Chemiluminescence detection of gangliosides by thin-layer chromatography

Sheryl L. Arnsmeier and Amy S. Paller[1]

Departments of Pediatrics and Dermatology, Children's Memorial Institute for Education and Research, Northwestern University Medical School, Chicago, IL 60614

Summary We describe a method for the detection of gangliosides on thin-layer chromatography plates using enhanced chemiluminescence. In contrast to previously published colorimetric techniques, the use of chemiluminescence to detect antibody binding to gangliosides has increased sensitivity and simplicity. In addition, the use of chemiluminescence lacks the hazards of radioactivity, and produces a permanent record.—**Arnsmeier, S. L., and A. S. Paller.** Chemiluminescence detection of gangliosides by thin-layer chromatography. *J. Lipid Res.* 1995. **36:** 911–915.

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Gangliosides are sialylated glycosphingolipids on the membranes of most eukaryotic cells. Detection of differences in the content of surface gangliosides between normal and neoplastic cells has recently led to novel intervention with anti-ganglioside antibodies (1, 2). In addition, gangliosides themselves have been used effectively to treat neurologic abnormalities, such as spinal cord injury (3). Gangliosides represent a small fraction of the membrane lipid of most cells, and techniques to quantitate specific gangliosides are hampered by lack of sensitivity or rapid fading of visualized bands. The use of specific anti-ganglioside antibodies and radiolabeled secondary antibodies currently provides the most sensitive means of ganglioside detection.

We describe a sensitive, non-radioactive method for the quantitation of gangliosides on thin-layer chromatography plates using enhanced chemiluminescence (ECL) to detect antibody binding. The technique reliably detects 2.5 ng (1.8 pmol) of ganglioside, and produces a permanent record that is suitable for densitometric scanning.

MATERIALS AND METHODS

Gangliosides, antibodies, and plates

Gangliosides GM3 from dog erythrocytes (4), GM1, GD1a, GD1b, and GT1b from bovine brain (5), and GD3 from bovine buttermilk (6) were prepared by Folch's solvent partition and DEAE-Sephadex chromatography (7). The glycolipids were further purified by high-pressure liquid chromatography on a silica gel column using a chloroform–methanol–water solvent system (8). Gangliosides were quantitated by dry weight and colorimetric resorcinol assays as previously described (9). GM2 was purchased (Boehringer-Mannheim, Indianapolis, IN) and used without further purification. Mouse monoclonal antibodies were provided as follows: anti-GM3 and anti-GM1 by Dr. E. Bremer, Chicago (9), anti-GM2 (10, 11) by Dr. P. Livingston, Sloan-Kettering, NY (10), and anti-GD3 (R24) by Dr. K. O. Lloyd, Sloan-Kettering, NY (11). Goat anti-mouse antibody was purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Pre-coated silica gel 60 aluminum-backed thin-layer chromatography plates (Merck, Darmstadt, Germany) were used after heating at 100°C for 1 h. 4-Chloro-1-naphthanol and 5-bromo-4-chloro-3-indolyl phosphate/*p*-nitro blue tetrazolium colorimetric agents were purchased from Bio-Rad (Hercules, CA). 3-3'-Diaminobenzidine tetrahydrochloride and *o*-phenylenediamine were purchased from Sigma (St. Louis, MO).

