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Vibrio cholerae Choleragenoid. Mechanism of Inhibition of Cholera Toxin Action†

Pedro Cuatrecasas‡

ABSTRACT: Choleragenoid, a biologically inactive protein which is derived from the exotoxin of *Vibrio cholerae*, blocks the binding of ^{125}I-labeled cholera toxin to liver membranes as effectively as the parent toxin. The binding of ^{125}I-labeled cholera toxin to membranes which have been incubated with choleragenoid and subsequently washed is suppressed to the same extent as when the membranes are incubated with cholera toxin. The rate of dissociation of the cholera toxin–membrane complex is the same whether it is measured in the presence of cholera toxin or of choleragenoid. Choleragenoid does not elicit a lipolytic response in isolated fat cells. When the cells are preincubated for 15 min at 24° with choleragenoid, there is potent inhibition of cholera toxin induced lipolysis. The concentration of choleragenoid required to inhibit by 50% the lipolytic response of 0.2 μg/ml of cholera toxin is about 40 ng/ml (7×10^{-10} M). Choleragenoid exhibits virtually no inhibitory effect if the fat cells are incubated (15 min, 24°) first with cholera toxin. The ability of choleragenoid to block the lipolytic activity of cholera toxin is explicable in terms of its ability to block the binding of the toxin to the membrane receptor by its own occupancy of that receptor. Cholera toxin and choleragenoid are equally active in blocking the binding of ^{125}I-labeled choleragenoid to liver membranes. Preincubation of ^{125}I-labeled choleragenoid with gangliosides blocks the ability of the iodoprotein to bind to membranes. Incubation of membranes with gangliosides (followed by washing of the membranes) greatly increases the binding sites on the membrane for choleragenoid. The present studies indicate that cholera toxin and choleragenoid interact with the same receptor (probably gangliosides) on the cell surface, and that the properties of this interaction are very similar if not identical for both proteins. Choleragenoid is thus a structural analog of cholera toxin which appears to bind to the receptor with unaltered affinity but which produces a biologically unproductive complex with the receptor. It is suggested that the choleragenoid– and cholera toxin–receptor complexes as initially formed are both very similar and inactive, and that the toxin– but not the choleragenoid–receptor complex is capable of undergoing a subsequent rearrangement within the structure of the membrane which converts it into a biologically active form.

In the process of purification of an active exotoxin from *Vibrio cholerae*, Finkelstein and LoSpalluto (1969, 1970) have purified another protein which is immunologically identical with the toxin but which is lacking in biological activity in intestinal (Finkelstein and LoSpalluto, 1969, 1970) and fat cell (Vaughan *et al.*, 1970) preparations. This protein, which has been called choleragenoid (Finkelstein and LoSpalluto,

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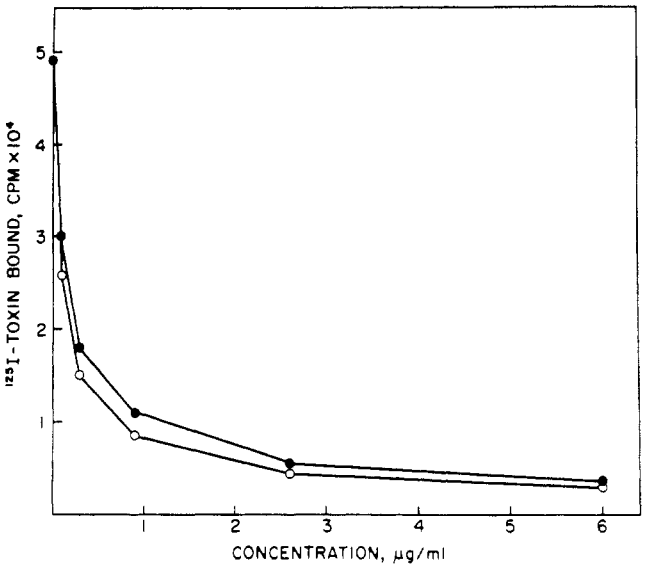

FIGURE 1: Effect of native cholera toxin (●) and choleragenoid (○) on the binding of ^{125}I-labeled cholera toxin to liver membranes. Membrane suspensions (92 μg of protein), in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin, were incubated for 3 min at 24° with the indicated concentration of cholera toxin or choleragenoid. ^{125}I-Labeled cholera toxin (9×10^4 cpm, 14 μCi/μg) was added to all the samples and the incubation was continued at 24° for 15 min. Specific binding to membranes was determined by filtration over cellulose acetate Millipore filters (Cuatrecasas, 1973a).

