Glycoconj J (2014) 31:573–585
DOI 10.1007/s10719-014-9539-5

Recognition of flavin mononucleotide, *Haemophilus influenzae* type b and its capsular polysaccharide vaccines by antibodies specific to D-ribitol-5-phosphate

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Received: 22 May 2014 / Revised: 22 July 2014 / Accepted: 22 July 2014 / Published online: 10 August 2014
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Abstract D-Ribitol-5-phosphate (Rbt-5-P) is an important metabolite in the pentose phosphate pathway and an integral part of bacterial cell wall polysaccharides, specifically as polyribosyl ribitol phosphate (PRP) in *Haemophilus influenzae* type b (Hib). The major objective of this study was to investigate whether an antibody specific to Rbt-5-P can recognize the PRP of Hib. D-Ribose-5-phosphate was reacted with proteins in the presence of sodium cyanoborohydride to obtain Rbt-5-P epitopes; 120 h reaction resulted in conjugation of ~30 and ~17 moles of Rbt-5-P/mole of BSA and OVA, respectively, based on decrease in amino groups, MALDI–TOF analyses, an increase in apparent molecular weight (SDS–PAGE) and glycoprotein staining. Immunization of rabbits with Rbt-5-P–BSA conjugate generated antibodies to Rbt-5-P as demonstrated by dot immunoblot and non-competitive ELISA. Homogeneous Rbt-5-P-specific antibody was purified from Rbt-5-P–BSA antiserum subjected to caprylic acid precipitation followed by hapten–affinity chromatography; its affinity constant is 7.1×10^8 M^{-1}. Rbt-5-P antibody showed 100 % specificity to Rbt-5-P, ~230 %, 10 % and 3.4 % cross-reactivity to FMN, riboflavin and FAD, respectively; the antibody showed ~4 % cross-reactivity to D-ribitol and <3 % to other sugars/sugar alcohols. Rbt-5-P–specific antibody recognized Hib conjugate vaccines containing PRP which was inhibited specifically by Rbt-5-P, and also detected Hib cell-surface capsular polysaccharides by immunofluorescence. In conclusion, Rbt-5-P–protein conjugate used as an immunogen elicited antibodies binding to an epitope also present in PRP and Hib bacteria. Rbt-5-P–specific antibody has potential applications in the detection and quantification of free/bound Rbt-5-P and FMN as well as immunological recognition of Hib bacteria and its capsular polysaccharide.

Keywords Capsular polysaccharide · Conjugate vaccine · *Haemophilus influenzae* type b · Immunogenicity · Polyribosyl ribitol phosphate · Ribitol-5-phosphate–specific antibodies

Abbreviations

BSA	Bovine serum albumin
FAD	Flavin adenine dinucleotide
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FMN	Flavin mononucleotide
Hib	*Haemophilus influenzae* type b
icELISA	Indirect competitive ELISA
KLH	Keyhole limpet hemocyanin
ncELISA	Non-competitive ELISA
OVA	Ovalbumin
PAS	Periodic acid–Schiff
PRP	Polyribosyl ribitol phosphate
Rbt	Ribitol
Rbt-5-P	Ribitol-5-phosphate
Rib-5-P	Ribose-5-phosphate
RSA	Rabbit serum albumin
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline containing 0.05 % Tween-20
Tetvac	Tetanus toxoid
TNBS	Trinitrobenzenesulfonic acid

Introduction

D-Ribitol-5-phosphate (5-*O*-phosphono-D-ribitol, L-ribitol-1-phosphate, Rbt-5-P), the reduced and phosphorylated form of

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D-ribose, is an important metabolite formed from the intermediates such as D-ribulose-5-phosphate and D-ribose-5-phosphate (Rib-5-P) in the pentose phosphate pathway [1]. Defects in pentose and polyol metabolism of some inborn metabolic disorders lead to accumulation of Rbt-5-P [2, 3]. Rbt-5-P is a constituent component of an important coenzyme of riboflavin (vitamin B2), *viz*., flavin mononucleotide (FMN; riboflavin-5′-phosphate) which contains an isoalloxazine ring linked to the C1′ of D-ribitol-5-phosphate [4]. Rbt-5-P is a precursor for the synthesis of ribitol-teichoic acid of cell walls in Gram–positive bacteria [5]. In some Gram–negative bacteria like *Haemophilus influenzae* type b (Hib), Rbt-5-P is present as polyribosyl-ribitol-phosphate (PRP) capsular polysaccharide, which is a linear copolymer of repeating units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate [$(C_{10}H_{19}O_{12}P)_n$], wherein n ranges from 6 to 10 [6, 7].

Hib infection is the leading cause of bacterial meningitis and pneumonia, the most serious infectious disease among infants and young children, leading to severe and permanent neurological defects in survivors. In India alone, an estimated 72,000 pneumonia deaths have been attributed to Hib [8, 9]. Since 1980's, after the development of the first conjugate vaccine against Hib, many new safe and effective carbohydrate-protein based conjugate vaccines were produced by chemical conjugation of polysaccharides to protein carriers [10, 11] which elicited antibodies similar to those seen in natural response; converting T-cell-independent to T-cell-dependent response early in life leads to immunological memory and enhanced immune response by booster doses of the vaccine thereby increasing immunity [12, 13].

Currently, there are four licensed Hib vaccines available for prophylactic use [14]; these contain PRP conjugated to a carrier protein, and include PRP-D (PRP conjugated to diphtheria toxoid), PRP-OMP (PRP conjugated to the outer membrane protein of *Neisseria meningitidis*), HbOC (PRP conjugated to CRM_{197}, a cross-reacting mutant diphtheria toxoid), and PRP-T (PRP conjugated to tetanus toxoid). These vaccines differ in structure and size of the polysaccharide, type of carrier protein, linkage between the protein and polysaccharide, and the ratio of polysaccharide to protein; some differences also exist in the elicited immune response [15, 16]. Though, most of the conjugate vaccines are produced from poly- or oligosaccharide extracts purified from microbial cultures, chemically synthesized oligosaccharide-protein conjugates have also been used as candidate vaccines in laboratory animals and human clinical trials [15, 17–19]; further, the synthetic oligosaccharide-protein conjugate Hib vaccine (the Cuban vaccine QuimiHib®) has already been licensed for immunization in several developing countries [6]. However, till date, there have been no attempts on the generation of specific antibodies to Rbt-5-P or the ribosyl–ribitol phosphate unit for investigating their recognition of the capsular polysaccharide of Hib.

Based on our earlier studies on the immunogenicity of sugar alcohols (erythritol, mannitol and xylitol) [20], antibodies were specifically generated to D-ribitol which recognized riboflavin as well as the capsular polysaccharide of Hib [21]. Therefore, it appeared interesting to investigate whether antibodies with specificity to Rbt-5-P could also recognize the PRP of Hib. The reactive carbonyl form (open-chain form, ~1 %) of Rib-5-P [22, 23] can be coupled to free amino groups of proteins at a slightly alkaline pH by reductive amination [24]. Reductively aminated Rib-5-P group, being acyclic, resembles Rbt-5-P structurally resulting in Rbt-5-P epitopes on the carrier protein (Fig. 1), which could potentially be used as a novel, simple and cost-effective immunogen, and the proposed antibodies to Rbt-5-P may find utility in the immunological detection of Hib bacteria and its vaccines.

In this investigation, Rbt-5-P conjugate of a carrier protein was prepared and used as the immunogen to produce polyclonal antibodies in rabbits. Antibody specific to Rbt-5-P was purified by hapten affinity chromatography followed by its characterization in terms of specificity and cross–reactivity with various sugar alcohols, Rbt-5-P, riboflavin and its analogs. Further, the utility of antibodies specific to Rbt-5-P for the detection of PRP of Hib and its conjugate vaccines was evaluated. The structures of some important compounds referred to in this study are given in Fig. 2.

