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Structure, Function, and Antigenicity of Cholera Toxin

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Chemical modification of intact cholera toxin or its B subunit by either partial nitration or reduction and alkylation did not result in significant loss of biological activity as determined by measurement of cyclic AMP in Chinese hamster ovary cells. Complete nitration or succinylation in the presence of guanidine hydrochloride resulted in complete loss of biological activity and significantly affected the immunoreactivity of the toxin and B subunit. Compositional analyses of both the isolated α and γ chains of the toxin were typical of globular proteins and did not reveal significant hydrophobicity. Analysis of antigenic relationships by radioimmunoassay indicated a partial crossreactivity between the α chain and the B subunit of cholera toxin. Since previous structural studies of the β chain of cholera toxin indicated chemical similarity with the glycoprotein hormones [Kurosky et al. Science 195:299 (1977)], radioimmunoassay procedures were employed to investigate for possible crossreactivity. No evidence of crossreactivity between cholera toxin subunits and subunits of ovine luteinizing hormone was found.

Key words: cholera toxin, chemical modification, amino acid composition, antigenic relationships, radioimmunoassay, adenylate cyclase, ovine luteinizing hormone

The stimulation of adenylate cyclase by cholera toxin in a wide variety of cell types has prompted considerable interest regarding the transmembrane events signaled by the action of the toxin on cells. Recent studies have shown that the toxin possesses NADase activity (NAD^+ nucleosidase activity, EC 3.2.2.5) that is capable of ADP-ribosylation of some acceptor protein directly or indirectly related to adenylate cyclase action [1, 2]. This reaction appears to be analogous to that observed for diphtheria toxin which causes inhi-

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bition of protein synthesis as a result of ADP-ribosylation of elongation factor II [3, 4]. Studies of the effects of cholera toxin on GTP hydrolysis in turkey erythrocyte membranes have led to the proposal that activation of adenylate cyclase is the result of inhibition of guanosine-triphosphatase [5]. The unique properties of cholera toxin make it a useful tool for probing membrane-protein interaction. This was further emphasized in the results which indicated that cholera toxin had some chemical similarity to the glycoprotein hormones. The amino-terminal region of the β chain of the toxin was shown to be chemically similar to a segment of the β chains of thyrotropin, chorionic gonadotropin, luteinizing, and follicle stimulating hormones [6, 7].

Cholera toxin is a protein comprised of three unique polypeptide chains ($\alpha\gamma\beta_n$) (where n = 4 to 6 [8]). The toxin molecule is diagramatically represented in Figure 1. The A subunit, and more specifically the α chain, possesses the adenylate cyclase-stimulating site, whereas the B subunit contains the membrane-binding sites. There is strong evidence which indicates that the toxin binds specifically to the ganglioside galactosyl-N-acetyl-galactosaminyl(N-acetylneuraminyl)galactosylglucosylceramide (G_{M1}) on the cell membrane [9–12]. Evidence by Craig and Cuatrecasas [13] and Fishman et al [14] indicated that the toxin molecule is multivalent and capable of binding between five and six molecules of G_{M1}. The primary structure of the toxin β chain has been completed [8, 15] but little is presently known of the primary structures of the α and γ chains of the A subunit. This report furthers the chemical characterization of cholera toxin and examines the effects of chemical modifications on antigenic and biological activity.

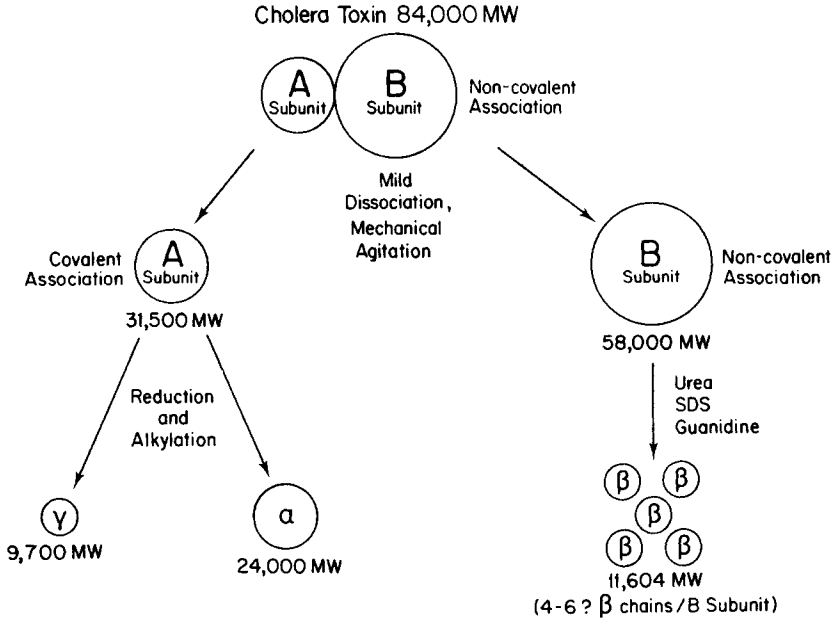

Fig. 1. Diagrammatic representation of the subunits of cholera toxin. See Kurosky et al [8] regarding nomenclature.