1969, 1970), appears to be smaller (mol wt 58,000) and less anionic than cholera toxin (mol wt 84,000) (LoSpalluto and Finkelstein, 1972). More recent evidence (Finkelstein *et al.*, 1971a,b) indicates that choleragenoid is derived from the cholera toxin molecule, thus explaining the immunological identity of these two proteins.

In the course of studies on the mechanism of action of cholera toxin (Cuatrecasas, 1973a–c) it was discovered that the biological activity of cholera toxin as well as the binding of ^{125}I-labeled cholera toxin to cells and membranes are strongly inhibited by choleragenoid. The present studies describe the nature of this inhibition and indicate that although choleragenoid and cholera toxin bind with very similar properties to essentially the same cell membrane sites, the choleragenoid–receptor complex is incapable of triggering the events which must subsequently occur and is thus biologically inactive. It has been suggested (Cuatrecasas, 1973c) that the initial cholera toxin–receptor complex is itself biologically inactive and must undergo a major transition within the structure of the membrane to be transformed into an active complex. Within this framework it is possible that the structural modifications in the choleragenoid molecule do not significantly modify its interaction with the cholera toxin receptor but do prohibit the transition of the receptor complex into an active state.

Experimental Procedure

Cholera toxin (lot 1071), purified by the method of Finkelstein and LoSpalluto (1970) and obtained from Dr. R. S. Northrup, SEATO Cholera Research Program, was prepared under contract for the National Institute of Allergy and Infectious Diseases by Dr. R. A. Finkelstein, The University of Texas Southwestern Medical School, Dallas, Tex. Purified choleragenoid was generously provided by Dr. R. A. Finkelstein. Bovine brain gangliosides (fraction II) were purchased from Sigma.

TABLE I: Binding of Cholera Toxin to Liver Membranes Which Have Been Washed after Incubating with Choleragenoid and Cholera Toxin.[a]

Treatment of Membranes	Binding of ^{125}I-Labeled Cholera Toxin (cpm)
None	14,500
Choleragenoid, 2 μg/ml	3,800
7 μg/ml	1,700
Cholera toxin, 2 μg/ml	4,400
10 μg/ml	1,900

[a] Suspensions of liver membranes (1.8 mg of protein/ml) were incubated at 24° for 20 min in Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin in the absence and presence of cholera toxin or choleragenoid. The samples were then diluted 25-fold with the same buffer (4°) and centrifuged at 30,000*g* for 20 min. After repeating the washing procedure once, the pellets were resuspended in the same buffer and 0.2-ml samples (containing 80 μg of protein) were incubated for 20 min at 24° in the presence of ^{125}I-labeled cholera toxin (3.2×10^4 cpm).

The methods used in the preparation of ^{125}I-labeled cholera toxin (5–20 μCi per μg) have been described (Cuatrecasas, 1973a). Choleragenoid was iodinated (16 μCi/μg) and purified by essentially the same procedures. The molecular weight of cholera toxin was taken to be 84,000 and that of choleragenoid 58,000 (LoSpalluto and Finkelstein, 1972). The $E_{1\,cm}^{1\%}$ (280 nm) was assumed to be 11.41 for cholera toxin and 9.56 for choleragenoid (LoSpalluto and Finkelstein, 1972). Isolated fat cells were prepared from male Sprague-Dawley rats (90–120 g) by the method of Rodbell (1966). Lipolysis was studied by determining the concentration of glycerol in the medium by the method of Ryley (1955). Liver membranes were prepared by homogenization and differential centrifugation in 0.25 M sucrose (Cuatrecasas, 1972; Illiano and Cuatrecasas, 1972). Membrane protein was determined by the method of Lowry *et al.* (1951) after heating at 100° for 30 min in 1 M NaOH; bovine albumin was used as the standard.