Thin-layer chromatography and immunostaining

Five hundred pg to 5 μg of ganglioside standards of GM3, GM2, GM1, GD3, GD1a, GD1b, or GT1b were applied to thin-layer chromatography (TLC) plates and run in a solvent system of chloroform–methanol–0.2% calcium chloride 55:45:10 (v/v/v). After development, the plates were dried, dipped in n-hexane containing freshly made 0.4% polyisobutylmethacrylate, and allowed to air dry. To further identify GD1a, GD1b, and GT1b, gangliosides run on TLC plates were incubated with 50 mU/ml of *Clostridium perfringens* neuraminidase in 0.1 M sodium acetate, pH 4.8, at 37°C for 1.5 h (12) to convert these more highly sialylated gangliosides to GM1 and allow detection with anti-GM1 antibody. The plates treated with neuraminidase were washed with phosphate-buffered saline (PBS). For immunostaining, the plates were treated for 20 min with 5% non-fat dried milk (Carnation Co., Los Angeles, CA) in PBS with moderate shaking at room temperature and then incubated with primary antibodies. Primary antibodies were diluted in PBS with 2% bovine serum albumin as follows: IgM anti-GM3 1:20, IgM anti-GM1 1:500; IgG anti-GD3 1:100; and IgM anti-GM2 1:2500, and incubated with the TLC plates overnight at 4°C with gentle shaking. Plates were shaken moderately in washes of PBS containing 0.1% Tween-20 (PBS-T) and 0.05% magnesium chloride, first with two 10-min washes and subsequently 10 min washes. After reblocking with 5% milk, the plates were dipped in wash once, then treated with horseradish peroxidase-conjugated goat anti-mouse antibody, diluted 1:1000, for 30 min at room tem-

Abbreviations: ECL, enhanced chemiluminescence; PBS, phosphate-buffered saline; PBS-T, PBS with 0.1% Tween-20; TLC, thin-layer chromatography.

[1]To whom correspondence should be addressed at: Division of Dermatology, # 107, The Children's Memorial Hospital, 2300 Children's Plaza, Chicago, IL 60614.

perature. Because of the overnight incubation, the silica gel tended to be less adherent by the second day. As a result, gentle shaking was used with secondary antibody. Plates were rinsed well with PBS-T containing 0.05% magnesium chloride, with gentle shaking in washes, changed every 5 min for 1 h. It was critical that all blocking and detection buffers were prepared on the day of the study.

For ECL detection, the plates were then incubated for 60 sec with a 1:1 (v/v) mixture of reagents 1 and 2 (luminol and hydrogen peroxide), provided in the ECL kit for Western blotting (Amersham, Arlington Heights, IL) (0.125 ml/cm²) (13). The plate was drained and wrapped in plastic (SealWrap). Binding was visualized by autoradiography (X-Omat AR film) after 5 to 60 sec of exposure, depending upon the antibody. For each anti-ganglioside antibody, exposure times of 5, 15, 30, and 60 sec were studied, and the shortest period that eliminated background with best visualization of the band was selected. The intensity of bands was read by laser densitometry (Helena Quick Scan Jr., TLC).

To compare the sensitivity of ECL with previously described non-radioactive antibody techniques, detection of binding using horseradish peroxidase- and alkaline phosphatase-conjugated antibodies and chromogenic agents, including 4-chloro-1-naphthanol (14), 3-3'-diaminobenzidine tetrahydrochloride, *o*-phenylenediamine, and 5-bromo-4-chloro-3-indolyl phosphate/*p*-nitro blue tetrazolium (15), was also studied as previously described without modifications.

