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Keyhole limpet hemocyanin contains Gal(β1-3)-GalNAc determinants that are cross-reactive with the T antigen

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Abstract Keyhole limpet hemocyanin (KLH) is widely used as a carrier molecule to enhance immune responses to administered antigens, and for immunotherapy of bladder and renal carcinoma. In the present study we show, using lectin and antibody binding studies, that native KLH contains Gal(β1-3)GalNAc-bearing oligosaccharides, and that immunization with KLH in Lewis rats induces the production of anti-Gal(β1-3)GalNAc antibodies. This might explain the beneficial effect of KLH in bladder cancers that express cross-reactive Gal(β1-3)GalNAc determinants or the T antigen.

Key words Keyhole limpet hemocyanin (KLH) · T antigen · Immunotherapy

Introduction

Keyhole limpet hemocyanin (KLH) is a high-molecular-mass glycoprotein antigen that is used as a carrier molecule for peptide or carbohydrate antigens in immunization of humans and experimental animals. KLH conjugates of several tumor carbohydrate antigens, including Gal(β1-3)GalNAc, which is expressed in bladder carcinoma cells and other adenocarcinomas [16], and GD2 or GM3 gangliosides, which are expressed by melanoma cells [4], have been used to immunize against the tumor cells [2, 10, 11]. In its native form, KLH has also been used for immunotherapy of bladder and renal carcinoma, where its effects have been ascribed to general immunostimulatory mechanisms [14]. Despite its widespread clinical and experimental use, little is known about its structure or about its immunostimulatory mechanisms [4].

In the course of our studies of anti-glycoconjugate antibodies in neurological diseases [13], we found that the lectin peanut agglutinin (PNA), which binds specifically to terminal Gal(β1-3)GalNAc determinants [12], and human anti-GM1 and anti-AGM1 antibodies, which cross-react with Gal(β1-3)GalNAc [13], also bind to native KLH. In this paper we show that native KLH possesses oligosaccharides containing terminal Gal(β1-3)GalNAc residues and that immunization with KLH induces antibodies that cross-react with the Thomsen-Friedenreich (T) antigen.

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Materials and methods

Reagents

KLH (60 mg/ml in 50% glycerol) was obtained from Calbiochem (San Diego, Calif.). Gal(β1-3)GalNAc linked to human serum albumin (Tα) was purchased from Biocarb (Lund, Sweden), and Gal(β1-3)GalNAc linked to bovine serum albumin (Tβ) was obtained from Pierce (Rocquford, Ill.). Biotinylated PNA and avidin–horseradish-peroxidase were from Vector (Burlingame, Calif.). Peroxidase-conjugated goat antibodies to rat IgG and IgM were purchased from Jackson immunochemicals (West Grove, Pa.) and peroxidase-conjugated antibodies to human IgM were from Organon-Teknika (Durham, N.C.). GM1, AGM1 and other reagents were from Sigma chemical Co. (St Louis, Mo.).

ELISA

Antibody and PNA binding was quantified by enzyme-linked immunosorbent assay (ELISA) [17]. Microwells in round-bottomed

microtiter plates (Fisher, Springfield, N.J.) were coated with 1 μg KLH, Tα or Tβ in 100 μl 0.1 M $NaHCO_3$ pH 8.0 overnight, or with 0.5 μg GM1 or AGM1 in 100 μl methanol, which was then evaporated. Wells were then saturated with 250 μl 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 4 h and 100 μl serially diluted biotinylated PNA, or human or immunized rat serum in ELISA solution containing 1% BSA in PBS was added in duplicate and incubated overnight at 4 °C. The human sera were obtained from patients with motor neuropathies and contained high titers of monoclonal IgM anti-GM1 or AGM1 antibodies, which cross-react with Gal(β1-3)GalNAc. Wells coated with BSA only served as controls for each dilution. Plates were washed five times in 1% BSA/PBS, and wells containing biotinylated PNA were counterstained with peroxidase-conjugated avidin, wells containing human sera were counterstained with peroxidase-conjugated affinity-purified antibodies to human IgM, and wells containing rat serum were counterstained with affinity-purified, peroxidase-conjugated antibodies to rat IgG or IgM. After a 2 h incubation at 4 °C, the plates were washed and 100 μl developing solution, containing 0.05 M Na_2HPO_4, 0.024 M sodium citrate pH 5.0, 0.06% *o*-phenylenediamine and 0.008% H_2O_2, was added. Reaction products were measured spectrophotometrically at 450 nm in a Biotek enzyme immunoassay reader. The absorbances (*A*) were plotted against log(serum dilutions) and the titer for each specimen was taken as the highest dilution in which the absorbance reading was 0.1 unit greater than in the corresponding BSA-coated wells.

