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GLYCOLIPID-LECTIN INTERACTIONS: DETECTION BY DIRECT BINDING OF ^{125}I-LECTINS TO THIN LAYER CHROMATOGRAMS

David F. Smith

Department of Biochemistry and Nutrition
Virginia Polytechnic Institute and State University
Blacksburg, VA 24061

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Summary. Glycolipids that bind ^{125}I-labeled lectins are detected by autoradiography after thin layer chromatography of glycolipid standards or crude lipid extracts. Soybean agglutinin, Bandeiraea simplicifolia I isolectins A_4 and B_4, and Helix pomatia lectin are used to detect corresponding cell surface, glycolipid receptors in human and bovine erythrocytes. When lipid extracts from A and AB erythrocyte stroma are analyzed with Helix pomatia lectin, a polymorphic expression of blood group A glycolipid determinants is detected. The Bandeiraea simplicifolia isolectins react weakly with human erythrocyte glycolipids but bind at least 4 glycolipids in bovine stroma extracts. Soybean agglutinin reacts with glycolipids in all erythrocytes analyzed. This technique extends lectin specificity studies from inhibition analyses in aqueous systems using available, known structures to identification of specific, lectin-binding glycolipids in crude lipid extracts of cell membranes.

Introduction. Cell surface glycolipids that bind cholera toxin (1,2) or tumor specific monoclonal antibodies against carbohydrate antigens (3,4) can be detected on thin layer chromatograms using modifications of an autoradiographic assay described by Magnani et al. (1). This technique combines the separating power of thin layer chromatography in non-aqueous systems with the sensitivity and selectivity of radioimmune binding of carbohydrate-specific proteins in aqueous systems. As described in the present paper, this method can be used to detect cell surface glycolipids that specifically bind lectins.

Materials and Methods. Soybean agglutinin, Bandeiraea simplicifolia I isolectins A_4 and B_4 and Helix pomatia lectin were purchased from Sigma, St. Louis, MO. Galactosylceramide[1], lactosylceramide, trihexoside ceramide and globoside were purchased from Supelco, Bellefonte, PA. Human erythrocytes were obtained as out-dated red cell concentrates from Appalachian Red Cross Blood Center, Roanoke, VA. Bovine blood, anticoagulated with heparin

1. Galactosylceramide, Galβ1-1'Cer; lactosylceramide, Galβ1-4Glcβ1-1'Cer; trihexoside ceramide, Galα1-4Galβ1-4Glcβ1-1'-Cer; globoside, GalNAcβ1-3 Galα1-4Galβ1-4Glcβ1-1'-Cer.

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(10 mg/liter blood), was obtained at slaughter from the Department of Food Science and Technology.

Erythrocytes were washed three times with 0.01 M Sodium phosphate, 0.14 M NaCl, 0.003 M KCl, pH 7.2 and membranes were prepared after hypotonic lysis in 0.005 M NaH_2PO_4, pH 6.8 (5). Lipid extracts were prepared from lyophilyzed membranes using a procedure developed for the extraction of brain gangliosides with minimal protein contamination (6). Briefly, 5 ml distilled H_2O (4°C) are added to lyophilyzed membrane (250 mg). The mixture is homogenized (4°C) for 30 sec using a Tissuemizer, (Tekimar Co., Cincinnati, OH) and added to 13 ml of methanol with constant stirring at room temperature. Chloroform (6.5 ml) is added, and stirring is continued for 30 min at room temperature. The suspension is centrifuged (2,000 xg, 20 min), and the pellet is subjected to the same procedure. The supernatants are combined, evaporated to dryness under reduced pressure, and dissolved in chloroform-methanol (2:1) so that 1 ml of solution is equivalent to 100 mg lyophilyzed membrane.

Lectins are dissolved (1 mg/ml) in 0.1 M NaH_2PO_4, pH 6.8 and stored at -20°C until used. $CaCl_2$ (0.1 mM) is included in buffers used with the Bandeiraea simplicifolia isolectins. Lectins are radiolabeled by a modification of an Iodogen-catalyzed reaction (7). Polypropylene screw-cap micro tubes (Sarstedt, Princeton, NJ) are coated with 25 µl of an IODO-GEN™ (Pierce Chem. Co., Rockford, IL) solution (1 mg/ml in CH_2Cl_2) by rotating in a 37°C water bath until solvent is evaporated. Lectin (20 µl) and $Na^{125}I$ (0.5 mCi, 5 µl, Amersham, Arlington Heights, IL) are added and the tubes are rotated every 2 min during 15 min incubation at room temperature. The ^{125}I-labeled lectins are separated from $Na^{125}I$ by gel filtration on PD-10 prepacked columns (Pharmacia, Uppsala, Sweden) equilibrated in 0.01 M NaH_2PO_4, 0.14 M NaCl, 1% polyvinylpyrrolidone (M_r 40,000, Sigma, St. Louis, MO), pH 7.2. The specific activities of labeled lectins are as follows: Helix pomatia lectin, 25 µCi/µg; Bandeiraea simplicifolia $I\text{-}A_4$, 12 µCi/µg; $-B_4$, 20 µCi/µg; and soybean agglutinin, 11 µCi/µg.