MATERIALS AND METHODS

Cholera Toxin and Subunit Purification

Cholera toxin, purified from fermenter cultures of Vibrio cholerae (Inaba strain 569B), was identical to the toxin previously furnished by the National Institute of Allergy and Infectious Diseases as judged by electrophoretic mobility, amino acid sequence analysis, and biological activity (8). The A and B subunits of cholera toxin were prepared by gel filtration on a Bio-Gel P-60 column eluted with 0.2 M sodium formate buffer, pH 3.5, containing 5.2 M guanidine-HCl [16]. The A subunit was then resolved into α and γ chains by gel filtration on the same P-60 column after reduction of disulfide bonds with 2-mercaptoethanol and alkylation with iodoacetamide [17]. Polyacrylamide gel electrophoresis of [^{125}I]-labeled A subunit, α chain, β chain, and γ chain, as well as sequence analysis, indicated these proteins to be homogeneous [18].

Preparation of Antiserum

Rabbits (groups of five) were immunized separately with 50 μg of cholera toxin, 200 μg of A subunit, 200 μg of B subunit, and 200 μg of α chain. Each protein preparation was emulsified in Freund's complete adjuvant and injected by a combination of intraperitoneal, intramuscular, and subcutaneous routes. Blood samples were drawn every 3 wk. A booster injection of antigen at the same respective protein concentration was administered whenever antibody titers began to decrease, as determined by passive hemagglutination using toxin sensitized erythrocytes [19]. All sera were stored at $-20°$.

Radioimmunoassay

A radioimmunoassay procedure using polyvinyl chloride microtiter plates and a double-antibody system was employed as we described previously [18]. This analysis was used to investigate the antigenic relationships of the subunits of the toxin. In addition, the assay was used to analyze for posible cross-reactivity between the toxin subunits and the α and β subunits of ovine luteinizing hormone. Protein preparations were radiolabeled using the chloramine T procedure of Greenwood and Hunter [20]. Antisera were diluted to bind 50% of the total radioactivity (about 10,000 cpm) of the radiolabeled antigen being assayed. In one series of experiments unlabeled α chain, A subunit, B subunit, and intact toxin were each allowed to compete for the binding of [^{125}I]-labeled α chain to anti-α chain sera. In a second series of experiments unlabeled α and β subunits of ovine luteinizing hormone (obtained from the Hormone Distribution Program, National Institute of Arthritis, Metabolism, and Digestive Diseases) were each allowed to compete for the binding of [^{125}I]-labeled A subunit to anti-A subunit serum and [^{125}I]-labeled B to anti-B subunit serum.

Chemical Modifications

Half-cystinyl residues were modified according to Cleland [21] by reduction with dithiothreitol and alkylation with iodoacetamide. A 2-mg sample of either cholera toxin or B subunit was reacted with a 50-fold molar excess of iodoacetamide over half-cystine. The reaction was terminated by addition of excess 2-mercaptoethanol. Alkylated proteins were dialyzed against Tris-HCl buffer containing 0.01 M Tris-HCl, 0.2 M NaCl, 0.001 M EDTA, 0.0001 M NaN_3, pH 7.5, and were stored at 4°.

Amino groups were modified by succinylation according to Klotz [22]. A 100-fold

molar excess of succinic anhydride over amino groups was reacted with 2-mg samples of cholera toxin or B subunit. Samples were dialyzed against water, lyophilized, and dissolved in the Tris-HCl buffer.

The tyrosyl residues in 2 mg of toxin or B subunit were modified by nitration using tetranitromethane (TNM) both in the absence and presence of 5 M guanidine-HCl [23]. A 10-fold molar excess of TNM to tyrosyl residues was used and the samples were dialyzed against the Tris-HCl buffer.

Cyclic AMP Determination

The biological activity of cholera toxin was determined by measuring intracellular cyclic AMP levels in Chinese hamster ovary (CHO) cells (K-1) after incubation with toxin for 3 h. Culture conditions were similar to those described by Guerrant et al [24] with the exception that Ham's F-12 medium was substituted for F-10. The phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine (MIX), was added to the F-12 medium (0.05 mM) prior to incubation with toxin. After incubation with toxin for 3 h, the cells were treated with 5% trichloroacetic acid (TCA) in 0.1 N HCl and centrifuged. The TCA in the supernatant was subsequently extracted with ether. Cyclic AMP levels in the extracted supernatants were quantitated by the method of Gilman [25]. The protein content of the TCA precipitate was determined by the method of Bradford [26].

The biological activities of B subunit and modified B subunit were measured by the ability of B subunit to inhibit the accumulation of cyclic AMP in CHO cells incubated with cholera toxin. Preliminary experiments were carried out to determine the optimal incubation conditions for the competitive inhibition assay. In each experiment the B subunit was diluted to different concentrations in F-12 medium containing 1% fetal calf serum (FCS) and 0.05 mM MIX. All washes and incubations with cholera toxin (10 ng/ml) were done using the same medium. A control incubation of cells treated only with toxin was included for each experimental condition. After three hours of incubation the cells were washed twice with 1.0 ml of phosphate buffered saline and the reaction terminated by addition of 1.0 ml of TCA. Cyclic AMP levels were assayed as described above.

