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STUDY OF THE CHEMICAL RECEPTOR FOR DIPHTHERIA TOXIN IN CELL MEMBRANES

Yu. V. Vertiev and Yu. V. Ezepchuk

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Adsorption of diphtheria toxin on immobilized preparations of cell membranes was studied in the presence of gangliosides. A mixture of gangliosides completely suppresses adsorption of the toxin on membranes of cells both sensitive (HeLa cells, macrophages) and resistant (L cells) to its action. Gangliosides treated with neuraminidase are less effective. Immobilized gangliosides effectively adsorb diphtheria toxin in the presence of protective colloid and of the detergent Triton X-100. On the basis of these results gangliosides can be regarded as the receptors of diphtheria toxin.

KEY WORDS: *diphtheria toxin; receptors; membranes; gangliosides.*

The chemical nature of cell receptors has recently been established for a number of bacterial toxins. For cholera, staphylococcal, tetanus, and botulinus toxins gangliosides have been shown to perform the receptor function in the cell [6, 8-10, 11, 13]. Data on the receptor for diphtheria toxin are not available, although the molecular structure and mechanism of action of this toxin have been studied in much greater detail than those of other toxins [3]. Meanwhile, experiments using the "viroimmunotest" enabled the writers to show that diphtheria toxin is fixed on cell membranes [2].

The object of this investigation was to study the effect of gangliosides on interaction between diphtheria toxin and cell membranes.

EXPERIMENTAL METHODS

Diphtheria toxin was obtained by the method of Gill and Dinius [7] and the concentration of its toxic protein was determined spectrophotometrically [12]. Membranes from HeLa and L cells and from guinea pig macrophages were obtained and immobilized on Sephadex G-25 by the method described previously [2]. Bovine serum albumin (from Koch-Light, England) and gangliosides (from Sigma, USA) were immobilized on Sephadex G-25 by Cuatrecasas' method [5] and designated Seph–BSA (Sephadex–bovine serum albumin) and Seph–BSA–G (Sephadex–bovine serum albumin–gangliosides), respectively. A purified preparation of neuraminidase from NAG vibrio [1] was immobilized on Sepharose 4B (from Pharmacia, Sweden) by Cuatrecasas' method [4]. The

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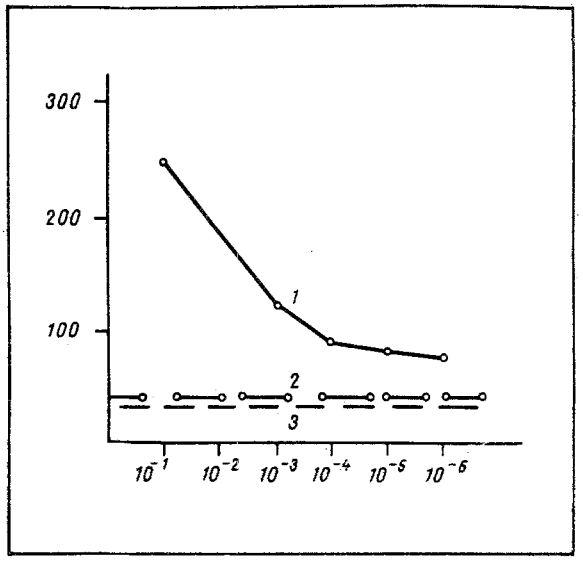

Fig. 1. Adsorption of diphtheria toxin on macrophage membranes. Abscissa) diphtheria toxin, in mg/ml; ordinate) number of plaques; 1) toxin; 2) control; 3) toxin + gangliosides.

gangliosides were dissolved in 0.1 M phosphate buffer, pH 7.0. To obtain monosialoganglioside Gm_1 (according to Svennerholm's classification [15]) the mixture of gangliosides in a concentration of 1 mg/ml was treated for 4 h at 37°C with the immobilized neuraminidase preparation in a concentration of 240 enzyme units in 0.1 ml. Absorption of diphtheria toxin on the membrane was determined by the "viroimmunotest" [2]. Usually 10 µl gangliosides in different concentrations were preincubated for 30 min at 37°C with an equal volume of diphtheria toxin (100 ng/ml). To 10 µl of this mixture, 30 µl of immobilized membranes was added. After incubation (30 min, 37°C) the immobilized membranes were washed 8 times, with 10 ml 0.15 M NaCl solution containing 0.2% gelatin each time, and to it was added 10 µl of phage–antitoxic immunoglobulin conjugates. The mixture was incubated at 37°C for 24 h, after which it was rinsed with 10 ml buffer 8 times. After the last rinsing, 7 ml of melted agar containing a culture of *Escherichia coli* was added to the residue of membranes and the whole sample was transferred to petri dishes. The number of plaques on the lawn was counted after incubation for 18 h at 37°C. Full details of the method were described previously [14].

EXPERIMENTAL RESULTS

As was stated previously [2], experiments with the viroimmunotest showed that diphtheria toxin is fixed on cell membranes and, under certain conditions, interacts firmly with them. To identify the structures responsible for this interaction experiments were carried out to study the effect of gangliosides on this process (Fig. 1). Figure 1 shows that preincubation of diphtheria toxin with gangliosides leads to total suppression of adsorption of the toxin on the membranes. Suppression of adsorption was observed with all concentrations of toxin tested.

Later, in experiments with membranes of different types of cells (macrophages, HeLa and L cells), irrespective of the sensitivity of these cells to diphtheria toxin gangliosides were found to inhibit adsorption of the toxin on the membranes of each type (Table 1).

