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The potential of dextran-based glycoconjugates for development of *Helicobacter pylori* vaccine

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Abstract We have recently demonstrated that synthetic glycoconjugates based on delipidated lipopolysaccharide (LPS) of *Helicobacter pylori* and containing an α(1-6)-glucan chain induced broadly cross-reactive functional antibodies in immunized animals. To investigate the candidacy of α(1-6)-glucan as an alternative vaccine strategy we prepared glycoconjugates based on dextrans produced by lactic acid bacteria *Leuconostoc mesenteroides* B512F and consisting of linear α(1-6)-glucan chains with limited branching. Three dextrans with averaged molecular masses of 5,000 Da, 3,500 Da and 1,500 Da, respectively, were modified with a diamino group-containing linker and conjugated to a carrier protein, tetanus toxoid (TT) or diphtheria toxoid (DT), and their immunological properties investigated. The conjugates were immunogenic in both rabbits and mice and induced specific IgG responses against α(1-6)-glucan-expressing *H. pylori* LPS. Studies performed with post-immune sera of mice and rabbits immunized with dextran-based conjugates demonstrated cross-reactivity with LPS from typeable and non-typeable strains of *H. pylori* and selected mutants. The post-immune sera from rabbits that received the conjugates exhibited functional activity against α(1-6)-glucan-positive strains of *H. pylori.* These data provide evidence that dextran-based conjugates may offer a simplified approach to the development of carbohydrate-based vaccines against *H. pylori*.

Keywords Helicobacter pylori · Conjugate vaccine · Dextran · Bactericidal assay

Introduction

Helicobacter pylori infection affects close to half of the world's population and is particularly common in developing countries as well as Indigenous populations of North America [1–3]. *H. pylori* is associated with low socio-economic status and overcrowded living conditions [4] and believed to be acquired in childhood during the first 18 months following the birth. The infection usually persists for life although transient infections are known to occur in children [4, 5]. The majority of *H. pylori*-infected individuals remain asymptomatic but 10 %–15 % develop peptic ulcers and 1–3 % will develop gastric carcinoma [6]. *H. pylori* increases the relative risk for gastric cancer by at least six-fold [7]. The infection is commonly treated with antibiotics in combination with a proton pump inhibitor but the efficacy of treatment is rapidly decreasing due to a rise in antibiotic resistance [8].

Previous vaccine strategies against *H. pylori* have been focused on the use of inactivated whole cells, recombinant proteins, and, more recently, DNA-based approaches [9, 10]. Our group has been interested in the development of a conjugate vaccine based on lipopolysaccharide (LPS), a major cell surface component of *H. pylori*. We have identified a common antigenic LPS core epitope, α(1-6)-glucan [11, 12], and have recently demonstrated that synthetic glycoconjugates based on a truncated *H. pylori* LPS devoid of Lewis antigens and containing an α(1-6)-glucan chain induced broadly cross-reactive bactericidal antibodies in immunized animals [13]. Linear α(1-6)-glucan chains are known to occur in dextrans produced by lactic acid bacteria *Leuconostoc mesenteroides* [14]. In this study, we sought to explore the utility of dextran-based glycoconjugates as an alternative vaccination strategy against *H. pylori.*

Materials and methods

Bacterial strains and growth conditions

H. pylori strain 26695 was obtained from Dr. R. Alm (Astra Zeneca, Boston, MA), *H. pylori* O:3 isolate was from Dr. J. Penner, SS1 from Dr. A. Lee (The University of New South

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Wales, Sydney, Australia), PJ1 and PJ2 clinical isolates were from Dr. W. Conlan, NRC. LPS from PJ1 and PJ2 was previously shown to be Lewis-negative and contain α(1-6)-glucan [15, 16] while LPS of strain SS1 was shown to be α(1-6)-glucan-negative [15, 17]. Construction by allelic exchange of *H. pylori* 26695 HP0826::Kan, O:3 HP0826::Kan and SS1 HP0826::Kan, O-chain deficient mutants, was described previously [18]. The insertional inactivation of HP0159 and HP0479 genes was described [19, 20] and LPS of *H. pylori* 26695 HP0159::Kan and 26695 HP0479::Kan were shown to be O-chain-deficient and lacking α(1-6)-glucan [19]. Cells were grown at 37 °C on antibiotic-supplemented Columbia Blood agar (Difco) plates containing 7 % horse blood in microaerophilic environment for 48 h as previously described [20]. For growth of *H. pylori* 26695 HP0826::Kan, O:3 HP0826::Kan and SS1 HP0826::Kan, 26695 HP0159::Kan and 26695 HP0479::Kan Columbia Blood agar/horse blood plates and Brucella broth, containing 10 % fetal bovine serum, were supplemented with kanamycin (20 μg/mL), in addition to other antibiotics [20].

LPS isolation and delipidation

The wet cell mass obtained by centrifugation of the bacterial growth was washed twice successively with ethanol, acetone and light petroleum ether and air-dried and LPS extracted by hot phenol-water extraction procedure and purified as described previously [16, 21]. LPS was obtained from the aqueous phase after extensive dialysis and lyophilization. *H. pylori* LPS was further purified by ultracentrifugation (105,000 x g, 4 °C, 12 h), and the pellet was suspended in distilled water and lyophilized.

Purification and characterization of dextrans

Dextran T5 (MW 5 KDa), Dextran T3.5 (MW 3.5 KDa) and Dextran T1.5 (MW 1.5 KDa), all from Pharmacosmos A/S (Holbaek, Denmark), were dialyzed against distilled water using dialysis membrane, 1,000 Da cutoff (Spectra/Por, Spectrum Laboratories Inc., Rancho Dominquez, CA, USA), and lyophilized. The recovered material was characterized by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) spectrometry and methylation analysis [16].

MALDI-TOF

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were obtained using a 4800 MALDI TOF/TOF mass spectrometer (AB Sciex, Framingham, MA). For protein analysis, the instrument was set to the positive linear mode to detect high mass positive ions. The matrix used was sinapinic acid [3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid] (Sigma- Aldrich, St. Louis, MO). The ions were produced by diode pumped Nd:YAG laser, operating at a wavelength of 355 nm. Following mass analysis, the ions were detected by a micro channelled plate operating between 1.9 kV to 2.1 kV. The resulted signals were collected by a computer and analyzed by 4000 series Explorer, (AB Sciex, Framingham, MA). In general, for each laser shot, a mass spectrum between 15,000 and 150,000 mass to charge ratio (*m/z*) was collected. A total of 375 mass spectra were then summed and averaged to yield a resulting mass spectrum.

