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Note

Hydrolysis of all gangliosides, including GM1 and GM2, on thin-layer plates by *Vibrio cholerae* neuraminidase*

JACQUES PORTOUKALIAN* and BERNADETTE BOUCHON

INSERM U. 218, Laboratoire d'Immunologie, Centre Léon Bérard, 69373 Lyon Cedex 08 (France)

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Thin-layer chromatography (TLC) is a standard method for the separation of glycosphingolipid mixtures. Until recently, when low amounts of glycosphingolipids were available, a tentative identification depended mainly on differences in migration rates with various solvent systems, and visualization by specific spray reagents [2]. The fact that several different compounds display close migration rates has often led to erroneous conclusions. The staining of glycosphingolipids on thin-layer plates with toxins [3] and specific antibodies [4] has been a significant step towards the characterization of minute amounts of these compounds. Very recently, Samuelsson showed that fucosyl transferases are active on glycosphingolipid precursors on thin-layer plates [5]. This observation led us to investigate the activity of glycosyl hydrolases on glycosphingolipids after chromatography on thin-layer plates, with the aim of characterizing them with specific antibodies. We report here the data obtained on the effect of neuraminidases on gangliosides in solid-phase hydrolysis.

EXPERIMENTAL

Materials

Gangliosides GM2, GD3 and GD2 were from human malignant melanocytes [6]. Lacto-N-norhexaosylceramide and sialosyllacto-N-norhexaosylceramide were purified from human placenta [7]. Gangliotriaosylceramide (asialoGM2)

*The nomenclature of Svennerholm [1] was used throughout.

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was prepared from guinea-pig erythrocytes [8]. Gangliotetraosylceramide (asialoGM1) was recovered after mild acid hydrolysis of bovine brain gangliosides (0.1 *N* sulphuric acid, 80°C, 2 h).

Vibrio cholerae neuraminidase (E.C. 3.2.1.18) was purchased from Calbiochem-Behring (Paris, France). *Clostridium perfringens* neuraminidase was from Sigma (St. Louis, MO, U.S.A.).

Mouse monoclonal anti-N-acetyllactosamine was obtained as described previously [9]. Mouse monoclonal anti-gangliotriaosylceramide (anti-asialo-GM2) [10] was provided by Dr. W.W. Young, Jr. (Department of Pathology, University of Virginia Medical Center, VA, U.S.A.). Mouse monoclonal R24 anti-GD3 [11] was a gift from Dr. K.O. Lloyd (Sloan-Kettering Institute, New York, NY, U.S.A.). Rabbit anti-gangliotetraosylceramide (anti-asialoGM1) antiserum was kindly given to us by Dr. M. Iwamori (Department of Biochemistry, Faculty of Medicine, University of Tokyo, Tokyo, Japan).

Thin-layer chromatography

The gangliosides were applied to plastic-backed, silica gel 60 thin-layer plates (Merck, Darmstadt, F.R.G.). These plates can be cut easily, and visualization with orcinol or resorcinol reagents does not damage the gel, as is often the case with alumina-backed high-performance thin-layer plates. The plates were developed at room temperature over 5 cm in paper-lined tanks (Desaga, Heidelberg, F.R.G.) with chloroform—methanol—0.2% calcium chloride (60:35:8). The standard lanes were then cut and visualized with resorcinol reagent [2].

For two-dimensional thin-layer chromatography, 4 cm × 4 cm plates were used. After migration on the first dimension, the plates were treated with polyisobutylmethacrylate (Aldrich, France) and incubated with neuraminidase as detailed below. The plates were dried under a stream of air and developed twice in acetone to remove the plastic layer that might disturb subsequent migration of glycolipids. After drying, the plates were developed in the second dimension. Standard lanes were migrated in both dimensions.

Neuraminidase treatment of gangliosides on thin-layer plates

After drying under a stream of air, the plate was dipped in 0.05% polyisobutylmethacrylate in hexane [3] for 1 min and dried off with a stream of air. The plate was then stuck to a Petri dish (6 cm diameter) with double-sided sticking tape, placed on a rolling apparatus and covered with 5 ml of 0.1% calcium chloride in distilled water (pH 5.5). After 15 min, a solution of neuraminidase in the same buffer (0.1 U/ml for optimal activity) was added and hydrolysis was allowed to proceed at room temperature. After incubation, the enzyme solution was removed and the plate was washed with phosphate-buffered saline, pH 7 (PBS). Controls were performed on thin-layer plates without neuraminidase, and in test tubes.

