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The Detection of Picomolar Quantities of G_{D1b}, G_{T1b} and G_{Q1b} on Thin-Layer Chromatograms by the Direct Binding of ^{125}I-Labeled Tetanus Toxin, Fragment C

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The ^{125}I-labeled fragment C of tetanus toxin was found to bind specifically to the gangliosides G_{D1b}, G_{T1b}, and G_{Q1b} when applied to thin-layer chromatograms on which a mixture of gangliosides had been resolved. As little as 2.5 pmoles of these gangliosides could be detected by this method. In addition to factors determined by the sample, namely the amount and species of gangliosides present, optimal binding of the ^{125}I-labeled fragment C also depended upon the iodination procedure used to generate the probe, the toxin concentration, and the concentration, buffer type, pH, and ionic strength of the binding solution. This new technique was shown to be a sensitive method for the detection and identification of specific gangliosides originating from extraneural or neural cells.

Standard visualization techniques such as resorcinol [1] and orcinol [2] spraying of thin-layer chromatograms tend to be too insensitive for the ready detection of gangliosides present in cell samples of extraneural origin. The technology for using more sensitive probes such as radiolabeled bacterial toxins and monoclonal antibodies has recently

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Nomenclature: The gangliosides follow the nomenclature system of Svennerholm [Eur J Biochem (1977) 79:11-21]. G_{M3}, II^3NeuAc-LacCer; G_{D3}, $II^3(NeuAc)_2$-LacCer; G_{M1}, II^3NeuAc-GgOse$_4$Cer; G_{D1a}, IV^3NeuAc,II^3 NeuAc-GgOse$_4$Cer; G_{D1b}, $II^3(NeuAc)_2$-GgOse$_4$Cer; G_{T1b}, IV^3NeuAc, $II^3(NeuAc)^2$-GgOse$_4$Cer; G_{Q1b}, $IV^3(NeuAc)_2$, $II^3(NeuAc)_2$-GgOse$_4$Cer; G_{P1b}, $IV^3(NeuAc)_3$, $II^3(NeuAc)_2$-GgOse$_4$Cer.

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been applied to the study of gangliosides. Previous studies based on tissue distribution of toxin binding, toxicity studies, solid-state adsorption, and biochemical analyses indicated that tetanus toxin binds specifically to the gangliosides G_{D1b}, G_{T1b}, and G_{Q1b} [3-5] through a portion of the toxin's heavy chain polypeptide, designated fragment C [6-9]. In this paper we describe the use of ^{125}I-labeled fragment C as a sensitive probe for G_{D1b}, G_{T1b} and G_{Q1b} on thin-layer chromatograms. Using this technique the presence of a particular ganglioside in a mixture of gangliosides was demonstrated by autoradiography of radiolabeled toxin specifically bound to that ganglioside. The amount of the particular ganglioside was determined by comparison to standards and the species of ganglioside by the specificity of the toxin binding in combination with the location of the ganglioside on the chromatogram.

Materials and Methods

Materials

Tetanus toxin (Fragment C) was purchased from Calbiochem-Behring, USA, and was used without further purification. Carrier-free Na^{125}I was obtained from Amersham, USA. Iodo-beads® and Merck aluminium-backed silica gel 60 high-performance TLC plates were purchased from Pierce Chemical Co., USA. Poly(iso-butyl methacrylate)-beads were purchased from Polysciences, USA. Bovine serum albumin (Fraction V) was purchased from Miles Laboratories, USA. Kodak XAR-5 x-ray film was used for autoradiography. The mixed ganglioside TLC reference and individual ganglioside standards were prepared from human brain and were the gifts of Prof. Lars Svennerholm.

Preparation of Total Lipid Extract from Extraneural Cells

Madin-Darby canine kidney (MDCK) cells were cultured in reinforced Eagle's medium containing 10% fetal bovine serum until 80-90% of the surface was covered by the monolayer, then harvested as previously described [10]. The washed cell preparation was homogenized in a Potter-Elvehjem homogenizer at 4°C, then extracted by adding first methanol, and then chloroform to the aqueous homogenate to give a final solvent ratio of chloroform/methanol/water (4/8/3, by vol) [11]. After a 30 min extraction at room temperature, the sample was centrifuged for 10 min at 800×g. The extraction procedure was repeated on the 8000×g.min pellet and the extracts combined. The total lipid extract was desalted on a Sephadex G-25 column [12] before use in binding experiments.