NMR spectroscopy

^{1}H-NMR spectra were performed on a Varian 400 MHz spectrometer using standard software. All NMR experiments were performed at 25 °C using a 5 mm indirect detection probe with the ^{1}H coil nearest to the sample. The methyl resonance of acetone was used as an internal reference at δ 2.225 ppm.

Preparation of conjugates

Each dextran (10 mg) was dissolved in 250 μL of 0.2 M borate buffer, pH 9.0; this was added to a solution (250 μL) containing 1,8-diamino-3,6-dioxaoctane (25 μL) and sodium cyanoborohydride (10 mg) in 0.2 M borate buffer, pH 9.0, as described by Roy *et al.* [22]. The reaction was carried out for 5 days at 55 °C. The reaction product was purified by gel permeation chromatography on Bio-Gel P-2 column (Pharmacia) using distilled water as an eluant. Fractions were collected and analyzed for their carbohydrate and amino group content using phenol sulfuric acid method for neutral sugars [23] and free amino group content by trinitrophenyl sulfonic acid (TNBS) method [24] and by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

The amine group of the spacer molecule was further derivatized by reaction with 3-maleimidopropionic acid *N*-hydroxysuccinimide ester as described for LPS-based conjugates [13]. Briefly, the spacer-containing dextran (2 mg) was reacted with 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (BMPS; 2 mg; Sigma-Aldrich) in dry DMSO for 24 h at 22 °C. The solution was dialyzed against distilled water using dialysis membrane with molecular weight cut off of 1,000 Da (Spectra/Por) and lyophilized.

The glycoconjugate was obtained through thiolation of tetanus toxoid and addition of a thiolated protein to the maleimido-functionalized dextran as previously described [13, 25]. Conjugates were analyzed for their carbohydrate and protein content using phenol sulfuric acid method for neutral sugars [23] and BCA protein assay kit (Pierce), respectively, with glucose and BSA as standards. The efficiency of conjugation was confirmed by high performance liquid chromatography (HPLC; Agilent 1200 series, Agilent Technologies, Waldbronn, Germany) as described [13]. Three glycoconjugates, Dextran-5K-TT, Dextran-3.5K-TT and Dextran-1.5K-TT, were prepared.

A similar procedure was applied for preparation of Dextran-3.5K-DT conjugates. The glycoconjugate was obtained through addition of thiolated DT to the maleimido-functionalized Dextran-3.5K. Two conjugation reactions with starting ratios of carbohydrate to protein 55:1 (mol/mol) and 16:1 (mol/mol) were carried out, resulting in Dextran-3.5K-DT-I and Dextran-3.5K-DT-II, respectively. The efficiency of conjugation was confirmed by high performance liquid chromatography as described above for dextran-TT conjugates. The final conjugate was analyzed for its carbohydrate and protein content using phenol sulphuric acid method for neutral sugars [23] and BCA protein assay kit (Pierce), respectively, with glucose and BSA as standards, and by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. SDS-PAGE was performed by standard methods using precast 7.5 % polyacrylamide gel mini-Protean TGX (Bio-Rad Laboratories Inc.).

Immunogenicity of conjugates in mice and rabbits

The immunogenicity of conjugates was tested in mice and rabbits. Five 6–8 week old female CD1 mice were immunized intraperitoneally with appropriate conjugates. Each mouse received 2 μg or 10 μg of carbohydrate in 0.2 mL Ribi adjuvant per injection. The mice were boosted on day 21 and 42 and sera recovered after terminal heart puncture on day 51.

Three New Zealand white rabbits were immunized subcutaneously with appropriate conjugates. Each rabbit received 20 μg or 25 μg of carbohydrate in 0.5 mL Incomplete Freunds adjuvant. The rabbits were boosted on day 28 and 56 and sera recovered after exsanguination on day 65.

The level of anti-LPS antibody in serum was measured by ELISA in which purified LPS was used as a coating antigen (1 μg/well) [13].

Indirect immunofluorescent (IF) microscopy

Gastric adenocarcinoma cells (AGS) (ATCC CRL1739; American Type Culture Collection, Manassas, VA) cells were grown to confluency in 4-well Lab-Tek chamber slides (Nunc, Naperville, IL). *H. pylori* strain 26695, O:3, PJ2, SS1 or 26695 HP0159::Kan at 10^8 CFU/mL was added to each well, and the slides were incubated for 1 h at 37 °C. Each well was then washed three times to remove non-adherent bacteria and then fixed with methanol for 5 min and left to dry. All subsequent steps were carried out as described previously [12]. Fluorescent images were obtained using an Axiovert 200 M (Zeiss) inverted microscope.

Bactericidal assay

Plate-grown *H. pylori* cells were harvested and washed with 5 mL of PBS per plate. Following centrifugation, pellets were suspended in 25 mL of PBS. The final bacterial suspension was diluted in Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen, Grand Island, NY) at 4×10^6 CFU/mL. The assay was performed in a flat-bottomed microtiter plate (ICN) as described previously [13]. The control plate with bacteria and complement but without sera was used to calculate the percentage killing. The bactericidal activity was determined as the highest dilution of sera that caused 50 % killing.

Results

Purification and characterization of dextrans

Dextran T5 (MW 5 KDa), Dextran T3.5 (MW 3.5 KDa) and Dextran T1.5 (MW 1.5 KDa) were dissolved in distilled water (20 mg/mL), dialyzed using dialysis membrane with 1,000 Da cutoff (Spectra/Por) to remove lower molecular mass components and lyophilized. Dialyzed and lyophilized dextrans were subjected to structural characterization by methylation analysis and MALDI-TOF. Methylation analysis was consistent with the presence of a linear α(1-6)-glucan polymer with α(1-2)-, α(1-3) and α(1-4)-linked branches (Table 1). In addition, methylation analysis of Dextran-3.5 K confirmed the presence of a few α(1-3)- and α(1-)4-linked D-glucose residues [14]. MALDI-TOF analysis indicated the presence of a mixture of oligosaccharides of various lengths consistent with the consecutive addition of Hex residues (Fig. 1).