Immunostaining on thin-layer plates

After chromatography, the plate was treated with polyisobutylmethacrylate as above (unless already done before enzyme treatment) and placed in a 6-cm diameter Petri dish. The plate was incubated in 5 ml of antibody solution (PBS containing 2% bovine serum albumin and 0.1% sodium azide) overnight at 4°C

on a rolling apparatus. The antibody was then washed off with PBS twice for 5 min, and 5 ml of biotinylated anti-mouse immunoglobulin antibody (Amersham, Paris, France) diluted 1/250 in PBS were added. After 1 h at room temperature, the second antibody was dicarded and the plate was washed twice for 5 min with PBS. A solution of streptavidin—horseradish peroxidase was overlaid (5 ml of a 1/250 dilution in PBS) on the plate for 45 min at room temperature. After three 5-min washings with PBS, the bound peroxidase was visualized with a freshly prepared solution of chloro-4-naphthol (Merck) [12] (10 mg dissolved in 3 ml of methanol before addition of 15 ml of PBS and 5 μl of 30% hydrogen peroxide). Maximal blue staining was obtained after 30 min in the dark. The reagent was discarded and the plate washed with PBS before drying under a stream of air. The blue coloration was quantitated with a computer-assisted spectrodensitometer (CS-930 Chromatoscan, Shimadzu, Kyoto, Japan) set at a 570-nm wavelength. The coloration is stable for months if the chromatogram is kept protected with a glass plate.

RESULTS

Thin-layer plate treatment by *Vibrio cholerae* neuraminidase was carried out in distilled water containing 0.1% calcium chloride, which gives a pH of ca. 5.5. In the buffer generally used (i.e. 0.01 *M* acetate), regardless of pH, the silica gel peeled off the plate within a few hours, and this was also observed in distilled water. Control experiments in test tubes with 0.1 U/ml of 0.1% calcium chloride were performed in 24 h with total melanoma tumour gangliosides and rat brain gangliosides. Gangliosides GM3 and GD3 were readily hydrolysed, whereas GM2 and GD2 were not desialylated, as no asialoGM2 could be

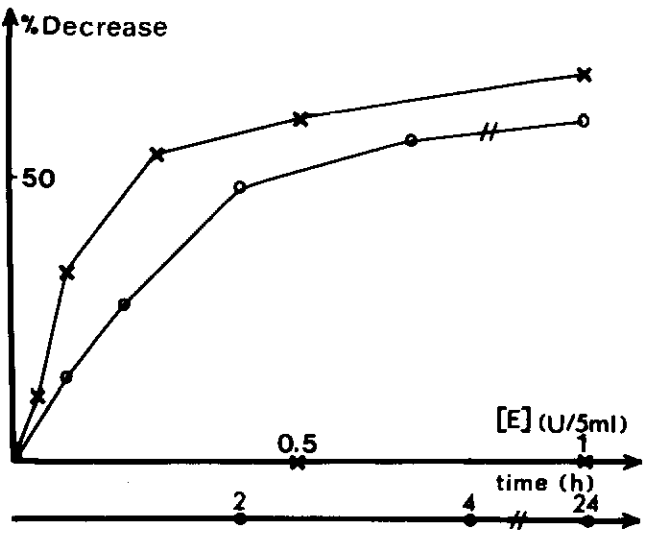

Fig. 1. Kinetics of GD3 hydrolysis by *Vibrio cholerae* neuraminidase on thin-layer plates, as monitored by the decrease of staining with anti-GD3 R24 mouse monoclonal antibody: (×) influence of enzyme concentration in a 4-h incubation; (○) time course of hydrolysis by the enzyme at 0.5 U in 5 ml. [E] = enzyme concentration. In three separate experiments, the variation range was 3—5%.

detected by immunostaining after TLC. *Clostridium perfringens* neuraminidase was able to hydrolyse GD2 in test tubes, as evidenced by strong staining with anti-asialoGM2, but no cleavage of purified GM2 could be seen. With rat brain gangliosides, neuraminidases from both sources gave mostly GM1 as the major degradation product, along with some GD1b, without trace of gangliotetraosylceramide (asialoGM1).