RESULTS

Compositional Analysis

The amino acid compositions of the α and γ chains are presented in Table I. These data were derived from duplicate analyses for each time interval.

Carboxyl-Terminal Analysis

Results from a time-course hydrolysis of cholera toxin γ chain with carboxypeptidase A are shown in Figure 2. Similar hydrolysis of the α chain did not release significant amounts of amino acid residues.

Radioimmunoassay

Competitive inhibition of radiolabeled α chain to anti-α sera is shown in Figure 3. Significantly, the B subunit was capable of partially competing for the binding of [^{125}I]-labeled α chain to anti-α sera. Intact toxin was less effective in competing for the binding of radiolabeled α chain to anti-α chain sera. Concentrations of up to 1.6×10^{-3} μmol/ml of α chain were found to be noninhibitory in this assay. The results of the competitive

TABLE I. Amino Acid Composition of Cholera Toxin A Subunit*

Amino acid	α Chain[a] residues ± SD	γ Chain[b] residues ± SD
CM – Cysteine	1.0 ± 0.02 (1)	0.9 ± 0.1 (1)
Aspartic Acid	26.1 ± 1 (26)	10.7 ± 0.6 (11)
Threonine[c]	7.0 ± 0.1 (7)	4.3 ± 0.3 (4)
Serine[c]	11.8 ± 0.2 (12)	7.9 ± 0.1 (8)
Glutamic Acid	22.1 ± 0.6 (22)	13.0 ± 0.2 (13)
Proline	19.2 ± 0.6 (19)	0 (0)
Glycine	22.7 ± 0.8 (23)	4.9 ± 0.1 (5)
Alanine	18.3 ± 0.8 (18)	1.0 ± 0.1 (1)
Valine	9.5 ± 0.2 (10)	3.2 ± 0.1 (3)
Methionine	2.3 ± 0.04 (3)	0.9 ± 0.04 (1)
Isoleucine	9.5 ± 0.2 (10)	4.7 ± 0.2 (5)
Leucine	15.0 ± 0.6 (15)	5.7 ± 0.1 (6)
Tyrosine[c]	16.1 ± 0.4 (16)	3.2 ± 0.1 (3)
Phenylalanine	5.5 ± 0.2 (5-6)	3.4 ± 0.1 (3)
Histidine[c]	10.5 ± 0.1 (10-11)	1.6 ± 0.1 (2)
Lysine	3.0 ± 0.1 (3)	7.8 ± 0.1 (8)
Arginine	14.1 ± 0.4 (14)	3.2 ± 0.02 (3)
Tryptophan[d]	2.0	
Glucosamine	0 (0)	0 (0)
Galactosamine	0 (0)	0 (0)
Total	214–216	77

*Mean values from duplicate 24-, 48-, and 96-hour hydrolyses. Performed on Beckman 121M and 119 analyzers [ω].
[a]Based on a molecular weight of 24,000.
[b]Based on one alanine (mol wt = 8,877).
[c]Extrapolated to zero time of hydrolysis.
[d]Determined after hydrolysis with mercaptoethanesulfonic acid [27].

binding of the α and β subunits of ovine luteinizing hormone with [^{125}I]-labeled A and B subunits of cholera toxin are shown in Fig. 4.

Cyclic AMP Determination

The observed increase in intracellular cyclic AMP in CHO cells incubated with varying concentrations of cholera toxin for 3 h at 37° was similar to that previously reported by Guerrant et al [24]. Maximal stimulation of adenylate cyclase occurred at a concentration of about 10 ng/ml of cholera toxin. The results obtained from three different experimental conditions used to evaluate the effect of B subunit on the accumulation of intracellular cyclic AMP in CHO cells incubated with cholera toxin are shown in Fig. 5. In experimental conditions A and B, the effect of B subunit on the cellular response to cholera toxin was similar. In experimental condition C, removal of the B subunit from the cells for the duration of the 3 h toxin incubation period resulted in decreased toxin competition by B subunit.

The effects of nitration, succinylation, and alkylation of cholera toxin on adenylate cyclase stimulation of CHO cells are indicated in Fig. 6. The effects of similar modification of B subunit on its ability to inhibit the action of cholera toxin are demonstrated in Fig. 7. Amino acid analysis confirmed that all of the half-cystinyl residues in both toxin and B

