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The Glycolipid Specificity of *Erythrina cristagalli* Agglutinin

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The interaction of ^{125}I-labeled *Erythrina cristagalli* agglutinin (ECA) with neutral glycosphingolipids on thin layer chromatograms was examined by the overlay technique followed by radioautography. The lectin bound to *para*-globoside with a sensitivity about 10 times higher than to lactosylceramide or globoside, in agreement with the specificity of the lectin for *N*-acetyllactosamine. The lower limit of detection of *para*-globoside was about 0.66 nmol. The specific binding of ECA to this glycolipid was confirmed by a highly sensitive enzyme-linked lectin assay (ELLA), utilizing the horseradish peroxidase-avidin-biotin system for detection of bound lectin. Overlays of neutral glycosphingolipid extracts from human erythrocyte membranes and from human granulocytes with ECA demonstrated that the lectin can be employed for the detection of small amounts of *para*-globoside in biological materials also in the presence of excess globoside. No staining was obtained when thin layer chromatograms of neutral glycosphingolipid extracts from rabbit erythrocyte membranes were overlayed with ^{125}I-ECA. After *in situ* treatment of the chromatograms with α-galactosidase, the lectin bound to several components, one of which had a mobility corresponding to that of the pentahexosylceramide Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer, the major neutral glycosphingolipid of rabbit erythrocytes, thus providing further evidence for the specificity of ECA for *para*-globoside.

Abbreviations: GSL, glycosphingolipid(s); CDH, lactosylceramide, Galβ4Glcβ1Cer; CTH, trihexosylceramide, Galα4Galβ4Glcβ1Cer; GLOB, globoside, GalNAcβ3Galα4Galβ4Glcβ1Cer; PG, *para*-globoside, Galβ4GlcNAcβ-3Galβ4Glcβ1Cer; AsG_{M1}, asialo-G_{M1}, Galβ3GalNAcβ4Galβ4Glcβ1Cer; FORS, Forsmann antigen, GalNAcα3-GalNAcβ3Galα4Galβ4Glcβ1Cer; CPH, pentahexosylceramide, Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer; ECA, *Erythrina cristagalli* agglutinin; SBA, soybean agglutinin; PBS, phosphate-buffered saline; PVP-40, polyvinylpyrrolidone M.W. 40 000; BSA, bovine serum albumin; HRP-avidin, horseradish peroxidase conjugated to avidin; ELLA, enzyme-linked lectin assay; ELISA, enzyme-linked immunosorbent assay; PMNL, polymorphonuclear leukocytes; HPTLC, high performance thin layer chromatography.

All sugars are in the D-configuration.

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Lectins are extremely useful tools for the detection, isolation and structural studies of glycoproteins, but their application to the investigation of glycolipids has until recently been limited. This was largely due to the lack of suitable methods for the examination of the interactions of glycolipids with lectins. The situation changed with the introduction in 1980 by Magnani *et al.* [1] of the solid phase autoradiographic ("overlay") technique for the detection of glycosphingolipids (GSL) on thin-layer plates after chromatography. Developed originally to reveal the interaction of GSL with toxins and antibodies, it was soon adapted to lectins as well. Another solid-phase method now available for the visualization of GSL-lectin interactions is ELLA (enzyme linked lectin assay) [2, 3]. This is an adaptation of ELISA (enzyme linked immunosorbent assay) and involves the binding of lectins to glycolipids immobilized on plastic surfaces by hydrophobic adsorption from solution [4]. In contrast to the overlay method, ELLA is not suitable for GSL mixtures but for individual, purified GSL components only. The introduction of the solid-phase methods opened the way for the wide application of lectins for the study of GSL.

