INTERACTION OF CHOLERA TOXIN AND TOXIN DERIVATIVES WITH LYMPHOCYTES*

I. Binding Properties and Interference with Lectin-Induced Cellular Stimulation

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The last years' research has provided considerable insight into the pathogenesis of cholera at the molecular level. The choleragenic agent has been identified to be a protein exotoxin of mol wt 84,000 which has been isolated and crystallized (1–3). Its toxic action seems to reside in its capacity to activate the adenylate cyclase system in the small bowel mucosa; in fact this ability is not restricted to intestinal cells but has been found with all mammalian cell types tested (4, 5).

Lymphocytes are particularly interesting cells to study with regard to events which relate initial surface binding of agents to cellular activation or deactivation. Such processes are involved in the action of specific antigens or mitogenic lectins. Recently a role of cyclic 3′5′-adenosine monophosphate (c-AMP) has been proposed for in vitro hypersensitivity reactions of immediate as well as delayed type (6, 7). A study of interactions between the potent adenylate cyclase activator cholera toxin and lymphocytes might therefore give information which could elucidate certain important aspects of lymphocyte function. Reports published during the progress of our study showing that lymphocytes respond to cholera toxin with a rise in c-AMP (8, 9) were consistent with this assumption.

It has recently been shown that the ganglioside G_{M1} in all likelihood constitutes the cell membrane receptor for the cholera toxin (10–13). This ganglioside seems to bind the toxin to the cell surface through the structure galactose β1-3-*N*-acetylgalactosamine β1-4 (*N*-acetylneuraminic acid [α2-3]) galactose β1-(11). It is also known that the toxin consists of two different types of subunits, heavy (H)[1] and light (L) (14). The available evidence suggests that the

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[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; Cer, ceramide (*N*-acylsphingosine); con A, concanavalin A; FITC, Fluorescein isothiocyanate; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; H, heavy subunit of toxin; L, light subunit of toxin; MEM, Eagle's minimum essential medium; NAN, *N*-acetylneuraminic acid; NGN, *N*-glycolylneuraminic acid; PBS, phosphate-buffered saline; SDS, sodium dodecylsulfate polyacrylamide.

intact toxin is built up by the noncovalent aggregation of one H chain and several, perhaps six or seven L chains. It is not well understood how the subunit arrangement of cholera toxin determines the biologic activity. It appears that the L type of subunit is responsible for the binding of the toxin to G_{M1} receptors (11),[2, 3] but at least as tested with the rabbit intradermal assay the additional presence of H subunit has been found important for the toxic activity (14). It was the aim of the present study to test the usefulness of the well-characterized mouse lymphocyte as target cell for more detailed investigations of how the cell-binding properties relate to the biologic action of the toxin, and what significance the H and L subunits have for binding and activity. We chose lymphocyte stimulation by the mitogenic lectin concanavalin A (con A), as the process to study possible interference with, since it represents an established cellular activation model where the stimulating agent resembles cholera toxin by being a subunit-composed protein which binds to defined cell surface receptors (15).

Materials and Methods

Mammalian Cells.—Thymus lymphocytes and spleen cells were obtained from 4–6-wk old CBA mice, which had been killed by cervical spine dislocation. The thymus and spleen were removed aseptically and cell suspensions prepared by squeezing the organs through a stainless steel grid into Eagle's minimum essential medium (MEM) or phosphate buffered saline (PBS). Human erythrocytes were obtained from an adult male donor and suspended in PBS.

Cholera Toxin, Toxoid, and Toxin Subunits.—Isolated cholera toxin (choleragen) and natural toxoid (choleragenoid) were prepared by Dr. R. A. Finkelstein, Dallas, Texas (1). Dr. R. Northrup distributed the toxin from the National Institute of Allergy and Infectious Diseases, Bethesda, Md. (Lots 1071 and 0572) and Dr. Finkelstein generously provided the toxoid. Stock solutions were prepared and stored as earlier (14).

Toxin was also fractionated in order to obtain samples with varying proportions of H and L subunits. A slight modification of a previously described fractionation procedure was used (14). A 200 μl sample of toxin, 5 mg/ml, was mixed with 100 μl of 1 M glycin-HCl, pH 3.8, and incubated at room temperature for 30 min. It was then subjected to filtration through a 15 × 600-mm Sephadex G-100 column using 0.1 M glycine-HCl, pH 3.8, as eluting buffer. The higher pH employed in this modified fractionation procedure gave a much better yield of the labile H subunit but less complete separation of this subunit from L-chain material, as estimated with Lowry protein determinations and sodium dodecylsulphate polyacrylamide (SDS) electrophoresis (14). The slowest filtering fraction (no. 34) contained pure L subunit material as judged from SDS electrophoresis. This was verified by immunodiffusion experiments where an antiserum to toxin (see below) which contained precipitating antibodies to the H subunit as well as to the L subunit was used.[2,3] In SDS electrophoresis tests also fraction 33 seemed to consist only of L subunits, but the immunodiffusion test revealed trace contamination with H subunit; it contained more material than fraction 34 and was therefore used as purified L subunit (fraction "L"). The faster-filtering fraction 28, which was rich in

[2] Holmgren, J. Immunologic and receptor-binding properties of cholera toxin and its subunits. Proceedings of the 9th U. S.-Japan Cholera Conference. U. S. Government Printing Office. In press.

[3] Holmgren, J., and I. Lönnroth. Immunochemical and receptor-binding properties of cholera toxin and its subunits. Manuscript in preparation.

H subunit (73%), was freed from the contaminating L subunit material by incubation in a series of polystyrene tubes, which had been coated with the ganglioside G_{M1} (16), which binds the L chains but not free H chains.[2,3] This procedure provided fraction "H", which contained pure H subunit as shown by SDS electrophoresis and only the faintest trace of L subunit in immunodiffusion analyses. Fractions with intermediate filtration rates (nos. 29–32) contained H as well as L subunits in substantial and varying proportions. The subunit contents in the toxin, toxoid, and toxin fractions are shown in Table V in the Results section.

Radioiodinated Proteins.—Cholera toxin and toxoid, and the lectin con A (Miles-Yeda, Rehovot, Israel) were labeled with ^{125}I by the chloramine T method (17). Carrier-free [^{125}I]Na was purchased from The Radiochemical Centre, Amersham, England. In a total volume of 25 μl, 25 μg of protein reacted for 1 min at room temperature with 0.5 mC of [^{125}I]Na (sp act > 14 mC/μg and 25 μg of chloramine T) (E. Merck AG, Darmstadt, West Germany). The reaction was stopped by adding 50 μg sodium metabisulphite, and after dilution with three volumes of distilled water the sample was filtered through a 5 × 60-mm Sephadex G-25 column. The yields of protein-bound radioactivity were approximately 10% for cholera toxin and toxoid, and 30% for con A. The molar ratio of iodine to protein was between 1:10 and 1:20.