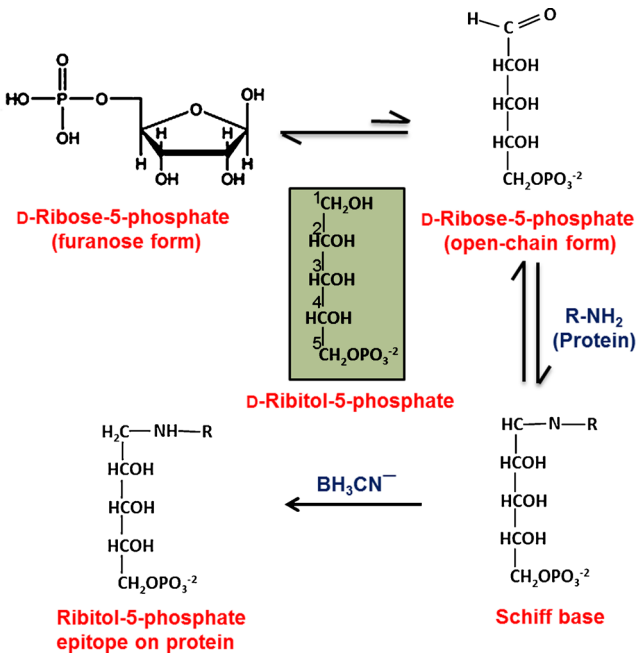

Fig. 1 Reductive amination reaction of D-ribose-5-phosphate with carrier protein to obtain ribityl-5-phosphate epitopes. The structure of Rbt-5-P with its carbon atoms numbered is shown in the center. In aqueous solution, Rib-5-P exists 34 % as the α-furanose form, 64 % as the β-furanose form, and ~1 % as the straight-chain hydrate [22, 23]

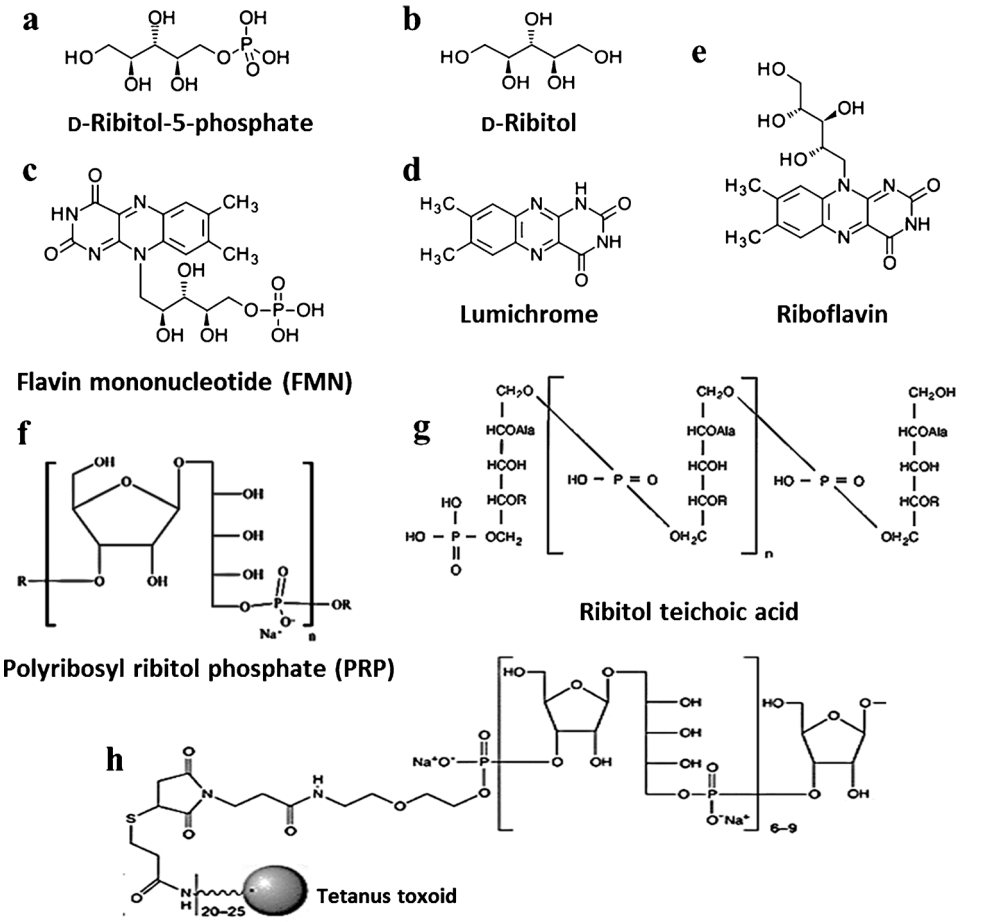

Fig. 2 Structures of selected compounds related to this study. **a** D-Ribitol-5-phosphate. **b** D-Ribitol. **c** FMN. **d** Lumichrome. **e** Riboflavin. **f** PRP of *Haemophilus influenzae* type b (n=6–10). **g** Ribitol-teichoic acid. **h** PRP tetanus toxoid conjugate

Materials and methods

D-Ribose-5-phosphate barium salt hexahydrate, bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), ovalbumin (OVA), rabbit serum albumin (RSA), riboflavin, flavin mononucleotide, flavin adenine dinucleotide (FAD), lumichrome (7,8-dimethylalloxazine), sodium cyanoborohydride ($NaCNBH_3$), Sepharose CL-6B and borane-pyridine complex solution were obtained from Sigma–Aldrich Chemical Co, St. Louis, MO. Periodic acid and trinitrobenzene-sulfonic acid (TNBS) were products of Hi-Media Laboratories, Mumbai, India. Schiff reagent (1-fluoro-2,4-dinitrobenzene) and caprylic acid (octanoic acid) were procured from Sisco Research Laboratories, Mumbai, India. Flat–bottomed 96–well ELISA microtiter plates were from Greiner Bio-One, Frickenhausen, Germany. Goat anti-rabbit IgG-alkaline phosphatase (ALP) conjugate, goat anti-rabbit IgG-fluorescein isothiocyanate (FITC) conjugate, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP–NBT), *p*-nitrophenyl phosphate (pNPP), Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) were products of Bangalore Genei, Bangalore, India. Hiberix™ (GlaxoSmithKline Biologicals s.a., Rixensart, Belgium), Sii HibPro™, Pentavac SD® and Tetanus toxoid (all manufactured by Serum Institute of India Ltd., Hadapsar, Pune, India) were procured from the local pharmacy. Hib bacterial strain (MTCC No. 3826) was obtained from Microbial Type Culture Collection (MTCC), CSIR–Institute for Microbial Technology (IMTECH), Chandigarh, India. All other chemicals and reagents were of analytical grade.

Hiberix™ and Sii HibPro™ are lyophilized vaccines containing PRP from Hib type b, covalently bound to tetanus toxoid. Pentavac SD® is a single-dose homogeneous preparation containing purified diphtheria and tetanus toxoids, inactivated whooping cough (pertussis) organism, highly purified, non-infectious particles of hepatitis B surface antigen and Hib capsular polysaccharide chemically conjugated to tetanus toxoid. Each single dose of vaccine (0.5 mL) is formulated to contain 10 μg of purified PRP covalently bound to 20–40 μg tetanus toxoid.

Preparation of Rbt-5-P by reduction of Rib-5-P

Since Rbt-5-P is not available commercially, it was prepared from Rib-5-P by reduction as per Egan *et al.* [7]. D-Ribose-5-phosphate barium salt hexahydrate was dissolved in distilled water (10 mg/mL) at 5 °C and the pH was brought to 2.5 with 1 N H_2SO_4; after 5 min, $BaSO_4$ precipitate was removed by centrifugation. To the resulting supernatant, 10 mg $NaBH_4$ was added and incubated at 25 °C for 6 h. Excess borohydride was neutralized by dropwise addition of 2 N acetic acid, and the boric acid formed was removed by repeated co-distillation with methanol [7]. Purity and molecular mass of Rbt-5-P was confirmed by using positive ESI in Q-Tof Ultima Globa mass spectrometer (Waters Associate, Manchester, UK) [25].