Iodination of Tetanus Toxin

^{125}I-Labeled tetanus toxin was prepared by two different radioiodination procedures. For both procedures, 50 μg of tetanus toxin, fragment C, at a concentration of 1 mg/ml in 0.1 M sodium phosphate buffer, pH 6.5 containing 0.5 M NaCl and 0.5% butanol was combined with 1.3 mCi of Na^{125}I. The first iodination reaction was initiated by the addition of 20 μl of freshly prepared chloramine-T (2.5 mg/ml) and was allowed to proceed for

30 sec at room temperature before quenching with 20 μl of freshly prepared sodium metabisulfite (5 mg/ml). 200 μl of 0.1 M sodium phosphate buffer, pH 7.5 containing 0.1% bovine serum albumin (BSA) was added to minimize non-specific protein adsorption during the ensuing gel filtration.

The second radioiodination procedure was performed as previously described for antiserum to insulin [13]. The reaction was initiated by the addition of two prewashed Iodo-beads to the toxin-radioiodide mixture and allowed to proceed in a 5 ml polystyrene centrifuge tube for 15 min at room temperature. The reaction was terminated by simply transferring the solution to a second tube. The two Iodo-beads left in the first tube were washed with 200 μl of 0.1 M sodium phosphate buffer, pH 7.5 containing 0.1% BSA and the washing was transferred to the second tube.

Carrier iodide (20 μl of 50 mM NaI) was added to both samples, and unreacted $Na^{125}I$ was removed by gel filtration on a Sephadex G-25 column equilibrated in 0.1 M sodium phosphate buffer, pH 7.5 containing 0.1% BSA. Except for the first experiment in which the binding activities of the two preparations were compared, the sample prepared by the Iodo-bead technique was used for the remainder of the study. This probe was found to be stable in its binding activity for at least 15 weeks when stored at 5°C.

Binding Technique

Ganglioside samples were chromatographed on thin-layer aluminium-backed plates using chloroform/methanol/0.25% aqueous KCl, 50/40/10 by vol, as the solvent system. The binding technique was based on that described by Magnani *et al.* for use with cholera toxin [14] with the following modifications. The chromatograms were air-dried, soaked for 30 sec in 0.05% poly(iso-butyl methacrylate) in mixed hexanes, and then allowed to dry. Prior to the binding step, the chromatogram was sprayed with a very fine mist of 40 mM Tris-HCl (except where another buffer is specified), pH 7.0, 1% BSA and 0.01% phenol red and then soaked for about 30 min in the same buffer solution so that the surface of the plate was completely hydrated. The binding solution consisted of the buffer solution used for hydration of the chromatogram plus 500-1750 ng of ^{125}I-labeled fragment C per ml of solution. 55 μl of binding solution per cm^2 of chromatogram area was used to overlay the plate. After 2 h at 4°C, the binding solution was pipetted off and any residual unbound toxin was removed by washing the plate four times with phosphate-buffered saline (10 mM sodium phosphate, 0.85% sodium chloride, pH 7.0). After the chromatogram was air-dried, it was exposed to x-ray film for 1-3 days. The end containing the solvent front was trimmed from the figures.

Results

Binding Specificity and Effect of Radioiodination Procedure

In Fig. 1 the binding specificities of ^{125}I-labeled fragment C generated by two different radioiodination procedures were compared. Lanes 1, 2 and 3 all contained the same