Results

Competition by Choleragenoid of Cholera Toxin Binding to Membranes. Choleragenoid can block very effectively the binding of ^{125}I-labeled cholera toxin to liver membranes (Figure 1). Displacement curves consistently demonstrate that on a weight basis the choleragenoid molecule is slightly more effective than native cholera toxin. Both proteins can displace more than 90% of the binding of ^{125}I-labeled cholera toxin if these proteins are added to the membranes before introduction of the iodotoxin. Very similar displacement curves to those described in Figure 1 are obtained if intact fat cells are used instead of liver membranes; in both cases choleragenoid is slightly more effective than cholera toxin. Considering that choleragenoid is of slightly lower molecular weight than cholera toxin (Lo-Spalluto and Finkelstein, 1972), these data suggest that the two proteins are binding with nearly the same affinity to identical sites on the cell membrane.

The data described in Figure 1 do not exclude the possibility that choleragenoid is inhibiting the binding of ^{125}I-labeled cholera toxin by directly combining with or inac-

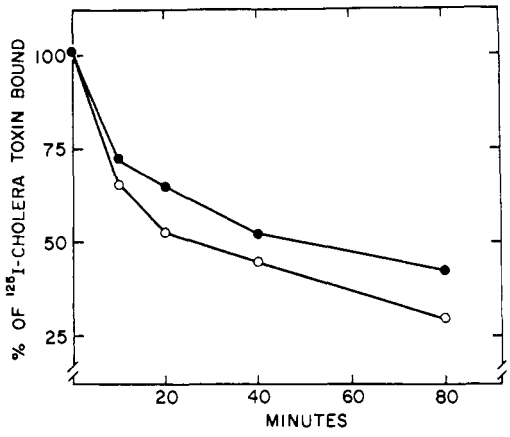

FIGURE 2: Effect of native cholera toxin (●) and choleragenoid (○) on the rate of dissociation of the ^{125}I-labeled cholera toxin–liver membrane complex. Membranes (250 μg of protein/ml) were incubated for 20 min at 24° with ^{125}I-labeled cholera toxin (4×10^5 cpm/ml) in Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin. Choleragenoid or cholera toxin (10 μg/ml) was added to the membrane suspensions (zero time) and the incubations were continued at 24° for the indicated time intervals.

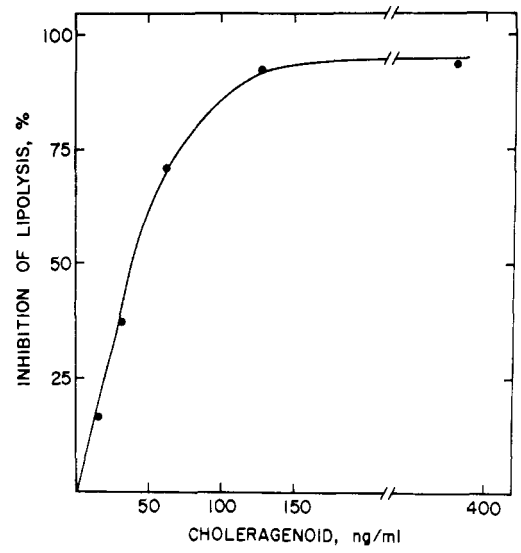

FIGURE 3: Inhibition of cholera toxin stimulated lipolysis in isolated fat cells by various concentrations of choleragenoid. Isolated fat cells (8×10^4 cells/ml) were incubated for 10 min at 24° in Krebs–Ringer–bicarbonate buffer containing 3% (w/v) albumin and the indicated concentration of choleragenoid. Cholera toxin (0.2 μg/ml) was added to all the samples and the cells were incubated at 37° for 150 min.

tivating the toxin molecule rather than by occupying its binding site on the cell membrane. For this reason experiments were performed in which the binding of ^{125}I-labeled cholera toxin to membranes was studied after thoroughly washing the membranes which had been previously incubated with the native proteins (Table I). In these conditions both proteins effectively inhibit binding, and choleragenoid is again at least as effective as cholera toxin. These experiments, which emphasize again the very tight nature of the interaction being studied, indicate that choleragenoid inhibits binding of cholera toxin by interacting directly with the membrane and that the affinity of this interaction is very similar or identical for both proteins.