Preparation of Rbt-5-P–protein conjugates by reductive amination

Rib-5-P (10 mM) was conjugated to a carrier protein, BSA (100 μM), or OVA (100 μM) by reductive amination [20, 21, 24] in the presence of a mild reducing agent, $NaCNBH_3$ (100 mM) in 0.2 M borate buffer (pH 8.0) and incubated at 37 °C to obtain Rbt-5-P-BSA or Rbt-5-P-OVA conjugates. Aliquots were taken at various time periods and the reaction was stopped by adjusting the pH to 4 with dilute acetic acid followed by dialysis (3 kDa mol. wt. cut-off membrane) against phosphate–buffered saline (PBS) at 4 °C. As appropriate controls, BSA or OVA were incubated in the absence of Rib-5-P. Protein estimation of conjugates was carried out using BSA as a standard [26]. Since the hapten is used in large molar excess compared to the protein amino groups, generally, unconjugated protein will be absent in the final protein conjugates. The molecular mass of BSA and OVA was taken as 66,433 and 44,300 Da, respectively (UniProtKB, www.uniprot.org; Sigma-Aldrich, www.sigmaaldrich.com).

Similarly, Rib-5-P (10 mM) was conjugated to KLH (5 mg) using $NaCNBH_3$ (100 mM) to obtain Rbt-5-P-KLH conjugates, in which ~50 % of the amino groups of KLH have been modified. Small molecular reactants were removed from the reaction mixture by adjusting the pH to 4 with dilute acetic acid followed by dialysis (3 kDa mol. wt. cut-off membrane) against phosphate–buffered saline (PBS) at 4 °C. Since KLH is a very large protein with its exact molecular weight unknown [27], it is difficult to calculate the molar ratio of hapten to carrier.

TNBS assay, SDS–PAGE and MALDI–TOF MS analyses of Rbt-5-P-protein conjugates

The number of ε-amino groups remaining unreacted in reductively aminated BSA or OVA samples was determined using TNBS reagent as previously described [20, 28]. The molecular mass of Rbt-5-P–BSA and Rbt-5-P–OVA conjugates (obtained after 120 h of reductive amination) was determined by MALDI–TOF MS [29] in a Kompact analytical SEQ MALDI–TOF mass spectrometer (Kratos, Manchester, UK).

The Rbt-5-P–BSA conjugates were analyzed by SDS–PAGE [30] followed by Coomassie blue staining, to observe if there is an increase in the apparent molecular mass of Rbt-5-P conjugates in comparison to control BSA. Periodic acid–Schiff (PAS) staining [31] following SDS–PAGE was also carried out to confirm the glycated nature of Rbt-5-P–BSA conjugates.

Immunization and immunochemical analyses of Rbt-5-P antiserum

Three New Zealand white male rabbits (*Oryctolagus cuniculus*), 7–12 months-old kept in the animal house facility of CSIR–CFTRI, Mysore were used for the immunization according to standard protocols [32], after obtaining approval from the institutional animal ethics committee (IAEC).

Initially, the immunogen, Rbt-5-P–BSA conjugate (~30 moles Rbt-5-P/mole BSA; 0.5 mL of 2 mg/mL conjugate) micro-emulsified with 0.5 mL of FCA was administered subcutaneously at 8–10 sites on the back of the animal. After 4 weeks, booster doses containing 0.5 mg of the immunogen micro-emulsified with FIA were administered intramuscularly at 15–day intervals. The animal was bled by marginal ear vein puncture one week before the first injection to obtain pre-immune serum and one week after each booster dose to obtain immune serum (antiserum). The pooled serum was stored in aliquots at −20 °C.

The antiserum titer was analyzed by dot-immunoblot [33] by spotting 1 μg of BSA, OVA and KLH along with their respective Rbt-5-P conjugates on nitrocellulose membrane. The blots were washed thrice extensively with Tris-buffered saline (TBS) containing 0.05 % Tween-20 (TBS-T) between each step. Blocking was carried out in TBS-T with 1 % gelatin (blocking buffer) for 2 h. The blots were incubated in Rbt-5-P–BSA antiserum diluted in blocking buffer (1:2,000, 1:10,000 and 1:20,000) for 2 h, followed by incubation with 1:5,000 dilution of goat anti-rabbit IgG-ALP conjugate for 1 h, and color development using NBT-BCIP solution.

The antiserum titer was determined by measuring the binding of serial dilutions of the antiserum to coated Rbt-5-P–KLH or Rbt-5-P–OVA by non-competitive ELISA (ncELISA) [34]. Microtiter wells were coated with 100 μL of Rbt-5-P–protein conjugate at 1000, 100, 50, 10 and 1 ng per well (for OVA conjugate with 15 Rbt-5-P moles/mole, these correspond to 315, 31.5, 15.75, 3.15 and 0.315 pmoles of the hapten, respectively) in 0.1 M carbonate-bicarbonate buffer, pH 9.6 by incubating at 4 °C. The wells were washed thrice between steps using PBS containing 0.05 % Tween-20 (PBS-T). Blocking was done using 1 % gelatin in PBS-T (blocking buffer) at 37 °C for 30 min. Antiserum diluted in blocking

buffer (1:10 to 1:100,000) was added (100 μL/well) and incubated at 37 °C for 2 h. Goat anti-rabbit IgG-ALP conjugate (1:5000 dilution in blocking buffer; 100 μL/well) was added and incubated at 37 °C for 1 h. Color development was done using pNPP (1 mg/mL; 100 μL/well) in 1 % diethanolamine buffer, pH 9.8, at 37 °C for 30 min. The reaction was stopped by the addition of 3 M NaOH (50 μL/well) and the absorbance was read at 405 nm in a microtiter plate reader (model 680, Bio-Rad Laboratories Inc., Hercules, CA).

The immune response of individual experimental rabbits following each dose of immunogen administration was determined after 10 days by ncELISA, using Rbt-5-P–KLH (100 ng/well) as coating antigen and 1:100 dilution of respective antiserum. A positive value in ELISA is defined as the absorbance value obtained with immune serum sample two-fold greater than that obtained in the case of pre-immune serum; the cut-off absorbance value was taken as 0.2. Antibody titer is defined as the reciprocal of the highest dilution that gave a positive value in ELISA.

Hapten-affinity chromatography of rabbit antiserum for purification of Rbt-5-P–specific antibodies

In order to obtain antibodies specific to Rbt-5-P, an affinity matrix was prepared by conjugating Rbt-5-P–KLH conjugate to Sepharose CL-6B that had been subjected to periodate oxidation as described elsewhere [21, 35]. KLH was used as the matrix protein as it is a non–homologous protein compared to BSA and contains large number of amino groups for conjugation [27].

Rbt-5-P–BSA antiserum was precipitated with caprylic acid to remove serum albumin [32], and the resulting supernatant was diluted 1:2 in PBS and loaded on Rbt-5-P–KLH–Sepharose CL-6B column (1.0 cm i.d. × 2.5 cm; 2 mL bed volume) that had been pre-equilibrated with PBS at 4 °C; the flow-through was recycled twice. After washing with 50 mL PBS, the bound antibodies were eluted with 0.2 M glycine-HCl buffer, pH 2.9; the eluate was neutralized immediately using 1 M Tris base. The purified antibody was concentrated using centricon-100 concentrators (100 kDa MWCO; Amicon, Beverly, MA), aliquoted and stored at −20 °C. The affinity-purified antibodies were analyzed by SDS–PAGE under both reducing and non-reducing conditions. The concentration of Rbt-5-P–specific antibody was calculated based on the reference value of $A^{0.1\%,\ 1\ cm}$ at 280 nm of 1.4 for rabbit IgG [32].

Measurement of antibody titer and affinity constant of Rbt-5-P–specific antibodies

Antibody titer of affinity-purified Rbt-5-P antibodies was obtained by two-dimensional checkerboard titrations, in which different amounts of the purified antibody (ranging from 1 to 100 ng) were titrated against varying amounts of the coating antigen (Rbt-5-P-KLH conjugate) in ncELISA as described in an earlier section.