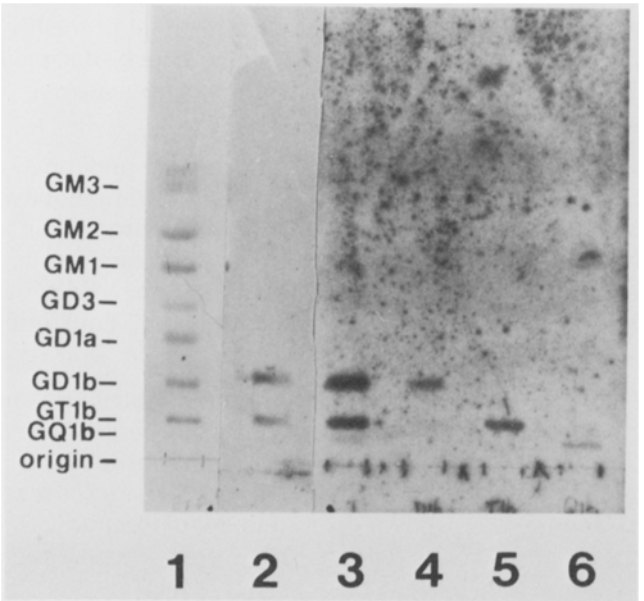

Figure 1. Effect of iodination procedure on the binding of ^{125}I-labeled tetanus toxin, fragment C to gangliosides. A mixed ganglioside TLC reference containing 6.08 nmoles of lipid-bound sialic acid was chromatographed in lanes 1-3. Lane 1 was visualized with orcinol reagent and lanes 2 and 3 probed by ^{125}I-labeled fragment C generated either by the procedure using chloramine-T (lane 2) or Iodo-beads (lane 3). The specific activity of the two probes was 8.9 and 8.1 μCi/μg, respectively. Lanes 4-6 contained 20 pmoles of G_{D1b}, G_{T1b} and G_{Q1b} reference gangliosides, respectively. These gangliosides were probed using tetanus toxin fragment C radiolabeled using the Iodo-bead procedure. The toxin concentration for overlaying lanes 2-6 was 1250 ng/ml of binding solution. Autoradiographs are shown for lanes 2-6.

mixture of gangliosides from brain. In lane 1 all of the gangliosides were visualized by orcinol staining. In lanes 2 and 3 specific gangliosides were visualized by autoradiography using probes generated by the chloramine-T (lane 2) and Iodo-bead (lane 3) radioiodination procedures. Although the specific activities of the two radiolabeled probes were virtually identical (8.9 and 8.1 μCi/μg of protein respectively), the toxin fragment iodinated with Iodo-beads was a more sensitive ganglioside probe than the toxin fragment iodinated with chloramine-T. Approximately 5 times the amount of G_{D1b} or G_{T1b} were needed to be detected by the latter probe.

The probe generated by the Iodo-bead method was used for lanes 4-6 and for all experiments throughout the remainder of the study. Lanes 4, 5 and 6 of Fig. 1 contained approximately 20 pmoles each of G_{D1b}, G_{T1b} and G_{Q1b} reference gangliosides, respectively, and were visualized by direct binding of this radiolabeled probe.

Effect of Binding Medium (Buffer Type and pH)

In addition to the amount and species of gangliosides present in the sample and the method used to generate the probe, the binding medium itself can greatly influence

