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Cholera Toxin B-Subunit Protects Mammalian Cells From Ricin and Abrin Toxicity

Carlo Delfini, Massimo Sargiacomo, Carla Amici, Goffredo Oberholtzer, and Maurizio Tomasi

Laboratorio di Biologia Cellulare, Istituto Superiore di Santità, Viale Regina, Elena 299, 00161 Rome, Italy

The glycoproteins ricin and abrin intoxicate cells by inhibiting protein synthesis. Pretreatment of HeLa cells with cholera toxin partially protects them from ricin and abrin activity. The involvement in this phenomenon of the various effects of cholera toxin, namely, redistribution of membrane receptors elicited from protomer B and increasing cyclic AMP concentrations induced by protomer A, were studied. Substances able to enhance cyclic AMP concentrations do not affect ricin and abrin activity, while protomer B alone protects cells. In addition, the effects of several lectins on ricin or abrin toxicity were examined. Almost complete prevention of ricin or abrin activity was obtained using concanavalin A (Con A) and wheat germ agglutinin (WGA). Conversely, neither succinyl Con A nor *Ulex europeus* agglutinin (UEA) affected the cellular response. Both protomer B of cholera toxin and Con A did not alter the binding of ricin or abrin; they seem to protect cells by altering membrane structure.

Key words: cholera toxin, abrin, ricin, inhibition of protein synthesis, protection effect, receptor redistribution

In recent years several proteins interacting with the carbohydrate moieties of cell surface glycoproteins and/or glycolipids have been isolated [1,2]. In a number of cases the receptor–ligand interaction induces a biologic response such as mitosis [1–5]. Although the mechanisms whereby the binding of lectins to cell surface receptors initiate the biologic effects are still unknown, multiple contacts between ligand and receptor as well as ligand-induced receptor clustering have been proposed to explain the complexity of the results [6]. In other instances, the binding is the first step of an intoxication process wherein the active protein crosses the membrane and reaches a cytoplasmic target [7]. The two-step mechanism seems to require the coupling in the same toxic molecule of two functional regions, ie, one binding region (B) joined throughout a disulfide bridge to another region possessing the toxic activity (A).

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Ricin and abrin are glycoproteins which act through this mechanism. They are composed of a subunit B (MW 35,000) which binds the toxin to galactose-containing receptors of the plasma membrane, and subunit A (MW 32,000) which blocks protein synthesis because of modification of the 60 S ribosomal subunit [7]. The two subunits are linked by a disulfide bridge [7]. In the case of cholera toxin, the binding region (protomer B) is formed by five identical β-subunits (MW 11,400), each of which specifically recognizes the oligosaccharide moiety of ganglioside G_{M1} [8]. The isolated multivalent protomer B produces redistribution of cell surface receptors with formation of patching and capping [9–11]. The binding region bears the active protein α (MW 21,000) which is joined through a disulfide bridge to polypepetide γ, and α-γ together form the protomer A. Reduction by the reagents of the disulfide bridge between α and γ splits cholera toxin into the active region α and binding region γ-β_5 [12]. The active α-region irreversibly modifies the guanosine triphosphate (GTP) regulatory protein of adenylate cyclase system and, thus, the catalytic component becomes permanently activated, producing high concentrations of cyclic AMP [13].

Previous studies examined whether ricin and cholera toxin could be competitive: Cholera toxin protects cells from ricin toxicity but ricin is unable to change the action of cholera toxin [14]. Similarly, the activity of other toxins as diphtheria toxin, ricin, and abrin can be inhibited by pretreatment of cell cultures with concanavalin A (Con A) [15,16]. Furthermore, the Con A-induced inhibition of ricin and abrin activity appears to interfere with the uptake of ricin and abrin into cells [16].

The aim of the present work was to establish to which functional region of cholera toxin (binding or enzymatic) could be attributed the protective effect on HeLa cells. The results presented here suggest that the protective effect is due to protomer B of cholera toxin, and appears to be related to its ability to cross-link receptors.

