TLC immunostaining characterization of *Clostridium botulinum* type A neurotoxin binding to gangliosides and free fatty acids

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The receptor structure of *Clostridium botulinum* neurotoxin type A was analysed by TLC immunostaining. GQ1b was found to be the most potent receptor, and the neurotoxin also bound to GT1b and GD1a, but not to GM3, GM2, GM1, GD3, GD1b and GT1a. Optimum binding of neurotoxin to the ganglioside appeared in 0.01 M phosphate buffer (pH 7.2) containing 0.2% NaCl. Higher and lower NaCl concentrations diminished neurotoxin binding to the ganglioside. In addition, the neurotoxin was able to bind to free fatty acids. Maximum binding was observed on stearic acid and neurotoxin binding to free fatty acids was not affected by NaCl concentration.

TLC *GQ1b ganglioside* *Anti-neurotoxin antibody* *Salt effect*

1. INTRODUCTION

Since the GM1 ganglioside was first demonstrated to be a cholera toxin receptor [1], several molecular species of gangliosides have been shown to participate in receptors for various bacterial toxins [2]. However, various analytical methods based on different principles such as inactivation of toxic activity, inhibition of toxin binding, conformational change of the toxin molecule, equilibrium dialysis, change in mobility on polyacrylamide gel electrophoresis and enzyme-linked immunoassay to gangliosides on plastic microtiter plates have sometimes given erroneous results mainly due to the unique physicochemical properties of gangliosides shown in micellar formation, negative charge, hydrophobic interaction, etc. Instead of the above indirect methods, direct binding analysis of toxin molecule to gangliosides on thin-layer plates [3] has made it possible to identify the receptor structure more clearly and to detect the receptor molecule quantitatively and sensitively. In fact, by application of this procedure, cholera toxin was shown not only to possess a strong affinity to GM1 but also to react with GD1b to a lesser extent (Takamizawa et al., unpublished). We also attempted to elucidate the receptor structure of *Clostridium botulinum* neurotoxin (type A), which binds to the presynaptic membrane on the neuromuscular junction [4], by a newly developed thin-layer chromatographic (TLC) immunostaining procedure.

2. MATERIALS AND METHODS

2.1. *Materials*

Gangliosides GM3, GM2, GM1, GD3, GD1a, GD1b, GT1a, GT1b and GQ1b were purified from human brain as in [5,6]. Commercially available free fatty acids were purified by reverse-phase high-performance liquid chromatography.

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2.2. *TLC immunostaining*

Gangliosides and free fatty acids were developed on silica gel-coated plastic plate (Polygram, Sil G, Macherey-Nagel, FRG) with chloroform, methanol, 0.5% $CaCl_2 \cdot 2H_2O$ (55:45:10, by vol.) and *n*-hexane, diethyl ether, acetic acid (80:30:1, by vol.), respectively and the plate was immersed in blocking buffer (1% ovalbumin, 1% polyvinylpyrrolidone and 0.02% NaN_3 in phosphate-buffered saline) at 37°C for 1 h. After washing the plate with 3% polyvinylpyrrolidone in phosphate-buffered saline, it was incubated with 0.5 μg/ml of *C. botulinum* neurotoxin (type A) at 37°C for 2 h. The toxin bound on the plate was detected with rabbit anti-*C. botulinum* neurotoxin (type A) antiserum and peroxidase-conjugated goat anti-rabbit IgG antiserum [7]. The substrate of peroxidase was 0.003% H_2O_2 and 0.003% 4-chloro-1-naphthol in 50 mM Tris-HCl buffer (pH 7.4) containing 200 mM NaCl. The intensity of the spot was determined with a dual-wavelength TLC densitometer at an analytical wavelength of 580 nm and a control wavelength of 710 nm (CS-910, Shimadzu).

3. RESULTS

3.1. *Optimum conditions for TLC immunostaining with* C. botulinum *neurotoxin (type A)*

Using 200 ng of GT1b as a receptor, the effects of pH, NaCl concentration and incubation time on *C. botulinum* neurotoxin binding (type A) were studied (fig.1). The highest degree of binding of toxin was observed in phosphate buffer (pH 7.2) containing 0.2% NaCl. Although the reaction at 37°C for 18 h gave an intense spot, incubation for 2 h was sufficient to quantitate the intensity of the spot with a TLC densitometer. Also, the intensities of the spots obtained by incubation at 37 and 5°C did not differ significantly.

