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A SEMI-SYNTHETIC GLYCOCONJUGATE ANTIGEN PREPARED BY CHEMICAL GLYCOSILATION OF PERTUSSIS TOXIN BY A MENINGOCOCCAL GROUP C OLIGOSACCHARIDE HAPTEN

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ABSTRACT

The preparation of a hybrid molecule obtained by chemical glycosilation of pertussis toxin (PT) is reported, with the purpose of obtaining a semi-synthetic glycoprotein antigen with bivalent antigenicity. The chemical glycosilation was performed using an oligosaccharide hapten derived from the purified capsular polysaccharide of *Neisseria meningitidis* group C. The semi-synthetic molecule was investigated by chemico-physical and immunochemical analysis. The oligosaccharide haptens appeared exposed on the surface of the carrier protein PT, since the glycoprotein inhibited the immunoprecipitate between a specific polyclonal antiserum and the native bacterial capsular polysaccharide. By contrast, the main antigenic regions recognized in the native protein PT by specific polyclonal antibodies appeared lost after the coupling procedure involving PT as the carrier protein.

INTRODUCTION

Pertussis toxin has been shown to be one of the most important protein antigens of *Bordetella pertussis* (1) involved in pertussis pathogenesis and immunity (2, 3). The use of this protein as immunogen involves a toxoiding procedure similar to that used for bacterial toxins such as diphtheria and tetanus, with the purpose of reducing some unwanted biological properties such as leukocytosis and lymphocytosis promotion, histamine sensitization and potentiation of insulin secretion. Recently, we have employed some protein carriers such as the non-toxic mutant protein CRM 197, serologically related to diphtheria toxin to deliver polysaccharide and oligosaccharide haptens in the form of semi-synthetic glycoconjugate antigens (4). A molecular model of these semi-synthetic compounds was able to induce a T-dependent immunological response in two animal models, specifically directed against the saccharide and the protein moiety of the hybrid molecule. The induced antibodies, which contained IgG class immunoglobulins were able to neutralize diphtheria toxin and recognize the polysaccharide capsule of the living bacteria belonging to the homologous serotype of the carried hapten (M. Porro et al., manuscript submitted for publication).

Based on these observations we considered the use of pertussis toxin as a potential carrier protein for oligosaccharide haptens and also considered the effect of glycosilation as a probe to reduce the toxicity of this bacterial toxin.

MATERIALS AND METHODS

Antigens and hapten

Purified capsular polysaccharide of *N. meningitidis* group C originated from a bulk powder for the preparation of human vaccine (Sclavo SpA, Siena, Italy) and the characteristics of chemical and serological purity complied with the WHO requirements for this antigen (5). The polydisperse molecular weight was investigated and characterized both by chemicophysical and immunochemical methods as previously reported (6). The hapten derived from the molecular structure of this polysaccharide was prepared by acidic pyrolysis in the following conditions : the polysaccharide was solubilized in a 5.5×10^{-4} M acetic acid solution at the concentration of 10 mg/ml in a sealed vial. Heating at the constant temperature of 100°C in an oil bath was performed for 6.5 h. The solution was then neutralized and the oligosaccharide purified by gel-chromatography on Sephadex G-15 (Pharmacia, Uppsala, Sweden). The chromatographic fractions showing chemical activity for sialic acid were pooled and sterile-filtered through a 0.22 μm membrane. An aliquot of this solution was characterized by gel-chromatography on Sephadex G-50 equilibrated in 0.2 M NaCl solution and appeared as a symmetrical gaussian curve showing a Kd = 0.46. The average molecular weight of this oligosaccharide appeared to be similar to that of a type 6A pneumococcal-derived oligosaccharide which showed a Kd = 0.48 on Sephadex G-50. This oligosaccharide was characterized by ^{13}C-Nuclear Magnetic Resonance (^{13}C-NMR) Spectroscopy (M. Porro et al., manuscript submitted for publication) and the physical dimensions were estimated in 8-10 monosaccharide residues. For the group C oligosaccharide hapten these dimensions would correspond to a structural formula similar to : (α-D-N,O-Acetyl Neuraminic Acid-2 → 9-α-D-N,O-Acetyl Neuraminic Acid)$_3$ (7) due to the different molecular weight of the N,O-Acetyl Neuraminic acid residue. The retention of N- and O-Acetyl groups in the structure of the oligosaccharide was detected by ^{13}C-NMR spectroscopy, using a Varian CFT 20 spectrometer (Varian Associated, Palo Alto, CA) operating at 20 MH_z in the pulsed Fourier transformed mode. Purified pertussis toxin was kindly provided by Dr. Y. Sato (Japan NIH, Tokyo, Japan). This antigen was obtained from *B. pertussis* Tohama phase I and had 12,000 Haptoglobin (Hp)-ELISA U/ml when titered using the reported procedure (8).

