EFFECT OF ANTITOXIN ON FIXATION OF TETANUS TOXIN BY SUBCELLULAR BRAIN STRUCTURES

N. G. Bondarchuk, G. N. Kryzhanovskii,* and A. Ya. Rozanov

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Experiments with purified tetanus toxin labeled with I^{131} showed that the toxin is fixed by the "unpurified mitochondrial" fraction of the guinea pig brain, which includes mitochondria, synaptosomes, and myelin fragments. The ability to fix labeled toxin is concentrated in the synaptosomes, is less strong in the myelin fragment, and weaker still in the mitochondria. Labeled toxin, if neutralized by antitoxin, is also fixed by these cell structures although to a rather lesser degree. If toxin and antitoxin are added simultaneously to the fraction of coarse mitochondria, no significant decrease in fixation of the toxin by the synaptosomes and myelin fragments is observed. The results confirm the previous hypothesis that the tetanospasmin molecule contains different centers responsible for fixation with nerve tissue and with antitoxin.

Previous investigations [1, 2] have shown that I^{131}-labeled tetanus toxin, neutralized with antitoxin, is fixed by protagon to the same degree as active tetanus toxin. The complex of gangliosides with cerebrosides from which protagon is formed, and which is responsible for the fixation of toxin by protagon, is a component part of the cell organelles of nerve tissue.

It was therefore decided to study the fixation of labeled tetanus toxin by subcellular fractions of the brain after its neutralization by antitoxin.

EXPERIMENTAL METHOD

Subcellular brain structures (synaptosomes, myelin fragments, mitochondria) and the "unpurified mitochondrial" fraction were isolated by the method of Gray and Whittaker [3]. A 10% guinea pig brain homogenate in 0.32 M sucrose was obtained with the aid of a Teflon electromechanical homogenizer. Each sample contained 100 mg protein of the homogenate or from 9 to 14 mg protein of "unpurified mitochondria."

Tetanus toxin (TT), purified by the method of Pillemer et al. [11] and obtained from dry toxin of the commercial strain No. 228 of the Leningrad Institute of Vaccines and Sera, was used. The I^{131}-labeled toxin was prepared from the TT by the method described earlier [3, 4]. The biological activity and specific radioactivity of the TT-I^{131} corresponded to that described previously [2], namely 62,500 LD_{50} and 400,000 counts/min/mg protein.

Freshly prepared TT-I^{131} was added to the brain homogenate at the rate of 1 μg to 1 mg protein of the homogenate (100 μg to homogenate from 1 g brain), and to the fraction of unpurified mitochondria at the rate of 1 μg TT-I^{131} to 1 mg protein of the fraction. The antitoxin – purified Diagerm-3 IEM tetanus antiserum – was diluted in 0.32 M sucrose and added simultaneously with the toxin in doses of 10, 100, and 1,000 i.u.

*Corresponding Member, Academy of Medical Sciences of the USSR.

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TABLE 1. Effect of Tetanus Antitoxin on Fixation of TT-I^{131} by Subcellular Structures when Added to Brain Homogenate

Experimental conditions	Protein of antiserum (mg)	Synaptosomes		Myelin fragments		Mitochondria	
		$M \pm m$	%	$M \pm m$	%	$M \pm m$	%
Homogenate + TT-I^{131}		1,58±0,12	100	1,16±0,13	100	0,74±0,04	100
Homogenate + TT-I^{131} + 10 i.u. antitoxin	1,25	1,33±0,15 $P_{0,23}$	84	0,97±0,12 0,35	83	0,75±0,03 0,5	102
Homogenate + TT-I^{131} + 100 i.u. antitoxin	12,5	1,21±0,16 $P_{0,11}$	76	0,98±0,12 0,35	84	0,64±0,03 0,09	85

Note. Here and in Table 2, fixed toxin (TT-I^{131}) shown in μg/mg protein of the fractions. The number of samples was 9.

(1.25, 12.5, and 125 mg protein, respectively) or sample of homogenate and 10 and 100 i.u. per sample of the "unpurified mitochondrial" fraction. In control experiments Diagerm-3 IEM antidiphtheria horse serum was used in doses of 1.25 and 12.5 mg protein, respectively.