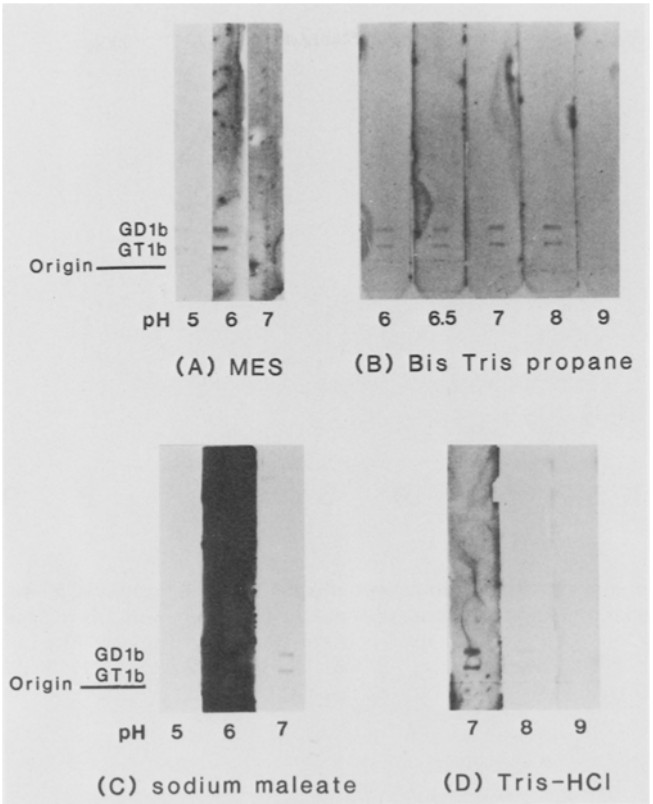

Figure 2. Effect of binding medium (buffer type and pH) on ^{125}I-labeled fragment C binding. A TLC reference containing approximately 44 pmoles of G_{D1b} and 44 pmoles of G_{T1b} was chromatographed in all lanes and then incubated with 1250 ng/ml of ^{125}I-labeled fragment C in the following buffer solutions: (A) MES, pH 5-7; (B) Bis-Tris-propane, pH 6-9; (C) Sodium maleate, pH 5-7; and (D) Tris-HCl, pH 7-9. All buffers were 30 mM and contained 0.1% BSA and 0.01% phenol red.

the resulting autoradiographic pattern. Four different buffer systems [2-(*N*-morpholino) ethanesulfonic acid (MES), sodium maleate, Tris-HCl and 1,3-bis(tris{hydroxymethyl}-methylamino)-propane (Bis-Tris-propane)] covering a pH range of 5.0-9.0 were investigated for their effect on the binding of the toxin to the mixed ganglioside reference. All buffers were used at a concentration of 30 mM and contained 0.1% BSA.

Fig. 2 shows that the amount of specific and of non-specific binding of ^{125}I-labeled fragment C was influenced both by pH and by the buffer system used to generate the pH. In the MES system (Fig. 2A), pH 6 gave maximal binding of toxin. In the Bis-Tris-propane buffer system (Fig. 2B), similar sensitivities were observed over a relatively broad pH range, pH 6-8. In the sodium maleate buffer system (Fig. 2C), no detectable binding was observed at pH 5, some binding but a high background at pH 6, and the clearest pattern of specificity at pH 7. The optimal ratio of specific to non-specific binding was observed using Tris-HCl at pH 7 (Fig. 2D). This buffer system and pH were used for the remainder of the experiments in this study.

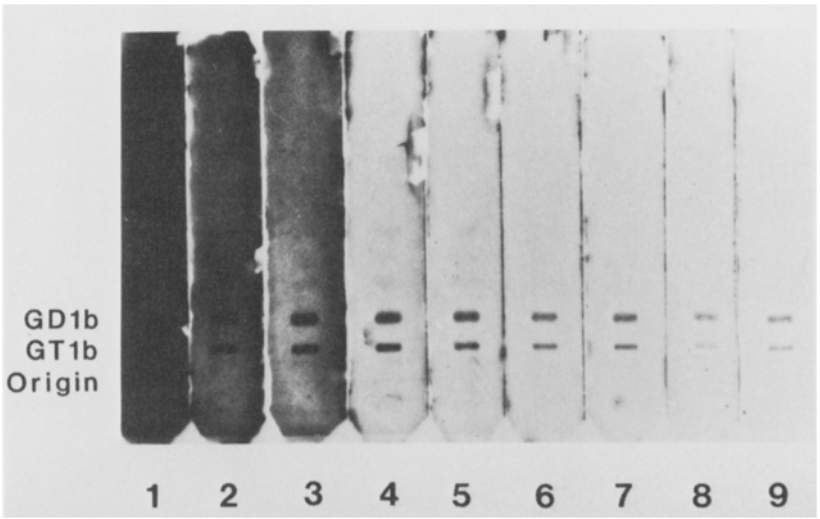

Figure 3. Effect of buffer concentration and ionic strength on ^{125}I-labeled fragment C binding. A mixed ganglioside TLC reference was chromatographed in all lanes and then incubated with Tris-HCl buffers at pH 7 at varying concentrations or ionic strengths. The toxin concentration was 1250 ng/ml. The buffer concentrations were as follows: lane 1, 10 mM; lane 2, 20 mM; 3, 30 mM; lane 4, 40 mM; lane 5, 50 mM; lane 6, 75 mM; lane 7, 100 mM; lane 8, 192 mM; and lane 9, 30 mM Tris-HCl plus 150 mM NaCl.

