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Biochimica et Biophysica Acta 1741 (2005) 1 – 3
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Rapid report

Different response of the knockout mice lacking b-series gangliosides against botulinum and tetanus toxins

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Received 16 February 2005; received in revised form 11 April 2005; accepted 25 April 2005
Available online 13 May 2005

Abstract

We assessed the response in knockout mice lacking the b-series (G_{D2}, G_{D1b}, G_{T1b} and G_{Q1b}) gangliosides against *Clostridium botulinum* (types A, B and E) and *tetani* toxins. We found that botulinum toxins were fully toxic, while tetanus toxin was much less toxic in the knockout mice. Combining the present results with our previous finding that tetanus toxin and botulinum types A and B toxins showed essentially no toxic activity in the knockout mice lacking both the a-series and b-series gangliosides (complex gangliosides), we concluded that the b-series gangliosides is the major essential substance for tetanus toxin, while b-series gangliosides may be not the essential substance for botulinum toxins, at the initial step during the intoxication process in mouse.

Keywords: Botulinum toxin; Tetanus toxin; Toxin; *Clostridium*; Ganglioside; Receptor; Knockout mouse

Tetanus [1] and botulinum [2] toxins produced by *Clostridium tetani* and *botulinum* exhibit very strong neurotoxicity that causes the blockage of neurotransmitter release [3–5]. The steps toward the blockage of neurotransmitter release by these toxins are believed to involve the specific binding and the penetration of the toxin into neural cells, followed by the proteolytic cleavage [3–5] of SNARE complex proteins that are the fundamental machinery for fusing presynaptic membrane and synaptic vesicles. We have previously found [6] that the gangliosides located on the surface of synaptic membranes are the major essential substance in neural cells for the intoxication of both tetanus toxin and botulinum types A and B (also type E; unpublished data by Kitamura) toxins in mice. This conclusion was derived from the evidence that both toxins did not show their toxic activity in the knockout mice [6] lacking complex gangliosides (G_{M1}, G_{D1a}, G_{D1b}, G_{T1a} G_{T1b} and G_{Q1b}) [7] as the result of the disruption of the β1,-4-*N*-acetylgalactosaminyltransferase (G_{M2}/G_{D2} synthase; EC 2.4.1.92) gene. Following our report [6], our finding was confirmed by a report describing that complex gangliosides at the neuromuscular junction are the membrane receptor for botulinum neurotoxin, using the same knockout mice lacking complex gangliosides [8]. Therefore, we theorized that both tetanus and botulinum toxin recognize and bind with gangliosides on the presynapses as the initial step of intoxication, before the penetration of the toxins into the neural cells [6]. The identification of the substances (receptor) binding the toxins as a first step on the way into neural cells is crucially important to understanding the disease caused by the toxins. However, the identification of a specific molecular species of ganglioside has not been successful in a previous work [6]. Therefore, we have chosen to use knockout mice lacking b-series (G_{D3}, G_{D2}, G_{D1b}, G_{T1b} and G_{Q1b}) gangliosides, resulting from a disruption of the alpha 2,8 sialyltransferase (GD3 synthase) gene [9]. These knockout mice were deficient in b-series gangliosides, which commonly contained 2 mol of sialic acid residues at internal sites on the gangliosides, but retained a-series gangliosides, which commonly contained 1

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doi:10.1016/j.bbadis.2005.04.005

mol of sialic acid residue at an internal site on the gangliosides, including $G_{M2,}$ G_{M1}, G_{D1a} and G_{T1a}. The knockout mice lacking either complex type gangliosides or b-series gangliosides showed no defects in brain morphology and organogenesis [7,9].

We examined the response of the knockout mice lacking b-series gangliosides against *Clostridium botulinum* (type A, B and E) and *tetani* toxins. The assessments of toxic activity were judged by measuring death time (survival time) after intravenous injection of toxin in mice. This bioassay was based on the linear relationship between the logarithm (log) of the survival time (min) and the log of the toxin dose (lethal dose (LD_{50})) for botulinum toxin (see [10]) and toxin dose (μg toxin protein/mouse) for tetanus toxin (see [11]). These bioassay methods have been accepted in this field.

One of the three types of toxins, type A, B or E, was injected into the knockout mice and wild type mice. The results from botulinum types A, B and E are shown in Table 1. There was essentially no difference in toxic effects between the wild and the knockout mice.

We injected tetanus toxins intravenously into the homozygous (knockout) mice and into wild type. The result with tetanus toxin is shown in Table 2. The wild type mice quickly succumbed to the toxic effects, while the knockout mice showed a much lower toxic effect (Table 2). These results indicated that the toxic activity estimated by the average survival time [11] was 61.7 μg/mouse for wild type and 0.9 μg/mouse for knockout mice. This implies that the knockout mice require about 70 times higher concentration of the toxin protein than do the wild type mice for a lethal dose; that is, the toxin does not exert its activity effectively in the knockout mice.