Effect of Choleragenoid on the Rate of Dissociation of the Toxin–Membrane Complex. It has previously been demonstrated that the initial cholera toxin–membrane interaction is reversible (Cuatrecasas, 1973b,c). This presented an opportunity to test in a different way the possibility that choleragenoid and cholera toxin bind to the same membrane site by essentially the same mechanism. The rate of dissociation of the ^{125}I-labeled cholera toxin–membrane complex is very similar whether native cholera toxin or choleragenoid is used to study this process of dissociation (Figure 2). Since under these conditions native cholera toxin acts directly by binding to the free receptors, thus preventing reassociation of the ^{125}I-labeled toxin which spontaneously dissociates from the complex, the similarity of the two dissociation curves strongly suggests that the process of receptor association of choleragenoid is very similar to that of cholera toxin. Furthermore, these data also indicate that choleragenoid does not inhibit cholera toxin binding by actively disrupting the toxin–receptor complex or grossly increasing the rate of dissociation of the complex. By all criteria studied choleragenoid and cholera toxin appear to bind to the same membrane site with essentially identical properties.

Effect of Choleragenoid on Cholera Toxin Induced Lipolysis. In agreement with the report of Vaughn *et al.* (1970), choleragenoid (1 ng to 10 μg/ml) under a variety of conditions of incubation could not be demonstrated to have lipolytic activity on isolated fat cells. Despite the apparent ability of choleragenoid to bind very strongly to the cholera toxin receptor, this interaction does not lead to activation of the biological response.

In harmony with the ability of choleragenoid to effectively inhibit the binding of cholera toxin to intact cells or membranes, choleragenoid is very potent in inhibiting the lipolytic response of fat cells to cholera toxin (Figure 3). The lipolytic activity of 0.2 μg/ml of cholera toxin is completely inhibited by prior incubation of the cells with concentrations of choleragenoid greater than 0.2 μg/ml. The concentration of choleragenoid required to achieve 50% inhibition of lipolysis is about 40 ng/ml (7×10^{-10} M), which is very near the dissociation constant which has been estimated (Cuatrecasas, 1973a) for the initial cholera toxin–fat cell complex.[1] These results, which generally reinforce the earlier evidence (Cuatrecasas, 1973a–c) that the techniques used to study the ^{125}I-labeled cholera toxin–cell interaction measure biologically significant receptor interactions, indicate that choleragenoid can block the action of cholera toxin by competitively occupying the same receptor and by forming an inactive complex with it.

In the experiments just described (Figure 3) the fat cells were incubated with choleragenoid before addition of cholera toxin. It was important to determine whether choleragenoid could reverse the lipolytic effects of the toxin if it were added to the cells after the toxin. Choleragenoid produces virtually no inhibition of lipolysis if it is added after the fat cells have been exposed for 15 min at 24° to cholera toxin (Figure 4). The lipolytic response under these conditions is completely blocked if the choleragenoid is added before to toxin. Some inhibition (about 30%) of toxin-induced lipolysis can be detected if choleragenoid is added 5 min after addition of cholera toxin under conditions similar to those described in Figure 4. These results are quite consistent with the very high affinity of the interactions being studied and with the identity of mem-

[1] As discussed elsewhere (Cuatrecasas, 1973a,b), the data from Figure 1 cannot be used to accurately estimate dissociation constants since in the experiments which utilize liver membranes, unlike those with fat cells, there is nearly complete binding of the toxin present in the medium.