Gangliosides and Related Neutral Glycosphingolipids.—Dr. L. Svennerholm, Göteborg, Sweden provided a series of pure gangliosides and related neutral glycosphingolipids, including the G_{M1} ganglioside which is the likely tissue receptor for the cholera toxin. The following substances, prepared and characterized as reported (11), were used: globoside, GalNAcβ1-3Galα1-4Galβ1- 4Glc-Cer; G_{A1}, Galβ1-3GalNAcβ1-4Galβ1-4Glc-Cer; tetrahexoside-GlcNAc, Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer; G_{M3}, NANα2-3Galβ1-4Glc-Cer and NGNα2-3Galβ1-4Glc-Cer; G_{M2}, GalNAcβ1-4(NANα2–3)Galβ1-4Glc-Cer; G_{M1}, Galβ1-3GalNAcβ1-4-(NANα2–3)Galβ1-4Glc-Cer; G_{D1a}, NANα2–3Galβ1-3GalNAcβ1-4(NANα2–3)Galβ1-4Glc-Cer; G_{D1b}, Galβ1-3GalNAcβ1-4(NANα2-8NANα2–3)Galβ1-4Glc-Cer; G_{T1}, NANα2–3Galβ1-3GalNAcβ1-4(NANα2–8NANα2–3)Galβ1-4Glc-Cer.

α-Methyl-D-Glucoside.—The specific oligosaccharide inhibitor of con A, α-methyl-D-glucoside, was purchased from Sigma Chemical Company, St. Louis. Mo.

Antisera.—Antiserum to cholera toxin was prepared in a rabbit. Three injections of 30 μg of toxin were given subcutaneously with intervals of 3 wk between each injection; the bleeding was taken 2 wk after the last injection. This antiserum contained precipitating antibodies against the L as well as the H subunits of the toxin as shown by double diffusion-in-gel technique.[2,3] Rabbit antiserum to mouse immunoglobulin was purchased from Behringwerke AG, Marburg-Lahn, West Germany. Fluorescein isothiocyanate (FITC)-labeled sheep antiserum to rabbit immunoglobulin was obtained from SBL, Stockholm, Sweden. All antisera were absorbed three times with CBA mouse erythrocytes before use.

Binding of Cholera Proteins to Cells Studied by Immunofluorescence.—Immunofluorescence studies of binding of cholera toxin, toxoid, and toxin subunits to mammalian cells were performed with mouse thymus lymphocytes or human erythrocytes suspended in PBS to a density of 10^7 cells/ml. To 100 μl of cell suspension was added 100 μl of cholera protein, when not otherwise stated at a concentration of 1 μg/ml. After incubation for 15 min at room temperature the cells were washed twice with PBS, and resuspended in 100 μl of PBS. The rabbit anti-cholera toxin antiserum diluted 1:4 in PBS was added in a volume of 100 μl, and 15 min later the cells were washed and resuspended as before. 100 μl of FITC antirabbit immunoglobulin diluted 1:4 in PBS was added, and after another 15 min at room temperature the cells were again washed and suspended in PBS, and 10 μl of the cell suspension placed under a cover slip on a microscopic slide. Fluorescence microscopy and photography was performed with a Leitz fluorescence microscope equipped with a Ploem illuminator and a Leitz automatic microscope camera (Leitz GMBH, Ernst Wetzlar, West Germany).

Binding of Radioiodinated Proteins to Cells.—Studies of the association of radioactive toxin,

toxoid or con A to mammalian cells were performed with mouse thymus lymphocytes suspended in MEM supplemented with 1 % bovine serum albumin (BSA), pH 7.2, to a density of 5×10^7 cells/ml. 200 μl of the cell suspension was incubated, when not otherwise stated at 37°C for 30 min, with [^{125}I]toxin, [^{125}I]toxoid, or [^{125}I]conA in the presence or absence of these proteins nonlabeled. The cells were washed twice on Millipore EAWP filters (Millipore Corp., Bedford, Mass.) with cold PBS, pH 7.2, and the filter with the cells transferred to a test tube. The radioactivity of the tube was measured in a gamma ray spectrometer. All experiments were performed in triplicates and the results are expressed as mean values for bound radioactivity/million cells in percentage of the added radioactivity. Since both [^{125}I]toxin and [^{125}I]-toxoid bound to some extent to the filters and since this effect was concentration dependent, the experiments were repeated without cells. The obtained values were used to transform filter-retained radioactivity into specific cell-bound radioactivity. The data were diagrammed according to Scatchard (18) where r/c is plotted against r using the function $r/c = nK - rK$ where r represents the mean number of cholera protein molecules bound per cell; c the free concentration of cholera protein; n the mean number of molecules of cholera protein bound to the cell surface when it is saturated; K the effective equilibrium constant for binding of cholera protein to the cell surface (the sort ml/molecule is converted to the more common liters/mol by multiplication with 6.02×10^{20}). The equation is valid provided that the reaction is bimolecular, i.e. one cholera protein molecule binds only to one receptor site on the cell surface, which is an unproven assumption. Per cent dissociation of toxin and toxoid was studied after incubation of the labeled protein (100 μg/ml) with the thymocytes (5×10^6 cells/ml) at 37°C for 15 min. After centrifugation and washing twice, the cells were again incubated in the MEM-BSA medium and portions of 100 μl, i.e. 1×10^6 cells, were filtered after various times through Millipore EAWP filters, and the retained radioactivity measured.

Lymphocyte Stimulation Tests.—Mouse thymocytes or spleen cells were suspended in MEM. The cells were washed once by centrifugation and suspended in RPMI 1640 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% human serum (Flow Laboratories Svenska AB, Solna, Sweden), 100 IU/ml of penicillin and 100 μg/ml of streptomycin. The cell density was 5×10^6/ml. 200-μl portions of the cell suspension were placed in the wells of Falcon Microtest II plates (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and added with 10 μl of conA, 100 μg/ml, or phytohemagglutinin (Difco Laboratories, Detroit, Mich.) diluted 1:10. Cholera toxin, toxoid, or toxin fractions as well as gangliosides, other glycosphingolipids or antiserum were also added in volumes of 10 μl. Cultivation of the cells was performed at 37°C under a gas mixture consisting of 10% CO_2, 7% O_2, and 83% N_2. After 3 days, 2 μC of [^{3}H]thymidine (The Radiochemical Centre) was added in a volume of 10 μl. The cultivation was continued for another 18 h when the cells were harvested and placed on Millipore EAWP filters. Washing was performed with 2 × 10 ml of PBS, 10 ml of 5% trichloroacetic acid and 10 ml of 95% ethanol. The filters were then dried, dissolved in 1 ml of Soluene (Hewlett Packard Sverige AB, Bromma, Sweden) and radioactivity measured by scintillation counting in Permablend (Hewlett Packard Sverige) dissolved in toluene. All experiments were performed in triplicates and results are expressed as mean values of one or several experiments.

