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INTERACTION OF POKEWEED MITOGEN WITH POLY(*N*-ACETYLLACTOSAMINE)-TYPE CARBOHYDRATE CHAINS*,†

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ABSTRACT

The carbohydrate-binding specificities of pokeweed mitogen (PWM) were studied by interaction between ^{125}I-PWM and purified glycoproteins. Human erythrocyte-membrane, band-3 glycoprotein bound ^{125}I-PWM, but other glycoproteins containing triple-branched (tri-antennary)-complex-, double-branched (bi-antennary)-complex-, hybrid-, high-mannose, or small-mucin-type carbohydrate chains failed to bind ^{125}I-PWM. Pretreatment of human erythrocytes with endo-β-D-galactosidase prevented ^{125}I-PWM binding to the erythrocytes, as well as to band-3 glycoprotein from these cells. Poly(*N*-acetyllactosamine) glycopeptides and complex-type glycopeptides purified from band-3 glycoprotein and porcine thyroglobulin, respectively, were labeled at their nonreducing, terminal D-galactosyl or 2-acetamido-2-deoxy-D-galactosyl groups by D-galactose oxidase–sodium borotritide treatment and examined for interaction with agarose-immobilized PWM isolectins. From the binding behavior of these labeled glycopeptides on columns of immobilized-PWM isolectins, and from the interaction of various glycoproteins bearing known carbohydrate chains with ^{125}I-PWM, it was concluded that all three major PWM isolectins (Pa-1, Pa-2, and Pa-4) bind specifically to poly(*N*-acetyllactosamine) structures.

INTRODUCTION

Glycoprotein heterosaccharide chains consisting of repeating disaccharide units of D-galactose and 2-acetamido-2-deoxy-D-glucose were first described by Lloyd and Kabat[1] for the blood-group substance of ovarian cyst fluid. Similar carbohydrate chains have subsequently been found in various tissues and their secretions[2]. Glycans of higher-molecular weight having a similar repeating unit [poly(*N*-acetyllactosamine- or poly(*N*-acetyllactosamino)glycan] have been found in human

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erythrocyte membranes[3–5], as glycolipids[3], and as one of the major carbohydrate chains of the band-3 glycoprotein[6]. The core portion of the carbohydrate chains from band-3 glycoprotein possesses the same structure as other asparagine-linked carbohydrate chains[7], and it carries the blood-group I*i* antigenic activities[6,8]. Similar carbohydrate chains have been reported to serve as differentiation markers of erythroid cells[9] and teratocarcinoma cells[10,11].

Lectins have been used as structural probes for various carbohydrate chains, and we have developed a technique to determine the structural features of various types of carbohydrate chains by the binding of different ^{125}I-labeled lectins after separation of these glycoproteins on polyacrylamide gels[12]. However, poly(*N*-acetyllactosamine)-specific lectins suitable for such analyses have not been described until now, and the study of pokeweed mitogen for this purpose is described herein.

RESULTS AND DISCUSSION

Such glycoproteins as porcine thyroglobulin, bovine serum fibronectin, human serum transferrin, human lactoferrin, bovine fetuin, human α_1-acid glyco-