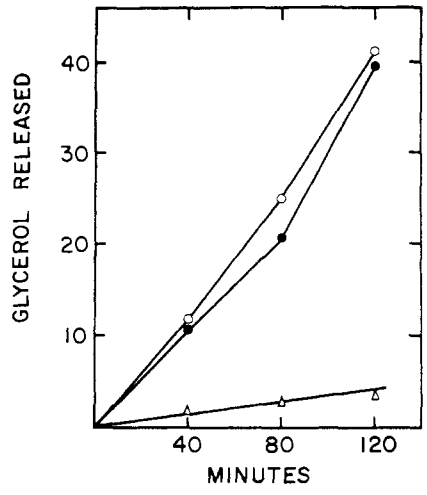

FIGURE 4: Inability of choleragenoid to reverse the lipolytic effect of cholera toxin when fat cells are preincubated with the toxin. Fat cells (about 5×10^5 cells/ml) were incubtaed in Krebs–Ringer–bicarbonate buffer containing 3% (w/v) albumin for 15 min at 24° with 1 μg/ml of cholera toxin (●, ○), or with 1 μg/ml of choleragenoid (△). The cells were washed thoroughly with the same buffer and incubated for 60 min at 37° in the absence of other additions (○) or in the presence of 1 μg/ml of choleragenoid (●) or cholera toxin (△). The cells were washed again and the lipolytic response was studied by incubating at 37° for the indicated time periods. Lipolysis is presented as micromoles of glycerol released per millimole of triglyceride. The lipolytic response of cells processed by the same procedures but in the absence of choleratoxin or choleragenoid was nearly the same as that described by △.

brane receptors for choleragenoid and cholera toxin. Despite the apparent ability of choleragenoid to permit dissociation of the toxin–membrane complex (Figure 2), it is not surprising that during a prolonged incubation at 37° biologically significant dissociation does not appear to occur (Figure 4) since it is known that at this temperature the toxin–membrane interaction becomes markedly stronger as the period of incubation is increased (Cuatrecases, 1973c).

Binding of ^{125}I-Labeled Choleragenoid and the Effect of Gangliosides. It was of interest to determine if the interaction of choleragenoid with cell membranes resembles that of cholera toxin (Cuatrecasas, 1973b) with respect to the ability of gangliosides to inhibit binding. Preincubation of ^{125}I-labeled choleragenoid with crude brain gangliosides or with ganglioside G_{M1} completely abolishes the binding of the iodoprotein to liver membranes and to fat cells in a manner very similar to that described earlier (Cuatrecasas, 1973b) for ^{125}I-labeled cholera toxin. Equally important, incubation of membranes with gangliosides followed by thorough washing of the membranes to remove the gangliosides in the medium results in a very substantial increase in the binding of ^{125}I-labeled choleragenoid to the membranes (Table II). In analogy with the results obtained with ^{125}I-labeled cholera toxin (Cuatrecasas, 1973b), the increase in ^{125}I-labeled choleragenoid binding is most pronounced if the concentration of the iodoprotein is high (Table II), indicating that ganglioside treatment of membranes increase the total number of binding sites for this protein. The binding of ^{125}I-labeled choleragenoid to membranes is inhibited by native cholera toxin and by choleragenoid in a way nearly identical with the inhibition which these proteins exhibit on ^{125}I-labeled cholera toxin binding (Figure 1). These results illustrate further the striking similarities in the properties of binding of these two proteins to what is probably the same receptor.

TABLE II: Effect of Preincubating Liver Membranes with Gangliosides on the Enhancement of Binding of ^{125}I-Labeled Choleragenoid.[a]

^{125}I-Labeled Choleragenoid Added (cpm)	Binding of ^{125}I-Labeled Choleragenoid (cpm)	
	Native Membranes	Ganglioside Membranes
3.6×10^4	7,760	11,800
1.4×10^5	17,800	38,400
7.2×10^5	50,000	199,000

[a] Liver membranes (1.4 mg of protein/ml) were incubated for 60 min at 24° in Krebs–Ringer–bicarbonate buffer, 0.1% (w/v) albumin, in the absence and presence of 0.1 mg/ml of crude bovine brain gangliosides. The membrane suspensions were then diluted tenfold with ice-cold buffer and centrifuged at 36,000*g* for 15 min, and the pellets were suspended in a small volume of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin. The membranes were homogenized (Polytron) for 5 sec and diluted to a final membrane protein concentration of 0.14 mg/ml. Samples (0.2 ml) of these suspensions were incubated with various concentrations of ^{125}I-labeled choleragenoid (16 μCi/μg) for 15 min at 24°.