Cell Viability Tests.—Cell viability was evaluated with the trypan blue exclusion test (19).

Demonstration of Mobility of Surface Immunoglobulin and of Cell-Bound Toxin ("Cap Formation").—For studies of surface immunoglobulin mobility a suspension of mouse spleen cells was prepared as described above for lymphocyte stimulation tests and the cell density adjusted to 2×10^7 cells/ml. To 100 μl of this suspension was added 100 μl of rabbit anti-mouse immunoglobulin and the cells were incubated at 37°C for 30 min. The cells were then washed twice with PBS containing 10 mM NaN_3 to inhibit further membrane mobility (20). After suspension in 100 μl of PBS the cells were incubated with 100 μl of FITC-labeled anti-rabbit immunoglobulin at room temperature for 15 min. After washing, the cells were mounted and examined as in the immunofluorescence studies described above.

For studies of cholera toxin receptor mobility, similarly prepared mouse thymus cells were incubated with cholera toxin, 2 μg/ml, at 37°C for 15 min, washed, and incubated with rabbit anticholera toxin antiserum, diluted 1:4, 1:10 or 1:20 at 37°C for another 30 min. The cells were then washed with PBS-NaN_3, and incubated with FITC-labeled antirabbit immunoglobulin and examined as described above.

Skin Toxicity Assays.—The rabbit intradermal test of Craig (21) was used for determination of the skin toxic activity of cholera toxin and its derivatives. This assay measures the skin capillary permeability increasing ability of toxin and is visualized by the dermal deposition of intravenously injected blue dye. All dilutions were done with a borate-gelatin buffer, pH 7.5 (3.1 g H_3BO_3, 7.0 g NaCl, 2.0 g gelatin, H_2O ad 1,000 ml), and each material was tested in three animals. The activity is expressed as number of blueing doses per nanogram of protein.

RESULTS

Interference of Cholera Toxin and Toxoid with Lymphocyte Stimulation and Lymphocyte Viability.—Mouse thymocytes were cultured in the presence of con A and the degree of stimulation was assayed by measurements of the [^{3}H]thymidine incorporation. The effect of cholera toxin and toxoid on this stimulation was assayed after addition of varying amounts of these proteins to the cultures simultaneously with the con A. Fig. 1 shows the results of three different experiments, each performed in triplicates. Both the cholera toxin and the toxoid inhibited the lymphocyte stimulation by con A but the toxin

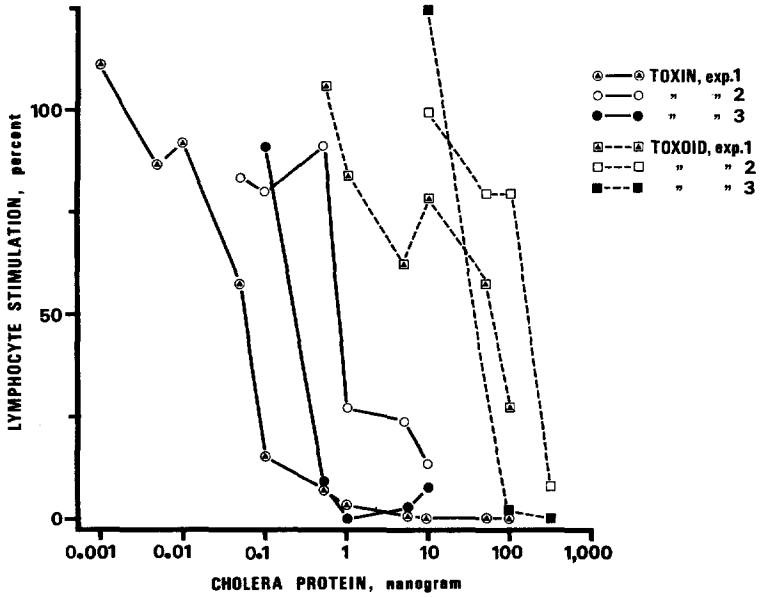

FIG. 1. Effect of cholera toxin and of natural toxoid on thymocyte stimulation by con A. Each value is mean of triplicate cultures and represents radioactivity of cell-incorporated [^{3}H]thymidine in percentage of that of cultures stimulated with con A in the absence of cholera protein. Subtraction of radioactivity of nonstimulated control cultures was done before the percentage calculation.

was about 300 times more potent on a weight basis than the toxoid. The mean amounts required for half-maximal inhibition were for toxin 300 pg and for toxoid 100 ng, i.e. 1.7×10^{-11} mol/liter and 9×10^{-9} mol/liter, respectively. Toxin caused complete inhibition at a mean concentration of 5×10^{-11} mol/liter, which corresponds to 10 bound molecules per cell.

The effect of toxin on the stimulation of mouse spleen cells by phytohemagglutinin was also determined. Also in this instance the toxin caused inhibition of the lectin-induced stimulation; 600 pg (3.4×10^{-11} mol/liter) gave half-maximal inhibition.

In order to study whether the inhibitory effect of toxin was due to cell death, the viability of thymocytes and spleen cells incubated with con A in the presence or absence of cholera toxin was determined by dye exclusion tests. There was only a very small increase of dead cells in the presence of toxin (Table I).

TABLE I

Trypan Blue Exclusion Test of the Influence of Cholera Toxin on the Viability of Con A-Stimulated Mouse Thymus and Spleen Cells

Day of culture	Cells showing staining with trypan blue			
	Spleen cells		Thymus cells	
	With toxin*	Without toxin	With toxin	Without toxin
	%		%	
1	18	16	35	24
2	35	34	62	54
3	38	33	72	68
4	45	43	86	83

* Concentration, 1 μg/ml.