Thus, soybean agglutinin (SBA), shown to bind preferentially to globoside and trihexosyl ceramide, has been utilized to examine GSL from bovine erythrocytes [5] and to reveal differences in the surface glycolipids of cultured adrenergic and cholinergic sympathetic neurons [6]. The lectin from *Helix pomatia* did not bind to neutral GSL from human B and O type erythrocytes, but bound to five components in lipid extracts from A and AB type cells [5]. This is in agreement with the A blood group specificity of the lectin and confirms the polymorphic expression of blood group active glycolipids. The B_4 lectin from *Vicia villosa* bound strongly to globoside but not to other major neutral GSL, such as lactosylceramide, trihexosylceramide and *para*-globoside [7]. In extracts from erythrocytes of the rare blood group Cad the lectin bound to a ganglioside apparently characteristic of these cells, in line with the specific interaction of the lectin with Cad erythrocytes. The strong binding of peanut agglutinin to asialo-G_{M1} has been demonstrated in two independent studies [2, 8], in accordance with the specificity of the lectin for Galβ3GalNAc, the terminal disaccharide of this glycolipid. Other lectins investigated for their interaction with GSL's are wheat germ agglutinin and the α-L-fucose specific lectin from *Ulex europeus* [2].

In previous studies we characterized the sugar specificity of the lectin from *Erythrina cristagalli* (ECA) with respect to a large number of mono- and oligosaccharides, as well as glycopeptides [9-12]. Here we report on the interaction of the lectin with authentic neutral GSL, with glycolipid extracts from human and rabbit erythrocytes, as well as from human polymorphonuclear leukocytes (granulocytes). We also compare the glycolipid specificity of ECA with that of SBA, both lectins belonging to the GalNAc/Gal specificity group [13]. A preliminary report of these experiments has been presented [14].

Materials and Methods

Chemicals and Reagents

The lectins from *E. cristagalli* [9] and soybean [15] were purified by affinity chromatography as described. Carrier-free $Na^{125}I$ was obtained from The Radiochemical Centre, Amersham, England. Polyvinylpyrrolidone M.W. 40 000 (PVP-40), bovine serum albumin

(BSA), lecithin, cholesterol, 2,2'-azino-di(3-ethylbenzthiazoline) sulfonic acid, sodium taurocholate and α-galactosidase from coffee beans were purchased from Sigma (St. Louis, MO, USA). Horseradish peroxidase-avidin (HRP-avidin) was obtained from BioYeda, Rehovot, Israel. Sephadex G-50, Sephadex LH-20, DEAE-Sephadex A25, Ficoll-Hypaque and Percoll were from Pharmacia, Uppsala, Sweden. Iatrobeads were from Iatro Laboratories (Tokyo, Japan) and polyisobutylmethacrylate from Polysciences, Warrington, PA, USA. Biotinylated ECA [16] was a kind gift from Dr. E.A. Bayer from our Department. Individual glycosphingolipid standards were gifts from Drs. H. Egge, S. Hakomori, K-A. Karlsson, B. Kniep, S. Kundu, and Y. Nagai.

Iodination

Iodination of the lectins was carried out by a modification of the chloramine T method [17]. The protein (50 μg) was dissolved in 100 μl sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS) and 0.5 mCi of $Na^{125}I$ was added. The reaction was started by the addition of 20 μl of freshly prepared chloramine T solution (0.5 mg/ml) and was allowed to proceed for 1 min at room temperature. It was stopped by the addition of 20 μl sodium metabisulfite (0.5 mg/ml), followed by 20 μl of potassium iodide (10 mg/ml). Excess $Na^{125}I$ was removed by gel filtration on a Sephadex G-50 column (1 × 10 cm) in equilibrium with 0.05 M sodium phosphate buffer, pH 7.4, containing 0.1% BSA. The iodinated lectins (7-10 × 10^6 cpm/μg) were stored in the same buffer at -20°C.