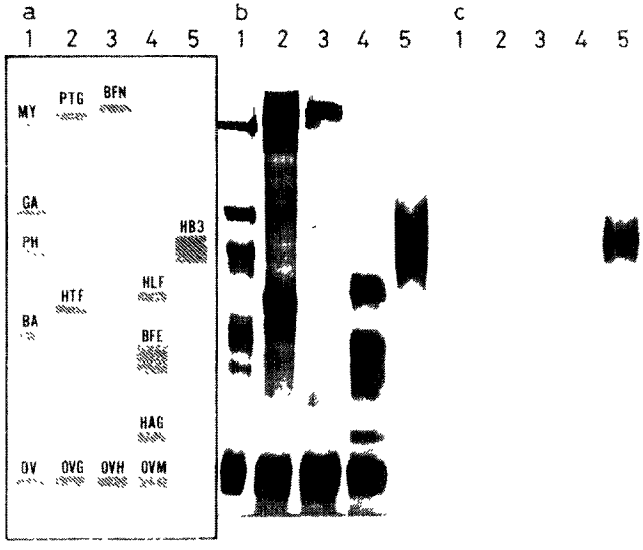


Fig. 1. Electrophoresis of various glycoproteins in 7% polyacrylamide gels in the presence of 0.2% sodium dodecyl sulfate. (a) Theoretical migrating positions of glycoproteins and molecular-weight markers. Lane 1: MY, myosin (M_r ~200 000), GA, β-D-galactosidase from *Escherichia coli* (M_r ~116 000), PH, phosphorylase b (M_r ~93 000), BA, bovine serum albumin (M_r ~66 000), and OV, hen ovalbumin (M_r ~45 000). Lane 2: PTG, porcine thyroglobulin, HTF, human transferrin, and OVG, hen ovalbumin with galactosylated, hybrid-type carbohydrate chains. Lane 3: BFN, bovine serum fibronectin, and OVH, hen ovalbumin with hybrid-type carbohydrate chains. Lane 4: HLF, human lactoferrin, BFE, bovine fetuin, HAG, human β_1-acid glycoprotein, OVM, hen ovalbumin with high-mannose-type carbohydrate chains. Lane 5: HB3, human erythrocyte band-3 glycoprotein. (b) Silver staining pattern of slab gel. (c) Autoradiogram of the same slab gel as in (b) after treatment with ^{125}I-labeled PWM.

protein, subfractionated hen ovalbumins (OVG, OVH, and OVM), and human erythrocyte band-3 glycoprotein (HB3) were separated by polyacrylamide-slab-gel electrophoresis in the presence of sodium dodecyl sulfate. When these glycoproteins, which included complex-type (porcine thyroglobulin, bovine fibronectin, human transferrin, human lactoferrin, bovine fetuin, and HB3), hybrid-type (OVG and OVH), high-mannose-type (porcine thyroglobulin and OVM), small-mucin-type (bovine fetuin), and poly(*N*-acetyllactosamine)-type (HB3) carbohydrate chains, were stained with ^{125}I-labeled pokweed mitogen (^{125}I-PWM), only HB3 bound ^{125}I-PWM before (Fig. 1) or after (not shown) mild-acid treatment to remove sialic acid[13]. This observation is consistent with the findings of Yokoyama *et al.*[14] who showed that HB3 strongly inhibits PWM binding to erythrocytes, even though the structures of its carbohydrate chains were not known at the time. Our results and those of Yokoyama *et al.*[14] suggest that the structure of at least one of the heterogeneous carbohydrate chains[6,7] of HB3 is involved in lectin binding.

Since Yokoyama *et al.*[14] reported that the core portion of the complex-type carbohydrate chains may be important for lectin binding, we examined the effect, on the binding to PWM, of removal of the poly(*N*-acetyllactosamine) portion of the carbohydrate chains with endo-β-D-galactosidase. Human erythrocytes were surface-labeled by D-galactose oxidase treatment followed by reduction with sodium borotritide. When membranes from untreated and endo-β-D-galactosidase-

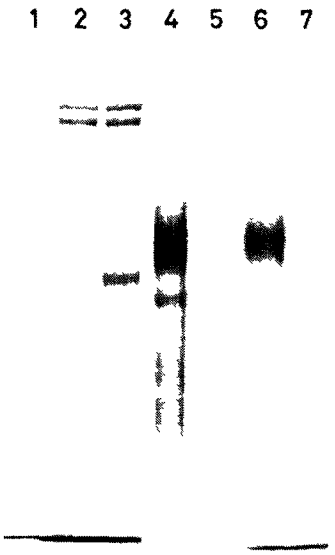


Fig. 2. Polyacrylamide-gel electrophoresis of human erythrocyte membranes in the presence of sodium dodecyl sulfate. All the erythrocytes were labeled by D-galactose oxidase and sodium borotritide treatment. Lane 1: molecular-weight-marker proteins as in Lane 1, Fig. 1. Lanes 2, 4, and 6: untreated erythrocytes. Lanes 3, 5, and 7, endo-β-D-galactosidase-treated erythrocytes. Lanes 2 and 3: Coomassie Brilliant Blue staining. Lanes 4 and 5: fluorograms produced after immersion with Enhance (New England Nuclear). Lanes 6 and 7: autoradiography after staining with ^{125}I-labeled PWM.

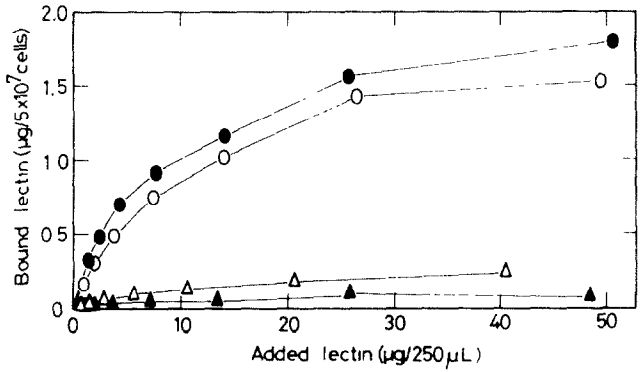
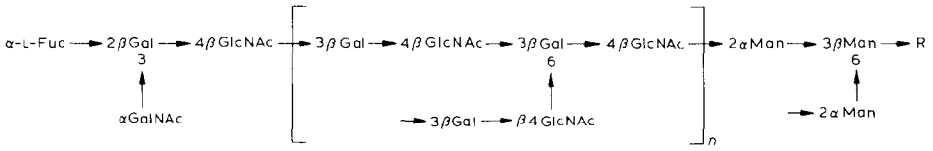