Binding Properties of Toxin and Toxoid to Lymphocytes.—

Immunofluorescence studies: Mouse thymocytes were incubated with cholera toxin or toxoid and thereafter treated with rabbit anticholera toxin and FITC-labeled antirabbit immunoglobulin serum. Binding of toxin could be demonstrated when the concentration of toxin was 100 ng/ml or more, and of toxoid when the concentration was about the same. Two patterns of binding were observed with both toxin and toxoid (Fig. 2). The fluorescence was distributed either as a continuous ring around the cell (Fig. 2 *a* and *c*) or as discrete areas on the cell (Fig. 2 *b*, *d*, and *e*).

Mixing toxin, or toxoid, 1 μg/ml, with an equal volume of the ganglioside G_{M1}, 100 ng/ml, before the incubation with thymocytes resulted in complete inhibition of toxin or toxoid binding as revealed by immunofluorescence. No binding of toxin to human red blood cells could be demonstrated by immunofluorescence.

Studies with radioiodinated toxin, toxoid, and con A: It was assessed that radioiodinated toxin, toxoid, and con A all bound to mouse lymphocytes.

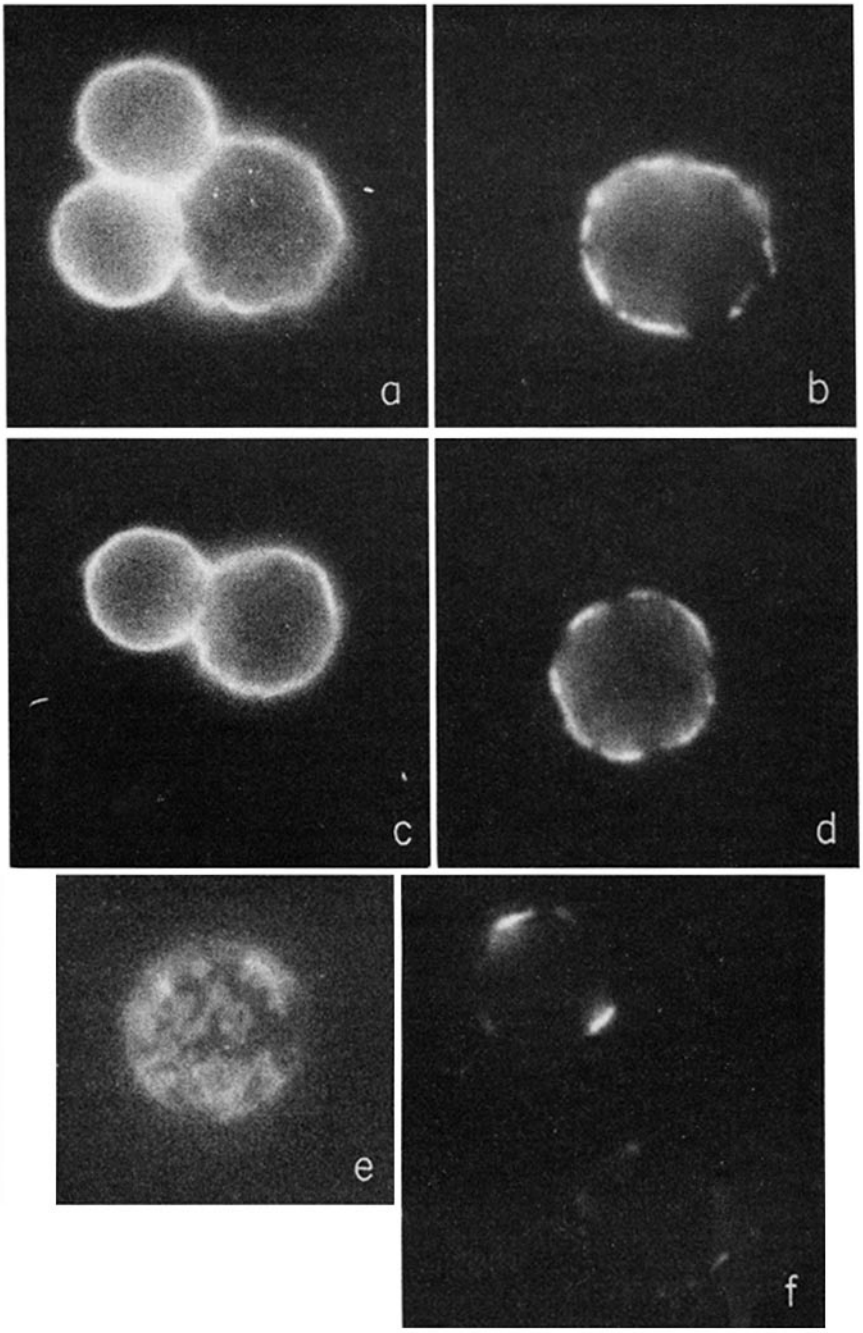

FIG. 2. Binding of cholera toxin, toxoid, and toxin subunits to mouse thymus cells as demonstrated by immunofluorescence. Concentration of toxin and its derivatives was 1 μg/ml and incubation was carried out for 15 min at room temperature. (*a*) Binding of cholera toxin showing fluorescent surface-located rings. (*b*) Binding of cholera toxin showing surface-located fluorescent patches. (*c*) Binding of cholera toxoid showing fluorescent rings. (*d*) Binding of cholera toxoid showing fluorescent patches. (*e*) Same as (*d*) but focus is on the upper surface of the cell. (*f*) Binding of purified L subunit preparation showing weakly fluorescent patches. Purified H subunit did not bind demonstrably. × 2,500.

Dilution experiments where the labeled and unlabeled toxin was mixed in various proportions but the total toxin amount held constant, showed that the radioiodination had not influenced the cell-binding properties. Nor had the labeling affected the skin toxic activity of the toxin.

The binding properties of toxin to cells were studied in two experiments with different batches of [^{125}I]toxin. A fixed concentration of [^{125}I]toxin, 50 ng/ml, was mixed with different concentrations of unlabeled toxin and incubated with lymphocytes for 30 min. It was found that in the low concentration range toxin bound in constant proportions to the cells; in higher concentration, the binding of toxin to the cell surface became a saturable process (Fig. 3 *a*). IC_{50}, i.e. the concentration leading to half-maximal inhibition of the percentage-bound [^{125}I]toxin, was 400 ng/ml in one experiment and 550 ng/ml in the other one (4.8×10^{-9} and 6.6×10^{-9} mol/liter). For more detailed evaluation of the data a curve was drawn from the mean values of the two experiments (dotted line, Fig. 3 *a*) and all points transcribed to a Scatchard plot (Fig. 3 *b*). From the latter diagram the mean number of toxin molecules bound per cell (n) was calculated and found to be 4×10^4 molecules/cell. This value was also obtained from both of the original curves in Fig. 3 *a*. The association constant K_A was 7×10^8 liters/mol when calculated from the averaged curve (Fig. 3 *b*), and 3×10^8 and 1×10^9 liters/mol in the individual experiments.