MATERIALS AND METHODS

Concanavalin A was purchased from Miles Laboratories (Elkhart, Indiana); wheat germ agglutinin (WGA) from Miles-Yeda (Rehovot, Israel); *Ulex europeus* agglutinin I (UEA) and succinylated concanavalin A (Suc Con A) from Vector Laboratories (Burlingame, California); epinephrine, oxytocin, vasopressin and butyryl-cAMP were obtained from Sigma Chemical Co. (St. Louis, Missouri); theophylline from Merck (Darmstadt, West Germany); and insulin from Eli Lilly (Firenze, Italy); L [4,5-^{3}H] leucine (182 Ci/mmole) and sodium [^{125}I] iodide (13–17 mCi/μgI) were purchased from Radiochemical Centre (Amersham, England).

The B protomer of cholera toxin (B) and cholera toxin were a generous gift from Dr. Tayot, Institute Merieux, France and were purified as described previously [17]. Ricin and abrin were prepared according to Nicolson and Blaustein [18].

HeLa cells were maintained in monolayer in Minimum Eagle's Medium (MEM) containing 10% fetal calf serum, as described by Benedetto et al. [19]. Unless otherwise indicated, all operations were performed at 37°C.

Cells were grown in 24-well tissue culture Linbro plates seeded with 3×10^5 cells per well and reaching confluence after 24 hr. Confluent monolayers were washed three times and incubated for 1 hr with serum and leucine-free MEM containing the agents indicated. After three washings, the cells were treated for 3 hr with the toxin

(ricin or abrin) at the concentrations shown. To measure protein synthesis, washed monolayers were exposed to tritiated leucine (0.2 μCi/ml) for 1 hr, then rinsed five times with 0.9 NaCl and lysed with 2% sodium dodecyl sulfate (SDS)–0.1M NaOH: the extract was precipitated with 10% trichloroacetic acid (TCA), collected on 0.45-μm Millipore filters and, after addition of 10 ml of Bray's solution, counted for radioactivity in Ultrobeta LKB 1210.

Toxins were iodinated by the Enzymobead Radioiodination Reagent (Bio-Rad Laboratories, Richmond, California) procedure; iodinated toxins were purified by Sephadex G-25 superfine column chromatography; the column was equilibrated and eluted with 0.05 M potassium phosphate buffer, pH 7.8; the labeled toxin recovered in the excluded volume was dialyzed at 4°C against the same buffer (four changes) to remove the last traces of free iodine.

RESULTS

It is well established that the inhibition of protein synthesis by ricin in HeLa cells is time, temperature, and toxin concentration dependent [20,21]. Thus, first we defined the conditions of incubation that would produce a rate of inhibition of protein synthesis by ricin and abrin that agents, such as cholera toxin, would be able to prevent.

Figure 1 shows that 60% inhibition of protein synthesis was obtained with exposure of HeLa cells to 0.05 μg/ml ricin or 0.005 μg/ml abrin for 3 hr at 37°C.