The kinetics of ganglioside cleavage by *Vibrio cholerae* neuraminidase were determined with GD3 as substrate. After migration of 1-μg aliquots of GD3 on thin-layer plates, 4-h incubations were carried out with increasing amounts of neuraminidase in 5 ml of buffer as described in Experimental. The release of sialic acid was estimated by the disappearance of immunostaining with R24 monoclonal antibody to GD3. Fig. 1 shows that treatment with 0.25 U of neuraminidase already strongly decreases the staining. The data obtained at an enzyme concentration of 0.5 U in 5 ml of buffer, with different incubation times up to 24 h, are presented in Fig. 1. A plateau was found after 2 h with 50% diminution, and over a 24-h period, staining was lowered by 65%. In these experimental conditions, complete disappearance of staining could only be achieved by raising the amount of enzyme to 2 U in 5 ml and incubating for 48 h, although such total hydrolysis was possible using lower amounts of ganglioside.

The time course of *Vibrio cholerae* neuraminidase hydrolysis of gangliosides

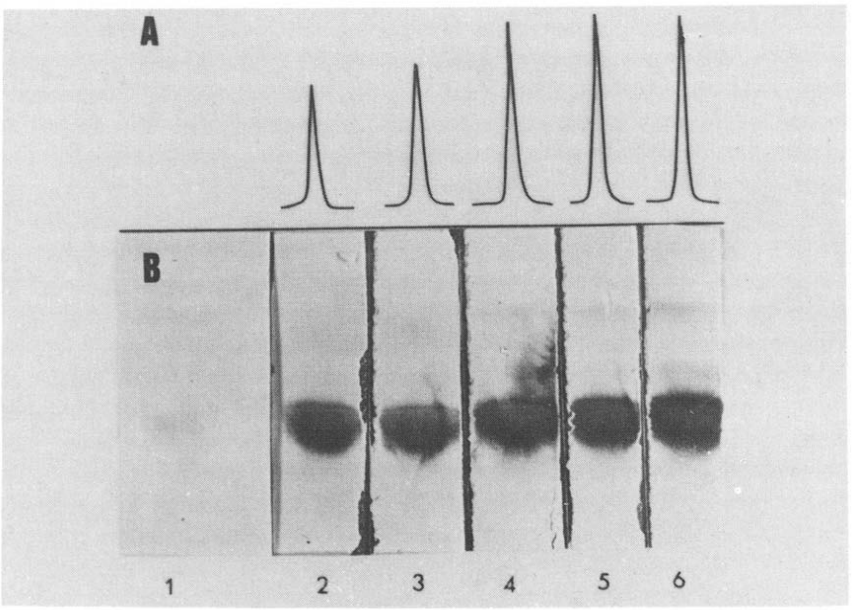

Fig. 2. Thin-layer chromatography of 0.5 μg of sialosyllacto-N-norhexaosylceramide in chloroform—methanol—0.2% calcium chloride (60:35:8), then peroxidase-linked immunostaining by mouse monoclonal anti-N-acetyllactosamine after treatment of the plate with *Vibrio cholerae* neuraminidase (0.5 U in 5 ml) for increasing lengths of time. (A) Scanning densitometry of the immunostains. (B) 1 = Resorcinol · HCl visualization of the ganglioside (2 μg); 2 = 0.5-h incubation with neuraminidase; 3 = 1-h neuraminidase; 4 = 2-h neuraminidase; 5 = 4-h neuraminidase; 6 = 24-h neuraminidase. No staining was observed without prior neuraminidase treatment of the plate.

on thin-layer plates was also studied using human placenta sialosyllacto-N-norhexaosylceramide as substrate (Fig. 2). Five spots of 500 ng each were applied to a plate, which was developed and then enzyme-treated. Neuraminidase action was monitored by the appearance of staining with mouse monoclonal anti-N-acetyllactosamine, which does not bind to this ganglioside but is strongly reactive with the asialo compound [9]. Whereas no staining was observed in the control experiment omitting neuraminidase (not shown), the effect of neuraminidase is already noticeable after 30 min of contact and the maximum is reached within 2 h of incubation, as can be seen in Fig. 2.