A similar study was done with the cholera toxoid (Fig. 3 *c* and *d*). IC_{50} ranged from 200 to 280 ng/ml (3.6×10^{-9} to 4.9×10^{-9} mol/liter), n was found to be 4×10^4 molecules/cell which agrees with the value of the toxin, and K_A between 1×10^9 and 7×10^9 liters/mol (mean 4×10^9 liters/mol).

The association experiment with toxin and lymphocytes was repeated at +5°C under otherwise identical conditions. The temperature difference did not significantly change the binding pattern of toxin which shows that metabolic events probably do not influence on the extent of binding. The same was found for toxoid.

In order to see whether the fraction of [^{125}I]toxin which remains in the supernate had different cell-binding properties than the fraction which had bound to the cells, 1 μg ^{125}I-toxin was incubated with 10^8 cells in 10 ml MEM-BSA for 15 min at 5°C. After centrifugation the medium which still contained 94% of the [^{125}I]toxin was mixed in portions with various amounts of unlabeled toxin and tested for binding to thymus cells as previously. The binding curves were almost identical to those obtained in the experiments shown in Fig. 3 *a* and *b*. The same experiment with toxoid gave results very similar to those in Fig. 3 *c* and *d*.

The kinetic of the binding to thymocytes was very similar for [^{125}I]toxin and [^{125}I]toxoid (Fig. 4). A very rapid binding was observed, with both proteins being complete within 5 min at 37°C.

When bound to the lymphocytes the dissociation tendency of both toxin and toxoid was very small. Less than 1% of the radioactivity was lost during incubation up to 1 h from lymphocytes saturated with [^{125}I]toxin or [^{125}I]toxoid.

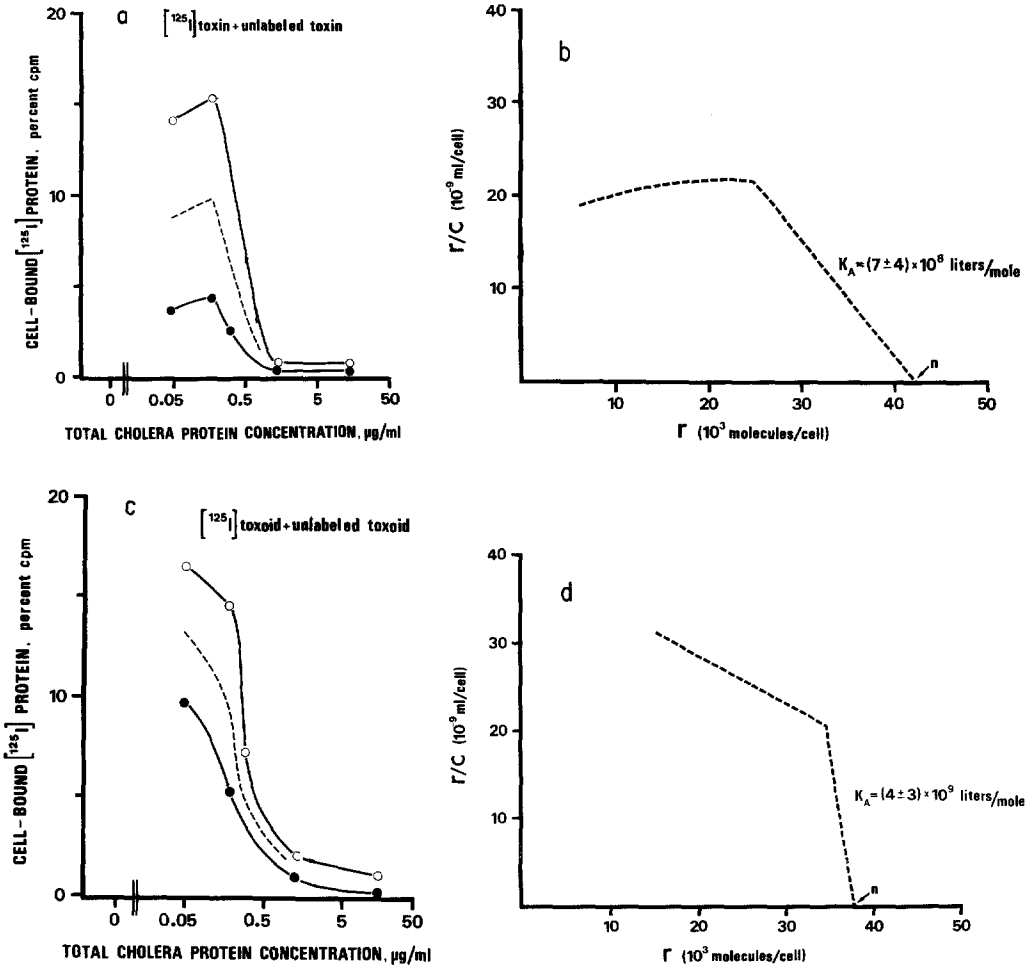

FIG. 3. Binding properties of toxin and toxoid to thymocytes. (*a*) Cell-bound radioactivity as a function of total concentration of added unlabeled toxin and [^{125}I]toxin. (○—○), experiment 1; (●—●), experiment 2; (---), average curve drawn from experiment 1 and 2. (*b*) Scatchard plot of the average curve in (*a*), (n = the mean number of molecules bound to a cell saturated with toxin; K_A = the association constant). (*c*) and (*d*) correspond to (*a*) and (*b*) using unlabeled toxoid and [^{125}]toxoid.

In order to investigate whether the cholera toxin is competing with con A for the same cellular binding sites, 100 ng of [^{125}I]con A was mixed with various amounts of unlabeled toxin or con A, and incubated with the thymocytes at 37°C for 30 min. Table II shows the results. The nonlabeled con A prevented the binding of [^{125}I]con A whereas the cholera toxin did not affect the cellular binding of [^{125}I]con A.