3.2. *Interaction of* C. botulinum *neurotoxin (type A) with gangliosides*

Under the optimum condition determined above, *C. botulinum* neurotoxin (type A) was reacted with 0.01–0.64 nmol of various gangliosides to clarify the structural requirement of the neurotoxin type A receptor. As shown in fig.2, GQ1b was shown to be the most reactive

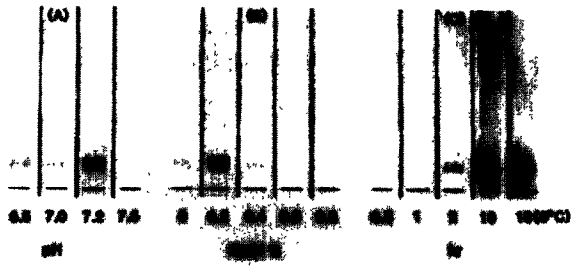

Fig.1. Effects of pH (A), NaCl concentration (B) and incubation time (C) on *C. botulinum* (type A) neurotoxin binding to GT1b. 200 ng GT1b developed on a TLC plate were reacted with 0.5 μg/ml of *C. botulinum* neurotoxin. Reaction media: (A) 0.01 M phosphate buffer at various pH values containing 0.2% NaCl, (B) various concentrations of NaCl in 0.01 M phosphate buffer (pH 7.2), (C) 0.01 M phosphate buffer (pH 7.2) containing 0.2% NaCl.

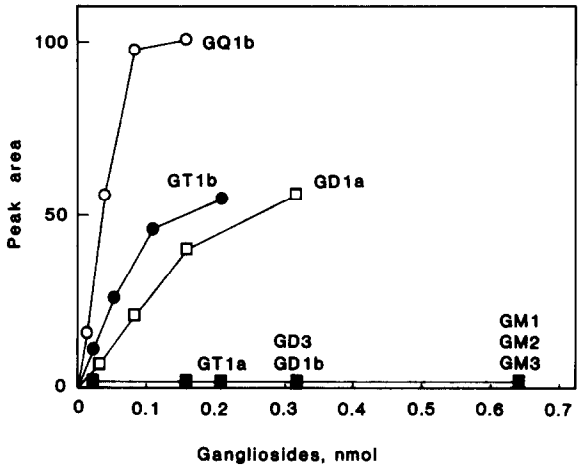

Fig.2. Binding of *C. botulinum* (type A) neurotoxin to various gangliosides. Analysis was performed according to the method described in the text.

with the toxin, and GT1b and GD1a were also potential receptors for the toxin. However, unexpectedly, GD1b and GT1a, both of which showed distinct neutralizing activity for the in vivo toxicity [8], were not able to be bound with the toxin. The toxin did not bind to GM3, GM2, GM1 and GD3.

3.3. *Interaction of* C. botulinum *neurotoxin (type A) with free fatty acids*

In a separate experiment, we found that lipid extracts from human milk fat globule membrane

(MFGM) neutralized the in vivo toxicity of the toxin (Takamizawa et al., unpublished) and investigated the component involved in the neutralization. As shown in fig.3, lipid extracts from human milk contained cholesterol ester, triglycerides, free fatty acids and cholesterol, among which only free fatty acids showed binding

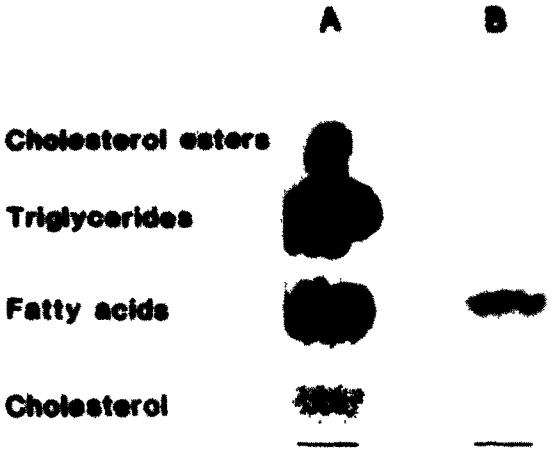

Fig.3. Binding of *C. botulinum* (type A) neurotoxin to lipids from human milk.

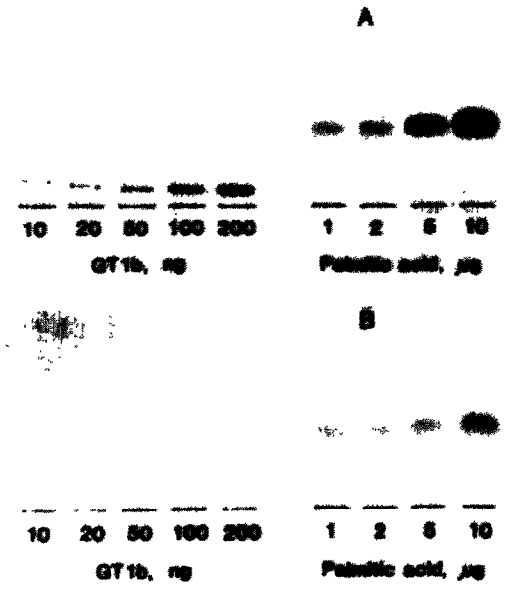

Fig.4. Binding of *C. botulinum* (type A) neurotoxin to GT1b and palmitic acid in 0.01 M phosphate buffer containing 0.2% (A) and 0.8% (B) NaCl.