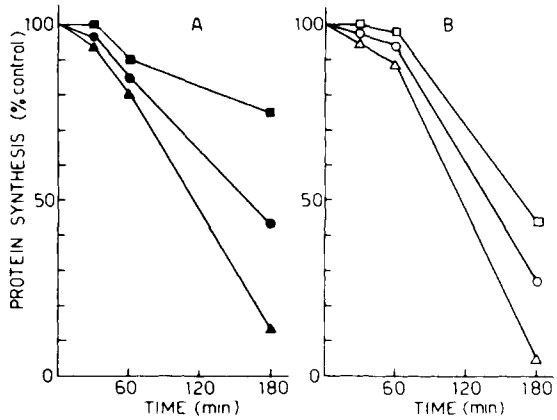

Fig. 1. Inhibition of protein synthesis induced by ricin (A) and abrin (B). Cells were treated with 0.5 ml ricin (■-■-■, 0.01 μg/ml; ●-●-●, 0.05 μg/ml; ▲-▲-▲, 0.10 μg/ml) and abrin (□-□-□, 0.005 μg/ml; ○-○-○, 0.010 μg/ml; △-△-△, 0.050 μg/ml). At the indicated times, monolayers were pulsed for 1 hr with 0.5 ml medium containing 0.2 μCi/ml of tritiated leucine and washed as described in Materials and Methods. Then cells were dissolved in sodium dodecyl sulfate (SDS)-NaOH and protein content was determined by Lowry method. 10^6 cells contained 295 ± 15 μg of protein. Protein synthesis values are given as the percentage of incorporated leucine referenced to control. Control exhibited 55,500 cpm/10^6 cells ± 4,500. Data represent the mean of at least five experiments.

Figure 2 shows that under cholera toxin treatment, 0.05 μg/ml of ricin inhibits the protein synthesis by 20% instead of by 60% as in untreated cells.

The addition of cholera toxin to HeLa cells induces a slight enhancement (2- to 2.5-fold) of cyclic AMP levels. In cell lines such as Friend erythroleukemia, cholera toxin produced a more extensive increase of cyclic AMP [11], but HeLa cells possess very few receptors of cholera toxin [22], so that a small number of molecules of cholera toxin bound to the cell surface may explain the low increase in cyclic AMP. Hence, the question of whether increased cyclic AMP is responsible for the increased resistance of HeLa cells to ricin and abrin toxicity was examined on cells treated with the substances listed in Table I, all of which modulate cyclic AMP levels [23]. A direct observation of the activity of cholera toxin α-subunit is not possible because of the inability of the α-subunit alone to produce an effect on intact cells [13].

As depicted in Table I, none of the substances tested prevented the inhibition of protein synthesis in ricin- or abrin-treated cells; extensive variations of cyclic AMP concentration caused by the substances employed were observed (the values are reported in Table I).

Insulin, theophylline, epinephrine, oxytocin, and vasopressin act reversibly on the adenylate cyclase system [23], while cholera toxin permanently activates this system [13]. Thus, the possibility that the interaction of cells with ricin and abrin could abolish the hormonal effects might exist. To further document this point, butyryl cyclic AMP was used. This substance enters the cells and maintains a high concentration of cyclic AMP for long periods but was ineffective in preventing ricin or abrin action.

These data seem to exclude the involvement of a hormone-like activity of cholera toxin [24] in its protective effect, and they rather suggest that protomer B, which has lectin-like activity [25], plays a role in this process. Experiments performed with pure protomer B were designed to exclude or confirm this hypothesis.

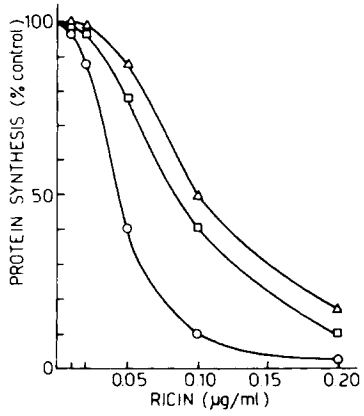

Fig. 2. Protection effect of cholera toxin and its protomer B of ricin induced inhibition of protein synthesis. Cells were preincubated for 1 hr with 2 μg/ml of cholera toxin (△-△-△), 2 μg/ml of protomer B (□-□-□), serum and leucine-free MEM (○-○-○), then washed and treated with different concentrations of ricin for 3 hr. Protein synthesis was carried out as described in Materials and Methods and calculated as in legend to Fig. 1.