Studies of surface immunoglobulin mobility and toxin receptor mobility—"*cap-*

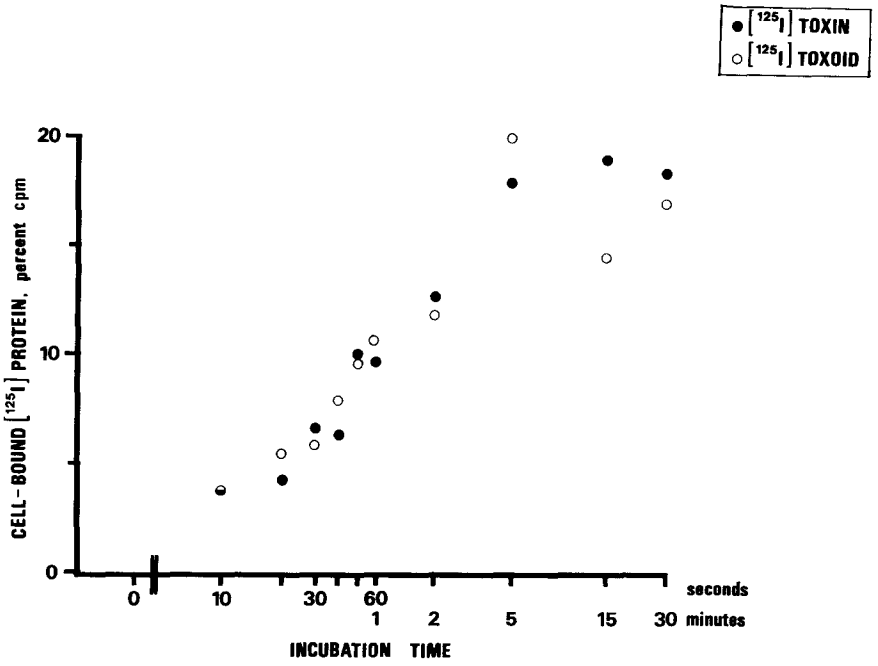

FIG. 4. Cell-bound [^{125}I]toxin and [^{125}I]toxoid as a function of the incubation time. A cholera protein concentration of 50 ng/ml was used, and incubation performed at 37° C.

TABLE II

Study of Possible Competition between Con A and Cholera Toxin in Binding to Mouse Thymus Lymphocytes

Competing protein			Cell-bound [^{125}I]Con A*
	μg	*μmol/l*	%
—	0	0	15.8
Con A	0.2	0.01	17.5
Con A	2	0.11	12.4
Con A	20	1.11	2.8
Cholera toxin	2	0.12	16.7
Cholera toxin	20	1.19	16.6

* Mean of triplicates; [^{125}I]con A was tested in an amount of 0.1 μg.

formation": Surface immunoglobulin was demonstrated on mouse spleen cells by means of immunofluorescence. It could be found that the surface immunoglobulin was localized to one pool of the cell on about 85% of cells, when the cells had been incubated with antimouse immunoglobulin serum. This "cap-formation" was not significantly affected by preincubating the cells with either cholera toxin or toxoid at a concentration of 1 μg/ml for 30 min (Table III).

TABLE III

Influence of Cholera Toxin and Toxoid on Cap Formation of Surface Immunoglobulin on Mouse Spleen Cells

Pretreatment	Cells showing caps
	%
—	84
100 ng cholera toxin	82
100 ng cholera toxoid	83

Attempts were also done to demonstrate cap-formation of cholera toxin bound to thymocytes. It was found that cells preincubated with toxin and then with rabbit antitoxin diluted 1:4, 1:10, or 1:20, showed caps after treatment with antirabbit immunoglobulin only in 6, 5, and 8% for the different antiserum dilutions. Thus it seems that the mobility of the thymocyte receptors for toxin is more restricted than the mobility of spleen cell surface immunoglobulin.

Effect of Gangliosides and Antiserum in Lymphocyte Stimulation Tests.—Mouse thymocytes were cultured in the presence of con A and an inhibitory amount, 10 ng, of cholera toxin. Different pure gangliosides or related neutral glycosphingolipids were added in varying amounts at the same time as the toxin, and the degree of lymphocyte stimulation determined by measurements of [^{3}H]thymidine incorporation. The results are found in Table IV. The only glycosphingolipid which blocked the inhibitory effect of cholera toxin on lymphocyte stimulation was the G_{M1} ganglioside which was effective in amounts equal to or above the amount of toxin present. In similar experiments also antiserum to cholera toxin blocked the lymphocyte stimulation inhibitory action of the toxin (Table IV).

It was controlled that the G_{M1} ganglioside and the antiserum to cholera toxin in the effective concentrations did not per se affect the stimulatory power of con A on lymphocytes. It was also assessed that α-methyl-D-glucoside, the inhibitor of con A, and con A itself had no effect on the skin activity of cholera toxin even when tested in high concentrations.

Binding of Cholera Toxin Subunits to Thymocytes and Effect of Subunits on Lymphocyte Stimulation.—Binding of purified L and H subunits to mouse thymocytes was studied by immunofluorescence. The L subunit was found to bind to thymocytes when applied to the cells in concentrations of 1 μg/ml or more (Fig. 2*f*). Binding of purified H could not be demonstrated by immunofluorescence.

The effect of the purified L and H subunits (fractions H and L) on thymocyte stimulation by con A was studied and compared with the effect of toxin fractions containing both subunits in different proportions (fractions 28 and 31). The separated subunits were much less potent in inhibiting the lymphocyte stimulation than the fractions containing both subunits (Fig. 5). In general, the capacity of the cholera toxin and its derivatives to inhibit lymphocyte stimulation correlated with the skin toxic activity of the proteins. This is

TABLE IV

*Influence of Gangliosides, Neutral Glycosphingolipids, and Antitoxin Antiserum on the Inhibitory Effect of Cholera Toxin on Con A-Induced Thymocyte Stimulation**

Test substance	Amount	Decrease of the inhibition caused by toxin‡
	ng	%
G_{M1}	100	100
	10	100
	1	2
	0.1	0
G_{D1a}	100	15
	10	0
	1	0
G_{A1}	100	10
	10	0
	1	0
G_{D1b}	100	0
G_T	100	0
G_{M2}	100	0
G_{M3}	100	0
G_{M3}-NGN	100	0
Tetrahexoside-GlcNAc	100	0
Globoside	100	0
Antitoxin	Dilution	
	1:200	100
	1:2,000	100
	1:20,000	12

* 1×10^6 cells, 1 μg of con A and 10 ng of toxin; total volume 0.2 ml.

‡ Mean of three to nine cultures.

shown in Table V, where it is also seen that the biologic activity of the toxin fractions containing both H and L subunit material is similar to that of the intact toxin, whereas the activity of the separated subunits is more like that of the toxoid.