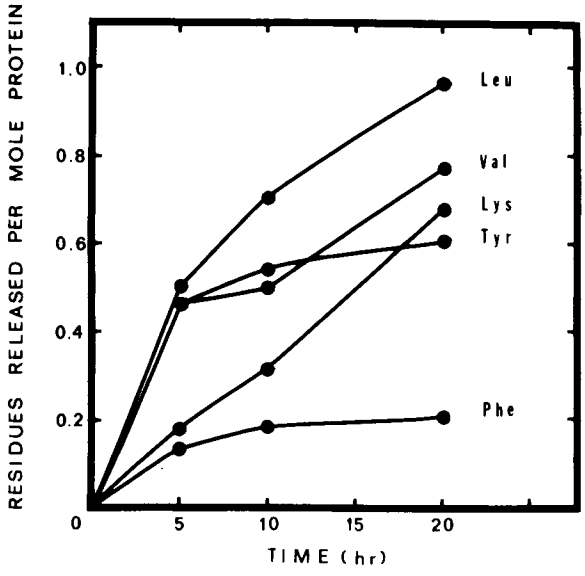

Fig. 2. Carboxypeptidase A hydrolysis of the γ chain of cholera toxin (enzyme to protein ratio of 1:50 (w/w)) at 37° in 0.2 M N-ethylmorpholine acetate buffer, pH 8.5 [28]. Hydrolysis was terminated by lowering the pH to 3 with formic acid. Samples were lyophilized, deproteinized, and subjected to amino acid analysis.

subunit were completely alkylated by reaction with iodoacetamide. Similarly, the reaction of tyrosyl residues in toxin and B subunit with TNM in the presence of guanidine-HCl was complete as evidenced by amino acid analysis and absorption of nitrotyrosyl at 428 nm at pH 9. Nitration in the absence of guanidine was determined to be about 30% for both proteins. Succinylation in the presence of guanidine and 100-fold excess succinic anhydride resulted in complete modification of the amino groups of both toxin and B subunit. Reaction of succinylated proteins with dansyl chloride [29] confirmed complete reaction of ε-amino groups and the amino terminal residues. Automated sequence analysis of succinylated β chain revealed complete blockage of the amino terminus.

Immunoreactivity of Modified Toxin and B Subunit

The effects of chemical modification of the antigenic determinants of toxin and B subunit were analyzed by radioimmunoassay as shown in Figures 8 and 9, respectively.

DISCUSSION

Compositional analysis of the α and γ chains of the A subunit of cholera toxin indicated these proteins to be reasonably typical of globular proteins and did not reveal any abnormal levels of hydrophobic residues. The γ chain is devoid of both proline and tryptophan. On the basis of one alanyl residue per molecule the molecular weight of the γ chain approximates the value of 9,700 previously reported [15]. The composition of the α chain is slightly higher in arginine (6.5%) and is lower in lysine (1.4%) when compared with the arginyl and lysyl values reported for average protein compositions, 3.9% and 7%, respectively [30]. From the molecular weight of the β chain obtained from sequence analysis (11,604) [8] and assuming five β chains per B subunit, the calculated molecular

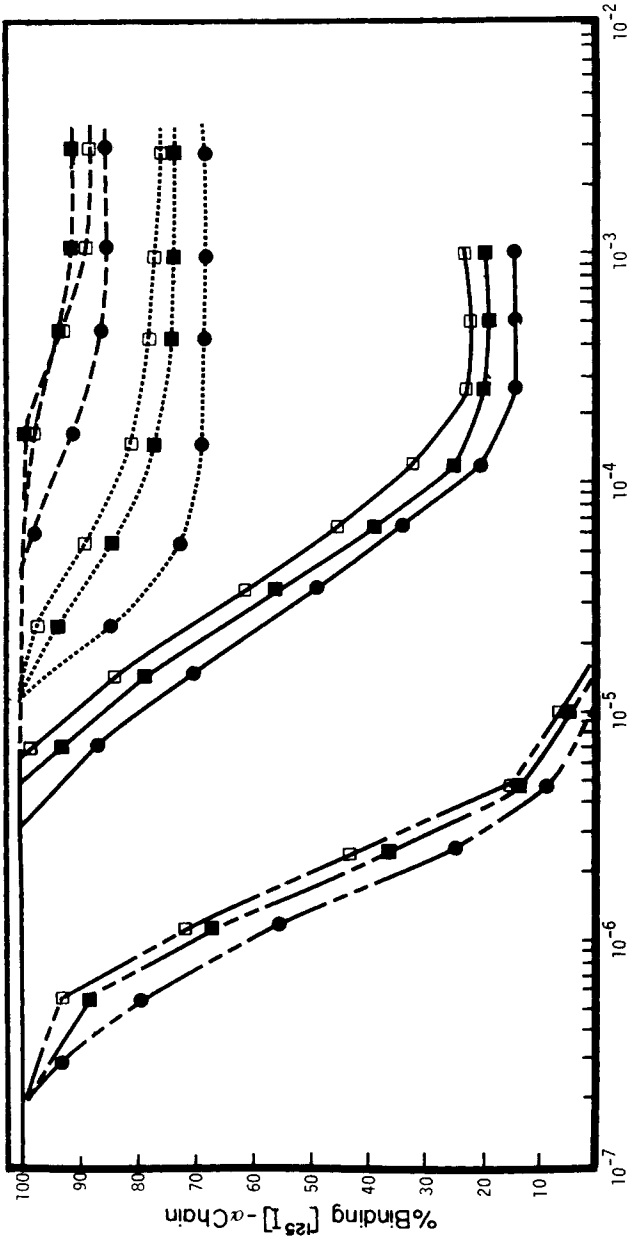

Fig. 3. Competitive inhibition of radiolabeled toxin α chain to three anti-α chain sera (●, ■, □) by unlabeled α chain (— · · · —), A subunit (——), B subunit (- - - - -), and intact toxin (– – –). Rabbit sera were titrated by radioimmunoassay and diluted to bind 50% of the radioactivity of the added radiolabeled toxin α chain.