The affinity constant of the purified Rbt-5-P–specific antibodies was calculated using a computer program (Ab_affi program written in GW-BASIC [36], which is a simple and reliable method for calculating the affinity constant based upon the law of mass action. Rbt-5-P–KLH conjugate was coated using a series of two–fold dilutions (1000, 500, 250 and 125 ng/100 μL) such that the final concentration was one-eighth of the starting concentration. KLH (1000 ng/100 μL) as control coating antigen lacking the hapten, and 25 ng/100 μL affinity-purified Rbt-5-P antibodies were used in ncELISA as described in an earlier section.

Specificity and cross-reactivity of Rbt-5-P–specific antibodies

Specificity and cross-reactivity of the affinity-purified antibodies were studied by indirect competitive ELISA (icELISA). Here, purified antibodies (25 ng/well) were pre-incubated with various concentrations (0.1 nM to 1 mM) of test compounds (competitive inhibitors like ethylene glycol, glycerol, various sugar alcohols and monosaccharides) at 37 °C for 2 h before adding to coated microtiter wells (Rbt-5-P–KLH, 100 ng/well). All other steps were followed as described in an earlier section. IC_{50} value of each compound was calculated by 4-parameter logistic nonlinear regression curve fit mode using Prism 5 software (GraphPad, San Diego, CA). IC_{50} value is defined as the concentration required for 50 % inhibition in ELISA values. The percent cross-reactivity for various compounds was calculated using the formula (IC_{50} for Rbt-5-P ÷ IC_{50} for test compound) × 100. Other compounds tested for cross-reactivity include L-lysine, riboflavin and its analogs (FMN, FAD and lumichrome).

Further, binding of Rbt-5-P–specific antibodies to Rbt-protein conjugates prepared as described previously [21] and binding of Rbt–specific antibodies [21] to Rbt-5-P–protein conjugates were evaluated by ncELISA; microtiter wells were coated separately with 100 ng/well ribitol–protein conjugates (Rbt–BSA, hapten content: 42.6 pmoles; Rbt–OVA, hapten content: 32.4 pmoles; or Rbt–KLH) and 100 ng/well Rbt-5-P–protein conjugates (Rbt-5-P–BSA, hapten content: 41 pmoles; Rbt-5-P–OVA, hapten content: 31.5 pmoles; or Rbt-5-P–KLH) along with the corresponding unconjugated proteins (BSA, OVA and KLH) as negative controls. Purified Rbt–specific antibodies [21] or Rbt-5-P–specific antibodies (25 ng/well) were used in ncELISA as described in an earlier section.

Detection of PRP of Hib vaccines by Rbt-5-P–specific antibodies

Microtiter wells were coated with 100 ng/well of one of the following: KLH, OVA, Rbt-5-P–KLH and Rbt-5-P–OVA (31.5 pmoles of Rbt-5-P content), while Hiberix™, HibPro™, Pentavac SD® and Tetvac were coated at 1000 ng/well concentration. ncELISA was carried out, as described in an earlier section, using purified Rbt-5-P–specific antibodies (25 ng/well) for the detection of PRP of Hib.

icELISA was carried out to determine the percent inhibition of Rbt-5-P–specific antibodies (25 ng/well) binding with PRP of Hib vaccines by Rbt-5-P. Antibodies were pre-incubated with various concentrations of Rbt-5-P (1 nM, 1 μM and 1 mM) at 37 °C for 2 h before adding the mixture to coated microtiter wells containing one of the following: 100 ng/well of Rbt-5-P–KLH conjugate, 1000 ng/well (with respect to PRP content) each of Hiberix™, HibPro™ and Tetvac. Further steps were carried out as described for ncELISA under 'Immunization and immunochemical analyses of Rbt-5-P antiserum'. Assuming 8 repeating units of ribosylribitol phosphate and a terminal Rbt or Rbt-5-P, the molecular mass of PRP was calculated as ~2940 Da; therefore, 1000 ng/well of Hib conjugate vaccine with respect to PRP represents 340 pmoles of Rbt-5-P moiety.

Indirect immunofluorescence detection of Hib by Rbt-5-P–specific antibody

The immunological detection of Hib cells by Rbt-5-P–specific antibody was carried out using indirect immunofluorescence method [37]. Colonies of Hib cultured in chocolate blood agar were suspended in PBS, and treated with formalin to kill the bacterial cells. One drop of the cell suspension was placed on a glass slide, air-dried and fixed with methanol for 15 min. The fixed smear was covered with 100 μL of Rbt-5-P–specific antibody (1 μg) and incubated at 25 °C for 30 min. The slides were washed thrice with PBS and air-dried. Finally, the smear was treated with 100 μL of 1:50 dilution of goat anti-rabbit IgG-FITC conjugate and incubated at 25 °C for 30 min. The slides were washed thrice with PBS, air-dried, coated with glycerine-PBS and observed under the fluorescence microscope (Nikon Eclipse Ni, Tokyo, Japan).

Statistical analyses

Statistical treatment of ELISA data was carried out using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using Prism 5 software (GraphPad, San Diego, CA).

Results

Characterization of Rbt-5-P–protein conjugates

Since Rbt-5-P is not available commercially and is absolutely essential for cross-reactivity studies, it was prepared by reduction of Rib-5-P as described by Egan *et al.* [7], and confirmed by mass spectrometry (Fig. 3a). Rib-5-P was conjugated to BSA as well as OVA by reductive amination to obtain Rbt-5-P epitopes on the respective proteins as outlined in Fig. 1. The degree of substitution was calculated by the decrease in the number of amino groups on the carrier protein as determined by TNBS assay, and is shown in Fig. 3b. After 120 h of reductive amination, ~32 and ~17 moles of Rbt-5-P have been conjugated per mole of BSA and OVA, respectively. MALDI–TOF analysis of 120 h Rbt-5-P–BSA conjugate showed a molecular mass of 72,573 Da; assuming a molecular mass of 66,433 Da for unconjugated BSA, the increase in molecular mass of 6,140 Da translates to ~29 moles of Rbt-5-P per mole BSA (Fig. 3c). This is in good agreement with the value obtained by TNBS assay.

SDS–PAGE analysis (reducing) of Rbt-5-P–BSA conjugates clearly showed an increase in apparent molecular weight as compared to control BSA (Fig. 3d). Since BSA is not a glycoprotein, the glycation of BSA as a result of reductive amination with Rib-5-P was detected by PAS staining, wherein Rbt-5-P–BSA conjugates appeared as pink bands (Fig. 3e) which confirmed the addition of glycans or saccharides to the carrier protein, BSA.

Immunochemical analyses of Rbt-5-P-BSA antiserum

Rabbit antiserum was analyzed for the specific antibody response by dot–immunoblot analysis (Fig. 4a). The immunoreactivity of antiserum was found to be positive for Rbt-5-P–BSA, Rbt-5-P–KLH, Rbt-5-P–OVA and BSA, but was negative for both OVA and KLH. The highest dilution of antiserum with which Rbt-5-P–KLH can easily be detected was found to be 1:20000, indicating the presence of specific antibodies to Rbt-5-P in the antiserum. Pre-immune serum at 1:2000 dilution failed to show any reactivity towards all the conjugates (Fig. 4a).

The immune response of individual experimental rabbit, ten days after each dose of immunogen administration showed that generation of Rbt-5-P–specific antibodies after the first booster dose and subsequent booster doses up to the 6th dose remained nearly the same as shown in Fig. 4b.