Thin-layer chromatography is carried out on plastic-backed Eastman chromagram sheets, 13179 Silica gel, (Kodak, Rochester, NY). The chromatography sheets (10 x 20 cm) are scored to form twenty 1 x 10 cm lanes. After samples (1-5 µl) are applied, the chromatogram is developed in a sandwich chamber (Kodak) as previously described (1) in solvents: (A) chloroform-methanol-water (5:4:1) or (B) chloroform-methanol-water (70:27:5). After drying, 1 x 10 cm strips are cut, sprayed with 0.01 M NaH_2PO_4, 0.14 M NaCl, 2% polyvinylpyrrolidone, pH 7.2 to produce even wetting and immediately placed in the same solution for 15 min at 4°C. After excess solution is drained from the strips, they are placed on a glass plate and layered individually with ^{125}I-lectin (50 µl/cm^2, 1.0 x 10^6 cpm/ml) and allowed to react for 3 hr at 4°C. The ^{125}I-lectin solution is removed from each strip and the strips are then transferred to tubes (15 x 125 mm) containing 0.01 M NaH_2PO_4, 0.14 M NaCl, pH 6.8 and allowed to stand for 2 min at 4°C. This step is repeated 5 times to remove excess labeled lectin. The strips are then air dried and autoradiography is carried out as previously described (4) using a Cronex intensifying screen (Dupont, Wilmington, DE) for 18 hr at -75°C.

Results. After thin layer chromatographic separation, glycolipid standards are analyzed for their interaction with four D-Gal- and/or D-GalNAc-binding, ^{125}I-labeled lectins. Soybean agglutinin selectively binds trihexoside ceramide and globoside; binding to lactosylceramide or galactosylceramide is not observed under these conditions (Fig. 1A, lanes 1 and 2). The human

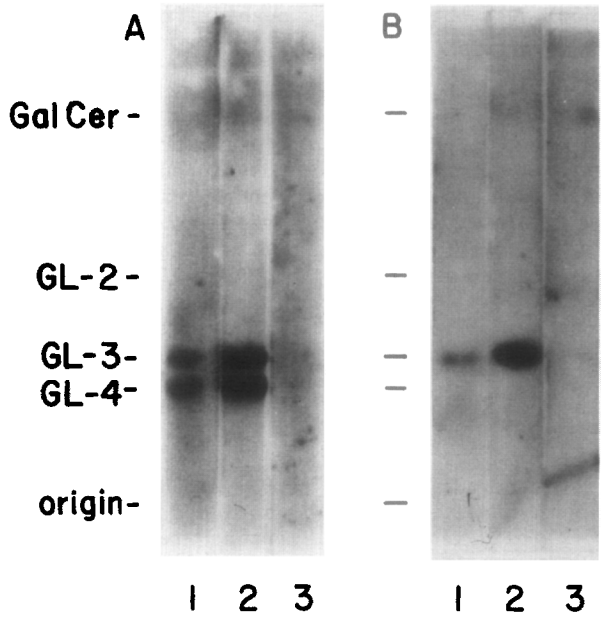

Fig. 1. Binding of ^{125}I-lectins to glycolipids on a thin layer chromatogram. (A) Lanes 1 and 2, autoradiograph of glycolipid standards (galactosylceramide, Gal-Cer; lactosylceramide, GL-2; trihexoside ceramide, GL-3; and globoside, GL-4) at 1 and 4 µg, respectively, after treatment with ^{125}I-soybean agglutinin; Lane 3, autoradiograph of glycolipid standards at 4 µg each after treatment with ^{125}I-*Helix pomatia* lectin. (B) Lanes 1 and 2, autoradiograph of glycolipid standards at 1 and 4 µg, respectively, after treatment with ^{125}I-*Bandeiraea simplicifolia* I isolectin A_4; Lane 3, autoradiograph of glycolipid standards at 4 µg each after treatment with ^{125}I-*Bandeiraea simplicifolia* I isolectin B_4. Chromatography was carried out in solvent A. Position of standards detected by orcinol spray reagent (11) are indicated.

blood group A specific, *Helix pomatia* lectin does not bind the glycolipid standards tested (Fig. 1A, lane 3). This is consistent with the absence in these structures of terminal α-D-GalNAc residues necessary for binding by this lectin (8). Trihexoside ceramide is the only glycolipid standard tested that binds either *Bandeiraea simplicifolia* I isolectins under the conditions of this assay (Fig. 1B).