Preparation and characterization of conjugates

Conjugates were prepared as described in Materials and methods. Briefly, reducing end glucose residue of dextran was reacted with 1,8-diamino-4,6-dioxaoctane by reductive amination procedure using sodium cyanoborohydrate [22]. The presence of a spacer was confirmed by ^{1}H-NMR spectroscopy by the appearance of a new proton resonance at 3.22 ppm, corresponding to CH_2NH_2 group (Fig. 2b). The amine group

Table 1 Methylation analysis of Dextran-5K, Dextran-3.5K and Dextran-1.5K

Sugar linkage	Relative molar ratios[a]		
	Dextran-5K	Dextran-3.5K	Dextran-1.5K
Terminal Glc	1	1	1
3-linked Glc	-	0.2	-
4-linked Glc	-	0.2	-
6-linked Glc	13	8.5	7
2,6-linked Glc	0.5	0.2	0.1
4,6-linked Glc	0.5	0.4	0.1
3,6-linked Glc	2	2	0.4

[a] Based on the detector response (total ion count)

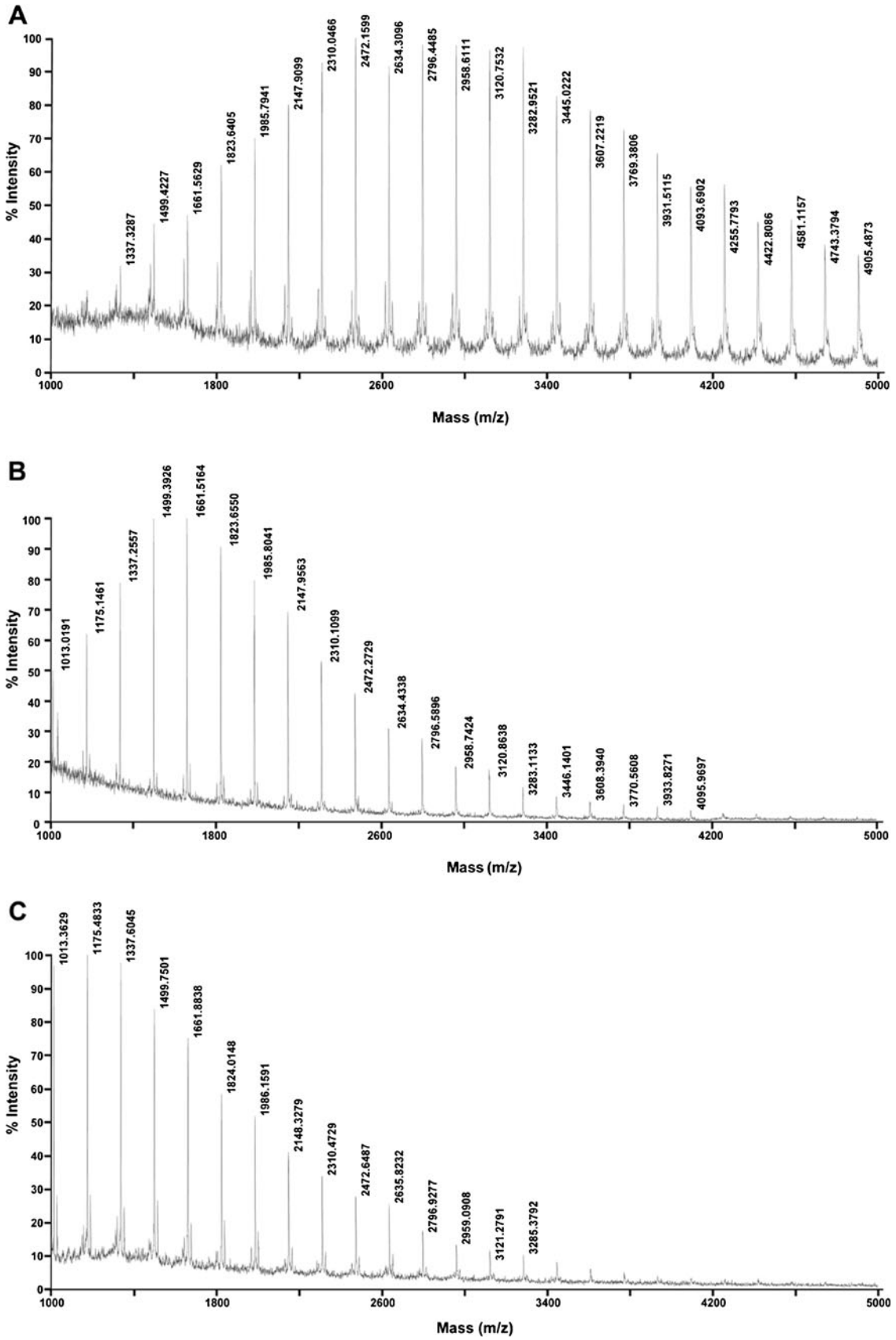

Fig. 1 MALDI-MS analysis of dextrans following dialysis and lyophilization: A Dextran-5K; B Dextran-3.5K; C Dextran-1.5K