Table 1
Sensitivity of wild type and knockout mice to *Clostridium botulinum* types A, B and E toxins

	Survival time (min)					Toxic activity (LD_{50}/ml)
Type A toxin						
Wild type	40	40	45	44	(42)	202,700
Homozygous	38	45	44	39	(41)	219,700
Type B toxin						
Wild type	44	45	58	55	(51)	57,000
Homozygous	44	40	47	70	(50)	59,800
Type E toxin						
Wild type	32	36	35	30	(33)	112,500
Homozygous	29	34	35	34	(33)	112,500

Homozygous is the knockout mice.
The average survival time of four mice is shown in parentheses.
The toxic activity (LD_{50}/ml) of botulinum toxins is estimated by the average survival time (see [10]).
The mice were 7–8 weeks from birth, 18–19 g of body weight.
The four mice were a mixture of both sexes.
Clostridium botulinum toxins. Type A neurotoxin was purified by the method described previously [13]. Type B toxin was purchased from Wako Pure Chemical Industries (Code no. 026-08181), Ltd. Osaka. Type E activated 12S toxin was purified by a method described previously [14].
The mice were injected with 0.1 ml of botulinum toxin solution (202,700 LD_{50}/ml for type A, 57,000 LD_{50}/ml for type B and 112,500 LD_{50}/ml for type E each) in a 10 mM phosphate buffer (pH 7.2) containing 0.85% of NaCl and 0.2% bovine serum albumin for starting toxin dose.

Table 2
Sensitivity of wild type and knockout mice to *Clostridium tetani* toxin

	Survival time (min)					Toxic activity (μg/mouse)
Wild type	55	56	53	62	(57)	61.7
Homozygous	212	190	169	212	(205)	0.9

Homozygous is the knockout mice.
The average survival time of four mice is shown in parentheses.
The toxic activity (toxin protein dose per mouse: μg/mouse) was estimated by the average survival time (see [11]).
The mice were 7–8 weeks from birth, 18–19 g of body weight.
The four mice were a mixture of both sexes.
Tetanus toxin was purified according to a method as described previously [15].
The mice were injected with 61.7 μg of tetanus toxin in 0.1 ml of 10 mM phosphate buffer (pH 7.2) containing 0.85% of NaCl and 0.2% bovine serum albumin for starting toxin dose.

These results show that in the intoxication process, the b-series gangliosides are the major essential substance for tetanus toxin activity. The b-series gangliosides may not be essential for botulinum A, B and E toxins at the equivalent initial step during the intoxication process in mouse.

There remains an open question whether botulinum toxins act via the a-series gangliosides alone, or via a- and b-series gangliosides independently, and that the over-expression of G_{D1a} in the brain by a disruption of the alpha 2,8 sialyltransferase (GD3 synthase) gene [9] accounts for retained toxicity of botulinum toxins in mice lacking b-series gangliosides. In the future, when mice lacking the terminal ganglioside sialyltransferase(s) and when other approaches are available, this matter can be solved.

Tetanus toxin still had a very low toxic activity in the knockout mice lacking b-series gangliosides (see Table 2), and tetanus and botulinum toxins also showed similar residual actions in previous results using the knockout mice lacking complex gangliosides [6]. This may be due to the presence of other minor route(s) without the involvement of the b-series (for tetanus toxin) and the a- and b-series (for botulinum toxin) gangliosides in the intoxication process for the toxins in mouse.

Our previous report [12] indicated that botulinum type A toxin interacted with gangaliosides in vitro and that these gangliosides bound to the toxins in vitro detoxified the neurotoxicity. Both G_{T1b} and G_{Q1b} in the b-series of gangaliosides showed the strongest detoxification ability in vitro, among various gangliosides [12]. These results of an in vitro experiment performed under non-physiological conditions are not consistent with the present results of in vivo experiments showing that the b-series of gangaliosides were not essential for botulinum toxins. However, gangliosides have a strong and specific affinity for the toxins under the low ionic strength in vitro [12].

The present results, together with the previous results [6], suggest that both the number and location of sialic acid residues in the carbohydrate backbone of a ganglioside molecule are important in the presynaptic membranes. It is conceivable that both botulinum and tetanus toxins are recognized and bound by gangliosides located on the presynaptic membranes, before the penetration of the toxin into the neural cells, in the intoxication process, from the initial step (receptor binding) to the final step (proteolytic cleavage of SNARE complex proteins), in mouse. While the characteristic symptoms in human induced by *Clostridium botulinum* and *tetani* toxins are apparently different, the studies in vitro on the mode of action of the two toxins at the molecular level showed no clear difference between these toxins, which have similar molecular structures, similar actions of proteolytic cleavage on target substances (SNARE protein complex) in the presynapes and similar binding with gangliosides in vitro [3–5]. However, the present in vivo results show that *Clostridium botulinum* and *tetani* toxins are clearly different in the response to the knockout mice lacking b-series (G_{D2}, G_{D1b}, G_{T1b} and G_{Q1b}) gangliosides.

Acknowledgements

We thank Dr. K. Onodera (Kogakuin University, Tokyo), Dr. K. Hashimoto (National Institute of Infectious Diseases, Tokyo) and Dr. K. Kusano (former National Institutes of Health, Bethesda) for their discussions and critical reading of the manuscript.

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