DISCUSSION

This study shows, as does a parallel study by Sultzer and Craig (22), that cholera toxin can inhibit mouse lymphocytes from being stimulated to DNA synthesis by mitogenic lectins. It further shows that the capacity of toxin and toxin derivatives to inhibit lymphocyte stimulation corresponded well to their activity in the skin test which is documented to reflect the choleragenic action of the toxin. The G_{M1} ganglioside specifically inhibited the toxin effect on lymphocytes, as it has previously done with the effect in skin, gut, and fat cells (10–13). In the light of the c-AMP increasing effect of cholera toxin on lymphocytes, it seems that the lymphocyte adds to the variety of target cells upon which the common key action of cholera toxin is activation of adenylate cyclase.

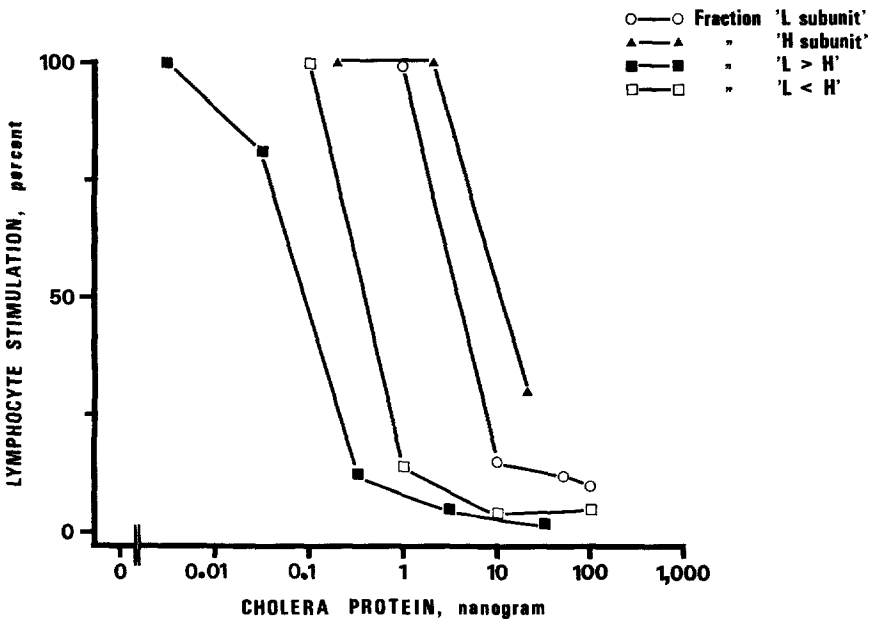

FIG. 5. Effect on con A-induced thymocyte stimulation of purified toxin subunits (L and H) and of toxin fractions containing both L and H subunits in different proportions (L > H = fraction 31, and L < H = fraction 28). Each value is mean of three to six cultures, and is calculated as described in the legend to Fig. 1.

TABLE V

Comparison of Skin Toxicity and Lymphocyte-Stimulation Inhibitory Activity of Cholera Toxin and Toxin Derivatives

Cholera protein	Subunit percent*		Skin blueing doses per nanogram‡	Lymphocyte stimulation-inhibitory doses per nanogram§
	H	L		
Toxin	33	67	4.4	3.3
Toxoid	0	100	0.005	0.01
Frac. H	100	0	0.1	0.1
Frac. 28	73.0	27.0	2.5	2.0
Frac. 29	36.5	63.5	7.5	N.D.‖
Frac. 30	10.0	90.0	9.3	N.D.
Frac. 31	1.6	98.4	5.1	12.5
Frac. 32	0.8	99.2	2.8	N.D.
Frac. L (33)	(0)	100	1.0	0.3
Frac. 34	0	100	0.6	N.D.

* Densitometric determination of protein-stained SDS electrophoresis plates, and qualitative control by immunodiffusion; parentheses indicate positive gel-precipitation reaction where densitometry was negative.

‡ Mean of 8–12 values in three rabbits tested over a 6-mo period.

§ Mean of six or nine values for half-maximal inhibition of con A-induced thymocyte stimulation determined in two or three separate experiments.

‖ N.D., not done.

The outcome of this activation is determined by the cell type and not by the toxin. This explains why toxin-treated fat cells release lipid (23), liver cells convert glycogen into glucose (24), adrenal cells produce steroid hormone (25), and lymphocytes can no longer be stimulated by con A, etc.

We were particularly interested in using the lymphocytes to test how the cell-binding properties relate to the biologic action of the toxin, and what significance the two types of subunits, H and L, have for binding and activity. We found by immunofluorescence tests that toxin, which probably consists of one H and six or seven L subunits, and a natural toxoid consisting exclusively of aggregated L subunits, both bound well to the surface of lymphocytes; purified L subunits also showed binding but purified H subunit did not. These findings infer that the cell-binding property of the toxin resides in the L type of subunit, and that the nontoxicity of the toxoid cannot be explained as an inability to bind to the target cells. However, it required more quantitative experiments with radioactive toxin and toxoid to clarify whether there exist differences between the proteins with regard to binding kinetics, binding strength or number of molecules that could bind to a single cell. We found no such difference to the advantage of toxin. Approximately 40,000 molecules of either protein could maximally bind to a single cell. The kinetics of the binding was identical and the affinity was not significantly different; in fact it tended to be higher for toxoid than for toxin, K_A being $7 \pm 4 \times 10^8$ and $4 \pm 3 \times 10^9$ liters per mole respectively. These data for the binding properties of toxin to thymus cells agree with those recently published by Cuatrecasas for binding to fat cells. A single fat cell could bind 20,000 molecules of cholera toxin, and the dissociation constant was estimated to 4.6×10^{-10} mols/liter (26). It seems documented by these results that toxoid binds equally as well as toxin to the cells. Additionally, the data suggest that the majority of molecules of both proteins bind with the same number of combining sites to the cell surface. In the low concentration range the binding curves for both toxin and toxoid flattened out in a characteristic manner. The explanation for this binding pattern is presently unknown, but might be elucidated by detailed quantitative studies between cholera toxin or toxoid and solid phase pure G_{M1} ganglioside which are in progress. However, the present study revealed that (*a*) the labeled and unlabeled cholera proteins bind equally well to the lymphocytes; (*b*) when attached to the cells, very little cholera toxin or toxoid dissociates into the medium; and (*c*) the bound and unbound fractions of the cholera proteins have similar binding properties to cells, since a toxin or toxoid solution which had been partially absorbed with cells, had the same binding properties as the original solution.