Deglycosylation and blocking studies

To determine whether PNA and the human anti-Gal(β1-3)GalNAc antibodies bind to oligosaccharides on KLH and whether they bind to the same determinants, PNA and the three human antibodies were tested for binding to deglycosylated KLH, and PNA was tested for its ability to block the binding of the human antibodies to KLH.

For the deglycosylation studies, KLH coated on ELISA plates was deglycosylated by periodate oxidation [18]. After saturation, the wells were rinsed with 50 mM sodium acetate buffer, pH 4.5, and exposed to various concentrations of sodium periodate (0–20 mM) in 50 mM sodium acetate buffer, for 1 h at room temperature in the dark. Plates were then rinsed briefly in acetate buffer and further incubated with 50 mM sodium borohydride in PBS for 30 min at room temperature, to reduce the aldehyde groups generated by the periodate oxidation and prevent nonspecific cross-linking of antibodies. Plates were then washed five times in BSA/PBS and incubated with serial dilutions of PNA, human antibodies, or serum from KLH-immunized rats. Binding was detected as described above.

In the antibody-blocking studies, KLH-coated wells were incubated with serial dilutions of PNA in 50 μl BSA/PBS for 2 h, and serum from patient 1, diluted 1:20 000 in 50 μl BSA/PBS was then added overnight. IgM binding was then determined as described above.

Carbohydrate analysis

The carbohydrate content of KLH was quantified by phenol/sulfuric acid spectrophotometric analysis [1]. To test for the presence of sialic acid, KLH (5 μg and 10 μg) was spotted on a silica gel TLC plate and sprayed with resorcinol spray reagent [8]. Similar quantities of a BSA-GM1 conjugate and BSA respectively served as positive and negative controls.

Rat immunization studies

Native KLH was diluted to 3 mg/ml and dialyzed against deionized water for 48 h. Female 8-week-old Lewis rats were immunized by subcutaneous injection of 100 μg KLH in 0.9% NaCl emulsified with Freund's complete adjuvant. Animals were boosted with 100 μg KLH in Freund's incomplete adjuvant on day 21. Preimmune sera and sera drawn on day 28 were assayed on the same day by ELISA for antibodies to KLH, Tα, Tβ, AGM1 and GM1.

Results

Binding of PNA and human anti-Gal(β1-3)GalNAc antibodies to KLH

PNA bound to KLH on ELISA plates at PNA concentrations of as little as 1 ng/ml (Table 1). It also bound to Tα at 1 ng/ml and to Tβ at 0.1 ng/ml. PNA binding to AGM1 was tenfold weaker than to KLH. Sera from two patients with high titers of AGM1 antibodies bound to KLH and to the two anomeric variants of the T antigen at titers comparable to their anti-AGM1 reactivity, whereas serum from patient 3, which had weak reactivity with AGM1, bound to KLH and to the T antigen at considerably lower titers (Table 1). Patient 1 reacted with the Tα and Tβ antigens at the same titers whereas patients 2 and 3 showed preferential reactivity to the Tβ antigen with fivefold lower reactivity with Tα.

Deglycosylation and blocking studies

Following deglycosylation of KLH, PNA no longer bound to the deglycosylated KLH (Fig. 1). The binding of the human anti-Gal(β1-3)GalNAc antibodies to KLH was likewise abolished by deglycosylation (data not shown). In the blocking experiments, PNA at concentration of 0.4 μg/ml and higher blocked the binding of patient 1 serum to KLH.

Carbohydrate analysis

Sugar content determination by the phenol/sulfuric acid assay showed the carbohydrate content of KLH to be approximately 1000–1200 sugar molecules/protein molecule (10%–12% w/w). KLH spotted on thin-layer chromatography plates did not stain with resorcinol spray reagent, whereas GM1-BSA conjugate was stained, indicating that KLH does not contain sialic acid.

Immunization studies

In animal experiments, five rats were immunized with KLH. Preimmune sera showed low titers (1:200) of IgM reactivity to KLH but not to GM1, AGM1 or the T antigens. Postimmunization sera showed development of high titers of anti-KLH antibodies, which were detectable at serum dilutions of over 1:50 000 for IgG

Table 1 Binding of peanut agglutinin (PNA) and IgM anti-Gal(β1-3)GalNAc antibodies to keyhole limpet hemocyanin (KLH) and other cross-reactive glycoconjugates including the glycopeptides Tα and Tβ, and the glycolipids GM1 and AGM1. The minimum concentration of PNA required for detection of binding, and the titers of the IgM for each antigen are presented