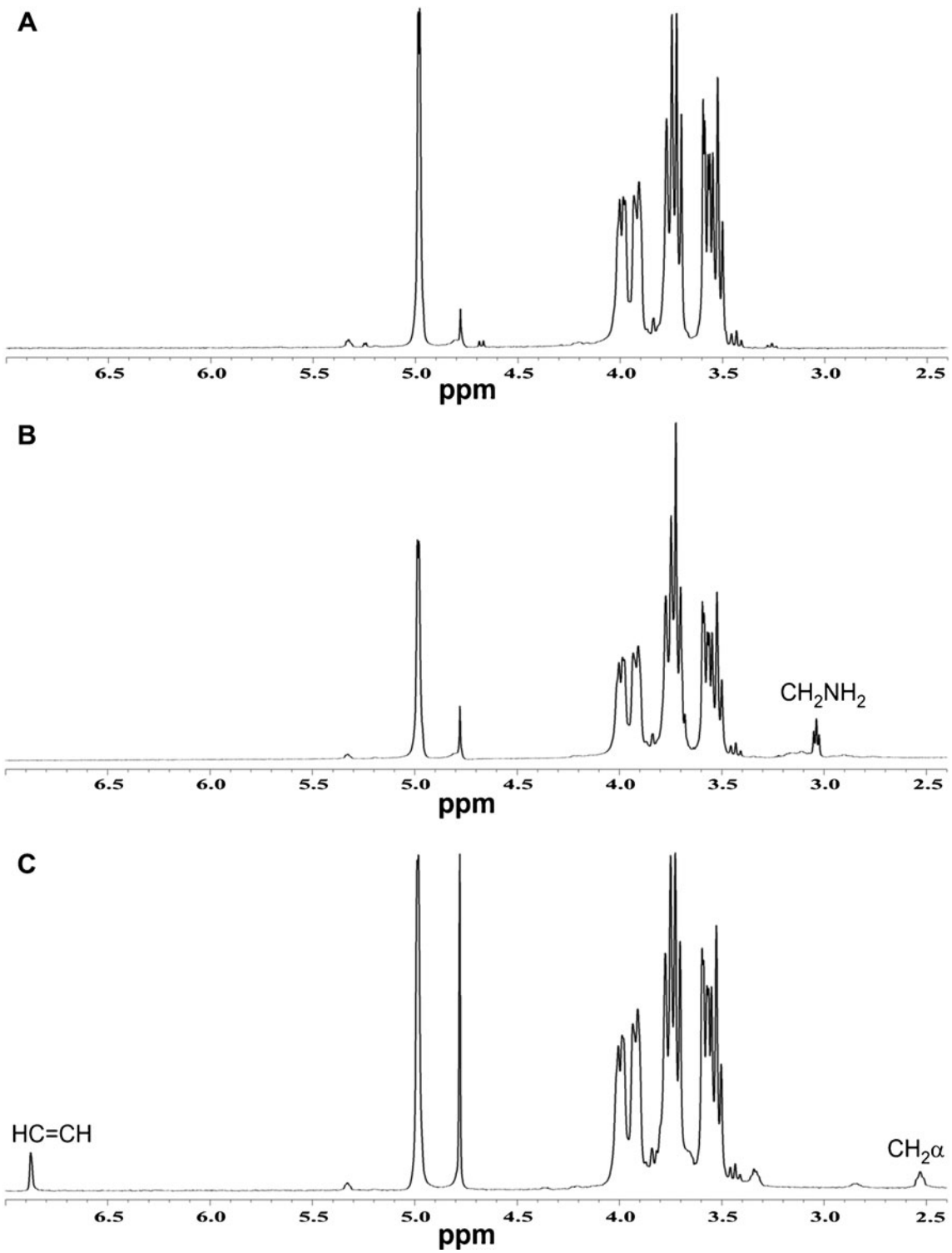

Fig. 2 ^{1}H-NMR spectrum at 25 °C of the Dextran-3.5K following dialysis and lyophilization (**a**), following 1,8-diamino-4,6-dioxaoctane group incorporation (**b**) and following β-maleimidopropionate incorporation (**c**)

of the spacer was further derivatized by reaction with 3-maleimidopropionic acid *N*-hydroxysuccinimide to yield maleimido-functionalized dextran, as confirmed by the presence of proton resonances at 2.55 ppm and 6.9 ppm, corresponding to $CH_{2\alpha}$ and CH=CH groups of β-maleimidopropionate, respectively (Fig. 2c). The glycoconjugate was obtained through thiolation of a carrier protein and addition of the thiolated protein to the maleimido-functionalized dextran as described

previously for LPS-based conjugates [13]. The efficiency of conjugation was monitored by HPLC. The ratio of carbohydrate to protein in Dextran-5K-TT, Dextran-3.5K-TT and Dextran-1.5K-TT ranged from 0.96:1 to 0.67:1 (w/w), and the yield ranged from 48 % to 38 %, based on the carbohydrate content (Table 2). All conjugates reacted equally well with α(1-6)-glucan-specific monoclonal antibodies by ELISA suggesting that the conformation of the glucan epitope was unchanged (data not shown).

To assess the immunogenicity of DT- and TT-based dextran conjugates, two conjugation reactions with starting ratios of carbohydrate to protein 55:1 (mol/mol, Dextran-3.5K-I) and 16:1 (mol/mol, Dextran-3.5K-DT-II) were carried out. The level of incorporation of Dextran-3.5K onto DT in each conjugate was determined by MALDI-MS (Fig. 3). Due to the molecular mass variability of saccharide chains attached glycoconjugates appeared as broad peaks of low intensity. MALDI-MS analysis of Dextran-3.5K-I was consistent with the incorporation of 4 and 8 carbohydrate molecules per one molecule of DT corresponding to fragment ions *m/z* 73,923 and *m/z* 80,802, respectively (Fig. 3b). MALDI spectrum of Dextran-3.5K-DT-II conjugate was consistent with the average incorporation of 2 carbohydrate molecules per one DT molecule (*m/z* 65,608) although a significant portion of the glycoconjugate was consistent with higher loading of carbohydrate per DT and contained 8.2 molecules of carbohydrate per 1 molecule of DT (*m/z* 81,381) (Fig. 3c). The ratio of carbohydrate to protein in two conjugates ranged from 0.93:1 to 0.34:1 (w/w), based on the carbohydrate content (Table 2). SDS-PAGE analysis carried out on Dextran-3.5K-DT-I and Dextran-3.5K-DT-II confirmed the conjugation of Dextran-3.5K to DT with DT-based glycoconjugates appearing as polydisperse bands of higher molecular masses than unconjugated DT (Fig. 4).

Table 2 Composition and yield of conjugates used in this study

Conjugate	Amount (μg/mL)		Final ratio (w/w)of CHO to protein	Yield (%)
	CHO[a]	Protein[b]		
Dextran-5K-TT	750	780	0.96:1	48
Dextran-3.5K-TT	780	1060	0.74:1	42
Dextran-1.5K-TT	690	1480	0.67:1	38
Dextran-3.5K-DT-I	1400	1500	0.93:1[c]	25
Dextran-3.5K-DT-II	950	2800	0.34:1[c]	31

[a] Amount of carbohydrate (CHO) was determined according to Dubois *et al.* [23]; D-glucose was used as a standard

[b] Amount of protein was determined by BCA test; BSA was used as a standard

[c] Molar ratio of CHO was determined using average molecular mass values based on average molecular mass of Dextran-3.5K corresponding to 2,634 Da, as determined by MALDI

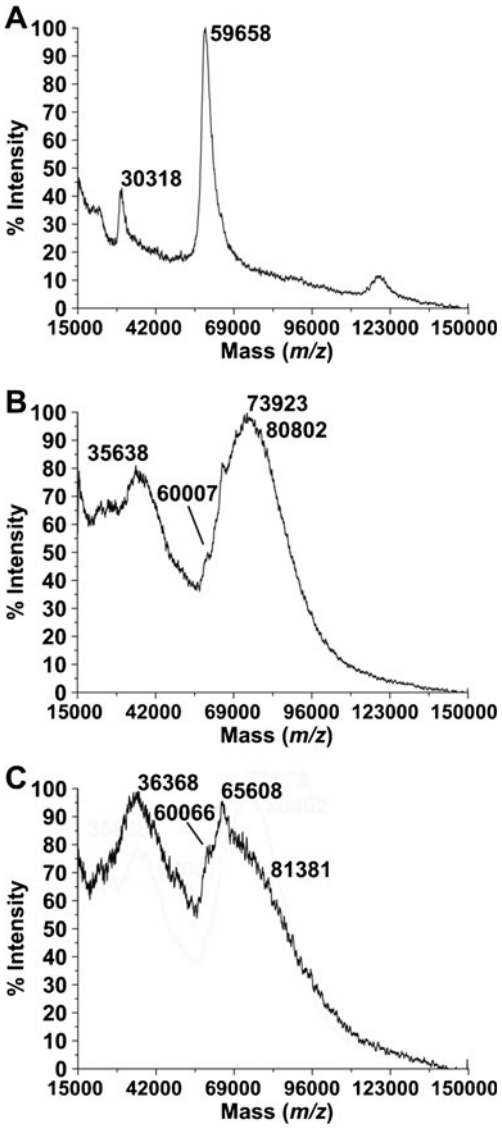

Fig. 3 MALDI-MS analysis of DT and Dextran-3.5K-DT conjugates: **a** DT; **b** Dextran-3.5K-DT-I; **c** Dextran-3.5K-DT-II. Observed positive ions at *m/z* 30318, *m/z* 36368 and *m/z* 35638 correspond to doubly charged ions