Isolation of Cells

Human erythrocytes were isolated from slightly outdated bank blood and rabbit erythrocytes from freshly drawn blood. Polymorphonuclear leukocytes (PMNL) were isolated from fresh human blood obtained from healthy donors. The buffy coat was diluted with an equal volume of PBS containing 5% dextran and sedimented at unit gravity for 30 min at 37°C. The leukocyte rich supernatant was collected, washed twice with PBS and resuspended in PBS. The cell suspension was further purified on a discontinuous Percoll gradient [18]. Purity of the cells was >95% as determined by Giemsa staining [19].

Hapten Inhibition Studies

Inhibition of the hemagglutinating activity of ECA by sugars was carried out as described previously [9].

Preparation of Erythrocyte Ghosts

Ghosts of human and rabbit erythrocytes were prepared by the method of Fairbanks *et al.* [20] and stored in lyophilized form.

Preparation of Neutral Glycosphingolipids

Erythrocyte ghosts or PMNL from one portion of blood were extracted sequentially with chloroform/methanol, 2/1, 1/1 and 1/2 by vol, and the extracts were combined and evaporated [21]. The dry residue was fractionated according to the method of Folch *et al.* [22], the lower phase was evaporated, the residue was dissolved in chloroform/me-

thanol/water, 30/60/8 by vol, and separated into neutral and acid glycolipids by chromatography on a DEAE-Sephadex A-25 column in acetate form [23]. The solvent system chloroform/methanol/water, 30/60/8 by vol, was used for elution of neutral GSL and chloroform/methanol/0.8 M aqueous NaOAc, 30/60/8 by vol, for the elution of gangliosides. The fraction containing the neutral GSL was evaporated to dryness, dissolved in 3 ml of 0.3 M KOH in methanol/water, 95/5 by vol, and submitted to methanolysis for 2 h at 37°C [23]. The insoluble residue was removed by centrifugation and the supernatant was desalted on a column (13.5 × 500 mm) of Sephadex LH-20 with the solvent chloroform/methanol/water, 5/5/1 by vol. Final traces of impurities were removed by chromatography on an Iatrobead column. The sample was applied in chloroform/methanol, 85/15 by vol, the column was washed with chloroform and elution of the purified GSL was done with chloroform/methanol, 1/1 by vol.

Quantification of Glycosphingolipids

Hexoses in total extracts of GSL were determined by the orcinol method [24] with a 2:1 mixture of galactose and glucose as a standard. The amounts of individual GSL were calculated from their content of carbohydrates determined by gas chromatography in the form of trimethylsilyl derivatives [25].

Lectin Binding Assay

Binding of ECA to GSL was measured by ELLA essentially as described by Molin *et al.* [2]. Briefly, the wells of polyvinylchloride microtiter plates were coated with 20 μl of serial dilutions of GSL in methanol containing lecithin (0.1 mg/ml) and cholesterol (0.06 mg/ml). The lipids were adsorbed by evaporating the solvent *in vacuo* at room temperature, after which the plates were incubated for 15 min with 200 μl of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.14 M NaCl, 1% BSA and 0.01% merthiolate (buffer A) to block unspecific binding of the lectin. The buffer was aspirated, 50 μl of the same buffer containing biotinylated ECA (5 μl/ml) was added to each well and the plate, covered with parafilm, was incubated overnight at 4°C. After washing the wells four times with 0.01 M sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl (buffer B), 50μl of an HRP-avidin solution (5 μg/ml) in buffer A was added. After 1 h at room temperature the plate was washed four times with buffer B, and 100 μl of a substrate solution [2,2'-azino-di(3-ethylbenzthiazoline) sulfonic acid, 0.25 mg/ml of 0.05 M citric acid-Na_2HPO_4 buffer, pH 4.0, containing 0.005% H_2O_2] was added. Incubation was carried out at room temperature for about 15 min and the absorbance at 405 nm read immediately in an Autoreader Model EL 310, Bio-Tek Instruments, Vermont, USA.