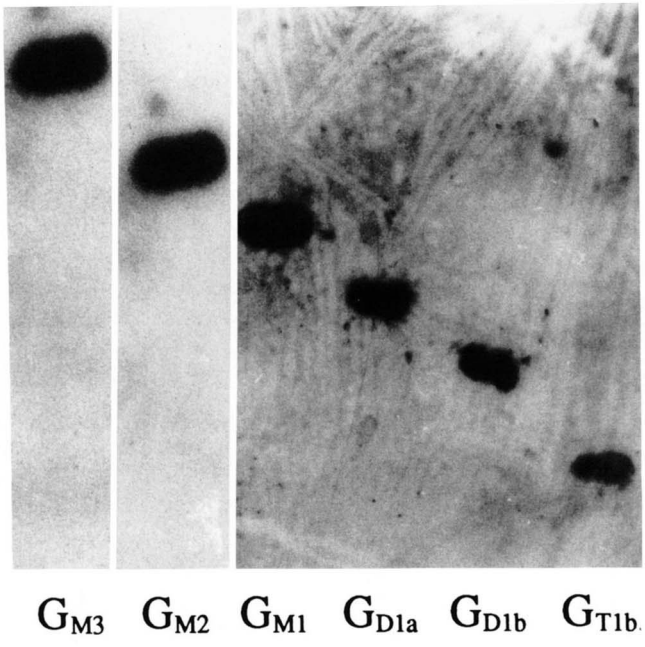

Fig. 1. Gangliosides GM3, GM2, GM1, GD1a, GD1b, and GT1b, 1 μg (900 pmol; 722 pmol; 647 pmol; 544 pmol; 544 pmol; 470 pmol, respectively) per lane, visualized by autoradiography after detection with enhanced chemiluminescence. The GM3, GM2, and GM1 were treated directly with primary monoclonal antibodies, while the GD1a, GD1b, and GT1b were incubated with neuraminidase and subsequently treated with anti-GM1 monoclonal antibody. Film exposure time for each ganglioside was as follows: GM2, GM1, GD1a, and GD1b, 5 sec; GM3 and GT1b, 30 sec.

RESULTS

The chemiluminescent technique presented in this report allows detection of several gangliosides, and theoretically can be used to locate any ganglioside or glycolipid for which an antibody is available (**Fig. 1**). This technique is extremely sensitive, and concentrations of ganglioside as low as 2.5 ng (1.8 pmol of GM2) were easily and reproducibly able to be identified and photographed (**Fig. 2**). At various times, we have been able to detect as little as 500 pg of ganglioside (0.36 pmol GM2), but the band has been too faint to be seen photographically. In our laboratory, the techniques with the colorimetric agents 4-chloro-1-naphthanol, 3-3'-diaminobenzidine tetrahydrochloride, *o*-phenylenediamine, and 5-bromo-4-chloro-3-indolyl phosphate/*p*-nitro blue tetrazolium only detected a minimum of 100 ng (72 pmol) ganglioside GM2 when run concurrently with the chemiluminescence testing. The measured reflection densities of bands generated by ECL as measured by densitometry correlated well with the quantity of plated ganglioside.

Clostridium perfringens neuraminidase cleaves the terminal sialic acid residues from polysialylated and oligosialylated gangliosides, leaving GM1, which is detectable with the anti-GM1 monoclonal antibody or with the β-subunit of cholera toxin. Pretreatment of the TLC plate with neuraminidase prior to primary antibody incubation allowed easy detection of gangliosides GD1a, GD1b, and GT1b with antibody using chemiluminescence. If anti-GM1 antibody is not available, commercially available horseradish peroxidase-conjugated β-subunit of cholera toxin (Sigma, St. Louis, MO) can be substituted, as the β-subunit binds to GM1.

The reduced background proved critical for maximal signal/noise ratio. Milk proved to be a more effective blocking agent than bovine serum albumin or gelatin. The addition of 0.05% magnesium chloride in the washing buffer, a modification from the procedure suggested by the manufacturer of the ECL kit, as well as the fresh preparation daily of all buffers, blocking agents, and antibodies, was found to reduce further the nonspecific background. The technique had good reproducibility, but depended upon careful attention to stringent conditions of washing.