The antiserum was also analyzed for its sensitivity in detecting both Rbt-5-P–KLH and Rbt-5-P–OVA conjugates using a checkerboard analysis by ncELISA wherein the concentrations of both antigen and antiserum are varied. The antiserum titration using 100 ng/well of coating antigen showed the presence of Rbt-5-P–specific antibodies sensitive

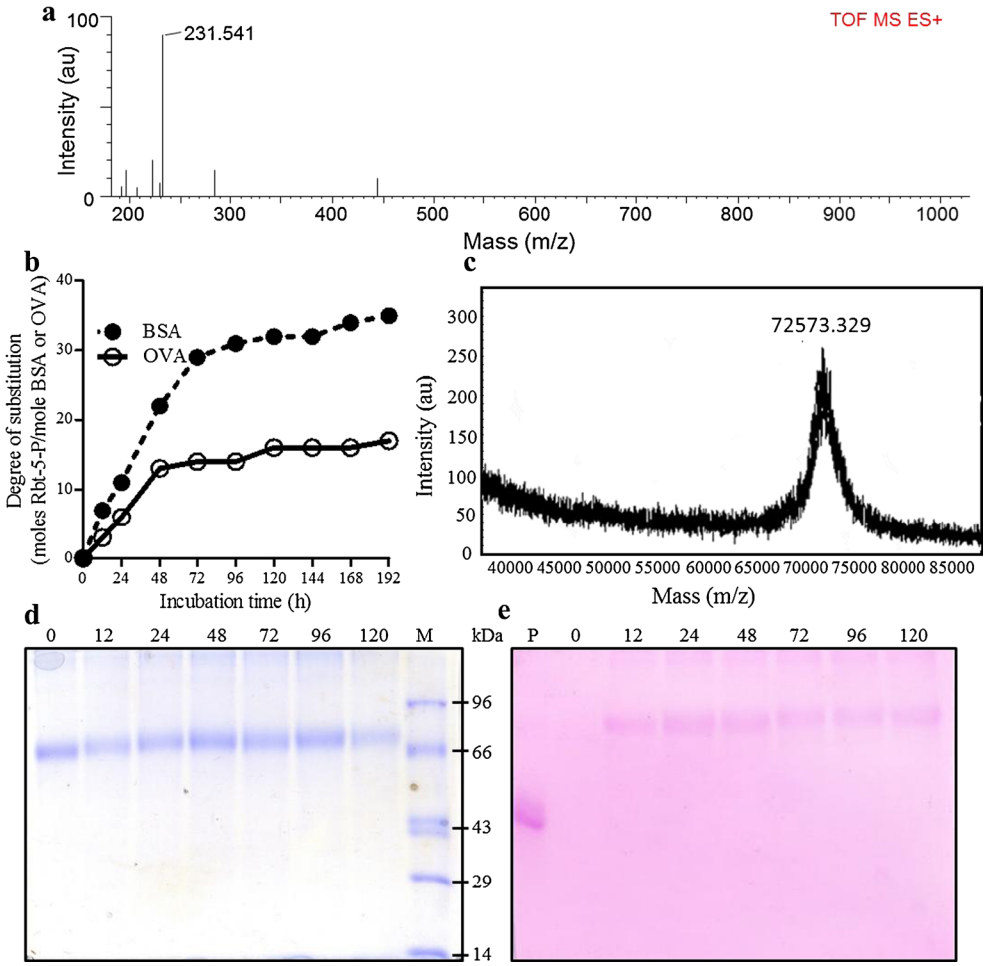

Fig. 3 Preparation Rbt-5-P, its protein conjugates and their characterization. **a** ESI-MS profile of purified Rbt-5-P. **b** Time course (TNBS assay) for the formation of Rbt-5-P–BSA or Rbt-5-P–OVA conjugates by reductive amination of BSA or OVA, respectively, with Rib-5-P. **c** MALDI-TOF-MS of Rbt-5-P–BSA obtained by reductive amination at 120 h. **d** Coomassie-stained SDS–PAGE (10 %, reducing) of Rbt-5-P–BSA conjugates; lane M: mol. wt. markers. The numbers at the top represent incubation times (in h) during the reductive amination reaction. **e** PAS-stained SDS–PAGE (10 %, reducing) of Rbt-5-P–BSA conjugates; lane P: positive control (ovalbumin)

at 1:1000 and 1:10000 dilutions for Rbt-5-P–KLH (Fig. 4c) and Rbt-5-P–OVA conjugate (Fig. 4d), respectively.

Characterization of hapten affinity-purified antibody indicates its specificity for Rbt-5-P

Purification of antibodies specific to Rbt-5-P from rabbit Rbt-5-P–BSA antiserum was carried out by hapten affinity chromatography on Rbt-5-P–KLH–Sepharose CL-6B; the elution profile showed a single peak with a yield of 155-160 μg protein/mL of rabbit antiserum. The affinity–purified preparation showed an IgG band around 160 kDa, and a 66 kDa protein band was observed as a contaminant (marked X) on SDS–PAGE under non-reducing conditions (Fig. 5a). Further, the 66 kDa contaminant protein subjected to in–gel trypsin digestion followed by peptide mass fingerprint analysis was confirmed as RSA (gi 372467098; PDB entry: 3V09_A), and the result was identical to that obtained using pure RSA as a positive control (data not shown).

Alternatively, caprylic acid precipitation of rabbit Rbt-5-P–BSA antiserum followed by hapten affinity chromatography on Rbt-5-P–KLH–Sepharose CL-6B resulted in homogeneous specific antibodies to Rbt-5-P with a yield of 60–65 μg/mL of antiserum. The affinity–purified antibody preparation showed a single band around 160 kDa on non-reducing SDS–PAGE (Fig. 5b).

Binding curve (Fig. 5c) obtained with checkerboard analysis using purified Rbt-5-P–specific antibodies and Rbt-5-P–KLH conjugate indicated that at an antibody concentration of 10 ng/100 μL for 100 ng/well coating antigen (Rbt-5-P–KLH) concentration, the antigen-antibody reaction is clearly evident. In order to obtain an optimal detection in icELISA for cross–reactivity studies, 25 ng of purified antibodies and 100 ng/well of coating antigen were considered for further analysis. The affinity constant of Rbt-5-P–specific antibodies as calculated