Figure 2 shows the pattern of the rate of protein synthesis when the cells were incubated with 2 μg/ml of protomer B and then treated with ricin or abrin. It is evident that protomer B prevents the inhibitory effects of protein synthesis of ricin and abrin but with an efficacy slightly lower than intact cholera toxin. However, the thresholds of protective effect elicited by protomer B and cholera toxin on HeLa cells were similar and were reached with 2 μg/ml and 20 min of preincubation (data not shown).

Previous studies have shown that the interaction of protomer B with cell membranes induces redistribution of membrane receptors [9–11], as do several multivalents lectins [6]. Thus, by using the lectins listed in Table II, we examined, on HeLa cells, the relationship between the clustering or redistribution of membrane receptors and the induced resistance to the ability of abrin to inhibit protein synthesis.

TABLE I. Effects of Some Substances Changing the c-AMP Level on Ricin-Induced Inhibition of Protein Synthesis*

Substances	Concentration	c-AMP (pmoles/10^6 cells)	Time of preincubation (hr)	Protein synthesis (%)
None	—	4.8 ± 6	—	40.0
Insulin	1 -100 μg/ml	2.7 ± 5	1	37.3
Theophylline	1 mM	18.9 ± 2.2	0.75	42.0
Epinephrine	0.1- 10 μg/ml	20.3 ± 1.8	1	44.0
Oxytocin	1 -100 μg/ml	29.3 ± 2.3	1	37.3
Vasopressin	1 -100 μg/ml	23.8 ± 1.9	1	36.3
Butyryl-c-AMP	0.2- 1.0 mM	101.5 ± 4.0	14	35.7

*Cells were preincubated with the indicated substances and treated with 0.05 μg/ml of ricin for 3 hr. Protein synthesis was carried out as described in Materials and Methods and calculated as in legend to Fig. 1. Protein synthesis values are the means of replicate determinations. No individual determination exceeded a 10% deviation from the mean.

TABLE II. Effect of Some Lectins on the Inhibition of Protein Synthesis Induced by Ricin and Abrin*

	Protein synthesis (%)		
	No treatment	Treatment with ricin	Treatment with abrin
None	100.0	42.4	43.5
CLT (2 μg/ml)	91.6	86.0	84.2
B (2 μg/ml)	107.9	83.4	81.3
Con A (10 μg/ml)	97.8	83.8	84.5
Con A (50 μg/ml)	101.3	90.5	86.2
WGA (10 μg/ml)	85.7	84.3	92.5
WGA (50 μg/ml)	102.8	87.6	93.8
Suc Con A (10 μg/ml)	84.4	43.3	45.6
Suc Con A (50 μg/ml)	83.8	48.5	47.2
UEA (10 μg/ml)	82.1	41.0	40.7
UEA (50 μg/ml)	78.5	43.5	44.2

*Cells were preincubated for 1 hr with the indicated lectins, then treated with ricin (0.05 μg/ml) or abrin (0.005 μg/ml) for 3 hr. Protein synthesis was carried out as described in Materials and Methods and calculated as in legend to Fig. 1. The values are the means of replicate determinations. No individual exceeded a 10% deviation from the mean.

As shown in Table II, pretreatment of HeLa cells with Con A and WGA induces an almost complete resistance to ricin and abrin action in the cells. Figure 3 depicts the concentration and time dependence of the Con A protective effect. In contrast, neither UEA nor Suc Con A were able to prevent toxin action. It should be pointed out that the dimeric derivative of Con A (Suc Con A), despite having the same sugar-binding specificity as Con A, is unable to cross-link membrane proteins. These data are consistent with the interpretation that the protective effect of Con A is not due to a competition between this lectin and ricin or abrin for similar cell surface receptors.

In order to further document this hypothesis, we studied the effects of lectin treatment on the binding of ricin and abrin to cells. As Figure 4 shows, intact cholera toxin, protomer B, and Con A do not change the number of ricin molecules bound to HeLa cells. In contrast, WGA causes a 30–35% decrease of associated radioactivity. Similar results were obtained with abrin. We point out that the ricin concentration used for binding were the same as used in the protein synthesis experiments.