Discussion

The present studies indicate that choleragenoid, a protein which is derived from the cholera toxin molecule and which is immunologically identical with it (Finkelstein and LoSpalluto, 1969, 1970; LoSpalluto and Finkelstein, 1972; Finkelstein *et al.*, 1971b), interacts with the cholera toxin receptor of cell membranes in a manner which is nearly indistinguishable from and perhaps identical with that of the toxin. The displacement of binding of ^{125}I-labeled cholera toxin or of ^{125}I-labeled choleragenoid to membranes by cholera toxin and by choleragenoid is nearly identical. Choleragenoid does not interact directly with the toxin and it does not simply perturb the membrane in a way which causes dissociation of the toxin–membrane complex. Choleragenoid, also like cholera toxin (Cuatrecasas, 1973b), appears to bind selectively to membrane gangliosides.

Despite the ability of choleragenoid to bind very effectively to the same receptors which are occupied by cholera toxin, the product of this interaction does not lead to a detectable biological response in fat cells or in intact gastrointestinal preparations (Finkelstein and LoSpalluto, 1969, 1970). These conditions form the basis for the very potent inhibitory properties of choleragenoid on the metabolic effects of cholera toxin.[2] Choleragenoid is thus a unique biologically inactive structural analog of cholera toxin which acts as an inhibitor of the toxin by virtue of competition with the toxin for binding to the receptor.

Because of the extraordinarily high affinity of these proteins for the same receptor, and because the strength of binding of the toxin molecule increases with increasing length and temperature of incubation (Cuatrecasas, 1973b), the net effect in a biological system depends to a critical degree on which protein is exposed to the cells first. Thus, if cholera toxin is added

[2] The inhibitory effect of choleragenoid is quite specific since epinephrine-induced lipolysis is not modified by preincubating fat cells with choleragenoid for 100 min at 37°.

to the fat cells 15 min before choleragenoid almost no inhibition of lipolysis is detectable, while reversal of this order of addition results in nearly complete inhibition (Figure 4). The inability of choleragenoid to reverse the metabolic effects of cholera toxin once the toxin has been incubated with the cells for more than 15 min resembles the inability of gangliosides to reverse the lipolytic activity of the toxin under similar conditions (Cuatrecasas, 1973b). Although choleragenoid and gangliosides both exhibit exceptional toxin-inactivating properties which are based on an ability to inactivate the receptor or the toxin, respectively, both of these can only act on the free toxin or the free receptor and they are both without effect on the toxin–membrane complex. Since the tenacious nature of the cholera toxin–cell interaction increases progressively during the course of the incubation (Cuatrecasas, 1973c), it is not surprising that choleragenoid and gangliosides are both ineffective in reversing the effects of the toxin once these are manifest.

Studies on the nature of the delay in the onset of action (lag period) of cholera toxin have led to the hypothesis that the initial toxin–receptor complex in the cell membrane is an inactive form of the complex (Cuatrecasas, 1973c). It has been suggested that this inactive complex may be transformed into a form which is active by a time- and temperature-dependent transition which may involve special structural reorientations or reorganizations of the complex within the plane of the membrane. Within this framework it is possible to postulate that choleragenoid may be capable of forming essentially the same type of inactive receptor complex which is initially formed by cholera toxin, but that the presumably small alteration in the structure of the choleragenoid molecule does not permit the complex to undergo the subsequent molecular rearrangements which are required to transform the complex into an active form. These possibilities are currently being tested by special microscopic techniques which allow direct visualization of the cholera toxin macromolecules on the surface and inner plane of the cell membrane.[3]

Acknowledgment

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[3] In collaboration with Dr. Vincent T. Marchesi.