Lectin Overlay of Thin-layer Chromatograms

TLC was done on aluminium-backed silica gel 60 high performance plates (Merck, Darmstadt, W. Germany) using chloroform/methanol/water, 60/30/5 by vol, as solvent. Glycolipids were revealed by iodine vapour and orcinol spray. Overlay of the thin layer chromatograms with the iodinated lectins was performed essentially as described [26]. After drying, the developed plates were dipped in a 0.1% solution of polyisobutylmethacrylate in ether, air dried and made hydrophilic by soaking for 2 h at 4°C in PBS containing 2% BSA, 2% PVP-40 and 0.01% NaN_3. The overlay was performed with radio-iodinated lectin, 70-80 μl per cm^2 of chromatogram area, 1×10^6 cpm/ml in the same buffer. The

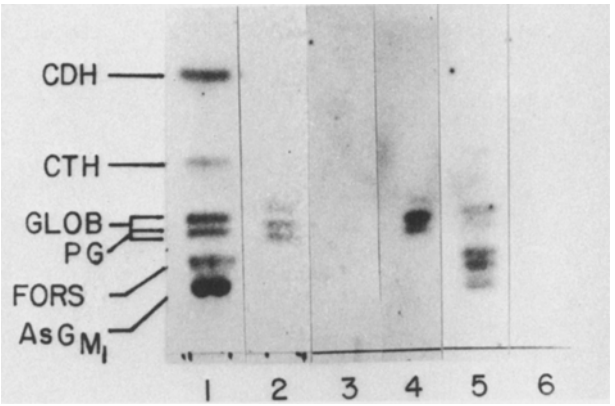

Figure 1. Binding of ^{125}I-labeled ECA and of ^{125}I-labeled SBA to GSL after HPTLC. Lanes 1,3,5; standard mixture containing lactosylceramide, trihexosylceramide, globoside, Forssman antigen and asialo-G_{M1}, 2.8 nmol each: lanes 2,4,6; *para*-globoside, 2.8 nmol. The chromatograms were developed with chloroform/methanol/water, 60/30/5 by vol. Lanes 1,2; staining with orcinol: lanes 3,4; autoradiogram of ECA overlay: lanes 5,6; autoradiogram of SBA overlay.

overlayed plates were incubated for 18 h at 4°C in a humidified atmosphere, washed in cold PBS containing 0.05% PVP-40 and exposed to Agfa-Gevaert Curix X-ray films for 18 h at -70°C. For quantification of binding, the plates were scanned in a Video Densitometer (Bio-Rad Model 620, Richmond, CA, USA) and the area corresponding to each spot was recorded.

α-Galactosidase Treatment of Glycolipids on Plates

After TLC the dried plates were dipped in a solution of 0.5% polyisobutylmethacrylate in ether (w/v) and air dried. They were then placed in a petri dish (5 cm diameter), covered with 2.5 ml 0.05 M sodium citrate buffer, pH 5.0, containing sodium taurocholate (1 mg/ml) and one unit of α-galactosidase, incubated for 1 h at room temperature and then for 3 h at 37°C without shaking. Fresh enzyme (one unit) was added and incubation continued at room temperature for 24 h. The plate was removed from the dish and gently washed 4-5 times with PBS.

Results and Discussion

The binding of ECA to commonly occurring neutral GSL is shown in Fig. 1. As can be seen, with the amounts used (2.8 nmol of each GSL), only *para*-globoside, can be detected. In contrast, SBA binds to several of the GSL (Fig. 1, lane 5). Upon longer exposure, lactosylceramide could also be discerned, in addition to the GSL seen in the Figure (not shown). In all cases tested, binding was completely abolished in the presence of 0.4 M lactose. The lower limit of detection of *para*-globoside by ECA was 0.66 nmol (Fig. 2). The intensity of staining with the lectin, measured by densitometric