Fig. 3. Binding of ^{125}I-labeled PWM to human erythrocytes (○) untreated adult erythrocytes, (●) adult erythrocytes after treatment with *Vibrio cholerae* neuraminidase (50 mU/0.5 mL of packed cells for 1 h at 37°); (▲) adult erythrocytes after treatment with endo-β-D-galactosidase (100 mU/0.5 mL of packed cells for 3 h at 37°); and (△) cord erythrocytes.

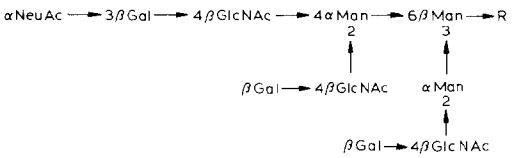
treated erythrocytes were compared after polyacrylamide-gel electrophoresis, the following results were obtained: (a) Staining with Coomassie Brilliant Blue R-250 indicated that, after endo-β-D-galactosidase treatment, HB3 accumulates at a faster moving position, in the broad band-3 region of untreated erythrocytes (Fig. 2), owing apparently to loss of the large (and heterogeneous) portions of the HB3 carbohydrate chains. (b) Fluorography of the gels showed that all labeled terminal β-D-galactosyl or 2-acetamido-2-deoxy-D-galactosyl groups of HB3 had been removed by the enzymic treament; in comparison, tritium-labeled, nonreducing, terminal groups of glycophorin A remained after enzymic treatment (Fig. 2). (c) ^{125}I-PWM bound to untreated HB3 and other components in the band 4.5 region, but failed to bind to any components after treatment of cells with endo-β-D-galactosidase (Fig. 2).

Similar results were obtained in experiments where the direct binding of ^{125}I-PWM to untreated and endo-β-D-galactosidase-treated erythrocytes was measured. After enzymic treatment with endo-β-D-galactosidase, ^{125}I-PWM failed to bind to erythrocytes, and sialidase treatment produced only a slight increase in the binding of ^{125}I-PWM (Fig. 3). Interestingly, the binding of ^{125}I-PWM to umbilical cord erythrocytes was much lower than that to adult erythrocytes (Fig. 3). Prior addition of poly(*N*-acetyllactosamine)-type glycopeptide (HB3 N-I, 2 μg) to PWM (10 μg/250 μL) inhibited 63% of the binding to 5×10^7 untreated, adult human erythrocytes (data not shown). Further inhibition studies have not been performed because of the limited availability of the purified glycopeptide.

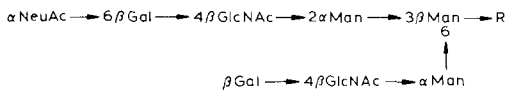
In other experiments, the major isolectins of PWM (Pa-1, Pa-2, and Pa-4) were purified and immobilized on agarose gels in order to examine their binding properties towards various glycopeptides. In order to confirm that the poly(*N*-acetyllactosamine) portion of the HB3 carbohydrate chain is important for interaction with PWM, three classes of glycopeptides including carbohydrate chains of poly(*N*-acetyllactosamine)-type (HB3 N-I, 1), triple-branched, complex-type (por-



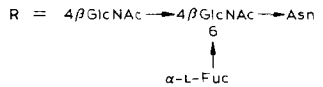
1 (HB3 N-I)



2 (PTG UB-I-a)