Effect of Buffer Concentration and Ionic Strength

The effects of buffer concentration and ionic strength of the binding medium were next examined as two related but separable factors. A concentration range of Tris-HCl from 10 mM to 192 mM was investigated (Fig. 3, lanes 1-8). In addition, to separate the possible effect of buffer concentration from that of ionic strength, a 30 mM solution of Tris-HCl containing 150 mM NaCl (lane 9) was tested as a binding medium. This buffer solution had the same ionic strength as the 192 mM Tris-HCl buffer solution (lane 8), and the same buffer concentration as the 30 mM Tris-HCl buffer solution (lane 3). Therefore, by experimental design, the effects of the two variables were assessed by comparing one pair of samples with equal Tris-HCl concentrations and unequal ionic strength (lanes 3 and 9) and another pair of samples with unequal Tris-HCl concentrations and equal ionic strength (lanes 8 and 9).

Fig. 3 shows that both the non-specific and the specific binding of ^{125}I-labeled fragment C decreased as the buffer concentration increased. The highest backgrounds were observed in the 10-30 mM Tris-HCl range (lanes 1-3). The intensities of the ganglioside bands which bind fragment C as visualized by autoradiography were relatively constant over a range of 10 to 50 mM Tris-HCl (lanes 1-5), then decreased.

The ganglioside mixture used in lanes 1-9 was the same as that used in Fig. 1A and contained approximately equal amounts of sialic acid in G_{M3}, G_{M2}, G_{M1}, G_{D3}, G_{D1a}, G_{D1b} and G_{T1b} and a trace of G_{Q1b}. The predominant gangliosides of the mixture visualized by binding of the probe were G_{D1b} and G_{T1b}. Optimal signal to noise ratio occurred with

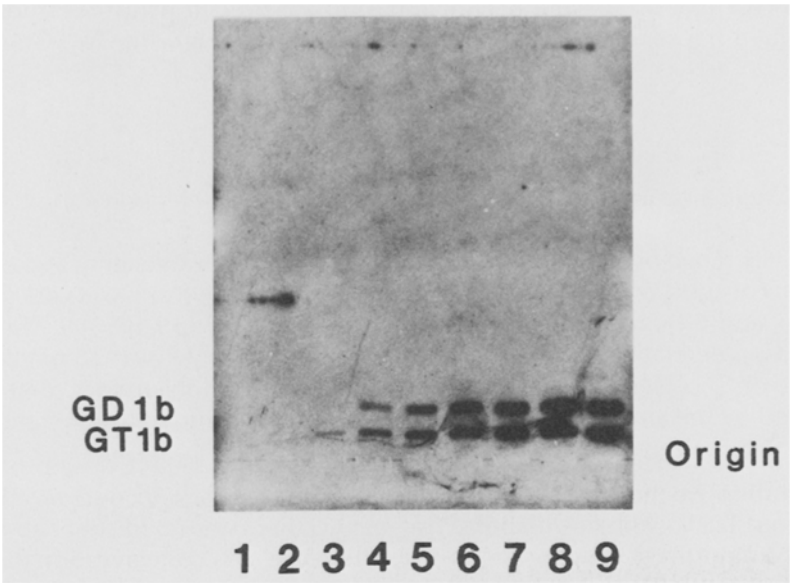

Figure 4. Effect of ganglioside concentration on the binding of ^{125}I-labeled fragment C to G_{D1b} and G_{T1b}. Varying amounts of an equimolar mixture of G_{D1b} and G_{T1b} were chromatographed and incubated with ^{125}I-labeled fragment C as described in Materials and Methods. The following amounts of G_{D1b} and G_{T1b} were applied: 0.5, 1.0, 2.5, 5.0, 10, 20, 40, 60 and 80 pmol to lanes 1-9, respectively.