Figure 2 shows ^{125}I-lectins binding to their corresponding glycolipid receptors in a total lipid extract of bovine erythrocyte stroma. No binding by *Helix pomatia* lectin is observed (not shown). Lipid extracts of human erythrocyte stroma from blood groups A, B, O and AB are chromatographed and analyzed for glycolipids that bind ^{125}I-labeled *Helix pomatia* lectin (Fig. 3A) and soybean agglutinin (Fig. 3B). *Bandeiraea simplicifolia* I

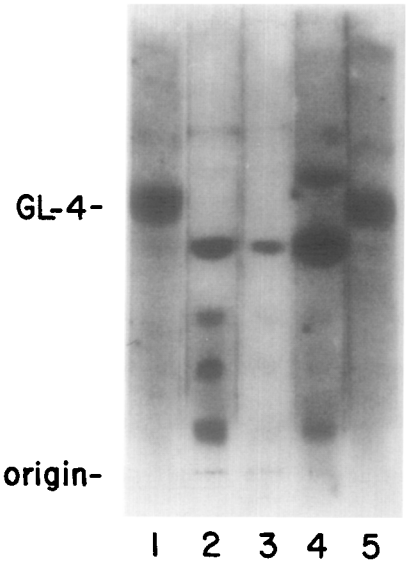

Fig. 2. Binding of ^{125}I-lectins to glycolipids on a thin layer chromatogram of total lipid extracted from bovine erythrocyte stroma. The globoside standard (5 µg) is detected with ^{125}I-soybean agglutinin (lanes 1 and 5). Lanes 2-4 are aliquots of total lipid extract equivalent to 8 µl packed bovine erythrocytes. Lane 2, treated with ^{125}I-*Bandeiraea simplicifolia* I-A_4; Lane 3, with ^{125}I-*Bandeiraea simplicifolia* I-B_4; Lane 4, with ^{125}I-soybean agglutinin. Chromatography is carried out in solvent B.

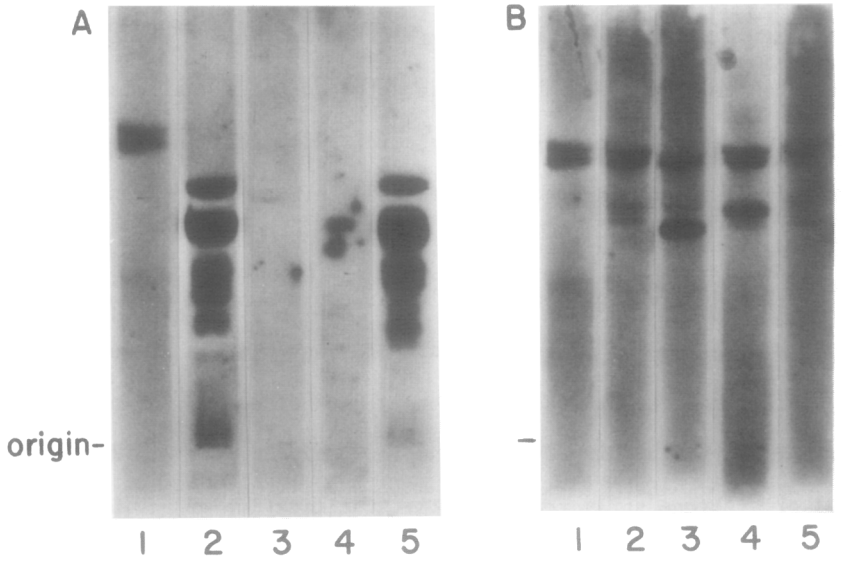

Fig. 3. Binding of ^{125}I-*Helix pomatia* lectin and ^{125}I-soybean agglutinin to human erythrocyte glycolipids. Standard (lanes 1) globoside and trihexoside ceramide (0.25 µg each) and aliquots of total lipid extracts equivalent to 10 µl of packed human A, B, O or AB erythrocytes are subjected to thin layer chromatography (solvent B) in lanes 2-5, respectively. (A) Autoradiograph after treatment with ^{125}I-*Helix pomatia* lectin. (B) Autoradiograph after treatment with ^{125}I-soybean agglutinin.

isolectins A_4 and B_4 demonstrated only very weak binding to glycolipids in extracts from human erythrocytes (data not shown). Binding by all labeled lectins is abolished by including 0.1 mM D-GalNAc in the ^{125}I-lectin solution.