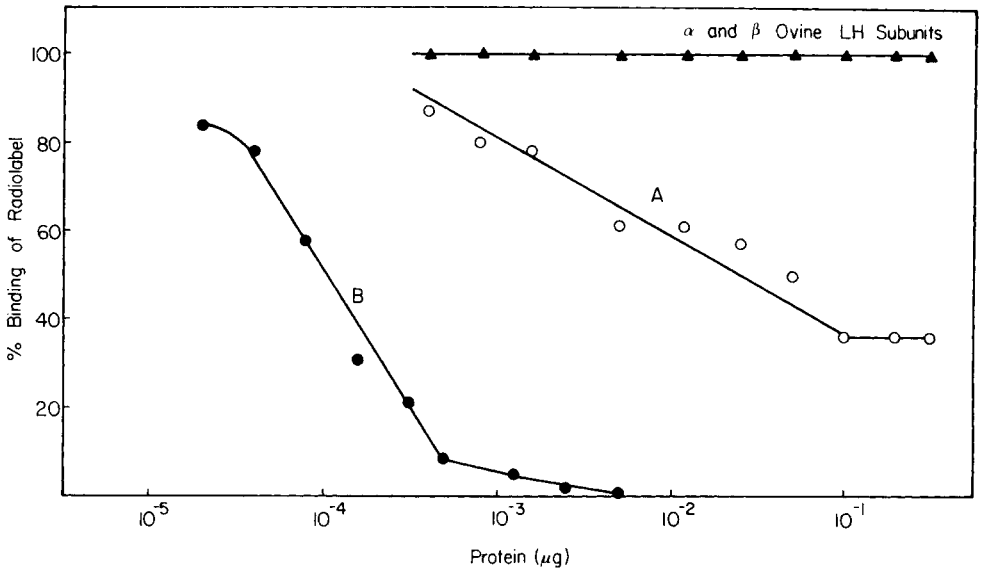

Fig. 4. Competitive inhibition of [^{125}I]-labeled A and B subunits of cholera toxin to anti-A and anti-B sera by α and β subunits of ovine luteinizing hormone (LH) and by unlabeled A and B subunits of toxin. Inhibition of radiolabeled A to anti-A serum by unlabeled A (○). Inhibition of radiolabeled B to anti-B serum by unlabeled B (●). Inhibition of radiolabeled A to anti-A or radiolabeled B to anti-B by α or β subunits of LH (▲).

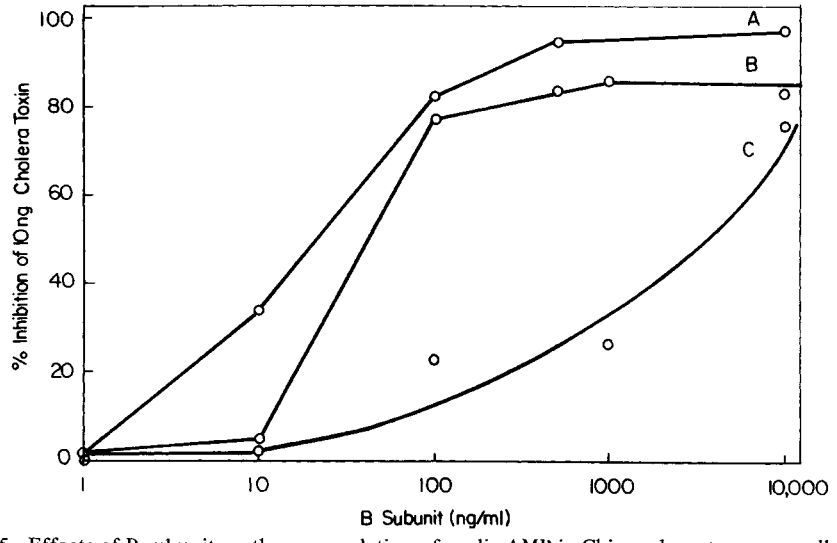

Fig. 5. Effects of B subunit on the accumulation of cyclic AMP in Chinese hamster ovary cells incubated with cholera toxin under three different incubation conditions. (A) B subunit was incubated with the cells for 15 min prior to the addition of cholera toxin. Both B subunit and toxin remained on the plates throughout the 3 h incubation. (B) The unbound B subunit was removed from the cells after 15 min and washed twice with 1.0 ml of medium. An aliquot of 2 ml of cholera toxin (10 ng/ml) was incubated with the cells for 15 min. After nonadsorbed toxin and medium were removed by aspiration, 2.0 ml of the F-12 medium containing MIX was added and the cells were incubated for an additional 3 h. (C) Insubation of the B subunit with the cells was allowed to continue for 15 min prior to removal of the unbound B subunit by aspiration and washing twice with 1.0 ml of medium. A 2.0-ml aliquot of medium containing cholera toxin was added to the washed cells and allowed to incubate for 3 h.