On thin-layer plates, the effect of *Vibrio cholerae* neuraminidase on gangliosides with sialic acid residues in the internal position was investigated. Human malignant melanocyte gangliosides, containing both GM2 and GD2 [6], were spotted and treated on thin-layer plates with neuraminidase after migration. The enzyme activity was monitored by staining with mouse monoclonal anti-gangliotetraosylceramide (anti-asialoGM2), which is unreactive to GM2 [10]. Along with some unspecific binding to contaminating acidic phospholipid (mostly directed to cardiolipin), often occurring with ascites fluids and antisera, the antibody reacted with GM2 and GD2 on the plate incubated with neuraminidase (Fig. 3). No staining was detectable in control experiments lacking the enzyme (not shown). An additional spot appearing above GD2 might reveal an O-acetylated derivative of GD2; this possibility is currently under investigation.

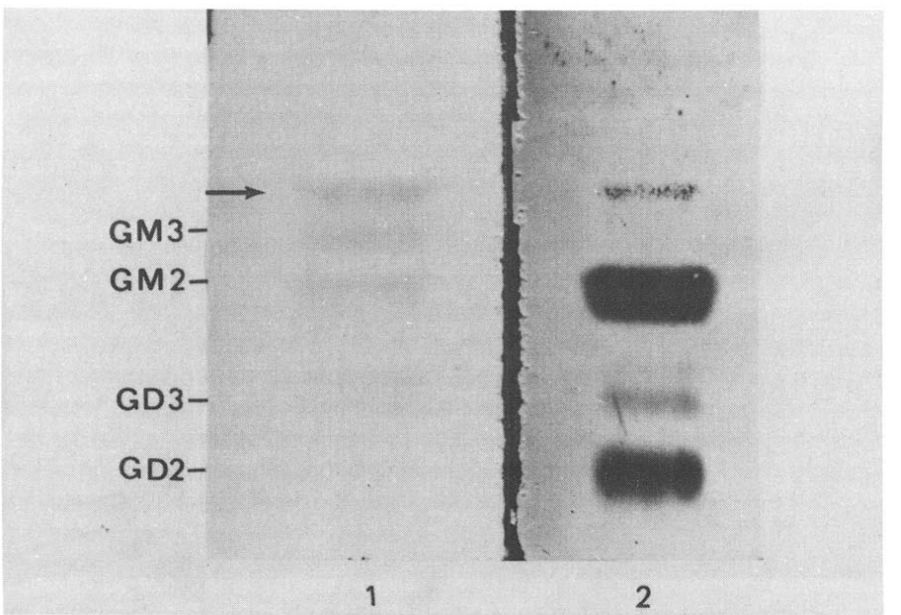

Fig. 3. Thin-layer chromatography of 1 μg of total melanoma gangliosides in the solvent described in Fig. 2, then peroxidase-linked immunostaining with mouse monoclonal anti-gangliotriaosylceramide (anti-asialoGM2) following plate treatment with *Vibrio cholerae* neuraminidase (0.5 U in 5 ml for 18 h). 1 = Resorcinol · HCl visualization of 2 μg of gangliosides (the arrow indicates acidic phospholipids); 2 = immunostaining of melanoma gangliosides after plate treatment by neuraminidase. Control without neuraminidase displayed staining on the phospholipids only (not shown).

