of the rhamnose residues in more than 80% of the cleared linkages.