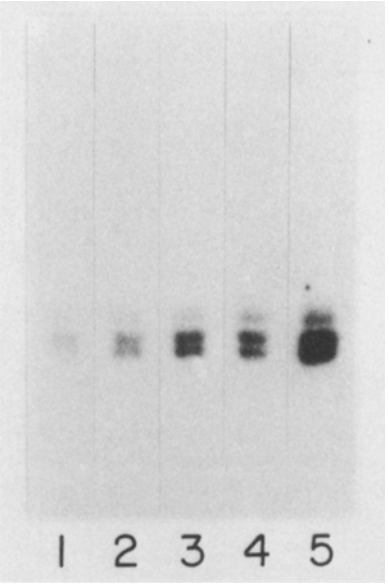

Figure 2. Autoradiogram of increasing amounts of *para*-globoside overlayed with ^{125}I-ECA. Lane 1, 0.66 nmol; lane 2, 1.3 nmol; lane 3, 2 nmol; lane 4, 2.6 nmol; lane 5, 3.3 nmol.

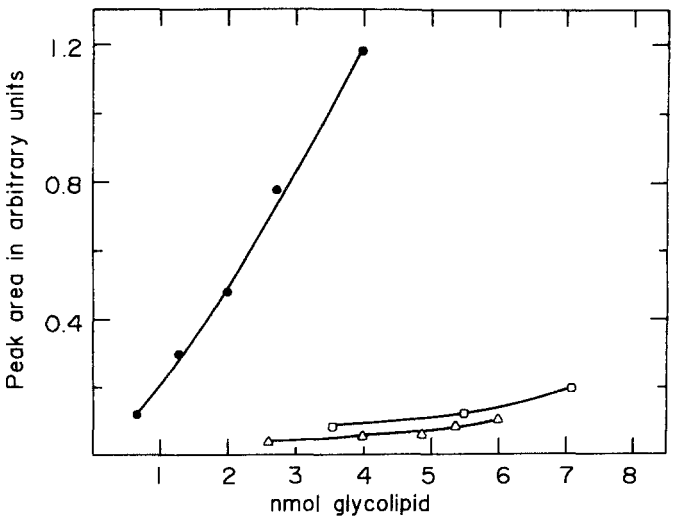

Figure 3. Densitometric scan of autoradiograms of increasing amounts of GSL overlayed with ^{125}I-ECA. ●, *para*-globoside; □, lactosylceramide; △, globoside.

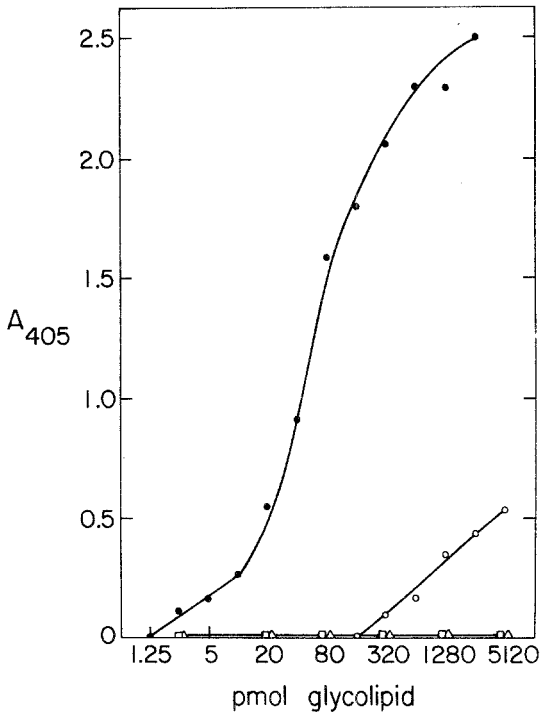

Figure 4. Binding of ECA to GSL measured by ELLA. ●, *para*-globoside; △, globoside; □, trihexosylceramide; ○, lactosylceramide.

scanning of the autoradiograms, gave a nearly linear response from 0.66 to 4 nmol (1-6 μg) of *para*-globoside (Fig. 3). The slope of the curve was very steep, indicating a high affinity of the lectin for this GSL. At about 3-4 nmol, weak staining of lactosylceramide and globoside could also be discerned on the densitometric scan. It should be noted, however, that the slopes of the density curves for these GSL, in particular that for globoside, are very gradual, indicating weak binding. No binding to trihexosylceramide was observed even at 6 nmol, the highest amounts examined.