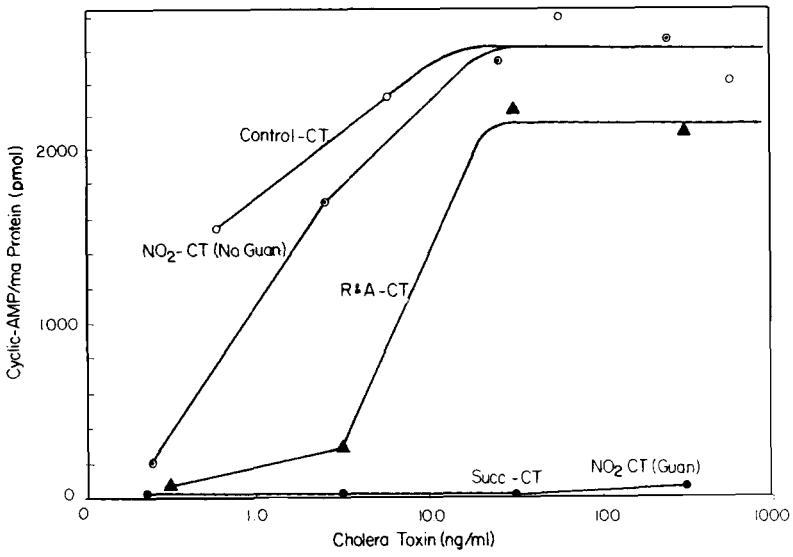

Fig. 6. Intracellular concentrations of cyclic AMP in Chinese hamster ovary cells incubated with chemically modified cholera toxin. Unmodified control toxin (○); partially nitrated toxin (⊙); reduced and alkylated toxin (▲); and completely succinylated or nitrated toxin (●).

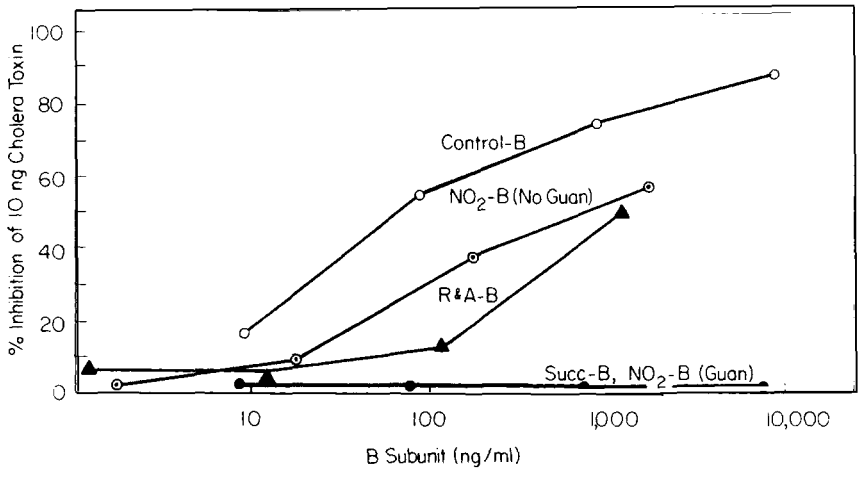

Fig. 7. Effects of modified B subunit on the accumulation of cyclic AMP in Chinese hamster ovary cells incubated with cholera toxin. Incubation conditions were the same as described for condition B in Figure 5. Unmodified control B subunit (○); partially nitrated B subunit (⊙); reduced and alkylated B subunit (▲); and completely succinylated or nitrated B subunit (●).

weight for the intact toxin is about 90,900. This is about 8% larger than the reported ultracentrifuge value of 84,000 [31].

Carboxypeptidase A hydrolysis of the α chain did not release a significant amount of any amino acid. Hydrolysis of the γ chain released several amino acids (Fig. 2) and leucine was deduced to be the carboxyl terminus. Although other explanations may be possible, the most likely sequence arrangement of the residues released from the γ chain by carboxy-peptidase A is (Phe, Tyr, Val) Lys-Leu-COOH.