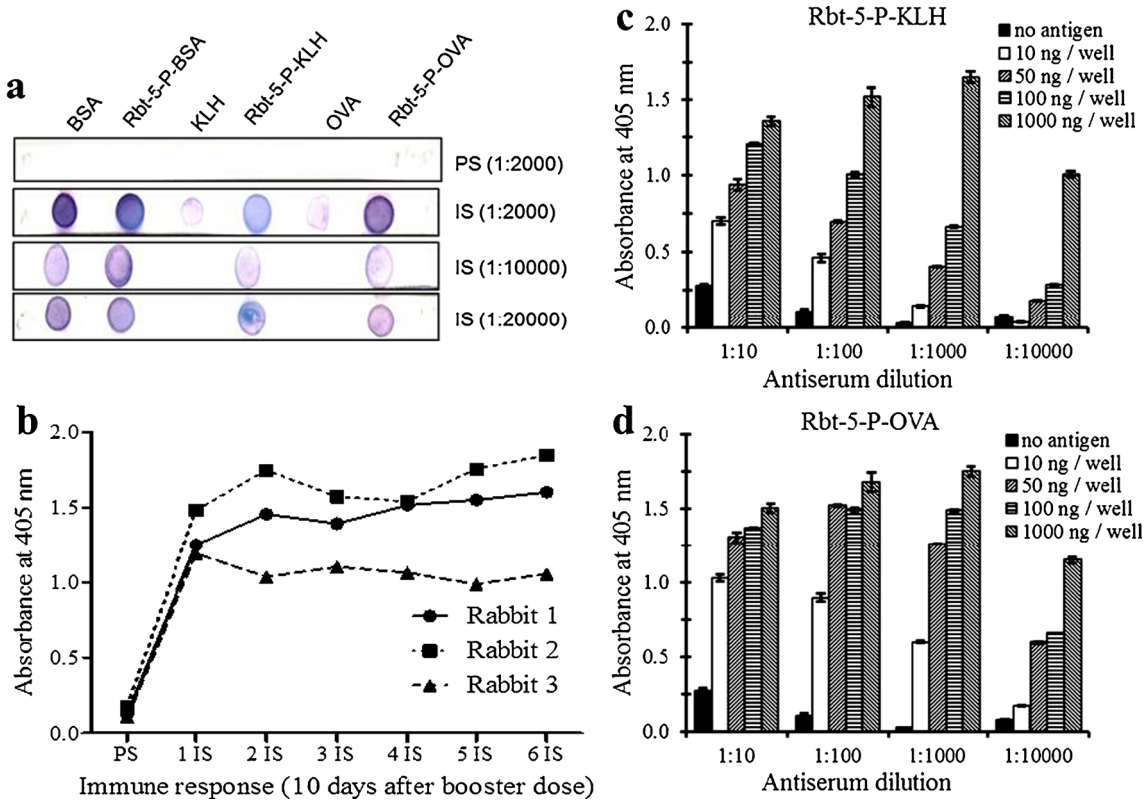

Fig. 4 Immunochemical analyses of rabbit Rbt-5-P–BSA antiserum. **a** Dot-blot on nitrocellulose membrane; antigen amount: 1 μg. PS, pre-immune serum; IS, immune serum; secondary antibody: goat anti-rabbit IgG-ALP (1:5000 dilution). **b** IgG immune response to Rbt-5-P-BSA immunization as measured by ncELISA. 1 IS to 6 IS refer to first immune serum to sixth immune serum. **c** and **d** Checkerboard analysis by ncELISA at different amounts of antigen (shown as inset) and antiserum dilutions; coating antigen: Rbt-5-P–KLH **c** or Rbt-5-P–OVA **d** Absorbance values are means of triplicates

using ncELISA was found to be 7.1×10^8 M^{-1}, which is comparable to the affinity constants of antibodies for other sugar alcohols like ribitol, erythritol and xylitol [21, 38, 39].

Cross-reactivity studies of Rbt-5-P–specific antibodies reveal high cross-reactivity for FMN and low cross-reactivity for ribitol, riboflavin and FAD

In order to demonstrate the specificity and cross-reactivity of purified Rbt-5-P antibodies, icELISA (Fig. 5d) was performed with various sugars, sugar alcohols, glycerol, ethylene glycol, L-lysine, riboflavin and its analogs (FMN, FAD and lumichrome). The percent cross–reactivity of Rbt-5-P-specific antibody towards these compounds is tabulated in Table 1. Based on the specificity of Rbt-5-P antibodies, the cross-reactivity for Rbt-5-P is taken as 100 %.

Rbt-5-P–specific antibodies showed ~4 % cross-reactivity with D-ribitol, 1-3 % cross-reactivity with mannitol, xylitol, sorbitol, D-ribose, D-xylose and Rib-5-P, and <1 % for all other compounds tested (galactitol, *meso*-erythritol, L-arabinitol, D-galactitol, ethylene glycol, glycerol, L-lysine and lumichrome). Interestingly, riboflavin-5-phosphate (FMN) containing Rbt-5-P group as an integral part of its structure showed maximum (~230 %) cross-reactivity. However, riboflavin, FAD and lumichrome showed only 10 %, 3 % and <1 % cross-reactivity, respectively. The cross-reactivity of Rbt-5-P antibody is 25-fold lower for Rbt compared to Rbt-5-P; it is interesting to note that a similar decrease (23-fold) is seen for riboflavin compared to its phosphorylated form, FMN (Table 1).

Evaluation of binding of Rbt-5-P–specific antibodies to ribitol-protein conjugates using ncELISA showed that they have minimal cross-reactivity with ribitol-protein conjugates as shown in Fig. 6a. Similarly, ribitol–specific antibodies generated as described earlier [21] showed that they have minimal cross-reactivity with Rbt-5-P-protein conjugates (Fig. 6b). However, BSA conjugates appear to have slightly more non-specific binding compared to OVA or KLH conjugates.

Detection of Hib conjugate vaccines and Hib bacterial cells by Rbt-5-P–specific antibodies

Detection of three commercial sources of Hib vaccine (Hiberix™, Sii HibPro™ and Pentavac SD® containing PRP conjugated to tetanus toxoid) was evaluated by ncELISA

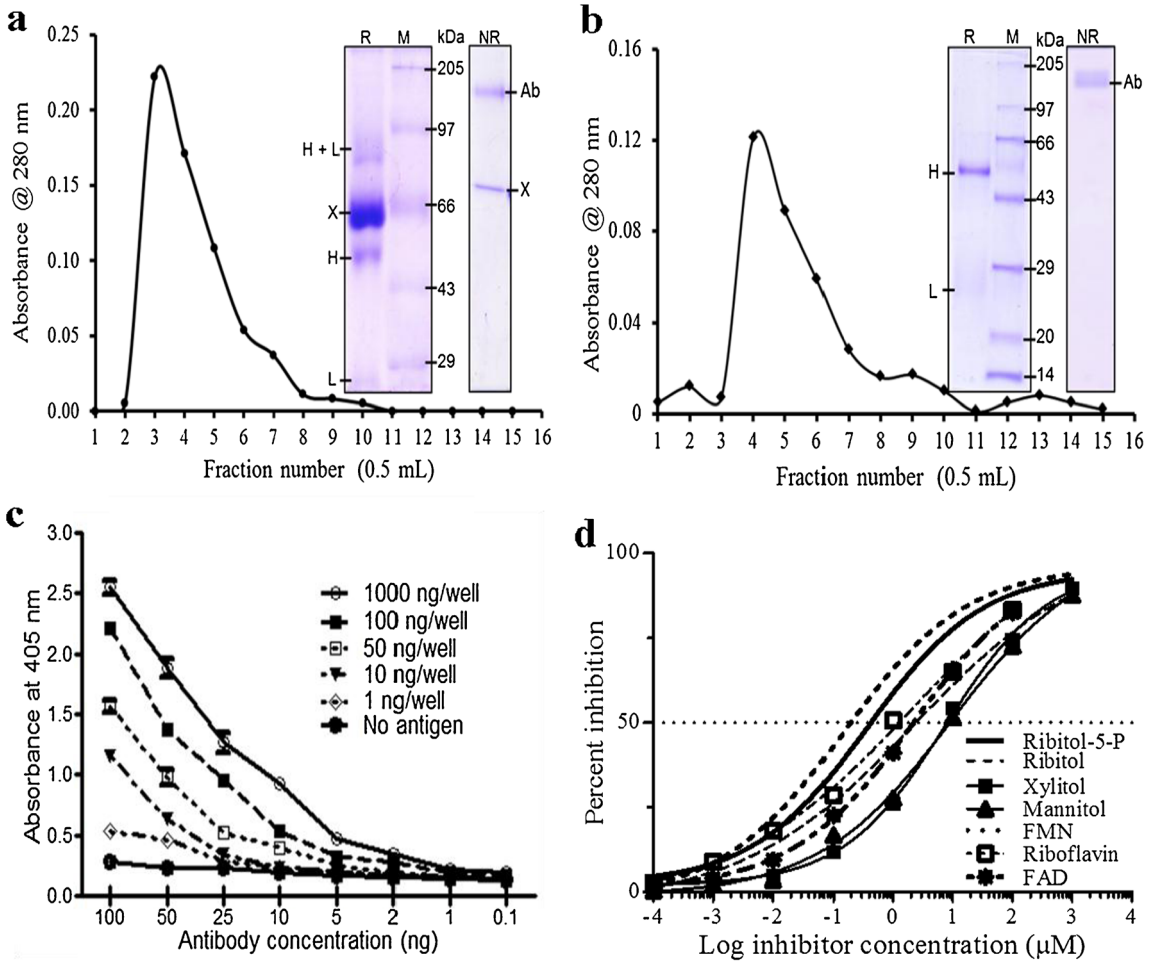

Fig. 5 Affinity purification and characterization of Rbt-5-P–specific antibodies. **a** Hapten affinity chromatography of rabbit Rbt-5-P–BSA antiserum on Rbt-5-P–KLH–Sepharose CL-6B. Inset: 10 % SDS–PAGE, Coomassie staining of affinity-purified Rbt-5-P–specific antibodies with 66 kDa contaminant (band X). Bands marked L and H represent light and heavy chains of the antibody, Ab. Lane R: reducing condition; lane NR: non-reducing condition; lane M: mol. wt. markers (reducing condition). **b** Caprylic acid precipitation of rabbit Rbt-5-P–BSA antiserum followed by hapten-affinity chromatography on Rbt-5-P–KLH–Sepharose CL-6B. Inset: 10 % SDS–PAGE, Coomassie staining of affinity-purified Rbt-5-P–specific antibodies. **c** Binding curves obtained by checkerboard analysis with Rbt-5-P–specific antibodies by ncELISA; coating antigen: Rbt-5-P–KLH; secondary antibody: goat anti-rabbit IgG-ALP (1:5000 dilution). Absorbance values are means of triplicates. **d** Specificity of Rbt-5-P–specific antibodies as determined by icELISA. Coating antigen: Rbt-5-P–KLH (100 ng/well). Purified antibodies specific to Rbt-5-P (25 ng in 100 μL volume) were pre-incubated with various concentrations of test compounds (as shown in inset) at 37 °C for 1 h, then transferred to ELISA wells. Other details are as given in panel **c**