The capacity to inhibit lectin-induced lymphocyte activation seemed to involve the presence of not only L but also H subunit. Thus the purified H and L subunits of toxin were much less active than fractions of toxin containing both subunits; it is very possible that the slight activity in the purified subunit preparations was due to trace contamination with the other subunit. This pattern was also seen in skin tests, thereby confirming our observations in a previous study (14). Finally, preliminary experiments indicate that whereas fractions containing both H and L subunits raise the c-AMP levels much in

lymphocytes, the separated subunits are almost inactive.[4] The proposal in a previous report that for skin toxic activity both H and L subunits are important (14) therefore seems valid also in the employed in vitro systems using lymphocytes.

The view is reasonable that it is the difference in subunit composition which causes the difference in biologic activity between toxin and toxoid. One function of the L subunit is probably as indicated by this and previous studies (11)[3] to confer binding of the toxin to cell surface G_{M1} receptors. We have recently shown that it is the oligosaccharide portion with its single sialic acid which is probably the initial binding site of G_{M1}, but at least for in vitro fixation the sphingosine portion also seems important, probably by stabilizing the initial binding between the toxin and the ganglioside (27).

The role of the H subunit is not known. Apparently it does not bind to the surface provided that the purified preparation tested by us was representative, and it does not seem to make the toxin better binding than toxoid to cell membranes. This does not exclude that membrane structures could exist which become accessible for the H subunit through rearrangements of membrane architecture after the attachment of the toxin to the cell surface. Since the H subunit seems to be important for the toxin action it is possible that this subunit, directly or via an intermediary reaction, represents the adenylate cyclase-activating portion of the toxin. Obviously, more information is required, e.g. of possible effects on both membrane and toxin structure which may be caused by the formation of the toxin-G_{M1} ganglioside complex, before it is meaningful to discuss how this activation could occur. However, the concept that one portion of a bacterial toxin is responsible for avid binding to cell membrane receptors, whereas a different portion exerts the direct action on cell metabolism, is not unique for cholera toxin. A similar hypothesis has been formulated for diphtheria toxin (28), and recent observations also suggest a distinction between "binding" and "toxic" sites for tetanus toxin (29, 30).

The observations in this study and in the report of Sultzer and Craig (22) that inhibition of lectin-induced stimulation by cholera toxin concerns thymus as well as spleen cells, and the mitogens con A, phytohemagglutinin and lipopolysaccharide, indicate that cholera toxin can interfere with DNA synthesis in T cells as well as B cells. They also suggest that the toxin does not compete with the surface receptors for the lectins; this was directly proven with radiolabeled con A which bound equally well to lymphocytes when toxin was present as when it was absent. It was also evident that the toxin does not exert the inhibitory effect by decreasing cell viability, nor does toxin react directly with con A. An interesting observation is that cholera toxin did not affect cap formation of immunoglobulin receptors on the surface of spleen cells when the cells were treated with anti-immunoglobulin antiserum. This indicates that

[4] Lönnroth, I., J. Holmgren, and C. Norrman. Interaction of cholera toxin and toxin derivatives with lymphocytes. II. Effect on intracellular cyclic AMP levels. Manuscript in preparation.

receptor mobility was not generally disturbed in lymphocytes to which toxin had bound. It may therefore be suggested that the toxin mediates its effect on the cells without interfering with the mobility of the lectin receptors or with the ability of the lectins to induce cap formation. It seems that toxin does not affect the events in lectin-induced lymphocyte activation which take place on the cell surface, but a later more central step as yet unrecognized. This is the more likely as the natural cholera toxoid tested had no effect on the stimulation despite it had almost identical binding properties as toxin to the cells. The known ability of cholera toxin but not toxoid to activate adenyl cyclase and thereby raise the intracellular levels of c-AMP in various cells, including lymphocytes, suggests that the step interfered with may be regulated, directly or indirectly, by c-AMP. The effect could relate to the observation that phosphorylation of histones by protein kinases in lymphocytes is regulated by c-AMP (31)

This work documents that studies of interaction between lymphocytes and cholera toxin may be of interest to elucidate important aspects of the mechanism of action of the toxin, as well as factors which control lectin-induced lymphocyte stimulation. They may also have relevance for certain immune functions of lymphocytes, since cholera toxin has been reported to decrease lymphocyte-mediated in vitro destruction of allogeneic cells (8, 9) and to have an adjuvant action on humoral antibody formation in vivo (32).

SUMMARY

The interaction of cholera toxin and a number of toxin derivatives, containing different proportions of light and heavy toxin-composing subunits (L and H), with mouse lymphocytes was studied. Experiments with [^{125}I]toxin showed that a single cell can rapidly, within minutes, bind up to 40,000 molecules of toxin, the association constant was estimated to $7 \pm 4 \times 10^8$ liters/mol, and binding was found to be very similar at 37°C and 5°C. Immunofluorescence studies revealed that the toxin attachment is located on the cell surface, and that purified L subunit but not H subunit binds to the cells. A natural cholera toxoid, built up by aggregated L subunits, showed almost identical binding properties as toxin to the cells. Pure G_{M1} ganglioside, the proposed membrane receptor structure for toxin, prevented entirely the cellular binding of both toxin and toxoid.

Cholera toxin in concentrations down to approximately 5×10^{-11} mol/liter (corresponding to 10 bound molecules/cell) inhibited thymus cells from being stimulated to DNA synthesis by concanavalin A (con A), and spleen cells from such stimulation by phytohemagglutinin. The G_{M1} ganglioside but not a series of other pure structurally related gangliosides and neutral glycosphingolipids neutralized this toxin activity. Toxin derivatives which, in similarity with toxin, possessed H as well as L subunits but in other proportions, were potent inhibitors of con A-induced thymocyte stimulation, whereas the natural toxoid (aggregated L subunits), purified toxin L subunit and purified toxin H subunit were up to 300-fold less active on a weight basis. The capacity of chol-

era proteins to inhibit con A-induced thymocyte stimulation correlated well with their activity in the rabbit intradermal toxicity assay.

The inhibitory action of cholera toxin on con A-induced thymocyte stimulation did not depend on decreased cell viability from the toxin treatment, nor was it caused by a reaction between toxin and con A. [^{125}I]con A bound equally well to the cells when toxin was present as when it was absent, which proves that the toxin did not compete for cellular con A receptors. Nor did the toxin seem to disturb the general mobility of membrane receptors or their ability to accumulate in caps.

It is concluded that the L type of subunit confers rapid and firm binding of cholera toxin to lymphocyte membranes, probably to G_{M1} ganglioside receptors. For biologic activity the additional presence of H subunit is important. One manifestation of toxin action on lymphocytes is inhibition of lectin-induced DNA synthesis; probably this effect relates to the ability of cholera toxin to raise the levels of intracellular cyclic 3′5′-adenosine monophosphate.

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