DISCUSSION

The original radiolabeled overlay technique for immunostaining free oligosaccharides on thin-layer chromatography plates was developed by Magnani in 1985 (16). As an alternative to the use of hazardous radioactive

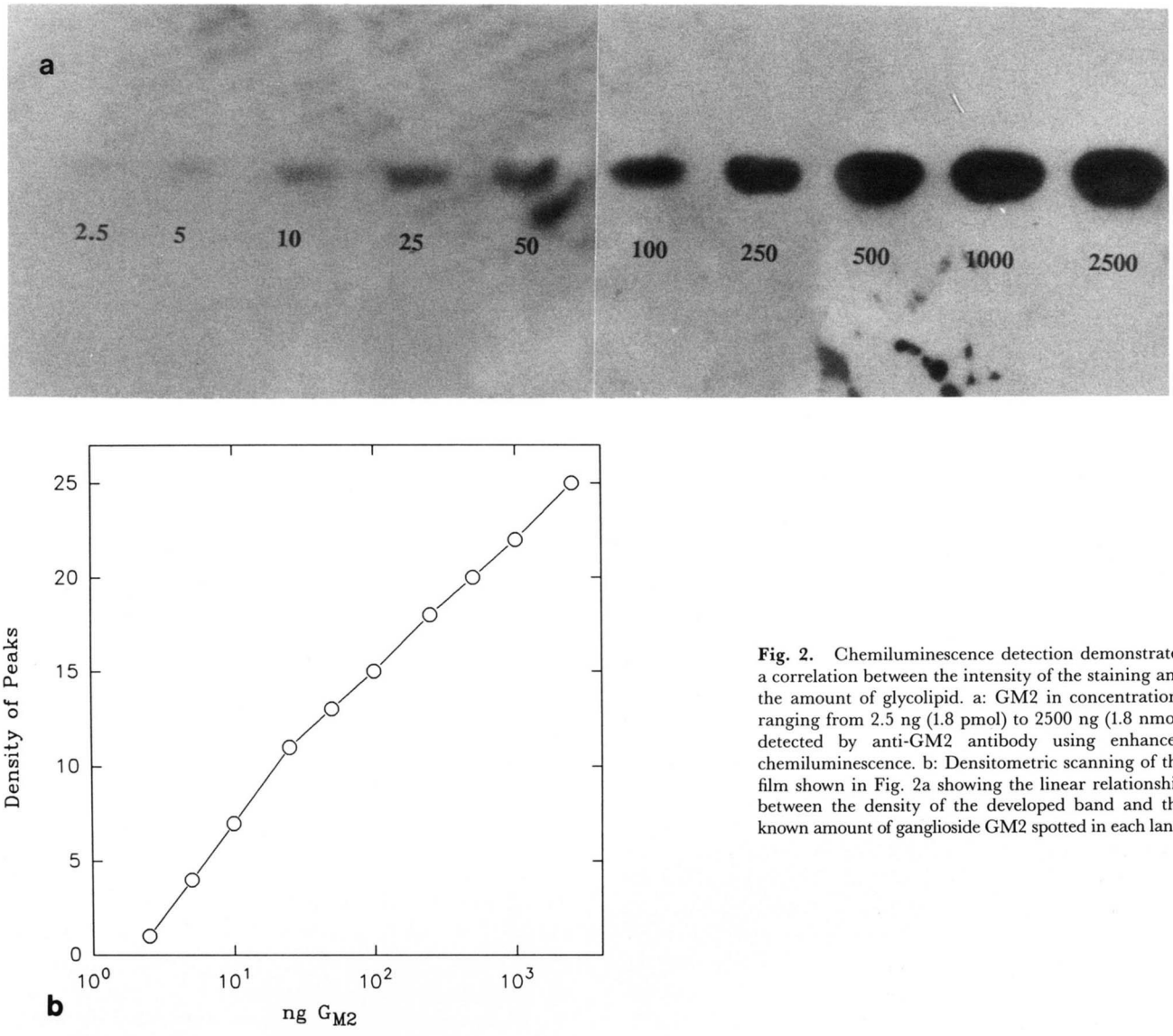

Fig. 2. Chemiluminescence detection demonstrates a correlation between the intensity of the staining and the amount of glycolipid. a: GM2 in concentrations ranging from 2.5 ng (1.8 pmol) to 2500 ng (1.8 nmol) detected by anti-GM2 antibody using enhanced chemiluminescence. b: Densitometric scanning of the film shown in Fig. 2a showing the linear relationship between the density of the developed band and the known amount of ganglioside GM2 spotted in each lane.