Immunogenicity of conjugates in mice and rabbits

All conjugates elicited IgG responses against *H. pylori* LPS from α(1-6)-glucan-positive *H. pylori* strains, 26695 HP0826::Kan and corresponding wild-type 26695, in both mice and rabbits after three injections. Control rabbits immunized with a mixture of relevant dextran and a protein carrier (with an adjuvant) showed no or low level specific responses to these LPS after three immunizations (Table 3).

Cross-reactivity studies were performed with rabbit post-immune sera against LPS from wild-type *H. pylori* strains representative of various LPS glycotypes [15, 16] and selected LPS mutants lacking either O-chain polysaccharide (26695 HP0826::Kan, and O:3 HP0826::Kan) or α(1-6)-glucan (SS1) or both (SS1 HP0826::Kan, 26695 HP0159::Kan) [18, 19]

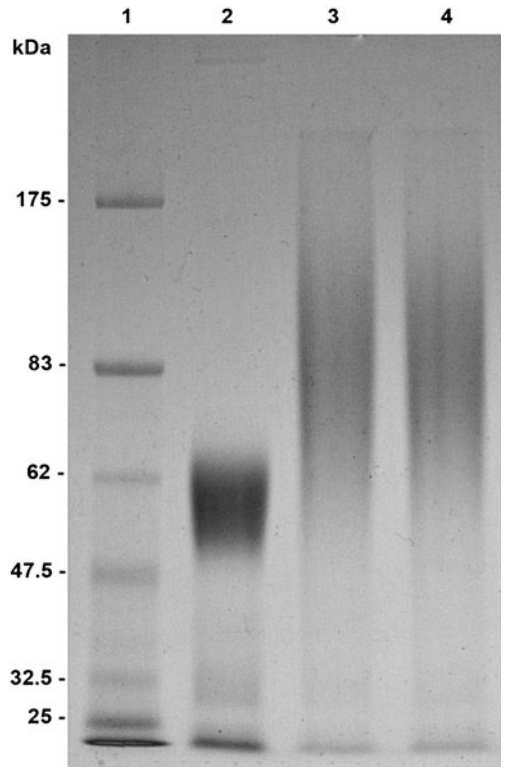

Fig. 4 SDS-PAGE analysis of Dextran-3.5K-DT conjugates: Lane 1, molecular weight markers; lane 2, DT protein; lane 3, Dextran-3.5K-DT-I (carbohydrate/protein ratio 0.93:1, w/w); lane 4, Dextran-3.5K-DT-II (carbohydrate/protein ratio 0.34:1 w/w)

(Fig. 5). Overall, higher immune responses were obtained for dextran-TT conjugates than for dextran-DT conjugates (Fig. 5). The reactivity of rabbit post-immune sera was indicative of the requirement for the presence of α(1-6)-glucan since only weak immune responses were obtained against LPS from α(1-6)-glucan-negative *H. pylori* strains, SS1, SS1 HP0826::Kan and 26695 HP0159::Kan [15, 18, 19] (Fig. 5). When post-immune sera of mice immunized with dextran-TT conjugates was screened against the same LPS panel, some cross-reactivity with LPS from α(1-6)-glucan-negative *H. pylori* strains SS1, 26695 HP0159::Kan and SS1 HP0826::Kan was observed (Fig. 6). To probe the binding specificity of mouse sera, inhibition ELISA was conducted with purified 26695 HP0479::Kan LPS, an isogenic mutant lacking O-chain polysaccharide, α(1-6)-glucan and an outer core region and expressing a linear backbone oligosaccharide structure partially capped with [GlcNAc, Fuc] [20]. Inhibition of binding of LPS from α(1-6)-glucan-negative *H. pylori* strains 26695 HP0159::Kan, SS1 and SS1 HP0826::Kan was observed (Table 4).

Indirect immunofluorescent (IF) microscopy of *H. pylori* binding to Dextran-3.5K-TT post-immune rabbit sera

To determine if sera obtained from rabbits immunized with dextran-based conjugates recognizes surface exposed epitopes

Table 3 Murine and rabbit serum IgG titres against *H. pylori* 26695 HP0826::Kan LPS and 26695 LPS elicited by conjugates

Immunogen	Immune serum	Serum endpoint IgG titre 1:	
		26695 LPS	26695 HP0826::Kan LPS
Dextran-5K-TT[a]	Mice		
	1	782	6,166
	2	7,586	35,481
	3	858	4,266
	4	832	977
	5	9,120	8,318
Dextran-5K-TT[b]	Rabbits		
	1	1,778	38,019
	2	158,490	1,659,600
	3	63,096	3,311,300
Dextran-5K, TT, IFA	1	0	0
	2	0	0
Control[c]	1	0	0
	2	0	0
Dextran-3.5K-TT[a]	Mice		
	1	8,318	50,119
	2	912	6,506
	3	807	5,089
	4	4,936	14,678
	5	940	5,934
Dextran-3.5K-TT[b]	Rabbits		
	1	6,610	81,283
	2	1,738	56,234
	3	33,113	4,786,300
Dextran-3.5K, TT, IFA	1	0	0
	2	0	0
Control[c]	1	0	16
	2	0	0
Dextran-1.5K-TT[a]	Mice		
	1	3,261	2,840
	2	1,097	871
	3	858	1,047
	4	871	884
	5	9,120	898
Dextran-1.5K-TT[b]	Rabbits		
	1	2,884	35,283
	2	6,918	85,114
	3	436,520	2,818,400
Dextran-1.5K, TT, IFA	1	0	11
	2	0	0
Control[c]	1	0	16
	2	0	0
Dextran-3.5K-DT-I[d]	Rabbits		
	1	1,016	38,019
	2	855	20,893

Table 3 (continued)

Immunogen	Immune serum	Serum endpoint IgG titre 1:	
		26695 LPS	26695 HP0826::Kan LPS
	3	44,668	1,096,500
Dextran-3.5K, DT, IFA	1	0	0
	2	13	50
Control[c]	1	0	0
	2	0	0
Dextran-3.5K-DT-II[d]	Rabbits		
	1	398	7,943
	2	36,869	336,250
	3	32,609	374,400
Dextran-3.5K, DT, IFA	1	0	0
	2	0	0
Control[c]	1	0	0
	2	0	0

[a] Each mouse received 2 μg of carbohydrate per injection

[b] Each rabbit received 20 μg of carbohydrate per injection

[c] Adjuvant (IFA) only

[d] Each rabbit received 25 μg of carbohydrate per injection

on the intact *H. pylori*, indirect IF microscopy studies were carried out with Dextran-3.5K-TT post-immune rabbit sera. Rabbit serum IgG bound to the surface of all representative wild-type *H. pylori* α(1-6)-glucan-positive strains tested, namely 26695, O:3 and PJ2, as indicated by fluorescently labeled bacteria bound to the surface of AGS cells (Fig. 7). No binding was observed with α(1-6)-glucan-negative *H. pylori* strains SS1 and 26695 HP0159::Kan.