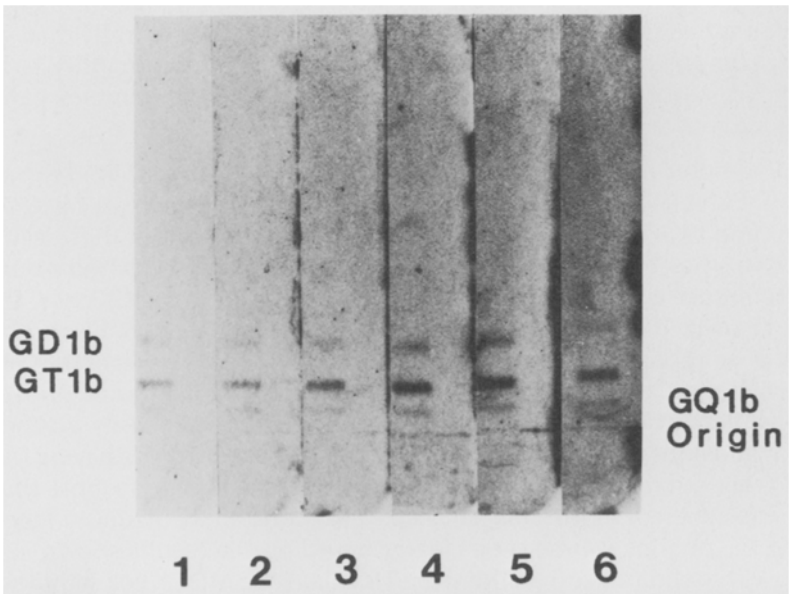

Figure 5. Effect of ^{125}I-labeled tetanus toxin fragment C concentration on binding behaviour. A mixed ganglioside TLC reference was chromatographed and then incubated with 40 mM Tris-HCl buffer, pH 7, containing the following concentrations of ^{125}I-labeled fragment C: 500, 750, 1000, 1250, 1500 and 1750 ng/ml in lanes 1-6, respectively.

40 mM Iris-HCl (lane 4). This buffer concentration was used in all further studies. In this lane, very faint traces of other bands were seen co-migrating with G_{M1}, G_{D3}, G_{D1a} and G_{Q1b}.

Effect of Ganglioside and Fragment C Concentrations on Detection Range

The sensitivity range of the ^{125}I-labeled fragment C probe for detecting and estimating the amounts of specific gangliosides is shown in Fig. 4. Known amounts (0.5 to 80 pmoles) of G_{D1b} and G_{T1b} reference gangliosides were applied in lanes 1-9. The minimal amount of G_{T1b} detectable by binding of ^{125}I-labeled fragment C was 2.5 pmoles, and of G_{D1b} (Fig. 4) or G_{Q1b} (see Fig. 6) 5 pmoles. Ganglioside amounts up to 60 pmoles could be estimated by the amount of ^{125}I-labeled fragment C bound in this system.

The effect of the concentration of ^{125}I-labeled fragment C (500-1750 ng protein/ml of binding solution) on the extent of binding is illustrated in Fig. 5. At concentrations greater than about 1250 ng/ml of ^{125}I-labeled fragment C there was no further substantial increase in the amount of ganglioside bound. Therefore, this concentration (lane 4) was used for the remainder of the study.

Application of the Probe to a Total Lipid Extract of Extraneural Origin

The samples used up to this point in the study were derived from neural tissue (human brain) and contained either a mixture of purified gangliosides or individual standards but no other types of lipids. The feasibility of using this probe to quantify gangliosides in crude fractions from extraneural tissue, in which the more complex gangliosides constitute $<0.5\%$ of the total lipid, was examined next.

The autoradiographic pattern that resulted from examining the total lipid extract of subconfluent MDCK cells by ^{125}I-labeled fragment C binding is shown in Fig. 6. A mixture of G_{D1b}, G_{T1b} and G_{Q1b} containing 5, 10, 20, or 40 pmoles of each of these gangliosides was streaked in lanes 1-4 as standards for quantitation. Lane 8 contained a mixed ganglioside TLC reference and lane 9 the purified gangliosides from MDCK cells. These references were visualized by resorcinol staining. Lanes 5-7 contained 0.6, 1.1, and 2.3 nmoles respectively of lipid-bound sialic acid present in the total lipid extract of the cell sample. In this very crude lipid extract, components migrating as glycolipids and phospholipids near the solvent front bound ^{125}I-labeled fragment C, but were easily distinguishable from true gangliosides by their location on the TLC plate. The bands having the double banding and migration behaviour of G_{M3}, which typically comprise more than 70% of the gangliosides present in the cell [10], also weakly bound the toxin. In lanes 6 and 7 the G_{D1b} and G_{T1b} bands can be seen clearly. Based on the results shown in Fig. 6, by comparison with standards run on the same TLC plate, the MDCK cell samples contained 0.16-0.20 nmoles of sialic acid/mg protein as G_{D1b} and 0.24-0.30 nmoles of sialic acid/mg protein as G_{T1b}. Trace amounts of G_{Q1b} were detected.