DISCUSSION

As cholera toxin induces ricin resistance on HeLa cells [14], we investigated whether the protective effect underlies a membrane structure modification or a metabolic change elicited by high cyclic AMP levels. The results show that protomer B of cholera toxin is able to protect HeLa cells to similar extent as intact cholera toxin. Thus, protomer B appears to play an essential role in the protective effect elicited by cholera toxin.

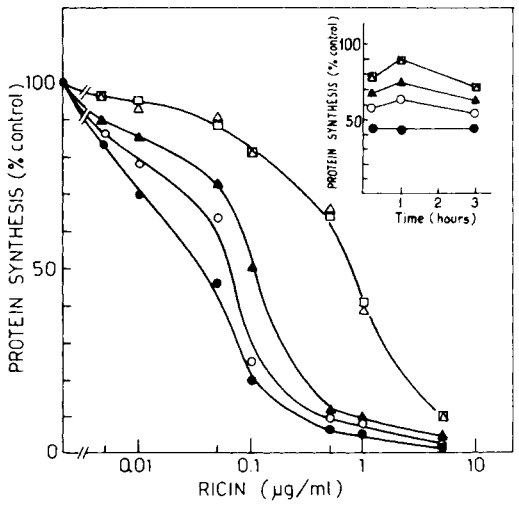

Fig. 3. Effect of Con A on ricin-induced inhibition of protein synthesis. Monolayers were incubated with increasing amounts of Con A in serum and leucine-free MEM and, after washing, were treated with ricin for 3 hr. Protein synthesis was carried out as described in Materials and Methods and calculated as in legend to Fig. 1. Effect of 1 hr incubation of the various amounts of Con A on protein synthesis induced by the indicated concentrations of the ricin. Inset shows the incubation time dependence of the various amounts of Con A on inhibition of protein synthesis induced by 0.05 μg/ml ricin (●-●-●, no Con A; ○-○-○, 1 μg/ml; ▲-▲-▲, 10 μg/ml; △-△-△, 50 μg/ml; □-□-□, 100 μg/ml).

This interpretation is further supported by the exclusion of protomer A activity in this phenomenon. Protomer A enhances cyclic AMP concentration by modifying the adenylate system, and the data presented have demonstrated that enhancement of cyclic AMP concentration does not induce any protective effect.

A comparison of the magnitude of the protective effect exhibited by cholera toxin and its protomer B reveals that subunit B is not as efficient as intact molecules,