Bactericidal activity of rabbit antisera

The post-immune sera obtained from rabbits immunized with Dextran-5K-TT, Dextran-3.5K-TT and Dextran-1.5K-TT showed significant functional activity against *H. pylori* 26695 HP0826::Kan mutant and corresponding wild-type strain 26695 (Table 5). No bacterial killing was observed with α(1-6)-glucan-negative *H. pylori* strain SS1. Prevaccination sera or sera obtained from rabbits immunized with a mixture of the corresponding dextran and tetanus toxoid (with an adjuvant) showed no or low levels of bactericidal activity against these strains. No functional activity against *H. pylori* strains tested was obtained with heat-inactivated complement

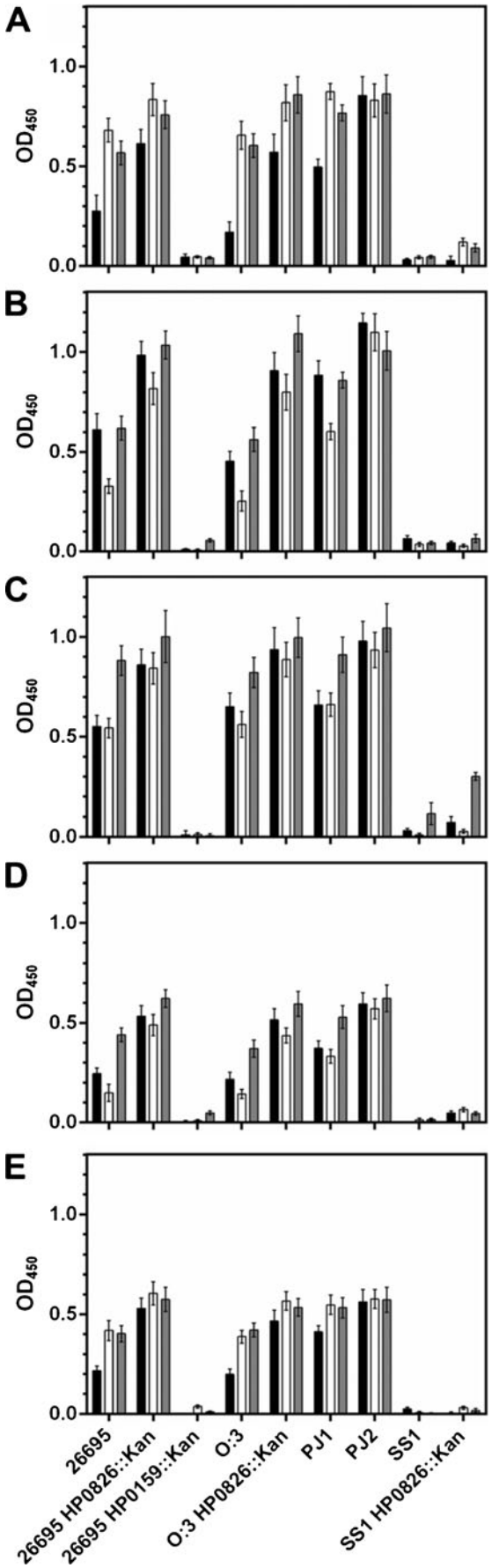

Fig. 5 Cross-reactivity of Dextran-5K-TT (**a**), Dextran-3.5K-TT (**b**), Dextran-1.5K-TT (**c**), Dextran-3.5K-DT-I (**d**) or Dextran-3.5K-DT-II (**e**) post-immune rabbit sera at 1:100 dilution in ELISA against a panel of purified LPS from wild-type *H. pylori* strains representative of various glycotypes and selected mutants as indicated. OD_{450}±10 %. The immunogenicity of conjugates was tested in groups of rabbits (three animals each per conjugate)