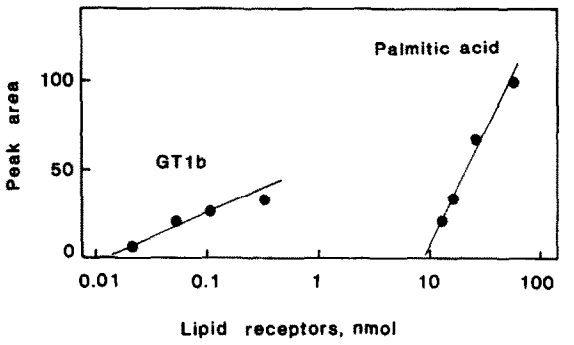

Fig.5. Binding of *C. botulinum* (type A) neurotoxin to GT1b and palmitic acid. Analysis was performed in 0.01 M phosphate buffer (pH 7.2) containing 0.2% NaCl.

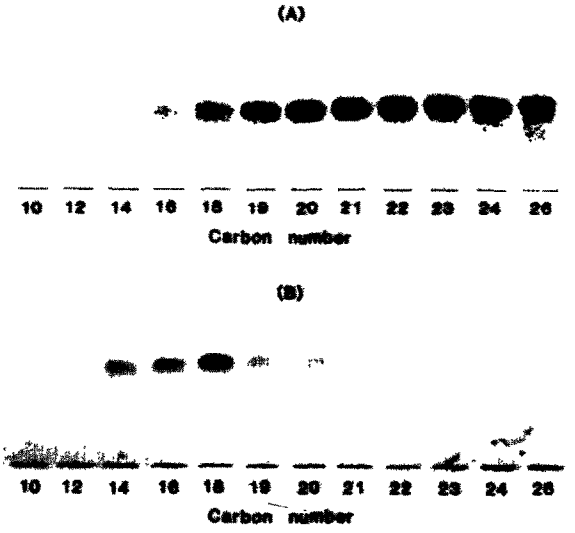

Fig.6. Binding of *C. botulinum* (type A) neurotoxin to free fatty acids of various chain lengths. 10 μg free fatty acids developed on a TLC plate were visualized with Cu-phosphoric acid reagent (A) and stained by TLC immunostaining with *C. botulinum* (type A) neurotoxin according to the method described in the text.

ability with the neurotoxin, indicating that carboxylic acid is involved in the toxin receptor. However, the binding property of free fatty acids with the neurotoxin was found to differ from that of ganglioside. Thus, the binding ability of ganglioside GT1b was completely lost at 0.8% NaCl, whereas that of free fatty acids was still retained (fig.4). At 0.2% NaCl, the amount of toxin binding to 10 nmol palmitic acid was equivalent to that to 0.03 nmol GT1b (fig.5). In addition, to

elucidate the binding characteristics of the neurotoxin to free fatty acids, we determined the degree of toxin binding to free saturated fatty acids with different chain lengths of 10–26 carbons (fig.6) and found that stearic acid showed stronger affinity with the neurotoxin than the shorter and longer fatty acids, indicating that hydrophobicity as well as the negative charge of free fatty acids is important for neurotoxin binding.

4. DISCUSSION

In place of the indirect analytical procedures, TLC immunostaining for the analysis of toxin receptor has become a useful method to characterize the fine structure of the receptor glycolipids and to determine the amount of receptor. By TLC immunostaining, *C. botulinum* neurotoxin (type A) was found to bind to GQ1b, GT1b and GD1a, but not to GT1a, GD1b, GM3, GM2, GM1 and GD3. Ganglio-*N*-tetraosyl ceramide as a core structure of gangliosides was essential for the receptor structure, and ganglio-*N*-tetraosyl ceramide with a disialosyl residue at the internal galactose required a mono- or disialosyl residue at the terminal galactose (e.g. GT1b and GQ1b). However, ganglio-*N*-tetraosyl ceramide with a monosialosyl residue at the internal galactose required a monosialosyl residue at the terminal (GD1a) for binding and the presence of a disialosyl residue at the terminal (GT1a) resulted rather in the loss of toxin binding. These results were somewhat different from those obtained from the detoxification experiment [8], which showed GT1b and GQ1b to be the most potent inhibitors and GD1a, GD1b and GT1a to be moderate inhibitors. In view of both results obtained with the different procedures, it is certain that GQ1b, GT1b and GD1a are definitely involved in the *C. botulinum* (type A) neurotoxin receptor. In addition, neurotoxin binding to free fatty acids was clearly demonstrated as shown in fig.6, although a much larger amount of fatty acids was necessary than that of the active gangliosides. Since this binding was not affected by NaCl concentration, the mode of binding for gangliosides and free fatty acids seemed to differ. It is evident that the binding of free fatty acids requires both the carboxylic acid and suitable hydrophobicity. However, for further characterization of the precise functional structure of the neurotoxin receptor, it is necessary to analyze the three-dimensional structure of carboxylic acid taken on a TLC plate. Analysis of synaptic membranes to clarify the native toxin receptor is now in progress in our laboratory.

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