3 (PTG UB-I-b)



cine thyroglobulin UB-I-a, **2**), and double-branched complex-type (porcine thyroglobulin UB-I-b, **3**) were examined for their interactions with the immobilized isolectins. Compound **1** bound to all three immobilized isolectins, but smaller, complex-type glycopeptides (**2** and **3**) did not (Fig. 4). Since the core portion of the three carbohydrate chains is the same, the binding of **1** to the PWM isolectins probably occurs through the peripheral portion of the carbohydrate chains. However, the nonreducing, terminal ABH blood-group antigenic structure of **1** is not likely to be involved in the binding of PWM because none of the PWM isolectins are blood-group ABO specific[15]. This conclusion is supported by the observation of Yokoyama *et al.*[14] that hexa-*N*-acetylchitohexaose interacts with PWM, although this lectin possess much greater affinity for HB3 than for the oligosaccharide. They noted that Pa-1 and Pa-2 isolectins possess similar binding specificities[14], and our results confirm their findings and suggest that the specificity of Pa-4 is also similar to that of Pa-1 and Pa-2. From our results, we conclude that the PWM isolectins recognize and bind to poly(*N*-acetyllactosamine). The lower binding to umbilical-cord erythrocytes than to adult erythrocytes suggests that the lectin preferentially binds to the highly branched, blood-group I-antigenic structure that includes the β-D-Glc*p*NAc-(1→6)-D-Gal sequence[16,17].

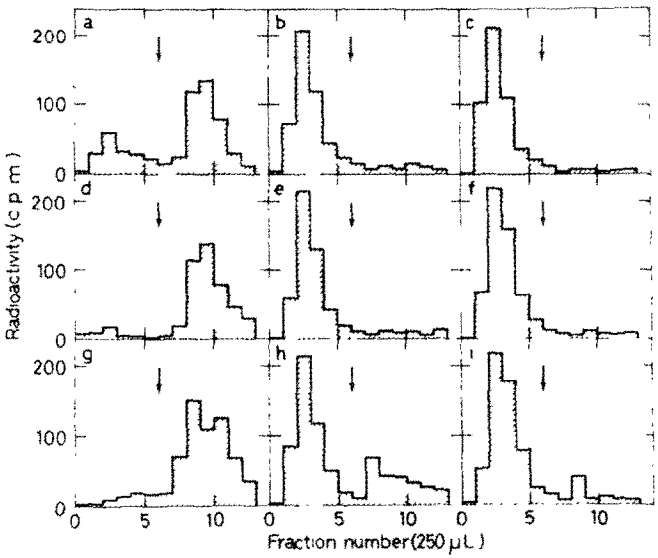


Fig. 4. Affinity chromatography, on PWM isolectins, of glycopeptides labeled by D-galactose oxidase–sodium borotritide treatment. (a), (b) and (c): Interactions of glycopeptides with Pa-1–agarose. (d), (e), and (f): interactions of glycopeptides with Pa-2–agarose; (g), (h), and (i), interactions with Pa-4–agarose. (a), (d), and (g), poly(*N*-acetyllactosamine) glycopeptides **1** (HB3 N-I); (b), (e), and (h), triple-branched, complex-type glycopeptide **2** (PTG UB-I-a), and (c), (f), and (i), double-branched, complex-type glycopeptide **3** (PTG UB-I-b). Arrow indicates change of elution buffer from DPBS to 0.2M $Na_2B_4O_7$ in 0.2M NaOH.

Three complimentary methods have been used to identify poly(*N*-acetyllactosamine) in glycoconjugates: (a) isolation of carbohydrate chains, followed by chemical characterization, (b) use of endo-β-D-galactosidase, and (c) immunochemical characterization by use of anti-blood group I serum. Here, we present another analytical tool, the use of PWM isolectins to probe the structure and the roles of poly(*N*-acetyllactosamine)-bearing glycoproteins. These isolectins may be useful for the separation and characterization of the functions of poly(*N*-acetyllactosamine)-bearing cell populations in mixtures of heterogeneous cells.

EXPERIMENTAL

Materials. — The source and preparation of porcine thyroglobulin, bovine fetuin, human transferrin, bovine fetuin, and subfractionated ovalbumins (OVG, OVH, and OVM), as well as the molecular-weight markers have been previously described[12]. In addition, the following glycoproteins were used: human lactoferrin obtained from Sigma Chemical Co. (St. Louis, MO 63178), human α_1-acid glycoprotein from Miles Laboratories (Naperville, IL 60566), and human erythrocyte, band-3 glycoproteins (HB3) prepared from Rh$^+$, A-type blood as described[6].