materials to detect antibody staining, colorimetric methods have been developed using secondary antibodies conjugated with enzymes, such as alkaline phosphatase visualized by 5-bromo-4-chloro-3-indolyl phosphate/*p*-nitro blue tetrazolium (15), and horseradish peroxidase developed with 4-chloro-1-naphthanol (14), 3-3'-diaminobenzidine tetrahydrochloride, or *o*-phenylenediamine. Detection of gangliosides with colorimetric agents, however, often causes significant nonspecific background and, with many of these chromogenic agents, produces a result that fades. Kniep and Mühlradt (17) have recently described a modified immunochemical technique that uses periodate oxidation and derivatization with digoxigenin-succinyl-ϵ-aminocaproic acid hydrazide (DIG) of glycolipids prior to detection by anti-digoxigenin antibody and 5-bromo-4-chloro-3-indolyl phosphate. This procedure requires a few hours longer than the chemiluminescence technique, and the maximal reported sensitivity is approximately equal to that of the chemiluminescence technique (2 ng of detected glycolipid).

Chemiluminescence, the emission of light as a result of a chemical reaction, is an alterative method of detection that obviates the need for radioactive labeling. Chemiluminescent methods have been used for detection of DNA in Southern blots and dot blots (18, 19), of proteins in enzyme-linked immunosorbent assays (20) and Western blots (21), and for in situ hybridization experiments (19). In the presence of hydrogen peroxide, horseradish peroxidase catalyzes the oxidation of luminol into radicals that emit light as they decay to ground state (22). The amount of light produced is proportional to the level of bound horseradish peroxidase.

In this report, we describe a simple and rapid method for the detection of glycosphingolipids by chemiluminescence on thin-layer chromatography plates. The exposure time before detection by this procedure is much shorter than the exposure time for colorimetric agents and certainly for radiolabeled reagents. Chemiluminescence assays also avoid the potential hazards of radiolabeled secondary antibodies, and the chemiluminescence secondary antibodies are stable for 6 to 12 months. A permanent record is produced that is suitable for scanning densitometry.

The sensitivity of non-isotopic detection systems is often limited by nonspecific background. By chemiluminescence, background is significantly diminished, allowing for greater sensitivity than standard detection techniques with horseradish peroxidase or alkaline phosphatase and chromogens. We were able to detect no less than 100 ng of gangliosides by using colorimetric agents. ECL allowed the visualization of as little as 500 pg of ganglioside GM2, and 2.5 ng of ganglioside routinely resulted in a band that was dark enough to be seen photographically. A limitation of this technique is the requirement of specific anti-ganglioside antibodies. However, commercially available cholera toxin may replace anti-GM1 monoclonal antibody and thus allows detection of several antibodies after treatment with sialidase.

Detection of gangliosides and other glycolipids by chemiluminescence has many potential clinical applications, such as monitoring anti-GM1 antibody levels in patients administered GM1 for spinal cord injury (3) or following serum levels of specific circulating shed gangliosides from tumor cells, e.g. in patients with neuroblastoma in which the levels of GD2 may correlate with neoplastic load (23). In addition, ECL for ganglioside detection may improve quantitation of circulating levels of anti-ganglioside monoclonal antibodies used therapeutically, including anti-GD2 antibody for neuroblastoma (1) and anti-GD3 antibody for melanoma (2). The detection of purified individual gangliosides was studied in this report. The sensitivity and selectivity of the chemiluminescent technique on gangliosides isolated from small samples of plasma or other biological samples needs to be tested.

The chemiluminescent detection method described in this paper produces a highly sensitive and simple non-radioactive method for the detection of gangliosides on TLC plates. Furthermore, it appears that visualization by chemiluminescence may be successfully applied to detect other glycolipids on TLC plates.

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