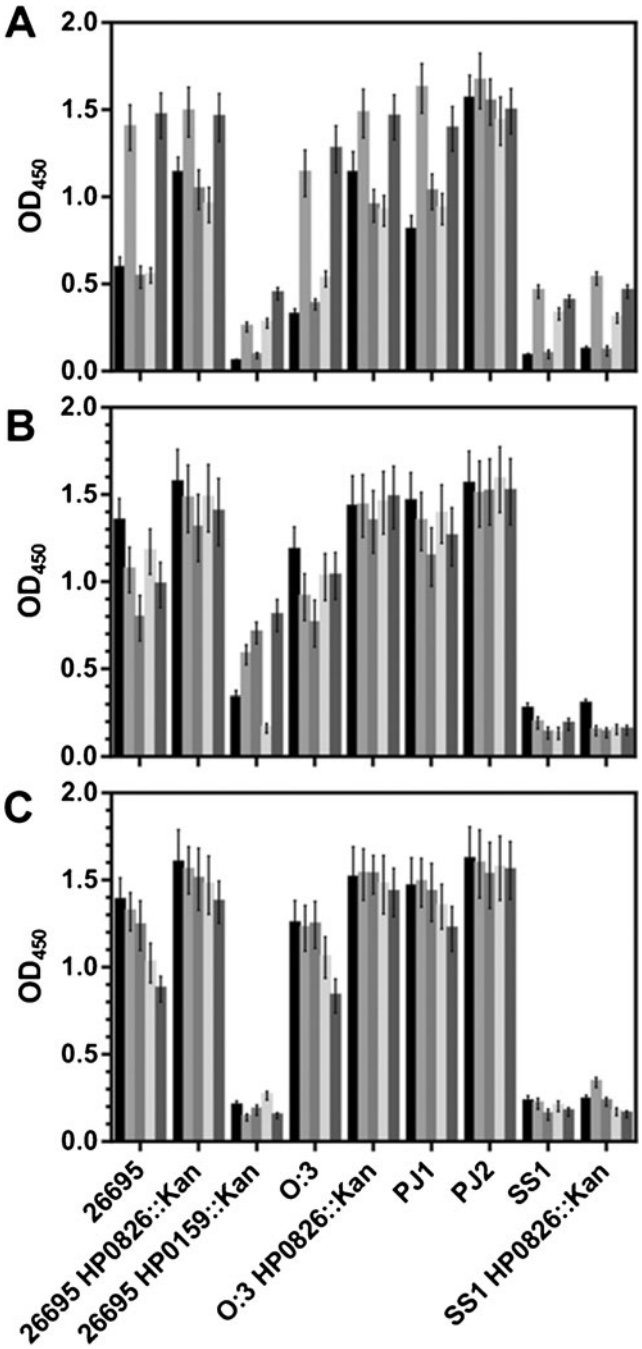

Fig. 6 Cross-reactivity of Dextran-5K-TT (**a**), Dextran-3.5K-TT (**b**), Dextran-1.5K-TT (**c**) post-immune mouse sera at 1:100 dilution in ELISA against a panel of purified LPS from wild-type *H. pylori* strains representative of various glycotypes and selected mutants as indicated. OD_{450} ± 10 %. The immunogenicity of conjugates was tested in groups of mice (five animals each per conjugate)

and without serum. Similar results were obtained for post-immune sera of rabbits immunized with Dextran-3.5K-DT-I and Dextran-3.5K-DT-II as compared to control sera. The highest bactericidal activity against wild-type strain 26695 of *H. pylori* was obtained with post-immune sera of rabbits immunized with Dextran-3.5K-DT-II (Table 5).

Table 4 Inhibition of mouse[a] antibody responses to LPS from α(1-6)-glucan-negative strains of *H. pylori* elicited by Dextran-5K-TT conjugate

Coating LPS	IC50[b]
26695 HP0159::Kan LPS	86
SS1 LPS	158
SS1 HP0826::Kan LPS	233

[a] Post-immune sera from mouse 5 immunized with Dextran-5K-TT

[b] Concentration of 26695 HP0479::Kan LPS (μg/mL) required for 50 % inhibition

Discussion

We have previously demonstrated that an α(1-6)-glucan epitope of the outer core region of *H. pylori* LPS is common in *H. pylori* isolates and is recognized by α(1-6)-glucan-specific bactericidal monoclonal antibodies, suggesting that α(1-6)-glucan outer core epitope can provide a target for protective immunity [12]. Subsequently, we explored the utility of the glycoconjugate, consisting of a modified LPS from *H. pylori* 26695 HP0826::Kan mutant [26] and a carrier protein, to elicit immune responses in immunized animals [13]. The ability of rabbit post-immune sera to recognize typeable and non-typeable strains of *H. pylori* and induce bactericidal antibodies suggested the possibility that glycoconjugate comprising α(1-6)-linked glucan alone and a suitable protein carrier would be sufficient to confer protection against *H. pylori.* We have employed commercially available dextrans from *L. mesenteroides* B512F that have been reported to produce mostly linear α(1-6)-linked glucan chains modified with short side-chains composed of consecutive α(1-6)-linked glucose residues [14]. Dextran-TT conjugates were prepared using the conjugation strategy previously developed for the synthesis of *H. pylori* LPS-based glycoconjugates. This approach is based on the incorporation of a diamino group-containing linker onto the carbohydrate antigen, followed by the introduction of maleimido functionality and coupling of the activated carbohydrate molecule to thiolated carrier protein yielding the desired glycoconjugate. This general strategy leads to synthesis of highly reproducible, stable and biologically active glycoconjugates [13].

To optimize the dextran size in the glycoconjugate, we sought to compare the immunogenicity of commercially available dextrans, namely Dextran T5 (average MW 5 KDa), Dextran T3.5 (average MW 3.5 KDa) and Dextran T1.5 (average MW 1.5 KDa), produced by *L. mesenteroides* B512F and consisting of polysaccharides with primarily linear α(1-6)-glucan chains with a limited branching as demonstrated by subsequent methylation analysis. Our previous findings have indicated the requirement for five to six contiguous α(1-6)-linked glucose residues for optimal recognition of the α(1-6)-glucan epitope [12]. Accordingly, all three dextrans were of a comparable size, with an average number of α(1-6)-linked

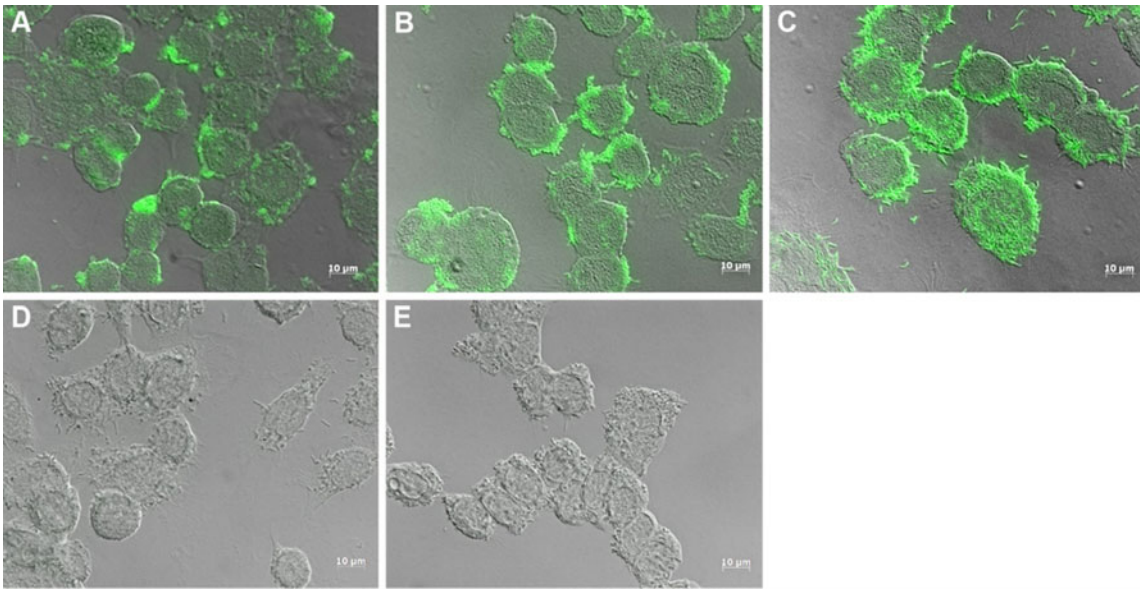

Fig. 7 Binding of Dextran-3.5K-TT post-immune rabbit sera to the surface of *H. pylori* on AGS monolayers, as visualized by indirect IF microscopy: **a** strain 26695; **b** strain O:3; **c** strain PJ2; **d** strain SS1; **e** strain 26695 HP0159::Kan. Scale bars, 10 µm

glucose residues ranging between 7 and 13 (Table 1). The dextrans were subjected to dialysis using dialysis membrane, 1,000 Da cutoff, which resulted in the removal of low-molecular-mass components and further optimization of the dextran size. Lyophilized dextrans were conjugated to TT using previously developed methodology, yielding Dextran-5K-TT, Dextran-3.5K-TT and Dextran-1.5K-TT conjugates, respectively.