Glycopeptides from HB3 were prepared by Pronase treatment (1 mg of Pronase per 5 mg of HB3) in 0.1M borate buffer (pH 8.0) containing 0.2% sodium

azide (1 mL) for 48 h at 37°. After gel filtration on Sephadex G-50 (1.0 × 100 cm column), the phenol–sulfuric acid-positive fractions [18] were pooled, lyophilized, and redigested with Pronase. After the second Pronase treatment, the elution profiles of the glycopeptides were almost identical to the pattern obtained[6] for neutral oligosaccharides from hydrazinolyzates of HB3. The high-molecular-weight carbohydrate chains that contain poly(*N*-acetyllactosamine) (corresponding to N-I) were collected and labeled at the nonreducing, terminal D-galactopyranosyl or 2-acetamido-2-deoxy-D-galactopyranosyl groups by treatment[19] with D-galactose oxidase and reduction with sodium borotritide. The labeled glycopeptide was further fractionated by affinity chromatography on *Ricinus communis* agglutinin I–agarose[6]. Bound glycopeptide, eluted with 0.2M lactose, was used in this study[6]. The average molecular weight of the glycopeptide has been determined as ~8000 by high-speed, gel-permeation chromatography with a system[20] equipped with twin columns (0.7 × 75 cm) of Fractogel-TSK (Toyopearl) HW-55(S) (MCB, Gibbstown, N.J. 08027).

Monosialoglycopeptides from porcine thyroglobulin were fractionated by ion-exchange chromatography after Pronase digestion[21], and labeled by treatment[19] with D-galactose oxidase–sodium borotritide. The labeled glycopeptides were further separated into double- (bi-antennary) and triple-branched (tri-antennary), complex-type glycopeptides on concanavalin A–Sepharose[22]. Their carbohydrate structures appear to be identical to those of the oligosaccharides UB-1-a and UB-1-b previously described[22].

PWM was prepared from pokeweed *(Phytolacca americana)* roots harvested in September at Point Blank, TX. The mitogen was purified and subfractionated into isolectin fractions Pa-1–5 according to Waxdal[15]. The yields of isolectins Pa-3 and Pa-5 were lower than those of the other isolectins, and they were not studied further. Isolectins Pa-1, Pa-2, and Pa-4 gave single bands on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate. PWM was iodinated by the method of Burridge[23] to produce a specific radioactivity of 8 c.p.m./ng of protein. PWM isolectins Pa-1, Pa-2, and Pa-4 were immobilized on Affi-gel 10 beads according to the manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA 94804). The amount of isolectin immobilized was calculated from the amount of unbound isolectin and was found to be 132, 196, and 270 μg/mL of packed agarose beads, respectively.

Enzymic treatment of human erythrocytes. — Adult human erythrocytes were labeled by treatment with D-galactose oxidase–sodium borotritide and then digested with endo-β-D-galactosidase *(Escherichia freundii,* Miles) as described by Fukuda *et al.*[24]. Sialidase (*Vibrio cholerae;* Calbiochem–Behring, La Jolla, CA 92037) treatment was performed with 50 mU of enzyme and 0.5 mL of packed erythrocytes in Dulbecco's phosphate buffered saline (DPBS, 1 mL) for 1 h at 37°.

Polyacrylamide-gel electrophoresis. — Polyacrylamide-gel electrophoresis and subsequent staining with ^{125}I-labeled PWM was carried out as previously described[12]. ^{125}I-PWM at a concentration of 5 μg/mL was used, and the incubation was for 12 h. Mixtures of glycoprotein standards, equivalent for 2 μg each, were

deposited on slab gels as indicated in the legend to Fig. 1. Erythrocyte membranes were prepared by hypotonic lysis in 5mM Tris · HCl buffer (pH 7.5), and then washed with the same solution and dissolved in electrophoresis sample-buffer containing 2% of sodium dodecyl sulfate at a concentration of 10^8 cell equivalents/15 μL; 15 μL of each sample was applied to the gel.

Binding of ^{125}I-labeled PWM to erythrocytes. — Binding experiments were performed in small glass tubes containing serially diluted, ^{125}I-labelled PWM and 5×10^7 erythrocytes in DPBS containing 0.5% of bovine serum albumin (BSA) (Sigma Chemical). Incubations were performed for 12 h. After washing 3 times with 0.5% BSA in DPBS to remove the unbound lectin, the radioactivity was counted with a Beckman Model 8000 gamma counter.

Affinity chromatography of ^{3}H-labeled glycopeptides on immobilized PWM isolectins. — Small columns (0.5 × 2.0 cm) of Pa-1, Pa-2, or Pa-4 isolectins immobilized on agarose were equilibrated with DPBS, and ^{3}H-labeled glycopeptide (~700 c.p.m., equivalent to ~200 ng) was loaded onto each column. After 30 min, elution was performed with DPBS and subsequently with 0.2M disodium tetraborate in 0.2M sodium hydroxide at a flow rate of ~1 mL/h. Each 250-μL fraction was collected and counted with NEN-Formula 963 (New England Nuclear, Boston, MA 02118) after being made neutral with 2M acetic acid, where necessary.

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