Discussion. The selectivity of soybean agglutinin for globoside and trihexoside ceramide over lactosyl- and galactosylceramide (Fig. 1A, lanes 1 and 2) was also observed in a direct binding assay of ^{125}I-soybean agglutinin to pure glycolipids in sodium dodecyl sulfate-polyacrylamide gels (9). Relative to soybean agglutinin Bandeiraea simplicifolia I isolectin A_4 reacts weakly with 1 µg trihexoside ceramide (Fig. 1B, lane 1); isolectin B_4 does not react (lane 3). As little as 0.25 µg of globoside or trihexoside ceramide is detected with ^{125}I-soybean agglutinin (Fig. 3, lanes 1).

Soybean agglutinin reacts with at least 3 glycolipids in the total lipid extract of bovine erythrocyte stroma; two of these glycolipids also bind isolectins A_4 and B_4 (Fig. 2, lanes 2-4). Although the isolectin B_4 reaction is much weaker than the A_4 reaction with the bovine stroma lipid extract, the pattern is identical (Fig. 2, lanes 2 and 3). Although no cross-contamination of subunits was detected after analysis of A_4 and B_4 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10), more detailed studies will be required to determine if A_4 and B_4 are reacting with the same glycolipids.

^{125}I-Labeled lectins can detect their corresponding cell surface, glycolipid receptors on thin layer chromatograms with an aliquot of total lipid extract equivalent to approximately 8 µl of packed bovine erythrocytes. At ten times this level of sample no glycolipids are detected by orcinol or anthrone spray reagent (11). Purification of bovine erythrocyte glycolipids is in progress to determine if the glycolipids detected by ^{125}I-lectins are major bovine erythrocyte glycolipids or if they represent previously undetected structures.

The polymorphic expression of blood group active glycolipids has been demonstrated by structural analyses of individual glycolipid variants

isolated from erythrocyte stroma (12-14). The polymorphic expression of blood group A active glycolipids is demonstrated with ^{125}I-Helix pomatia lectin (Fig. 3A, lanes 2 and 5). The aliquots of lipid extracts analyzed are equivalent to approximately 10 µl of packed human erythrocytes. The A-active glycolipids from types A and AB stroma are resolved into at least 5 variants. The autoradiograms of types A and AB are similar (Fig. 3, lanes 2 and 5), as expected, since both blood types carry the A-antigen on similar glycolipid species (15). No reaction is observed with type B stroma extracts and a single, weak reaction is seen with type O extracts. Although the Bandeiraea simplicifolia I isolectin B_4 has been described as a type B-specific lectin (16), its reaction with extracts of human stroma using this method is weak and not conclusive (data not shown).

In spite of soybean agglutinin's specificity for D-Gal and D-GalNAc (8), it does not bind the blood group active glycolipids detected by Helix pomatia lectin which have non-reducing α-D-GalNAc and α-D-Gal residues (compare Fig. 3A and 3B, lanes 2 and 5). This may be due to the extremely low amounts of blood group active glycolipids and higher affinity of the multivalent Helix pomatia lectin (8). Glycolipid receptors for soybean agglutinin are detected in all human erythrocyte stroma extracts which is consistent with its ability to agglutinate all human blood types (17). The fastest migrating glycolipids detected by ^{125}I-soybean agglutinin (Fig. 3B) co-migrate with trihexoside ceramide and globoside, the major glycosphingolipids of human erythrocyte stroma (18). Whether the differences in soybean agglutinin binding to glycolipids in extracts of type B and O (Fig. 3B, lanes 3 and 4) are individual variations or are blood group dependent requires further investigation.

Lectins can bind oligosaccharides of cell surface glycoproteins and glycolipids. In some cases the oligosaccharide structure involved is not the same as the best monosaccharide inhibitor of the lectin-binding reaction. This is certainly the case with the lectins of Phaseolous vulgaris (19), pea and lentil lectins (20) and concanavalin A (21) whose oligosaccharide

receptors have been structurally defined and found to be more complex than their best monosaccharide inhibitors (19-21). In these studies radiolabeled glycopeptides or free oligosaccharides were purified by lectin-affinity chromatography. This approach is not applicable to structural studies of glycolipid lectin receptors since glycolipids form mixed micelles in aqueous systems.

With the thin layer chromatogram binding assay, lectins can be used to detect, isolate and characterize their corresponding cell surface, glycolipid receptors in the same way monoclonal antibodies against glycolipid antigens (3,4,22,23) have been used to detect, isolate and identify tumor specific antigens (4,23).

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