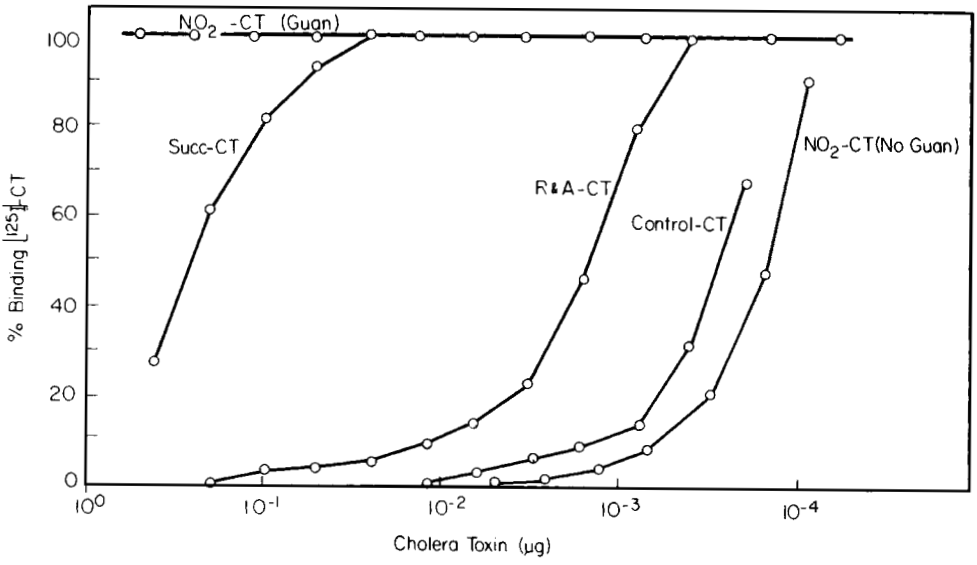

Fig. 8. Competitive inhibition of the binding of [^{125}I]-labeled cholera toxin to anti-toxin serum by unlabeled modified toxin. NO_2-CT (Guan)-completely nitrated toxin; Succ-CT-succinylated toxin; R&A-CT-reduced and alkylated toxin; Control-CT-unmodified control toxin; and NO_2-CT (No Guan)-partially nitrated toxin.

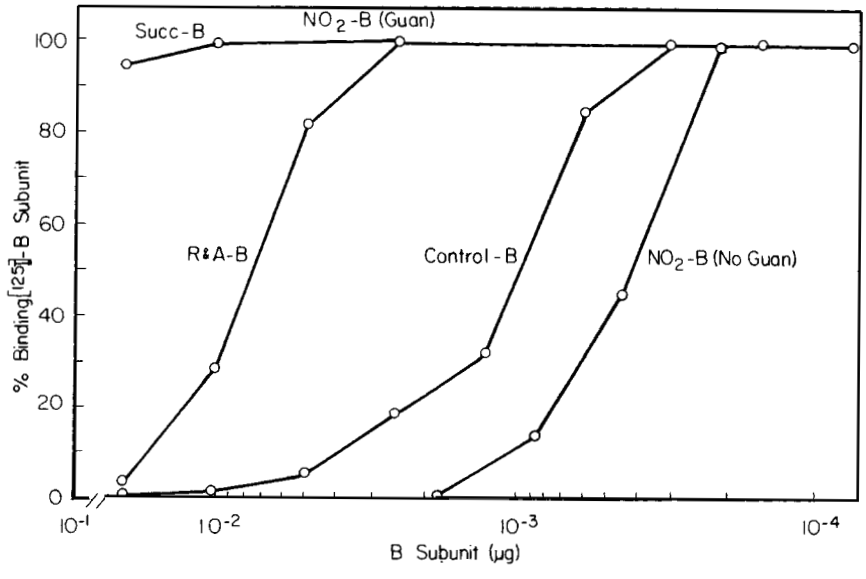

Fig. 9. Competitive inhibition of the binding of [^{125}I]-labeled B subunit to anti-B subunit serum by unlabeled modified B subunit. Succ-B-succinylated B subunit; NO_2-B (Guan)-completely nitrated B subunit; R&A-B-reduced and alkylated B subunit; Control-B-unmodified control B subunit; and NO_2-B (No Guan)-partially nitrated B subunit.

In view of the chemical similarity observed between the β chain of cholera toxin and the β chains of the glycoprotein hormones [6, 7] it was important to inquire about possible crossreactivity between toxin and hormone subunits. As evidenced in Fig. 4, no crossreactivity was observed between either the α or β subunits of ovine luteinizing hormone and the A and B subunits of cholera toxin. Other glycoprotein hormone subunits were not available for testing at this time. Conceivably, crossreactivity between the toxin and hormone subunits may be possible when antibodies raised to denatured proteins (reduced and alkylated) are employed [32].

