CHEMICAL MODIFICATION OF CHOLERA TOXIN FOR CHARACTERIZATION OF ANTIGENIC, RECEPTOR-BINDING AND TOXIC SITES

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1. Introduction

Recent studies have provided significant insight into pathogenetically important molecular events in cholera. A bacterial exotoxin, which is the diarrhoeogenic agent, has been isolated and characterized chemically [1]. It is an antigenic, neutral protein with a molecular weight of 84 000 [2] constituting a multichain aggregate of at least two types of subunits [3]. It binds avidly to mammalian cell membranes [4,5], probably to G_{M1} receptors [6–8]. Cell-binding is followed by activation of adenylate cyclase which causes various effects in different cell types [9,10].

It is important to clarify how the structure of cholera toxin determines the antigenic, the receptor-binding and the toxic properties. Recent studies by us have shown that one type of subunit, L, is responsible for the cell-binding and G_{M1} ganglioside-binding ability of the toxin and also for most of the antigenicity [3,5,6, 11]. The other subunit, H, is however, important for the biologic action and is also antigenic [3,5,11]. In the present study chemically-modified toxin derivatives have been prepared and investigated in an approach to characterize and perhaps differentiate between antigenic, G_{M1}-(receptor-)binding and toxic sites on cholera toxin. As a practical consequence, the work may serve as a guide in the search of a procedure for preparing a suitable toxoid for human immunization.

2. Material and methods

2.1. *Cholera toxin*

Isolated cholera toxin prepared by R. A. Finkelstein, Dallas, Texas [1], was obtained from the National Institutes of Allergy and Infectious Diseases, Bethesda, Md. Radiolabeling with ^{125}I was done as described [5].

2.2. *Protein modification*

Twenty commercially available reagents for amino acid residue specific modification of proteins were used. A 12 μM solution of cholera toxin in sterile Tris–EDTA buffer, pH 7.5 (0.01 M Tris, 0.0002 M Na_2-EDTA, 0.0006 M NaN_3 and 0.04 M NaCl) was diluted 1:10 in buffer containing the protein modifying reagent. The buffer and incubation time and temperature varied for different reagents; for those reagents found to have the most interesting effects on cholera toxin the incubation conditions are reported in table 2, and a more detailed description of these and the other reagents will be published elsewhere [12]. The reaction was stopped and the reactants separated by filtering the mixture through a small Sephadex G-15 gel column. The protein fraction was collected and diluted in Tris gelatin buffer, pH 7.5 (0.1 M Tris and 0.02%, w/v, gelatin) to a final concentration of 0.024 μM (2 μg/ml) cholera protein. It was divided in small aliquots which were stored at −20°C until tested.

For each reaction a control preparation was done, where cholera toxin was incubated under the appropriate conditions but without any modifying reagent, and gel filtered as above.

2.3. *Enzymatic treatment*

Three enzymes bought from Sigma were studied for their influence on cholera toxin: leucine-aminopeptidase type III (86 U/mg) at a final incubation concentration of 0.67 mg/ml, carboxypeptidase-A DFP (47 U/mg) at 7 mg/ml, and trypsin type III (100 U/mg) at 1 mg/ml. Each enzyme was incubated with 3.76 μM (0.33 mg/ml)

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cholera toxin at 37°C for 4 hr in 0.1 M *N*-ethylmorphiline–HCl, 0.001 M $MgCl_2$, pH 8.4, with or without 2 M urea. The reaction was stopped by 250-fold dilution of the sample with 0.2 M ammonium formate, pH 4.5, containing 0.2% gelatin (w/v). The sample was then immediately divided in small aliquots which were stored at −70°C until tested. Three types of controls were prepared: (i) a portion of the enzyme and toxin mixture taken at zero time; (ii) the enzyme and (iii) the toxin incubated separately under the same conditions as the test sample.

2.4. *Toxicity assay*

The toxic activity of the cholera toxin derivatives and their toxin control preparations was determined by testing multiple concentrations by the rabbit intradermal assay [13]. The effect of the chemical modification was evaluated by comparing the concentrations of test and control toxin preparations required for a blueing reaction of 7 mm in diameter (one blueing dose).

2.5. *Combined assay of G_{M1}- and antibody-binding*

A technique, G_{M1}-sorbent immunoassay, has recently been elaborated which measures a combination of the in vitro binding capacity of cholera toxin to polystyrene-attached G_{M1} ganglioside and the ability of the bound toxin to bind subsequently added specific antibody. The technique and the materials involved have been described [14]. Evaluation of the influence of the chemical modification in this and in the two following methods was done in analogy with the toxicity assay, i.e. by comparing the concentrations of test and control toxin preparations required for a significant reaction.