Further studies on the binding of ECA to neutral GSL were performed by ELLA using the biotinylated lectin and HRP-avidin (Fig. 4). This method is much more sensitive than the overlay technique and permitted the detection of as little as 2.5 pmol of *para*-globoside. Again, the slope of the curve was very steep and above 160 pmol, the response was out of scale. Binding to lactosylceramide was observed starting with 320 pmol of glycolipid. In contrast, no interaction with globoside or trihexosylceramide was seen even at 5 nmol, the highest concentration tested. The relative amounts of the various GSL detected differed in the two techniques (e.g. the ratio *para*-globoside:lactosylceramide was 5 for the overlay technique and 60 for ELLA), demonstrating that the results might depend on the assay system used - an observation also made by others [2]. Thus, comparisons of the interactions of a lectin with different glycolipids are valid only when made for the same type of assay.

Table 1. Inhibition by various sugars of the hemagglutinating activity of *Erythrina cristagalli* lectin.

Carbohydrate	Relative inhibitory activity[a]
Galactose	1.0
Fucose	0.5
N-Acetylgalactosamine	2.0
Galβ4Glc (lactose)	7.5
Galβ4GlcNAc (*N*-acetyllactosamine)	35
Galα4FucβOMe	4
Galα4GalβOMe	2.5
Fucα4GalβOMe	2
Fucα4FucβOMe	2

[a] The inhibitory activity of galactose is arbitrarily set as 1.0. The minimal galactose concentration required to give complete inhibition of 4 hemagglutinating doses of the lectin was 0.012 M.

The pronounced affinity of ECA for *para*-globoside is in agreement with the high specificity of ECA for *N*-acetyllactosamine (which constitutes the nonreducing end of the GSL) as determined previously by hapten inhibition experiments and by direct binding studies [9-11]. Similarly, the much weaker interaction with lactosylceramide and the even poorer binding to globoside are in line with the decreasing strength of binding to the lectin of lactose and *N*-acetylgalactosamine (the terminal non-reducing residues of lactosylceramide and globoside, respectively) [9-11]. However, the difference in the amount of *para*-globoside that can be detected with the aid of ECA, as compared to the other neutral GSL is larger than can be expected from the relative inhibitory activities of the various sugars. This could be due to the fact that the hapten inhibition assay is performed with sugars in solution, whereas in the studies described here the sugars are immobilized and, as a result, their steric configuration and accessibility may have been altered [2]. No data on the affinity of ECA for Galα4Gal, the terminal sequence of trihexosylceramide, have hitherto been available. In order to establish whether the lack of binding of ECA to this GSL reflects a very low affinity of the lectin for Galα4Gal, we carried out hapten inhibition studies with this and related disaccharides (Table 1). As can be seen, the inhibitory power of Galα4Gal is very similar to, and even slightly higher than, that of *N*-acetylgalactosamine. The lack of recognition of the disaccharide by the lectin under the conditions of the solid phase assay seems to stress the fact that the effect of immobilization on the interaction of a lectin with a carbohydrate varies with the carbohydrate under investigation.

The very high specificity of ECA for *para*-globoside as compared to that for globoside should make this lectin useful for the detection of small amounts of the former GSL in the presence of an excess of the latter. This is usually a task made difficult by the poor resolution of these compounds by commonly used chromatographic techniques. We tested the applicability of this approach with extracts of neutral GSL from human erythrocyte membranes rich in globoside and poor in *para*-globoside (> 60% and 4%, respectively, of neutral GSL [27]). Increasing amounts of the GSL were applied to thin-layer plates and chromatographed, and the dried plates were overlayed with radioiodinated ECA (Fig. 5). Already with 25 μg of neutral GSL, containing about 1 μg of *para*-globoside and 15 μg of globoside, *para*-globoside can be clearly identified.