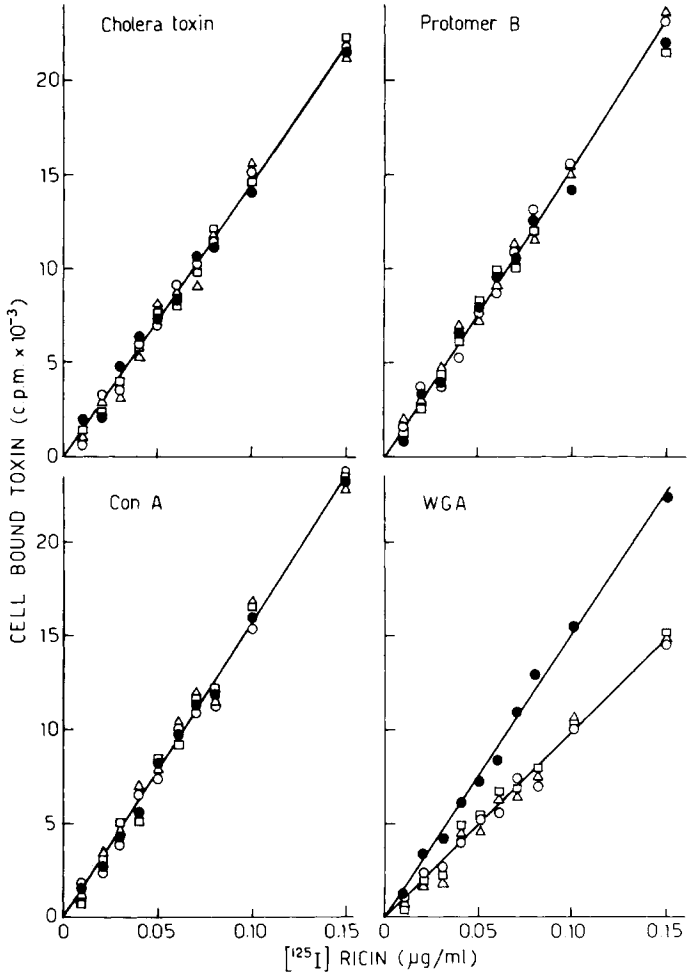

Fig. 4. Effect of preincubation with different lectins on the cellular binding of [^{125}I]-labeled toxin. Confluent monolayers were preincubated at 37°C for 1 hr with 0.5 ml of various concentrations of cholera toxin, protomer B, Con A, and WGA. Symbols for cholera toxin and protomer B are: ●-●-●, 0; ○-○-○, 0.5; △-△-△, 2.0; □-□-□, 10 μg/ml. Symbols for Con A and WGA are: ●-●-●, 0; ○-○-○, 1.0; △-△-△, 50; □-□-□, 100 μg/ml. After three washings cells were chilled in ice bath and incubated with 0.2 ml of phosphate-buffered saline (PBS) containing different amounts of labeled ricin (sp. act. 12 × 10^6 cpm/μg). Controls were performed by following the above procedure but adding 0.1 M lactose. After 10 min, cells were washed five times with cold 0.9% NaCl and lysed with 0.5 ml of 1 M NaOH at 95°C. The radioactivity was measured in Ultrogamma LKB 1280.

even by employing molar concentration of protomer B 20-fold higher than cholera toxin. Thus, in this event, protomer A might play some minor role.

Friedman and Kohn [26] showed that cholera toxin inhibits the ability of interferon to produce an antiviral state in cells. Belardelli et al [11] demonstrated that protomer B of cholera toxin is responsible for the inhibition of interferon action in Friend erythroleukemia cells. These authors concluded that the effect of protomer B is due to an induction of redistribution of membrane receptors.

These findings together support the hypothesis that a biochemical and morphologic alteration of plasma membranes, caused by cholera toxin, impairs the entry into HeLa cells of active molecules of ricin or abrin. The observations are difficult to reconcile with the accepted idea that cholera toxin interacts solely with ganglioside G_{M1}. However, the data rather suggest that the protective effect of cholera toxin seems to require the involvement of membrane proteins. A direct attachment of cholera toxin to glycoproteins in brush border cells has been described [27]. But other studies reported the exclusive role of ganglioside in cholera toxin interaction with brush border cells [28]. Nevertheless, indirect involvement of glycoproteins may be hypothesized. Membrane gangliosides have a high mobility [29]. This probably allows them to interact with a wide variety of membrane proteins. It is possible that the interaction of cholera toxin with ganglioside can, by secondary interaction of the latter with membrane glycoproteins, cause an effect similar to the direct cross-linking of glycoproteins. In other words, the oligosaccharide fraction of gangliosides may function as a mobile carbohydrate moiety of a glycolipid-protein receptor complex.

In order to shed more light on the above hypothesis, we investigated the effect on ricin and abrin activity of other multivalent lectins. It is known that Con A binds specifically to glycoprotein receptors on mammalian cells [2] producing several effects. These include patching and capping of receptors [6,30], morphologic alteration of the cells [31], and the formation of cytoplasmic vesicles and vacuoles [32]. The results presented here show that Con A, once bound to HeLa cells, prevents the blocking of protein synthesis yielded by ricin and abrin almost completely. The protective effect of Con A is not related to a change in ricin or abrin binding but appears to be due to a morphologic alteration of the cell surface.