Analysis by radioimmunoassay using unlabeled toxin and its subunits to compete for the binding of radiolabeled α chain to anti-α chain sera indicated partial crossreactivity between the B subunit and the α chain. About 30% of the [^{125}I]-labeled α chain could be displaced by B subunit at concentrations of 10^{-4} μmol/ml as shown in Fig 3. Cross-reactivity between the toxin subunits was reported previously [33, 34]; however, it was attributed to contamination although evidence to this effect was not presented. The subunit and chain preparations used in this study showed no evidence of contamination either by gel electrophoresis of radiolabeled or fluorescamine-reacted preparations or by automated sequence analysis [18]. Trace contamination, however, cannot be completely ruled out. It is possible that the observed crossreactivity relates to some inherent chemical similarity between the α and β chains. Curiously, in the reverse experiment, α chain was unable to compete for the binding of radiolabeled B subunit to anti-B serum. The reason for the absence of observable crossreactivity in this case when compared to the previous experiments using radiolabeled α chain and anti-α chain serum is presently unknown but may be related to the fact that the similar antigenic determinants within the α chain and B subunit have different immunogenicities. These results proved valuable in further establishing that the α chain preparation did not contain appreciable amounts of B subunit. It is important to point out that the competitive binding of B subunit to anti-α chain serum was not due to the aggregation of B subunit with α chain. When incubation mixtures of radiolabeled α chain with increasing amounts of B subunit were precipitated with anti-B serum (double-antibody system [18]) no radioactivity above background was observed in the immune precipitate; however, this serum was capable of binding significant amounts of radiolabeled B subunit. Furthermore, column gel filtration using Sephadex G-100 of incubation mixtures of radiolabeled α chain and B subunit confirmed the absence of any αB aggregation. Interestingly, intact toxin was found to compete poorly for the binding of radiolabeled α chain to anti-α chain sera which is consistent with the view that the A subunit is mostly buried within the intact toxin molecule [13].

The effect of B subunit on cholera toxin stimulation of adenylate cyclase in CHO cells is shown in Fig. 5. When both B subunit and toxin were incubated together for 3 h (curve A) approximately a 500-fold excess of B subunit was required to competitively inhibit the toxin. Similar results were obtained when B subunit and toxin were washed from the cells after 15 min of incubation (curve B). Removal of B subunit from the cells after 15 min followed by incubation of toxin for 3 h (curve C) required much larger concentrations of B subunit to achieve significant inhibition. This was due to competitive displacement of B subunit from the membrane by toxin. The fact that the cells bound B subunit (curve C) in excess of the amount necessary to prevent the action of the toxin indicated that the CHO cell membrane contained binding sites that were nonproductive to adenylate cyclase stimulation. Therefore, although the binding sites related to adenylate cyclase stimulation from 10 ng of cholera toxin were substantially saturated at a concentration of about 200 ng/ml of B subunit, the cells were still capable of binding greater amounts

of B subunit. King et al [35] have reported similar results with intact pigeon red cells. They found that about 40% of cell-bound toxin was relevant to the activation process.

It was somewhat surprising that reduction and alkylation did not affect significantly either the binding of B subunit to CHO cells or the adenylate cyclase stimulating-action of the toxin. However, both the binding of B subunit and the stimulation of adenylate cyclase by toxin could be abolished by either succinylation or complete nitration. Partial nitration (30%) of tyrosyl residues did not significantly modify the action of either toxin or B subunit. The tyrosyl residues nitrated may be internal residues since the hydrophobic character of tetranitromethane permits its access to buried groups [36]. It should be pointed out that exposure to 5.2 M guanidine-HCl followed by dialysis does not adversely affect the activity of B subunit [37] or toxin (Markel DE, unpublished observation). The difference in activity noted in the nitration experiments in the presence and absence of guanidine-HCl suggests that critical tyrosyl residues are required for the biological activity of toxin and B subunit. Toxin inactivation by nitration (or succinylation) does not necessarily reflect modification of residues important to adenylate cyclase stimulation but may simply reflect modification of membrane binding ability since the binding of B subunit is similarly altered. There is some evidence to indicate that the binding function of the toxin and the B subunit is dependent on the multivalent nature of the B subunit [12, 13]. Hence, modification of those residues committed to intersubunit interaction could result in the loss of quaternary structure of the B subunit and concomitant loss of binding.

Succinylation and complete nitration also abolished the immunoreactivity of the B subunit with anti-B serum as shown in Fig. 9. Succinylated or completely nitrated B subunit was unable to competitively inhibit the binding of radiolabeled B to anti-B serum. Similar results were obtained with modified whole toxin (Fig. 8) with the exception that the succinylated toxin was still capable of competing for the binding of radiolabeled toxin to antitoxin serum. In this case, competitive inhibition by succinylated toxin required about a 100-fold greater concentration at a 50% inhibition level when compared with untreated toxin. Thus, the analysis of the antigenic determinants of modified toxin and B subunit was consistent with the results obtained from measurement of biological activity and indicated that complete nitration or succinylation severely disrupted the structure of both toxin and B subunit.

Lönnroth and Holmgren [38] have surveyed a number of reagents to chemically modify cholera toxin employing micromethods for assaying toxicity, G_{M1} binding, and antibody-binding. Significantly, the reduced and alkylated toxin prepared by these workers was not active in the rabbit skin permeability assay but was still capable of binding to G_{M1} ganglioside as well as anti-toxin sera. Gill [39], on the other hand, employed the pigeon erythrocyte ghost assay and observed cholera toxin to be active after reduction and alkylation.

Clearly the elucidation of the action of cholera toxin on the cell membrane will provide worthwhile insights into the mechanism of cellular response to toxic effector molecules. A growing body of evidence suggests that the cellular interactions of a number of bacterial enterotoxins may occur through a common mechanism.

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