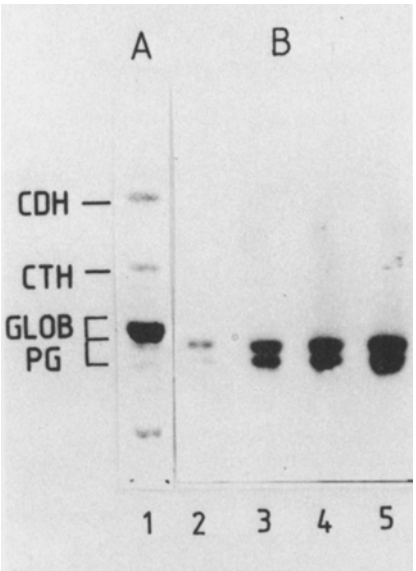

Figure 5. Binding of ^{125}I-ECA to increasing amounts of neutral GSL extracts from human erythrocyte membranes separated by HPTLC. Solvent system - chloroform/methanol/water, 60/30/5.5 by vol. Panel A: staining with orcinol: panel B; ^{125}I-ECA overlay. Amounts of GSL applied: lane 1, 20 μg; lane 2, 10 μg; lane 3, 20 μg; lane 4, 30 μg; lane 5, 40 μg.

In contrast to erythrocytes, human granulocytes contain lactosylceramide and *para*-globoside as their major neutral GSL [28]. Indeed, high performance thin layer chromatography (HPTLC) of purified extracts of neutral GSL from granulocytes showed two main bands by orcinol staining, corresponding to lactosylceramide and *para*-globoside (Fig. 6). Overlay with ECA followed by autoradiography revealed binding to the band migrating at the same rate as *para*-globoside. From the amounts of GSL applied to the thin-layer plates and the percentage of *para*-globoside in granulocyte glycolipids [28], the amounts of *para*-globoside in the samples were calculated to vary between 0.4 and 3.2 μg, well within the sensitivity limits of the method. In a control experiment, when the same amounts of GSL were chromatographed and overlayed with SBA, no staining was seen in the globoside/*para*-globoside region (data not shown).

We have also studied the interaction of ECA extracts of neutral GSL's from rabbit erythrocyte membranes. These membranes contain as their major neutral GSL trihexosylceramide and pentahexosylceramide, which has the structure of *para*-globoside substituted with a terminal α(1-3)-linked galactose [29]. Overlay with radioiodinated ECA of HPTLC plates of neutral GSL from rabbit erythrocytes gave no visible staining (Fig. 7). After *in situ* treatment of the plates with α-galactosidase, the lectin bound to a component with a mobility corresponding to that of the pentahexosylceramide, showing that the removal of the terminal non-reducing α-galactose yielded *para*-globoside as expected. Some slow-migrating GSL were also revealed after this treatment. These compounds probably correspond to the complex, branched GSL containing repeating *N*-acetyllactosamine units and terminal α(1-3)-linked galactose, present in rabbit

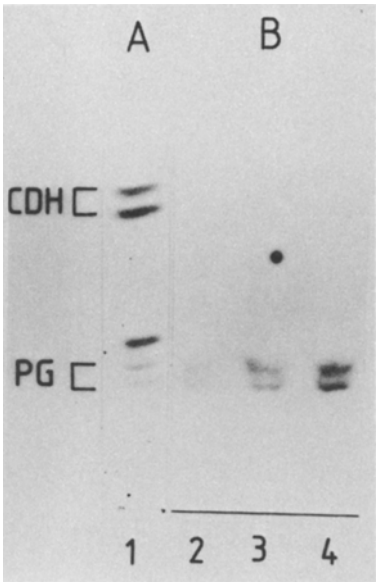

Figure 6. Binding of ^{125}I-ECA to increasing amounts of a neutral GSL extract from human granulocytes separated by HPTLC. Solvent system as in Fig. 5. Panel A: staining with orcinol; panel B: ^{125}I-ECA overlay. Amounts of GSL applied: lane 1, 9 μg; lane 2, 4.5 μg; lane 3, 9 μg; lane 4, 13.5 μg.