Species bound	Antigen				
	KLH	Tα	Tβ	GM1	AGM1
PNA (μg/ml)	0.001	0.001	0.0001	0.1	0.01
Antibodies					
Patient 1	1:102400	1:125000	1:125000	1:12800	1:205000
Patient 2	1:25000	1:25000	1:125000	1:16000	1:16000
Patient 3	1:6400	1:1000	1:5000	1:64000	1:1000

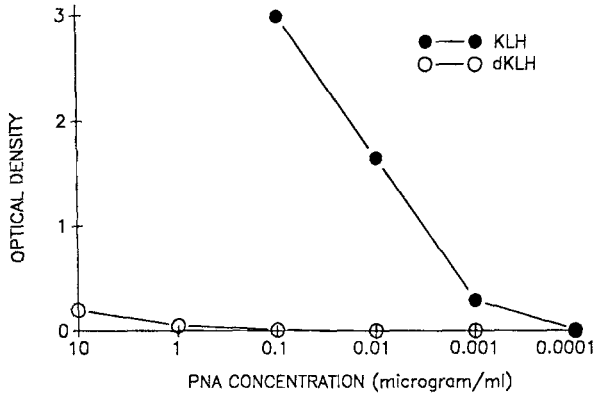

Fig. 1 Peanut agglutinin (PNA) binding to keyhole limpet hemocyanin (KLH) and to deglycosylated KLH (*dKLH*). ELISA plates were coated with KLH, or with deglycosylated KLH treated with sodium periodate. Serial dilutions of biotinylated PNA were then added to the wells containing native and deglycosylated KLH. The bound PNA was detected with peroxidase-conjugated avidin. As shown, periodate oxidation of KLH completely abolished the binding of PNA

Table 2 Titers of IgG antibodies to KLH, Tα, Tβ, GM1 and AGM1 in Lewis rats immunized with KLH in Freund's adjuvant

IgG	Antigen				
	KLH	Tα	Tβ	GM1	AGM1
Rat 1	1:50000	1:12800	1:3200	1:200	0
Rat 2	1:50000	1:12800	1:6400	1:800	0
Rat 3	1:50000	1:12800	1:12800	1:800	0
Rat 4	1:32000	1:8000	1:4000	1:200	0
Rat 5	1:32000	1:8000	1:4000	1:200	0

and 1:32000 for IgM. Titers of IgG anti-Tα and Tβ antibodies were 1:3200–1:12800 (Table 2). There was a slight preference for binding to Tα but the difference between Tα and Tβ anomers did not exceed a twofold dilution except in rat 1. IgM antibodies to the T antigens ranged from 1:800 to 1:3200 with a preference for the Tβ variant. All rats had low GM1 titers (1:200–1:800), but no anti-AGM1 antibodies were detected at dilutions of 1:200 or higher. The rat antibodies bound to deglycosylated KLH at titers of 30000, comparable to their anti-(native KLH) titers. Control rats immunized with CFA only, showed KLH reactivity at titers of up to 1:3200 and T antigen antibody binding at titers of not exceeding 1:800.

Discussion

This study demonstrates that native KLH is highly glycosylated and contains Gal(β1-3)GalNAc determinants that are recognized by PNA and human anti-Gal(β1-3)GalNAc antibodies. The blocking studies show that the human antibodies and PNA bind to the same oligosaccharides. The oligosaccharides are probably linked through a β linkage since IgM antibodies, which react better with Tβ than with Tα, also bind better to KLH. There is no evidence that KLH contains sialic acid or GM1-like oligosaccharides which are not normally associated with glycoproteins.

Immunization with KLH induces mostly antibodies that recognize peptide determinants of KLH and bind to the deglycosylated protein, but in addition there is a significant titer of antibodies that recognize the Gal(β1-3)GalNAc determinant or the T antigen. Although KLH reacts better with human antibodies that recognize the β-linked oligosaccharide as compared to the α-linked anomer, suggesting that Gal(β1-3)GalNAc in KLH is β-linked, immunization with KLH induces the formation of antibodies that recognize both the α- and β-linked anomers. Previous studies also showed that immunization with Tβ induces antibodies that bind equally well to both the α and β configurations [9].

It was previously shown that KLH has a carbohydrate epitope that is cross-reactive with *Schistosoma mansoni* [3], but the structures of the cross-reactive oligosaccharides have not been elucidated. Our findings suggest that an epitope of KLH is cross-reactive with the Thomsen-Friedenreich antigen present on bladder and other adenocarcinomas [6, 7], and that immunization with KLH elicits anti-(T antigen) antibodies. Since the T antigen conjugated to a carrier protein can induce both a humoral and a cellular immune response [5, 15], it could explain the effect of

KLH immunotherapy in bladder carcinoma. The presence of oligosaccharides in native KLH should also be addressed in studies utilizing KLH as a carrier for other oligosaccharide antigens [4].

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