Chemical activation of the oligosaccharide hapten

The group C oligosaccharide was chemically activated by introduction of a primary amino group, via reductive amination, at the hemiketal group of the terminal reducing unit of sialic acid.

NH_4Cl (42 mg/ml) was added to the solution containing the oligosaccharide (5.7 mg/ml) and the pH adjusted to 8 with 1M NaOH. Then, Na BH_3CN (15 mg/ml) was added and the solution kept at 37°C for 6 days in sealed vials. After this period, the solution was filtered through a 0.22 μm membrane and neutralized with 0.5M HCl. Gel-chromatography on Sephadex G-15 followed and the fractions chemically active for sialic acid and amino groups using the Habeeb method (8) were pooled and freeze-dried. Reconstitution of the freeze-dried product was done in dimetylsulfoxide and the solution was mixed drop-by-drop in a solution of N-hydroxysuccynimide diester of adipic acid in the same solvent, prepared according to the described procedure (10). The final stoichiometry in reactions between the amino-group activated oligosaccharide and the disuccynimidyl ester was equal to one. About 60% of the primary amino groups were transformed to monosuccynimidyl derivatives.

Chemical glycosilation of pertussis toxin

The solution containing the group C monosuccynimidyl ester-derived oligosaccharide in dimetyl sulfoxide was mixed to 3 mg of purified pertussis toxin solubilized in 10 ml of the same solvent. The reaction mixture was kept at room temperature overnight. After this period, the solution was purified by gel-chromatography on Sephadex G-100 and the fractions chemically active for protein and sialic acid content were pooled. Serial dialysis against sterile-filtered solutions of 0.1M NaCl/dimetylsulfoxide 50%, 0.1m NaCl/dimetylsulfoxide 25% and finally 0.5M NaCl in 0.1M PBS was done to eliminate traces of dimetylsulfoxide in the solution of the glycoconjugate antigen. The estimate of protein and sialic acid content in the semisynthetic molecule was performed by conventional chemical procedures.

Chemico-physical and Immunochemical characterization of the semi-synthetic glyco-conjugate antigen

SDS-PAGE in reducing conditions was performed according to the Laëmmli method (10) using an acrylamide gradient 3-12% (w/v) and silver staining of the gel as previously reported (11). The immunochemical reactivity of PT in both the native or conjugate form was tested using goat anti-PT immunoglobulins, kindly provided by Dr. J.L. Cowell (Center for Drugs and Biologies, FDA, Bethesda, MD) in an ELISA method performed as follows: polystyrene plates (COSTAR, Cambridge, MA) were coated overnight at 4°C by 100 µl of a PT solution at concentration of 500 µg/ml in PBS pH = 7.2. 100 µl of purified goat immunoglobulin fraction anti-PT (16.6 mg/ml) were then added as serial twofold dilutions in PBS pH = 7.2, containing Tween20 0.05% v/v and BSA 5% w/v, starting from a base dilution of 1/1000. Plates were incubated for 2 h at room temperature. 100 µl of a solution of rabbit antigoat IgG, conjugated with peroxidase (MILES, Rehovot, Israel) were added at a dilution of 1/1000 and incubated for 2 h at room temperature. Finally, 100 µl of a solution containing the substrate (7.5 mg of 2.2'-Azino-di-3-ethyl-benzothiazolin-sulphonate, Boehringer Mannheim, W. Germany) dissolved in 10 ml of 0.1M citrate buffer pH = 5.6 containing 0.34 ml H_2O_2 36% w/v) were added and the absorbance read at 405 nm after 30-60 minutes in a Titertek Multiskan apparatus. After each step, three 100 µl washings of five minutes each followed, using PBS pH = 7.2 containing Tween20 0.05% v/v. The immunochemical reactivity of the group C oligosaccharide hapten in both the native or conjugate form was investigated by testing the ability of the molecule to inhibit the immunoprecipitate between a specific horse polyclonal antiserum (kindly provided by Dr. C.E. Frasch, Center for Drugs and Biologies, FDA, Bethesda, MD) and the native *N. meningitidis* group C capsular polysaccharide. The inhibition of immunoprecipitation was detected by differential immunoelectrophoresis on agarose gel (M. Porro et al., manuscript submitted for publication); a new technique which combines the principles of «rocket» immunoelectrophoresis and of «affinity» electrophoresis (13).