2.6. *Assay of G_{M1}-binding*

Pure G_{M1} ganglioside was attached to polystyrene test tubes as described [14]. To the tubes was then added 0.25 ml of ^{125}I-labelled toxin (50 ng) in mixture with various concentrations of test or control toxin preparations. After incubation at 23°C for 2 hr, the radioactivity of the washed tubes was determined. The ability of the tast and control toxin preparations to compete with the binding of ^{125}I-labelled toxin to G_{M1} is a measure of their G_{M1}-(receptor-)binding potency.

2.7. *Assay of antibody-binding*

The antibody-binding ability of the modified toxin preparations was tested by means of the ELISA-inhibition assay described previously [15]. In principle, the ability of the test compared with the control toxin preparations to inhibit almost limiting dilution of antitoxin antiserum from binding to cholera toxin-coated polystyrene tubes is measured.

3. Results

The influence of the 20 protein-modifying amino acid specific reagents on the toxic, the G_{M1}-(receptor-)-binding and antibody-fixing properties of cholera toxin was studied. Table 1 shows that ten reagents, in a final concentration of 1 mM, had no effect on effect of these properties, whereas seven reagents had a parallel destroying effect on both the toxicity and the combination of G_{M1}-binding and antibody-fixation. When G_{M1}-binding and antibody-fixation were tested separately for the toxin derivatives obtained with these 17 reagents, a close but not complete association of the G_{M1}-binding and the antigenic sites was found. These toxin regions were particularly sensitive to reagents which have specificity for amino groups.

Three reagents had a differential effect on the toxicity and the combination of G_{M1}-binding and antibody-fixation (table 1). These substances, which have a reported specificity for arginine residues, abolished the toxicity without causing any decrease of the G_{M1}-binding ability (table 2). The antibody-binding capacity of the toxin also stayed intact, except after treatment with butandione when it was diminished (table 2).

Table 1
Influence on cholera toxin activities of protein-modifying amino acid specific reagents

Number of reagents	Effect on	
	Toxicity	G_{M1}- and antibody-binding
10	−	−
7	+	+
3	+	−

Table 2
Specification of reagents with differential effects on the toxic, G_{M1}-binding and antibody-fixing activities of cholera toxin

Reagent	Incubation conditions	Specificity	Effect on		
			Toxicity	G_{M1}-binding	Antibody-binding
Cyclohexanedione	1 mM in 0.05 M NaOH, 23°C, 1 hr	arginine	+	–	–
Phenanthrenequinone	1 mM in 0.05 M NaOH, 5% ethanol, 23°C, 1 hr	arginine	+	–	–
Butandione	1 mM in phosphate pH 7.0, 23°C, 3 hr	arginine	+	–	(+)

Table 3
Influence of specific proteolytic enzymes on cholera toxin activities

Enzyme		Effect on		
		Toxicity	G_{M1}-binding	Antibody-binding
Leucineaminopeptidase	a) without urea	–	–	–
	b) with urea	–	–	–
Carboxypeptidase	a) without urea	–	–	–
	b) with urea	+	–	–
Trypsin	a) without urea	–	–	–
	b) with urea	+	–	–

We also tested the effect of treating cholera toxin with two exopeptidases, i.e. leucine-aminopeptidase and carboxypeptidase which digest proteins from the amino- and carboxyterminal ends respectively, and with the endopeptidase trypsin. Neither of these enzymes had any influence even in high concentrations on the toxic or the G_{M1}- and antibody fixing activities of the toxin when tested in ordinary buffers, but in the presence of 2 M urea effects were observed (table 3). Both the carboxypeptidase and the endopeptidase abolished the toxicity but did not influence the G_{M1}- and antibody-fixing properties.

All of the described results have been verified with at least one further batch of each toxin derivative.

4. Discussion

The results demonstrate that those protein-modifying reagents which decrease the ability of cholera toxin to bind in vitro to the ganglioside G_{M1} reduce the toxic activity correspondingly. This is natural in the light of the indicated function of the G_{M1} ganglioside as membrane receptor for the toxin in various target cells. However, the toxicity was also destroyed by a few reagents which did not reduce the G_{M1}-binding ability. This agrees with evidence obtained by other means [3,5] that for expression of toxic activity are required peptide regions additional to those involved in cell-binding.

We have demonstrated that the L-type of subunit carries the G_{M1}-binding as well as most of the antigenic properties of cholera toxin [11]. In this study, it is shown that there is a close but not absolute association between these activities. It is of considerable interest that specific reagents have been found which can abolish the toxicity completely without affecting the antigenicity of the toxin. This type of reagents may prove useful for developing toxoids for immunization against cholera.

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