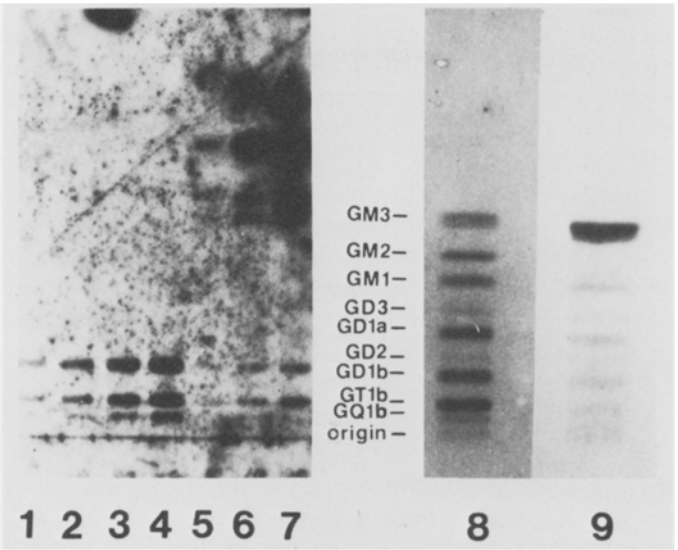

Figure 6. ^{125}I-Labeled fragment C binding to a total lipid extract of subconfluent MDCK cells. 5, 10, 20 or 40 pmoles of an equimolar mixture of G_{D1b}, G_{T1b}, and G_{Q1b} were streaked in lanes 1-4, respectively, as standards. Lanes 5-7 contained 0.6, 1.1 and 2.3 nmoles of lipid-bound sialic acid from a total lipid extract of subconfluent MDCK cells. Following chromatography, lanes 1-7 were exposed to the ^{125}I-labeled fragment C as described in Materials and Methods and the autoradiograph of these lanes is shown. The TLC mixed ganglioside (lane 8) reference and the purified ganglioside fraction of subconfluent MDCK cells (lane 9) were visualized with resorcinol reagent.

Discussion

Using the conditions elaborated in this study, the ^{125}I-labeled fragment C of tetanus toxin is shown to be a sensitive probe for G_{D1b}, G_{T1b} and G_{Q1b} on thin-layer chromatograms. It can detect pmolar amounts of these gangliosides in samples from extraneural tissue in which they represent $<0.5\%$ of the lipid. By comparing the intensity of the bands in the sample to standards on the same TLC plate, the actual amount of a particular ganglioside can be approximated in total lipid extracts. In contrast, standard chemical methods such as orcinol or resorcinol visualization require extensive purification of the gangliosides and nmolar quantities of the mixture for ready detection. Thus, the new probe is both simpler to use and more sensitive by 2 to 3 orders of magnitude than previously available traditional methods.

The general technique of using bacterial toxins as probes for specific gangliosides on TLC was recently introduced by Magnani *et al.* for the detection of G_{M1} by cholera toxin [14]. In the present study we chose to use fragment C rather than holotoxin because it is atoxic. The binding affinity of fragment C for the G_{1b} series (gangliotetraose backbone with two sialic acid residues attached to the internal galactose) of gangliosides is less than that of cholera toxin for G_{M1}. 2.5 pmoles of G_{T1b} and 5 pmoles of G_{D1b} or G_{Q1b} were needed as a minimum for detection by fragment C as compared to 70 fmoles of G_{M1} for