The results thus indicate that gangliosides compete with the receptors of the cell membranes, as a result of which adsorption of diphtheria toxin on the membranes is suppressed.

In the next series of experiments interaction of diphtheria toxin directly with gangliosides was studied. Immobilized gangliosides were used. The results given in Table 2 show that incubation of diphtheria toxin with immobilized gangliosides leads to binding of the toxin and gangliosides. A system not containing toxin was used as the control. Of all the conditions tested, the use of Triton X-100 (in a concentration of 0.1%) gave the greatest difference between the experimental and control tests. Some adsorption of toxin also was observed in the control variants, when only albumin was immobilized on the Sephadex. This fact can evidently be explained by the presence of lipid impurities in the albumin.

Since a mixture of gangliosides was used in the work, it is impossible to say precisely which ganglioside interacts specifically with diphtheria toxin. To answer this question with respect to monosialoganglioside, the mixture was treated with neuraminidase from *Vibrio chol-*

TABLE 1. Interaction between Diphtheria Toxin and Membranes of Different Cells in Presence and Absence of Gangliosides

Source of membranes	Number of plaques		
	toxin + membranes	toxin + gangliosides + membranes	control (membranes + gangliosides)
HeLa cells	272	27	32
L cells	170	20	25
Macrophages	320	40	45

TABLE 2. Adsorption of Diphtheria Toxin on Immobilized Preparations of Gangliosides

Preparation tested	Number of plaques			
	with triton X-100		without triton X-100	
	expt.	control	expt.	control
Seph–BSA–G	125	52	750	600
Seph–BSA	24	18	125	56

TABLE 3. Effect of Different Concentrations of Gangliosides on Adsorption of Toxin on Macrophage Membranes

Concentration of gangliosides, in μg/100 ng toxin, μg	Number of plaques	
	mixture of gangliosides not treated with neuraminidase	mixture of gangliosides treated with neuraminidase
10	40	60
1	42	170
0,1	42	175
0,01	160	180
Control	180	183

erae. This converts di- and trisialogangliosides into monosialoganglioside Gm_1 [5]. The results, given in Table 3, show that monosialogangliosides can evidently be eliminated from the list of hypothetical receptors of diphtheria toxin, although some degree of inhibition of adsorption of the toxin on the membrane was observed in the presence of gangliosides treated with neuraminidase. Further investigations are thus required to identify which ganglioside is the hypothetical receptor for diptheria toxin. Comparison of the results with data in the literature suggests that gangliosides perform a universal role in the reception of biologically active molecules.

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IMMUNOGENICITY OF POLYMERS OF H ANTIGEN OBTAINED BY DIFFERENT METHODS

V. L. Lyashenko, S. K. Aleksander,
L. I. Vaneeva, and G. B. Semenova

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The properties of two polymers obtained from flagellin by different methods — precipitation by ammonium sulfate followed by centrifugation or treatment with glutaraldehyde in solution followed by gel chromatography — were studied. Molecules of the former (POL) and the latter (GLUT) are similar in shape but POL has a higher molecular weight. The preparations contain a common H antigen and are similar in serologic activity. POL has very high "priming" activity for mice. GLUT is highly immunogenic only within a narrow dose range, is less immunogenic than POL, and differs from POL in forming a certain quantity of 7S antibodies.

KEY WORDS: *flagellin; immunogenicity; polymer antigen.*

One of the basic principles of modern immunology is that high-polymer molecules are much more immunogenic than low-polymer or monomer molecules. The concept of high polymerism is not completely quantitative: the relations between the properties of different polymers of the same protein and to what extent they depend on the method of preparation of the polymer are not yet known. Comparison of different polymers is essential to fill in the gaps of our knowledge of concepts such as "high immunogenicity," "T independence," and "tolerogenicity," used in connection with the degree of polymerization of antigens.

The object of this investigation was to compare polymers of flagellin obtained by two different methods.

EXPERIMENTAL METHODS

Three preparations of flagellin, namely flagellae (FLA), flagellin monomer (MON), and the spontaneously formed polymer (POL), were prepared by the methods described previously [1, 4]. The methods consisted of separating flagellae from bacterial cells of *Salmonella typhi* strain T-55-01 by intensive shaking of the culture and centrifugation, dissolving the flagellae at pH 3.5 (with the formation of MON), and treatment of the MON solution with 15% $(NH_4)_2SO_4$ (to precipitate POL). The glutaraldehyde polymer of flagellin (GLUT) was prepared by incubating MON in a concentration of 10 mg/ml in 0.1 M phosphate buffer, pH 6.8, with 0.025% glutaraldehyde for 30 min at 20°C [6]. The polymer was separated from unpolymerized protein on a column with Sephadex G-50. The preparations were purified by chromatography on a column with Sepharose 2B.

By marking the column with Newcastle desease virus (mol. wt. 7×10^7), mouse encephalomyocarditis virus (mol. wt. 10^7), dextran preparations with mol. wt. of 2×10^6, 5×10^5, and 4×10^4, and with deoxyribose with mol. wt. 134 daltons the molecular weight of the flagellin preparations could be determined.

The serologic specificity of the preparations thus obtained was determined by radial immunodiffusion tests with rabbit serum against flagellin. The serologic activity of the prep-

Department of Chemotherapy, All-Union Research Institute for the Search for New Antibiotics, Academy of Medical Sciences of the USSR. Department of Physical and Chemical Methods of Investigation, I. I. Mechanikov Moscow Research Institute of Vaccines and Sera, Ministry of Health of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR G. F. Gauze.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 85, No. 5, pp. 551-554, May, 1978. Original article submitted July 12, 1977.

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