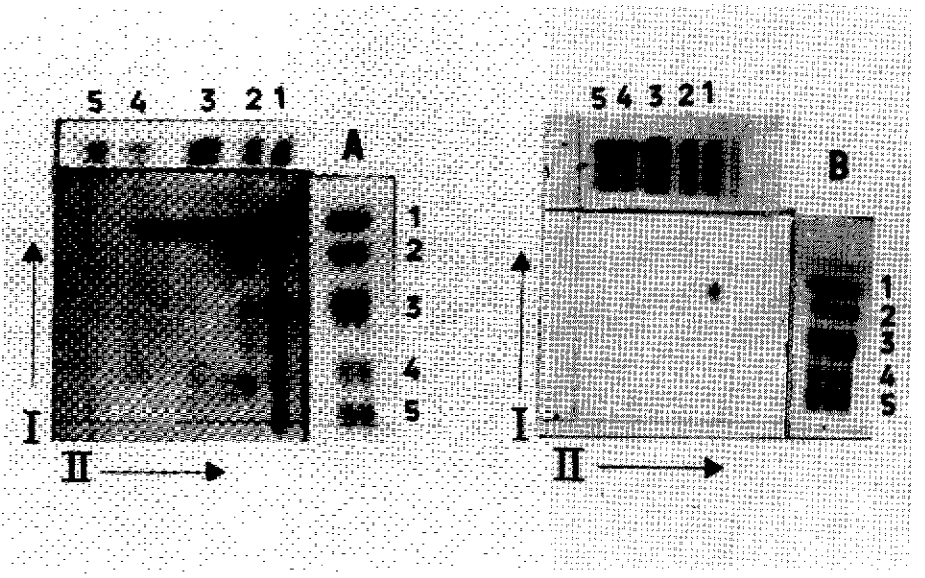

Fig. 4. Two-dimensional thin-layer chromatography of rat brain gangliosides and ganglio-tetraosylceramide (asialoGM1) in the solvent described in Fig. 2, with intermediate neuraminidase treatment of the plate (0.2 U in 5 ml, 18 h; see Experimental for details), then peroxidase-linked immunostaining with rabbit antiserum to asialoGM1. Standard lanes visualized with orcinol · H_2SO_4: 1 = asialoGM1; 2 = GM1; 3 = GD1a; 4 = GD1b; 5 = GT1b; I = first migration; II = second migration. (A) 5 μg of gangliosides and 2 μg of asialoGM1 spotted. (B) Control without neuraminidase: 2 μg of gangliosides and 0.2 μg of asialoGM1 spotted. The reactive spot migrating above asialoGM1 is unidentified.

Rat brain gangliosides, which contain a large amount of GM1 and GD1b, were also used as substrates for solid-phase hydrolysis with *Vibrio cholerae* neuraminidase. Immunostaining with rabbit antiserum, raised against ganglio-tetraosylceramide (asialoGM1), monitored ganglioside hydrolysis. However, because of the slight cross-reactivity of this antiserum with GM1 and GD1b (Fig. 4B), the gangliosides had to be separated on a second dimension after neuraminidase treatment to ensure proper identification of the products of hydrolysis. Fig. 4A shows that all asialoGM1-containing gangliosides were cleaved upon incubation with neuraminidase from *Vibrio cholerae*, including minor gangliosides that are not visualized by orcinol · H_2SO_4. GM1 was also a product of hydrolysis from several gangliosides and a stained tailing was observed on the second migration lane of asialoGM1 after neuraminidase. No asialoGM1 was released in the control experiment without the enzyme. Li et al. [13] reported some cleavage of GM1 by *Vibrio cholerae* neuraminidase in test tubes in the presence of sodium taurodeoxycholate, whereas in detergent-free buffer, GM2, but not GM1, was hydrolysed, and only by the enzyme from *Clostridium perfringens*. With the latter enzyme, in test tubes, we observed the conversion of GD2, but not GM2, into asialoGM2 in detergent-free buffer. Rauvala [14] showed that, in the absence of detergent, GM1 and GM2 below the critical micelle concentration are susceptible to *Clostridium perfringens* neuraminidase. It is thus likely that the enzymes from both sources hydrolyse the gangliosides on thin-layer plates without detergent

because of the low concentrations of substrate (nanogram range) required for the subsequent immunostaining procedure. The efficacy of *Vibrio cholerae* neuraminidase on GM2 and GM1 suggests that following adsorption of the gangliosides on the hydrophobic silica gel layer, the carbohydrate moiety is oriented outside, thus available for enzyme binding.

Further work is needed to determine whether the whole carbohydrate moiety of glycosphingolipids is susceptible to solid-phase enzymic hydrolysis on thin-layer plates. In our laboratory, preliminary experiments of cleavage with various glycosyl hydrolases have been successful on globoside, paragloboside and fucosyl GM1. The data presented in this paper show that this susceptibility should be useful to gain information on the structure of gangliosides, especially when available in small amounts.

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