cholera toxin, but both are high affinity probes. The results of the present study are consistent with the majority of previous studies which demonstrate that only members of the G_{1b} series of gangliosides are bound with high affinity by tetanus toxin [5, 6, 8, 15-17]. No evidence was found to support a previous claim that fragment C binds to G_{M1} and G_{D1b} with virtually the same affinity [7]. The specificity of the binding of fragment C to the G_{1b} series of gangliosides introduces it as a tool for structural identification. Because G_{D1b}, G_{T1b}, and G_{Q1b} are well separated on chromatograms, the same probe can be used simultaneously to identify each of these. Although they were not tested here, it is expected that this probe could also be used to identify higher homologs in the series, such as G_{P1b}. It should be noted, however, that low affinity binding of fragment C to G_{D1a}, G_{D3} and G_{M1} (Fig. 3, lane 4) and G_{M3} (Fig. 6, lanes 5-7) was observed when these were present in large amounts in the sample.

In addition to factors determined by the sample, namely the amount and type of ganglioside present, binding of fragment C to gangliosides on TLC was greatly influenced by the iodination method used to prepare the probe and the composition of the binding medium. Ledley *et al.* have reported that toxin labeled by the Bolton-Hunter procedure had 80% less neurotoxic activity [16]. In the present study it was discovered that ^{125}I-labeled fragment C prepared using chloramine-T was about 5 times less sensitive as a probe than fragment C prepared using Iodo-beads, even though the specific activities of the two probes were virtually identical.

The pH, buffer, and ionic strength of the binding medium also proved to be critical factors in determining the amount of toxin bound. It had been previously found that the pH optimum for the binding of ^{125}I-labeled fragment C to neural membranes was between 6 and 8 in Tris-maleate buffer [8]. In the present study optimal binding was achieved with Tris-HCl at pH 7, but not all buffers gave the same results. When MES was used at pH 7 there was no specific binding. With Bis-Tris-propane, specific binding was observed over a broad pH range. The behavior of these three biological buffers may be due to a common functional group. Each contains at least one amino group and at a pH where this carried a positive charge, specific binding occurred between fragment C and the G_{1b} gangliosides. With sodium maleate, a dicarboxylic acid buffer, the optimal pH range for binding was above the second pKa, where both groups were charged.

The ionic strength of the binding medium had a dramatic effect on the amount of non-specific binding observed. A 13-fold reduction in non-specific binding of fragment C was observed in 30 mM Tris-HCl at pH 7 when 150 mM NaCl was added to the binding medium (Fig. 3, lanes 3 and 9), while under the same conditions only a 4-fold reduction in specific binding was observed. Although the concentration of the toxin does contribute as a factor to the sensitivity of the system, good ratios of specific to non-specific binding were obtained over the entire range examined (500 to 1750 ng/ml).

The usefulness of the radiolabeled probe in detecting picomolar amounts of G_{D1b}, G_{T1b} and G_{Q1b} in crude fractions from extraneural tissue is demonstrated in Fig. 6. MDCK cells are derived from kidney which typically contains only 30-60 nmoles of lipid-bound sialic acid/g wet weight as compared with 3000-3500 nmoles/g wet weight found in adult grey matter [18], the cerebral tissue from which gangliosides are usually isolated as standards. In MDCK cells the G_{1b} series comprises less than 10% of the total gangliosides [10]. This again is in contrast to adult grey matter in which it comprises about 40% of the total [19]. Thus, extraneural tissue not only contains much less ganglioside than the

brain, but also is generally poorer in gangliosides containing two or more sialic acid groups. However, the amount of ganglioside that needs to be present to function as a receptor for a virus or toxin is minute. Although extraneural tissue has very small amounts of the higher gangliosides, there are sufficient quantities available for biological interactions. The MDCK cells used in this study were shown to be susceptible to Sendai virus infection on the day they were harvested for analysis. In previous studies [10], 2.5×10^7 cells were used to determine the amount of G_{D1b} or G_{T1b} by the resorcinol technique. In the present study the same information was obtained with only 2.6×10^5 MDCK cells by the use of radiolabeled fragment C. Thus, use of ^{125}I-labeled tetanus toxin fragment C as a very sensitive probe for G_{D1b}, G_{T1b} and G_{Q1b} will facilitate investigation of the distribution of the G_{1b} series in various cell types, their change in concentration in response to environmental factors, and ultimately their function within those cells.

Acknowledgements

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