using Rbt-5-P-specific antibodies. Hib conjugate vaccines were recognized by Rbt-5-P-specific antibodies compared to tetanus toxoid (Tetvac) which served as a negative control; the binding of these vaccine preparations was comparable to that of Rbt-5-P–conjugates of KLH and OVA, which served as positive controls (Fig. 7a). Further, the binding of Rbt-5-P-specific antibodies to Hiberix™ and Sii HibPro™ could be inhibited by D-ribitol-5-phosphate in a dose-dependent manner (Fig. 7b).

The ability of Rbt-5-P–specific antibody to detect Hib bacterial cells containing PRP capsular polysaccharide was confirmed by indirect immunofluorescence binding assay. Cell-surface localization of PRP was observed with intense green immunofluorescence signal in the case of Rbt-5-P–specific antibody (Fig. 8d) compared to that of pre-immune serum (Fig. 8b); corresponding images from phase contrast microscopy are shown in panels (c) and (a) of Fig. 8.

Discussion

Anti-carbohydrate antibodies with specificity for mono-, oligo- and polysaccharides have been generated by coupling the saccharide to carrier proteins using various chemistries [20, 21, 24, 40]. In the present study, the direct coupling of a reducing sugar (Rib-5-P) to proteins by reductive amination was used to obtain Rbt-5-P epitopes on carrier proteins. The

Table 1 Specificity and cross-reactivity of affinity-purified Rbt-5-P–specific antibodies

Test compound	IC_{50} (μM)	Percent cross-reactivity
D-Ribitol-5-phosphate	0.73	100.00
D-Ribitol	19.28	3.81
D-Mannitol	37.55	1.96
D-Xylitol	51.54	1.43
D-Sorbitol	69.76	1.05
D-Galactitol	158.01	0.47
meso-Erythritol	167.46	0.44
L-Arabinitol	811.13	0.09
D-Threitol	2.00[a]	0.04
Glycerol	5.49[a]	0.01
Ethylene glycol	9.25[a]	0.01
D-Ribose-5-phosphate	26.56	2.77
D-Ribose	59.08	1.24
D-Xylose	63.42	1.16
D-Arabinose	122.13	0.60
L-Lysine	2.94[a]	0.02
FMN	0.31	230.68
Riboflavin	7.29	10.08
FAD	21.37	3.44
Lumichrome	104.55	0.70

[a] These values are represented in mM

degree of substitution in terms of moles Rbt-5-P groups per mole of carrier protein was assessed by TNBS assay, an increase in apparent molecular weight by SDS–PAGE and MALDI–TOF analyses as well PAS staining for the glycated nature of conjugates. Rbt-5-P–BSA conjugate used as an immunogen in rabbits produced an excellent immune response as demonstrated by dot immunoblot and checkerboard ncELISA.

The hapten affinity purification of specific antibodies from Rbt-5-P–BSA antiserum on Rbt-5-P–KLH–Sepharose CL-6B affinity matrix showed the presence of an antibody band (160 kDa) and a 66 kDa contaminant protein, which was confirmed to be RSA by in-gel trypsin digestion and peptide mass fingerprint analysis. It is well known from earlier studies that both riboflavin and FMN bind to only a single site on serum albumin [41–44]. RSA contamination can be attributed to the bridging of ribityl-5-P moiety on the affinity matrix and the ribityl-5-P portion of FMN bound to RSA in rabbit serum by Rbt-5-P–specific antibodies. Complete removal of albumin from rabbit antiserum with caprylic acid precipitation followed by hapten–affinity chromatography yielded homogeneous specific antibodies to Rbt-5-P without the 66 kDa contaminant.

The affinity-purified antibodies showed marked specificity and sensitivity to Rbt-5-P, and low cross-reactivity towards various sugars and sugar alcohols as examined by icELISA. FMN containing Rbt-5-P group as an integral part of its structure showed maximum cross-reactivity (~230 %), higher than that seen for the hapten used for immunization. This may be due to the presence of hydrophobic residues in the proximity of the binding sites of Rbt-5-P–specific antibody interacting with the isoalloxazone ring of FMN in a manner analogous to those observed in the case of normal human IgGs [45, 46]; interestingly, riboflavin and FAD showed only 10 % and 3.5 % cross-reactivity, respectively. Lumichrome (a structural analog of FMN or riboflavin lacking the Rbt-5-P or Rbt group, respectively) and L-lysine showed <1 % cross-reactivity, although the ε-amino group of L-lysyl residues is involved in the formation of Rbt-5-P–BSA conjugate. Further, minimal cross-reactivity of Rbt-5-P–specific antibodies with Rbt-protein conjugates and non-recognition of lumichrome indicated that the Rbt-5-P group represents a single haptenic epitope of FMN. The magnitude of difference in cross-

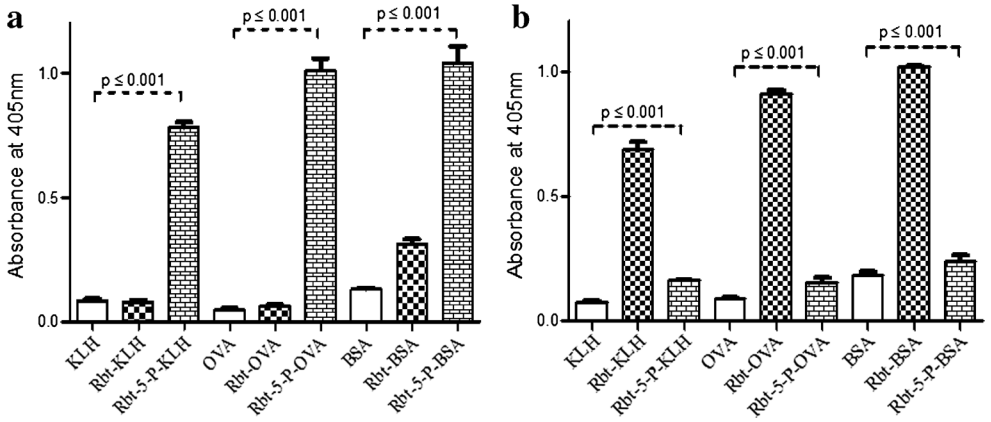

Fig. 6 Evaluation of cross-reactivity of **a** Rbt-5-P antibodies and **b** Rbt antibodies to protein conjugates of Rbt-5-P and Rbt by ncELISA. Coating antigens: 100 ng/well Rbt–protein conjugate (Rbt–BSA, Rbt–OVA or Rbt–KLH), 100 ng/well Rbt-5-P-protein conjugate (Rbt-5-P–BSA, Rbt-5-P–OVA or Rbt-5-P–KLH) and 100 ng/well unconjugated proteins (BSA, OVA or KLH as negative controls). Primary antibody: 25 ng/well Rbt-5-P-specific antibodies or 25 ng/well Rbt–specific antibodies; secondary antibody: goat anti-rabbit IgG-ALP (1:5000 dilution). Absorbance values are means of triplicates