Incubation of the homogenate with TT-I^{131} and antitoxin was carried out in 0.32 M sucrose, while the fraction of "unpurified mitochondria" was incubated with the toxin and serum in isotonic medium [6] containing 0.007 M phosphate buffer, pH 7.4, 0.2 M $MgSO_4$, 0.2 M Versene reagent (EDTA), 0.02 M ATP, 0.2 M sodium pyruvate, and 0.32 M sucrose solution for 10 min at 37°C. The mixture was then cooled to 0°C and the fractions of mitochondria, synaptosomes, and myelin fragments were then separated by differential centrifugation in a density gradient. The specific radioactivity was determined with a type SBT-13 counter and noise-free UMF-1500 apparatus. Protein was determined by Lowry's method [7].

EXPERIMENTAL RESULTS

The results of the experiments to study the effect of antitoxin on fixation of labeled toxin by subcellular structures in brain homogenate are given in Table 1. Ability to fix TT was most marked in synaptosomes, less so in the myelin fragments, and the least quantity of toxin was fixed by the mitochondria. This rule is in agreement with the results obtained by estimation of the fixed toxin biologically [8].

On neutralization of the TT with antitoxin the quantity of toxin fixed by the subcellular brain fractions was reduced (Table 1). This decrease, however, was comparatively small and was not statistically significant. If the dose of antitoxin added was in known excess over that neutralizing the toxin in the medium (100 i.u.), fixation of the toxin by the synaptosomes was reduced by 24%, and by the myelin fragments and mitochondrial fraction by 15%.

In the next experiments the effect of antitoxin on fixation by the fraction of "unpurified mitochondria," consisting of synaptosomes, myelin fragments, and mitochondria, was investigated. The results of these experiments (Table 2) showed that the differences observed in fixation of the toxin by synaptosomes, myelin fragments, and mitochondria were similar to those discovered in the previous series of experiments in which toxin was added to the whole homogenate.

Antitoxin, if added to the "unpurified mitochondria," reduced the fixation of labeled toxin. Under these circumstances, however, there was a smaller decrease in the fixation of neutralized tetanus toxin by the organelles compared with the first series of experiments. For instance, depending on the dose of antitoxin used, the synaptosomes fixed 99-90% of the neutralized toxin compared with the active toxin. It is important to emphasize that diphtheria antiserum, which is a partially proteolyzed horse globulin purified in the same way, had a similar effect on the fixation of tetanus toxin. The membranes of the corresponding cell organelles can evidently fix neutralized and active tetanus toxin equally well.

Presumably the differences in the affinity of organelles of the nerve cell for tetanus toxin depend on their content of gangliosides, the receptors of tetanus toxin in brain substance [9, 13, 14]. The subcellular fractions are known to contain different quantities of gangliosides (mitochondria 0.10 nmole, synaptosomes 0.30 nmole, myelin fragment 0.10 nmole N-ANA per gram fresh tissue) [12]. These same fractions fix TT differently, and as the results of these and previous investigations [10] show, the synaptosomes have the

TABLE 2. Effect of Tetanus Antitoxin on Fixation of TT-I^{131} by Subcellular Structures on Its Addition to the "Unpurified Mitochondrial" Fraction (MF)

Experimental conditions	Antiserum protein (mg)	Synaptosomes		Myelin fragments		Mitochondria	
		$M \pm m$	%	$M \pm m$	%	$M \pm m$	%
MF + TT-I^{131}		1,04±0,03	100	0,74±0,07	100	0,7±0,03	100
MF + TT-I^{131} + 10 i.u. antitoxin	1,25	1,03±0,07	99	0,61±0,05	83	0,59±0,04	85
P		0,96		0,15		0,06	
MF + TT-I^{131} + 100 i.u. antitoxin	12,5	0,94±0,05	90	0,63±0,05	84	0,59±0,05	85
P		0,09		0,2		0,09	
MF + TT-I^{131} + diphtheria antiserum	1,25	1,01±0,07	97	0,7±0,05	94	0,59±0,06	85
P		0,7		0,63		0,15	
MF + TT-I^{131} + diphtheria antiserum	12,5	0,96±0,04	92	0,62±0,05	84	0,56±0,07	80
		0,15		0,17		0,11	

greatest toxin-fixing ability. In this connection it is worth noting that gangliosides are a component mainly of the synaptic membranes [15]. However, other factors with a possible influence on the toxin-fixing properties of brain structures must not be forgotten. These other factors include the relationship between gangliosides and cerebrosides, and also between gangliosides and proteins in the complex [15]. Presumably under natural conditions the conformational state of the membranes fixing the toxin plays an important role in determining the toxin-fixing power of nervous structures.

The facts described above confirm the previous hypothesis [2] regarding the presence of different active centers in the tetanospasmin molecule which are responsible for fixation with the substrate (the receptor in nervous structures) and for binding with antitoxin. Presumably these centers are not only functionally but also spatially independent.

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