RESULTS

The chemico-physical characteristics of the glycoconjugate antigen, as investigated by SDS-PAGE and gel-chromatography, are shown in Fig. 1 and 2. The molecule appeared as a restricted polydispersed system as compared to the native protein without the presence of high molecular weight cross-linked material.

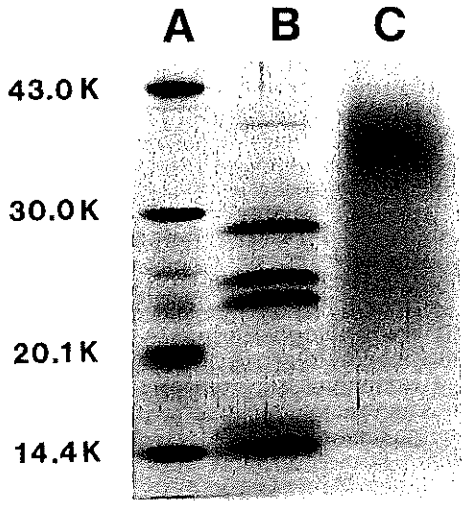

Fig. 1. Silver stained gel (12) showing the electrophoresis behavior of the semi-synthetic glycoconjugate (6 µg, lane C) as compared to the native PT (6 µg, lane B) using SDS-PAGE with an acrylamide gradient 3-12% w/v. Reference molecular weights are indicated in lane A.

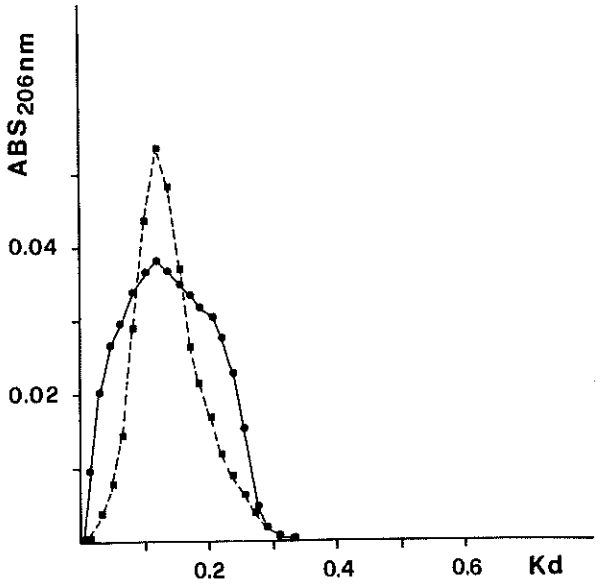

Fig. 2. Gel-chromatographic behavior on Sephadex G-100 of the glycoconjugate molecule (●——●) as compared to that of native PT (■ – –■).

In reducing conditions, native PT appeared as composed of five major subunits with molecular weights of 29,200 ; 25,100 ; 23,400 ; 15,800 and 14,800, respectively. Thus, the calculated molecular weight of the native PT was 108,300. In similar conditions the glycosilated protein gave evidence for two main diffused bands. The diffused aspect is typical of glycoprotein systems in SDS-PAGE. The stoichiometry of the semi-synthetic molecule was consistent with a ratio of sialic acid : protein = 0.12 (w/w). On a molar basis this value indicated about six oligosaccharide haptens bound for each mole of the carrier protein PT.