The conjugates were immunogenic in both rabbits and mice and induced strong, specific IgG responses against α(1-6)-glucan-expressing *H. pylori* LPS. Dextran-3.5K-TT and Dextran-1.5K-TT conjugates induced similar serum IgG titres in mice and rabbits against LPS from α(1-6)-glucan-positive *H. pylori* strains 26695 and 26695 HP0826::Kan, while Dextran-5K-TT induced slightly higher serum titres against the same LPS in rabbits (Table 3). Rabbit and mouse antisera were screened against a panel of LPS from typeable and non-typeable α(1-6)-glucan-positive *H. pylori* strains exhibiting strong and specific immune responses (Figs. 5 and 6). Furthermore, the broad cross-reactivity of the Dextran-3.5K-TT-induced rabbit sera was confirmed by indirect IF microscopy studies conducted with intact *H. pylori* cells adherent to AGS monolayers. These experiments demonstrated that rabbit serum IgG recognized surface-exposed epitopes on intact *H. pylori* cells of α(1-6)-glucan-positive strains 26695, O:3 and PJ2, representatives of typeable (26695 and O:3) and non-typeable (PJ2) strains while no binding was observed with α(1-6)-glucan-negative strains SS1 and 26695 HP0159::Kan (Fig. 7).

To evaluate the influence of different carbohydrate/protein loadings on the immunogenicity of glycoconjugates, we synthesized two Dextran-3.5-DT conjugates, Dextran-3.5K-DT-I and Dextran-3.5K-DT-II, with carbohydrate to protein ratios (w/w), 0.93:1 and 0.34:1, respectively. Interestingly, Dextran-3.5K-DT-II with lower carbohydrate loading was slightly more immunogenic in rabbits than Dextran-3.5K-DT-I (Table 3 and Fig. 5).

Table 5 Bactericidal activity against *H. pylori* strains 26695 HP0826::Kan, 26695 and SS1 with rabbit antisera elicited by dextran-based conjugates

Immunogen	Serum bactericidal titre (50 % killing)[e] against *H. pylori* 1:		
	26695	26695 HP0826::Kan	SS1
Dextran-5K-TT[a]	195±36	2,416±238	0
Pre-immune serum[b]	20±14	91±23	0
Dextran-3.5K-TT[a]	479±86	575±65	0
Pre-immune serum[b]	76±26	71±38	0
Dextran-1.5K-TT[a]	140±29	509±84	0
Pre-immune serum[b]	47±16	65±22	0
Dextran-3.5 K-DT-I[c]	398±77	331±83	0
Pre-immune serum[b]	25±18	16±8	0
Dextran-3.5 K-DT-II[c]	612±112	275±55	0
Pre-immune serum[b]	0	14±8	0
Control[d]	0	0	0

[a] Each rabbit received 20 µg of carbohydrate per injection

[b] Corresponding pre-immune control serum

[c] Each rabbit received 25 µg of carbohydrate per injection

[d] Heat-inactivated complement and without serum

[e] The reciprocal of the highest serum dilution causing 50 % killing was expressed as the bactericidal titre (BC_{50}). A bactericidal titer of an individual serum of≥100 was considered positive as compared to the pre-immune control serum

Mouse post-immune sera also exhibited some cross-reactivity against LPS from α(1-6)-glucan-negative *H. pylori* strains, namely SS1, SS1 HP0826::Kan and 26695 HP0159::Kan. It was suggested earlier by Kabat [27] that α(1→6)dextrans give rise to two major types of anti-α(1→6)dextrans, an antibody recognizing a cavity-type site that could fit terminal non-reducing glucose with four contiguous α(1-6)-linked glucose residues, in addition to an antibody that has a groove-type site recognizing a chain of six consecutive α(1-6)-linked glucoses excluding the non-reducing residue [27]. This principle is applicable to other polysaccharide/antibody systems and implies generation of two types of antibodies that either bind to internal in-chain antigenic determinant or the antigenic chain terminus [28]. Generation of murine antibodies specific to terminal glucose residues of dextran-TT conjugates could account for this cross-reactivity. To confirm these findings, inhibition ELISA was conducted with purified LPS of *H. pylori* 26695 HP0479::Kan. This LPS consists of a linear backbone oligosaccharide structure α-Glc-4-β-Gal-7-α-DDHep-2-α-Hep-3-α-Hep7*P*EtN-5-α-Kdo capped with [GlcNAc, Fuc] at α-DDHep and giving rise to two glycoforms (approximate ratio 1:1) which contain terminal non-reducing glucose residue [17]. *H. pylori* 26695 HP0479::Kan LPS blocked the binding of mouse immune sera to LPS from α(1-6)-glucan-negative strains SS1 HP0826::Kan, SS1 and 26695 HP0159::Kan with an inhibitory concentration 50 % (IC_{50}) of 233, 158 and 86 μg/mL, respectively, confirming the recognition of terminal non-reducing glucose residues in the LPS core by mouse immune sera (Table 4).

When post-immune sera of rabbits immunized with either conjugate was tested for bactericidal activity with α(1-6)-glucan-positive strains 26695 and corresponding mutant 26695 HP0826::Kan, Dextran-3.5K-DT-II post-immune sera exhibited the highest functional activity against strain 26695, whereas Dextran-5K-TT conjugate exhibited the highest bactericidal activity against Le negative 26695 HP0826::Kan mutant (Table 5). No detectable bactericidal activity was observed with α(1-6)-glucan-negative *H. pylori* strain SS1. The results were comparable with functional activity of rabbit post-immune sera previously generated against *H. pylori* LPS-based glycoconjugates, delipidated LPS-BSA (dLPS-BSA) and O-deacylated LPS-TT (LPS-OH-TT) [13].

In the present study, immunogenicity testing of glycoconjugates was carried out in the presence of IFA adjuvant for rabbit immunizations or Ribi adjuvant for immunization of mice. While these adjuvants are suitable for preclinical investigations in animal models, they are not acceptable for human use. Future testing of dextran-based glycoconjugate formulations will involve the use of alum, an adjuvant licensed for human applications.

In summary, dextran-based synthetic glycoconjugates may provide a simplified and cost-effective strategy for carbohydrate-based *H. pylori* vaccine development.

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