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Rapid report

Gangliosides are the binding substances in neural cells for tetanus and botulinum toxins in mice

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Abstract

We used the knockout mice lacking gangliosides and evaluated their response to tetanus and botulinum toxins. We found that tetanus toxin and botulinum type A or B toxin was less toxic in the knockout mice. We conclude that the toxins bind to the gangliosides on the synapses in the initial step of intoxication prior to penetration of the toxins into the neural cells. © 1999 Elsevier Science B.V. All rights reserved.

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Tetanus and botulinum toxins produced by *Clostridium tetani* and *Clostridium botulinum*, respectively, exhibit the strongest neurotoxicity and have been a threat to human health. Tetanus toxin invades from the wound, reaches nerve endings, and causes the blockage of chemical transmitter release. Wassermann and Takaki [1], a century ago, discovered that when an emulsion of brain tissue was mixed with tetanus toxin, the toxin was bound by the tissue. Subsequently van Heyningen in 1957 [2] confirmed the earlier observation and suggested that the component in neural tissues that fixed tetanus toxin in vitro was ganglioside [2,3].

Botulinum toxin (types A–G) causes blockage of the acetylcholine release at the neuromuscular junction and eventually death from asphyxiation due to diaphragm paralysis. In addition to the peripheral cholinergic synapses, the neurotoxin binds to the synaptosome of the central nervous system in vitro [4,5], particularly the presynaptic membrane [6]. The toxin-binding ability of synaptosomes decreases after treatment with neuraminidase, but not with trypsin [7].

The gangliosides that were thought to be the binding components for the toxin in vitro were separated into several molecular species and were pointed out to have numerous biological activities [8]. At the same time it was shown in vitro that the purified tetanus [9] and botulinum [10] neurotoxins interacted with the purified ganglioside G_{1b} series, particularly G_{T1b} or G_{Q1b} [10]. The steps toward blockage of

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transmitter release at the synapse are believed to involve specific binding, then penetration of neural cells by the toxin followed by proteolytic cleavage of SNARE complex proteins [11,12].

The characterization of the receptor is crucially important to understand how the toxin is recognized and penetrates. Despite a large number of in vitro experiments [4,5], the question has remained open whether the gangliosides as binding substances are truly part of receptors for tetanus and botulinum toxin in a whole body.

To solve this question we used knockout mice [13], lacking complex gangliosides including G_{M1}, G_{D1a}, G_{D1b}, G_{T1b} and G_{Q1b} because of disrupted $\beta_{1,4}$-*N*-acetylgalactosaminyltransferase (G_{M2}/G_{D2} synthase; EC 2.4.1.92) gene, for evaluating their responses to tetanus and botulinum toxins. These knockout mice were deficient in gangliosides but showed no defects in brain morphology and organogenesis [13].

We injected separately the same amount of tetanus, botulinum type A and B neurotoxin intravenously into the knockout mice and as control into wild type mice and assessed their survival time. The results from the tetanus study are shown in Table 1. The wild type mice quickly succumbed to the toxic effects while the homozygous mice showed a less toxic effect. The average survival time for wild type mice was 84 min, while that of knockout mice was 580 min. The knockout mice exhibited similar symptoms of paralysis leading up to their death as the wild type mice. There is a formula which has been accepted in this field to calculate the protein concentration from the survival time (see [14]). Assuming that this formula fits in the case of knockout mice the toxic protein, estimated by the survival time, in wild type mice was 17 μg/mouse and in knockout mice 0.03 μg/mouse. This means that the wild type mice were killed by lesser amounts of protein than the knockout mice.

Table 1
Sensitivity of wild type and knockout mice to tetanus toxin

	Survival time (min)				
Wild type	86	79	67	105	(84)
Homozygous[a]	540	540	660	540	(580)

The average survival time of four mice is shown in parentheses. The mice used were female, 23 weeks from birth and 23–28 g in body weight.

C. tetani neurotoxin was purified according to the method described previously [16]. The mice were injected with 0.1 ml of tetanus toxin solution in phosphate buffered saline containing 0.5% bovine serum albumin (this solution killed the mouse in 84 min).

[a]Knockout mice.

Table 2
Sensitivity of wild type and knockout mice to botulinum type A and B toxins

	Survival time (min)				
Type A toxin					
Wild type	55	55	55	60	(56)
Homozygous[a]	154	157	172	200	(171)
Type B toxin					
Wild type	46[b]	50[b]	53[b]	53	(51)
Homozygous[a]	107	107	137	106	(114)

The average survival time is shown in parentheses.

The mice used for type A toxin were 15 weeks from birth, 20–29 g of body weight. The mice used for type B toxin were 23 weeks from birth, 24–29 g of body weight. The mice were a mixture of female and male.

C. botulinum type A neurotoxin was purified by the method described previously [17]. *C. botulinum* type B toxin was purchased from Wako Pure Chemical Industries, Osaka. The mice were injected with 0.1 ml of botulinum toxin solution in phosphate buffered saline containing 0.5% bovine serum albumin (this solution killed the mouse in 56 min for type A toxin and in 51 min for type B toxin).

[a]Knockout mice.

[b]ddY mouse was used. (The toxin effect between the wild type mouse and the ddY mouse was not different.)

The results with botulinum types A and B are shown in Table 2. Injected with either sero type of the toxin, the wild type mice quickly succumbed to the toxin effects while the homozygous mice demonstrated resistance to the toxin. The knockout mice exhibited symptoms induced by the toxin similar to those of the wild type mice. The average survival time of wild type mice was 56 min, while that of knockout mice was 171 min for type A and that of wild type mice was 51 min, while that of knockout mice was 114 min for type B. In the case of botulinum toxins, LD_{50}/ml has been used (see [15]). The average lethal dose (LD_{50}) estimated by the survival time (see [15]) was 77 600 LD_{50}/ml in wild type mice, and 1900 LD_{50}/ml in the knockout mice for type A, and 57 000 LD_{50}/ml in wild type mice and 2400 LD_{50}/ml for type B in the knockout mice. This also

means that the knockout mice were killed by a larger amount of toxins than the wild type.

These results can be interpreted that tetanus and botulinum neurotoxins recognized the gangliosides in the intact animal. Because in the absence of endogenous gangliosides, the knockout mice appeared as if they were injected with a lesser amount of toxic protein.

Our present in vivo studies strongly suggest that the gangliosides in addition to existing in membranes form specific structure(s) that participate in the penetration of toxins into the neural cells.

It seems that the gangliosides are not essential for life maintenance because the knockout mice showed no apparent defect; however, our study suggests that it binds to toxins and/or other compounds that interact with gangliosides in vitro. The present results give some further hints on glycolipid biology research in the medical field, especially pathogenesis of neurotoxins.

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