The antigenic regions of PT were shown to be nonreactive with specific polyclonal antibodies (Table I) since a loss of specificity of more than 50% was determined by the ELISA method when compared with the specificity for native PT. By contrast, the oligosaccharide haptens bound to the protein carrier PT were recognized by polyclonal antibodies specific for the native group C meningococcal capsular polysaccharide (Table I). The specificity of these antibodies was found to be about 100 times higher for the bound oligosaccharides than for the same oligosaccharide in unbound form, but about ten times lower than that observed for the native polymer.

DISCUSSION

Many years ago, other researchers (14) used the approach of manipulating the immunological response of some animal species to oligosaccharide haptens by making use of carrier proteins for these molecules. They were able to induce a specific immunological response to a bacterial capsular polysaccharide by covalently linking an homologous synthetic oligosaccharide hapten to a carrier protein as serum albumin. It was later demonstrated that the immunological response to these kinds of molecules involves the stimulation of carrier-specific T-helper cells inducing anti-hapten antibody synthesis (15).

Table I. Immunochemical specificity of reference polyclonal antibodies for their respective native antigens as compared to that observed for the glycoconjugate molecule. The specificity of the meningococcal group C antiserum expresses the ratio between the MIC experimentally observed by differential immunoelectrophoresis for the native polysaccharide and that observed for the homologous derived oligosaccharide in unbound and conjugate form. The specificity of the goat anti-PT antiserum for the glycoconjugate was calculated assuming 100 as the specificity observed by measuring the absorbance value in the ELISA procedure for the native PT.

Reference Antiserum	Specificity for			
	Native polysaccharide	Derived hapten	Native PT	Glycoconjugate antigen
Horse meningococcal group C antiserum	1	1.06×10^{-3}		1.16×10^{-1}
Goat anti PT antiserum			100	10

These observations have recently induced several investigators to consider this technique in improving the immunogenicity of some bacterial capsular polysaccharides present in the modern meningococcal and pneumococcal vaccines for human use.

We have attempted to use a carrier protein such as PT for an oligosaccharide hapten derived from the purified group C meningococcal capsular polysaccharide, with the purpose of investigating the antigenic potential of this kind of hybrid molecule. In addition, the chemical glycosilation of pertussis toxin may be interesting as a probe to reduce the biological toxicity of this hemagglutinin of *B. pertussis*.

Using the reported scheme of reactions, we coupled a group C oligosaccharide to the primary amino groups of some basic amino acids of PT. The successful preparation of the glycoconjugate molecule was shown clearly with SDS-PAGE analysis performed in reducing conditions, where the protein in conjugate form showed the typical behaviour of a glycoprotein and the five subunits of the native PT (16) appeared modified. Absence of undesired high molecular weight cross-linked material was avoided using this procedure of coupling. The stoichiometry of the glycoconjugate was consistent for a molecular model in which each mole of the carrier protein PT supported approximately six oligosaccharide haptens derived from the group C meningococcal polysaccharide.

The *in vitro* immunochemical characterization gave opposite results for the antigenic characteristics retained by the protein carrier and by the haptens involved in the semi-synthetic molecule. In particular, the antigenic regions of PT were not significantly recognized by specific polyclonal antibodies. Although a more detailed analysis could be performed using monoclonal antibodies to PT, which at present are not available in our laboratories, these findings suggest that a lower substitution degree for the oligosaccharide haptens might be more suitable in retaining some antigenic properties of the carrier protein. On the other hand, the carried oligosaccharides were specifically recognized by polyclonal homologous antibodies, an observation indicating their exposure on the surface of the carrier protein. The increased specificity shown by polyclonal antibodies for the bound oligosaccha-

rides in respect to that for the unbound oligosaccharides was observed in our laboratories for other glycoconjugate molecules (M. Porro et al., manuscript submitted for publication). This could be explained by the observation that the specificity of polyclonal antibodies for carbohydrates in general, is also a function of their molecular weight (6, 17) and that in the case of a glycoconjugate the carrier protein can play a role as spatial support for several oligosaccharide haptens mimicking a higher molecular weight for the carbohydrate moiety than that really evidenced for the unbound oligosaccharides. This semi-synthetic antigen will be investigated for immunogenicity in animal models and for biological properties involving the toxicity of PT.

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