PROTAGON BINDING OF TETANUS TOXIN NEUTRALIZED BY ANTITOXIN

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Experiments using I^{131}-labeled purified tetanus toxin and protagon isolated from bovine brain showed that protagon binds tetanus toxins strongly in the presence of antitoxin (purified proteolyzed horse serum, brand "Diagerm-3" IEM) on incubation in physiological saline. Toxin preliminarily neutralized with antitoxin is bound by protagon to the same degree. It is concluded that, besides the toxophore group, the toxin molecule contains another two different functional groups, one responsible for binding of the toxin by the gangliosides of the brain, the other for linking with antitoxin.

Previous investigations [3, 4] showed that tetanus toxin, bound with protagon, can be neutralized by antitoxin and that in the course of such neutralization the toxin is not separated from the protagon. Since the protagon can be regarded as the unpurified physicochemical receptor of the toxin in the brain substance [6, 7], this problem is of considerable theoretical and practical importance.

The object of the present investigation was to study protagon binding of toxin preliminarily neutralized by antitoxin or neutralized with antitoxin actually during the binding process.

EXPERIMENTAL METHOD

Protagon was isolated by a slightly modified method of Tierfeld and Klenk, described previously [4]. Tetanus toxin (TT), obtained from dry toxin of the production strain No. 228 of the Leningrad Institute of Vaccines and Sera, purified by Pillemer's method [5], was used. I^{131}-labeled toxin was prepared from the TT by the method described previously [1, 2]. The activity of the toxin, determined by biological tests in albino mice, was 62,500 LD_{50}/mg protein and the specific radioactivity averaged 356,600 pulses/min/mg protein. The radioactivity of the TT–I^{131} and its complexes with protagon was determined with end-window type counters SI-2B of the B-1 apparatus and SBT-13 of the UMF-1500 apparatus, housed in lead for protection. The density of the residue on the stainless steel targets did not exceed 2 mg/cm^2.

The suspension of protagon in physiological saline (12.5 mg/ml) was obtained with a Teflon electromechanical homogenizer (2000 rpm, 2 min). The freshly prepared TT–I^{131} was added to the suspension in the proportions of 1 and 2 μg/mg protagon (50 and 100 μg toxin to 50 mg protagon) in 0.5 ml of 0.85% NaCl solution. Antitoxin (purified proteolyzed horse serum, brand "Diagerm-3") was diluted with 0.85% NaCl solution and added in a dose of 100 i.u. per sample in a volume of 0.5 ml. The total volume of the incubation medium was 5 ml. Preliminary neutralization of the TT–I^{131} (50 and 100 μg) was carried out by incubating the toxin with an excess of antitoxin (100 i.u.) in 1 ml physiological saline for 1 h at 37°C.

Incubation of the TT–I^{131} or neutralized TT–I^{131} with the protagon suspension continued for 45 min at 37°C, after which the mixture was rapidly cooled to 0° and centrifuged at 2°C (10,000 g, 10 min). The

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TABLE 1. Effect of Antitoxin on Binding of Different Doses of Toxin by Protagon

Toxin/protagon	Experiment	Number of experiments	Relative specific activity		
			M ± m	change (relative to control, in percent)	P
1 μg/1 mg	Protagon + TT–I^{131} (control)	8	16.8 ± 1.7	100	
	Protagon + TT–I^{131} + antitoxin	5	12.3 ± 1.3	72	0.10
	Protagon + TT–I^{131} preliminarily neutralized with antitoxin	5	13.5 ± 1.9	80	0.26
2 μg/1 mg	Protagon + TT–I^{131} (control)	8	20.9 ± 2.3	100	
	Protagon + TT–I^{131} antitoxin	8	14.8 ± 1.0	71	0.04
	Protagon + TT–I^{131} preliminarily neutralized with antitoxin	8	16.1 ± 0.8	77	0.08

TABLE 2. Effect of Antitoxin on Protagon Binding of Labeled Toxin (experiments of series II)

Experiments	Number of experiments	Yield of washed protagon		Relative specific activity		
		mg	%	M ± m	change (in percent of control)	P
50 mg protagon + 50 g TT–I^{131} (control) . . .	8	24.9	50	51.4 ± 4.0	100	
50 mg protagon + 50 g TT–I^{131} + antitoxin . . .	8	30.6	61	28.3 ± 3.7	55	0.004
50 mg protagon + 50 g TT–I^{131} preliminarily neutralized with antitoxin	8	31.5	63	28.9 ± 3.5	56	0.004

residue was washed three times by resuspension in 4.4 ml 0.85% NaCl solution and centrifugation for 10 min at 10,000 g under the same conditions.

The degree of binding of the TT–I^{131} was calculated as the relative specific radioactivity in the experiment of series I, as a percentage of the radioactivity of the original protagon, and in the experiments of series II as a percentage of the radioactivity of the washed-off protagon. In the latter case, losses of protagon during washing off were estimated gravimetrically.

EXPERIMENTAL RESULTS

The results of the experiments of series I, in which the effect of antitoxin on protagon binding of 2 doses of labeled toxin was studied, are given in Table 1. They show, first, that only about 20% of the added dose of labeled toxin was found in the washed protagon. The remaining toxin was evidently lost while the protagon was washed off; it probably also escaped together with the very tiny particles of protagon which, as special experiments showed, were not sedimented even by prolonged centrifugation. These experiments also showed that binding of TT–I^{131} increased only very slightly with an increase in the toxin: protagon ratio from 1 :1000 to 2 :1000.

The chief result of these experiments was the discovery that toxin is bound by protagon in the presence of an excess of antitetanus serum, and also that toxin already neutralized by antitoxin is bound by protagon. Neutralized tetanus toxin was fixed by protagon to the extent of only 20–23% less than the active toxin. Somewhat slower binding of toxin by protagon (28–29% less than in the control) was observed in the case of simultaneous neutralization by antitoxin.

In these experiments no allowance was made for loss of protagon incubated with the toxin during subsequent centrifugation and washing. These losses could have differed in different experiments. Accordingly, the experiments of series II were carried out to study the effect of antitoxin on protagon binding of

TT–I^{131}, in which the yield of protagon after washing was determined and binding of the toxin was calculated per milligram sedimented protagon (Table 2).

The results of these experiments showed that in the control (without antitoxin) only 50% of the protagon was sedimented from the suspension after washing four times. This sedimented protagon contained 51.4% of all the labeled toxin incubated. The comparison shows that all the active toxin was bound with protagon during incubation under these conditions. The experiments also showed that antitoxin (or proteins of antitetanus serum) increased the aggregation of the particles of the protagon suspension, with the result that the protagon yield on centrifugation rose from 50 to 61–63%.

The results of all these investigations indicate that toxin, neutralized by antitetanus serum, is bound by protagon. The intensity of binding is of the same order as the fixation of active toxin. Consequently, the affinity of toxin for the gangliosides of the brain [6–8] is changed only slightly by neutralization of the toxin with antitoxin. It can be concluded from these results that the centers in the toxin molecule responsible for its fixation with the gangliosides of the brain and for its binding with antitoxin are not the same. Allowing for the fact that toxoid, which is deprived of its toxicity, is also bound by protagon [7], it can further be concluded that the toxin molecule contains at least three functional groups: a toxophore group, an antigenic group, and a group responsible for fixation of the toxin by the receptor in the brain substance.

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