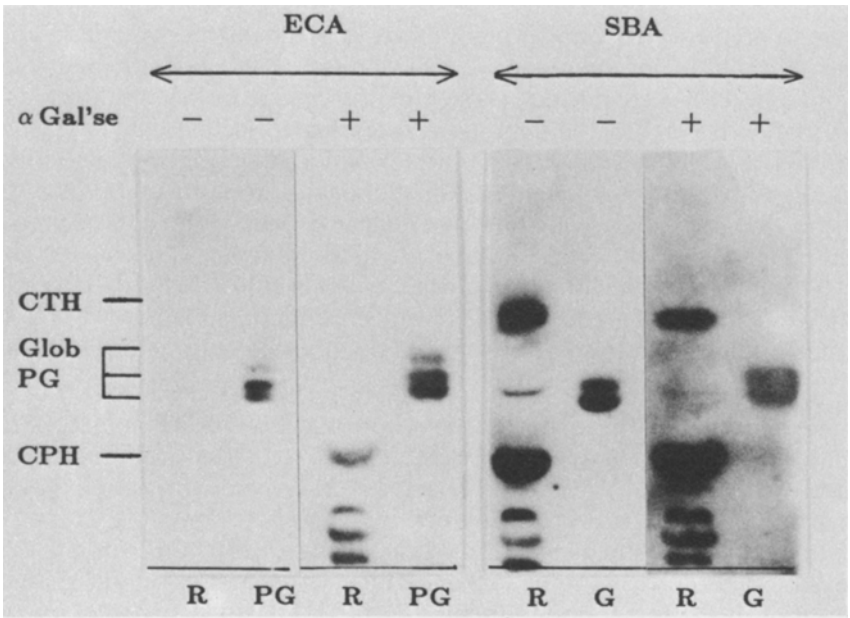

Figure 7. Binding of ^{125}I-ECA and ^{125}I-SBA to neutral GSL extracts from rabbit erythrocyte membranes before and after *in situ* treatment with α-galactosidase. Left panel: overlay with ^{125}I-ECA; right panel: overlay with ^{125}I-SBA. R, rabbit erythrocyte GSL; PG, *para*-globoside; G, globoside.

erythrocyte membranes [30]. After removal of the galactose and unmasking of the *N*-acetyllactosamine units, the GSL could be revealed by staining with ECA. In agreement with the results of the experiments with pure GSL standards, no labeling of trihexosylceramide was observed. In a control experiment, the plates before and after α-galactosidase treatment were overlayed with SBA (Fig. 7). Already on the untreated plates, SBA interacted with trihexosylceramide and the pentahexosylceramide, as well as with the slow-moving GSL. No change was observed after exposure of the plates to the enzyme.

With this experiment we have shown for the first time that α-galactosidase can be used for *in situ* removal of α-linked residues on thin-layer plates. Such *in situ* enzymatic cleavage of a glycosidic bond has been described previously for sialidase [31] but never performed with α-galactosidase. In comparison with sialidase, α-galactosidase treatment required more drastic conditions, i.e. 37°C versus room temperature and much longer incubation time, leading to increased "peeling" of the silica layer from the chromatographic plate. The peeling could be avoided by increasing the concentration of polyisobutylmethacrylate from 0.1% to 0.5%. This experiment also provides a convincing and independent demonstration for the specificity of ECA for *para*-globoside.

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