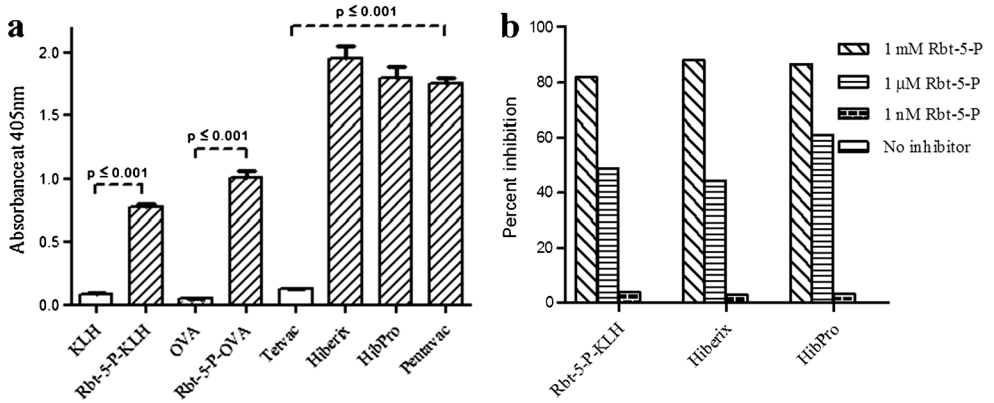

Fig. 7 Detection of Hib conjugate vaccines by Rbt-5-P–specific antibodies. **a** ncELISA using three different commercial vaccine preparations of PRP conjugate. Coating antigens: Rbt-5-P–KLH, Rbt-5-P–OVA, KLH, OVA (each at 100 ng/well) and test samples – Hiberix™, Sii HibPro™, Pentavac SD® and tetanus toxoid (Tetvac) (each at 1 μg PRP content/well corresponding to 340 pmoles of PRP). **b** icELISA for determination of the percent inhibition of Rbt-5-P-specific antibodies towards PRP conjugate. Coating antigen: 100 ng/well of Rbt-5-P–KLH or 1 μg PRP content/well of Hiberix™/Sii HibPro™. Purified Rbt-5-P–specific antibodies (25 ng in 100 μL) were pre-incubated with various concentrations (1 nM, 1 μM and 1 mM) of Rbt-5-P at 37 °C for 1 h, then transferred to ELISA wells. The percent inhibition for the sample labeled 'no inhibitor' is zero. Other details are as given in legend to Fig. 6

reactivity between Rbt-5-P and Rbt on the one hand, and that between FMN and riboflavin on the other clearly indicates that the phosphate group on the C5 of D-ribitol is very crucial for the specific binding of hapten (Rbt-5-P) to Rbt-5-P–specific antibody. The cofactors FMN and FAD occur in flavoproteins bound to the protein component either in a covalent or non-covalent manner [47, 48]. Since antibodies to Rbt-5-P also recognize FMN, they have potential utility as immunological reagents for the identification of flavoproteins containing FMN.

In this study, Rbt-5-P–specific antibody is used for the *in vitro* detection of PRP-containing glycoconjugate vaccines from different manufacturers including one combined vaccine (Pentavac SD®), and was found to be inhibited by Rbt-5-P in a dose-dependent manner indicating that Rbt-5-P–specific antibodies could be used diagnostically to ensure the integrity and stability of the Hib vaccines either alone or in combination with other vaccines. Although it has been shown previously that PRP conjugate vaccine of Hib is well recognized by Rbt-specific antibodies [21], the present data shows that it is equally well recognized by Rbt-5-P–specific antibodies. During structural studies on PRP from Hib, Egan *et al.* [7] found that phosphorus-containing end groups were present as D-ribosyl 2,3- and D-ribitol 4,5-cyclophosphates, as well as D-ribosyl C_3- and D-ribitol C_5-attached phosphate monoesters. It appears that the amount of free terminal Rbt-5-P is low in Hib conjugates and most is present as cyclic phosphates. Hence, the number of terminal Rbt-5-P epitopes available in the PRP conjugates may be very limited. In view of these aspects and the high content of Rbt-5-P moieties in PRP, it is likely that Rbt-5-P–specific antibodies may well recognize the internal Rbt-5-P of PRP. Moreover, Rbt-5-P–specific antibodies have low cross-reactivity with D-ribose (~1 %) and D-ribitol (~4 %) as seen from the present study.

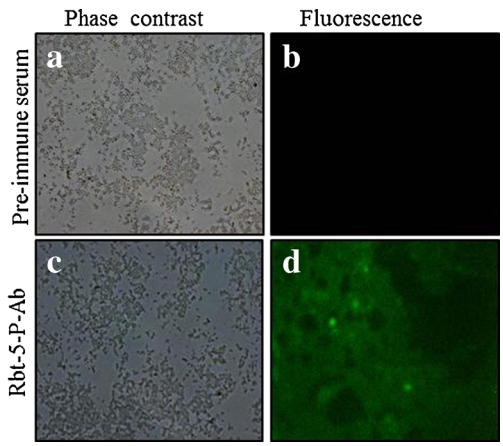

Fig. 8 Detection of Hib by indirect immunofluorescence. **a** and **b** Hib cultures treated with pre-immune serum and observed by **a** phase contrast and **b** fluorescence microscopy. **c** and **d** Hib cultures treated with Rbt-5-P-specific antibodies and observed by **c** phase contrast and **d** fluorescence microscopy. Magnification: 40 X; secondary antibody: goat anti-rabbit IgG-FITC conjugate (1:500 dilution)

The binding of Rbt-5-P–specific antibodies to PRP conjugate vaccines and immunologic recognition of Hib cell-surface macromolecules demonstrated that the Rbt-5-P–protein conjugate used as an immunogen possessed at least one relevant antigenic epitope of PRP. Hib conjugate vaccines consist of PRP capsular polysaccharide linked to a protein carrier. Such vaccines differ in the length of polysaccharide, the nature of the protein carrier and the linkage between the two. In general, although these various Hib conjugates are immunogenic and efficacious it is clear from various studies that they are not identical in performance [6, 8, 9, 12–14, 17, 18]. In view of the haptenic nature of Rbt-5-P which represents a monosaccharide of the ribosyl-ribitol phosphate unit, it would be interesting to investigate the immunogenicity of Rbt-5-P–protein conjugate in comparison to various Hib

conjugates which differ in the length of the polysaccharide, and to further test the bactericidal/protective activity of the resultant antiserum. Novel synthetic and biosynthetic methods for the preparation of glycoconjugates are opening new insights for the design of improved carbohydrate-based vaccines in terms of immune response and efficacy [49, 50].

In conclusion, Rbt-5-P–BSA conjugates carrying Rbt-5-P epitopes were prepared using reductive amination of BSA with Rib-5-P to obtain Rbt-5-P-specific antibodies in rabbits. Rbt-5-P–specific antibodies have been purified from Rbt-5-P–BSA antiserum on a hapten-affinity matrix and characterized in terms of specificity and cross-reactivity. Antibodies showed excellent specificity towards Rbt-5-P with minimal cross-reactivity towards Rbt; maximal cross–reactivity was seen with FMN indicating that the Rbt-5-P group is a unique haptenic epitope of FMN. Rbt-5-P–specific antibodies could be used for detection and quantification of Rbt-5-P, FMN and covalently bound FMN in biological samples. Further, the Rbt-5-P–specific antibodies showed diagnostic utility in the immunological identification of Hib bacteria and its PRP-containing glycoconjugate vaccines.

Acknowledgments We thank Dr. Ram Rajasekharan, Director, CSIR–CFTRI, Mysore for his keen interest and encouragement. We thank Ms. P. V. Bhavani, Microbiology Division, Defence Food Research Laboratory, Mysore for her assistance in indirect immunofluorescence assay. This work was supported by the Council of Scientific and Industrial Research (CSIR), New Delhi, India by an institutional project, MLP-0094 to YPV and a research fellowship from University Grants Commission (UGC), New Delhi to GR.

Conflict of interest The authors have declared that they do not have any conflict of interest.

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