These data parallel the findings of Middlebrook [15], who reported that Con A is able to block diphtheria toxin activity on Vero cells. Since the monovalent derivative, Suc Con A, does not change the pattern of inhibition of protein synthesis shown by ricin- and abrin-treated cells, we conclude that the tetravalent binding structure of unmodified Con A is the stringent requirement for cell protection. Although the coincidence of effects caused by cholera toxin and Con A might be fortuitous, both proteins appear to work on plasma membranes by similar processes. The findings strengthen the hypothesis that membrane proteins mediate the protective effect of cholera toxin.

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REFERENCES

1. Sharon N: In Marcus A (ed): "Lectins in Higher Plants in Biochemistry of Plants," Vol. 6. New York: Academic Press, 1981, pp 371–402.
2. Goldstein IJ, Hayes CE: Adv Carbohydr Chem Biochem 35:127, 1978.
3. Nicolson GL: Biochim Biophys Acta 458:1, 1976.
4. Ravid A, Novogrodsky A, Wilchek M: Eur J Immunol 8:289, 1978.
5. Fraser AR, Hemperly JJ, Wang JL, Edelman GM: Proc Natl Acad Sci USA 73:790, 1976.
6. Schreiner GF, Unanue ER: Adv Immunol 24:37, 1976.
7. Olsnes S, Pihl A: In Cuatrecasas P (ed): "The Specificity and Action of Animal, Bacterial and Plant Toxin." London: Chapman and Hall, 1976, pp 129–173.
8. Brady RO, Fishman PH: Adv Enzymol 50:303, 1979.
9. Craig SW, Cuatrecasas P: Proc Natl Acad Sci USA 72:3844, 1975.
10. Hansson HA, Holmgren J, Svennerholm L: Proc Natl Acad Sci USA 74:3782, 1977.
11. Belardelli F, Ausiello C, Tomasi M, Rossi GB: Virology 107:109, 1980.
12. Tomasi M, Battistini A, Araco A, Roda LG, D'Agnolo G: Eur J Biochem 93:621, 1979.
13. Gill DM: In Greengard P, Robison GA (eds): "Adv Cycl Nucl Res," Vol. 8. New York: Raven Press, 1977, pp 85–118.
14. Tomasi M, Delfini C, Sargiacomo M, Cardelli M, Oberholtzer G, D'Agnolo G: In Eaker D, Wadstrom T (eds): "Natural Toxins." Oxford: Pergamon Press, 1980, pp 457–462.
15. Middlebrook JL, Dorland RB, Leppla SH: Exp Cell Res 121:95, 1979.
16. Delfini C, Amici C, Belardelli F, Oberholtzer G, Sorrentino M: Exp Cell Res 142:427,1982.
17. Tayot JL, Holmgren J, Svennerholm L, Lindblad M, Tardy M: Eur J Biochem 113:249, 1981.
18. Nicolson GL, Blaustein J: Biochim Biophys Acta 266:543, 1972.
19. Benedetto A, Delfini C, Puledda S, Sebastiani A: Biochim Biophys Acta 287:330, 1972.
20. Sandvig K, Olsnes S: Exp Cell Res 121:15, 1979.
21. Olsnes S, Sandvig K, Refnes K, Pihl A: J Biol Chem 251:3985, 1976.
22. Fishman PH, Atikkan EE: J Biol Chem 254:4342, 1979.
23. Robinson GA, Butcher RW, Sutherland EW: "Cyclic AMP." New York and London: Academic Press, 1971.
24. Kohn LD: In Cuatrecasas P, Greaves MF (eds): "Receptors and Recognition," Series A, Vol. 5. London: Chapman and Hall, 1978, pp 133–212.
25. Richards RL, Moss J, Alvin CR, Fishman PH, Brady RO: Proc Natl Acad Sci USA 76:1673, 1979.
26. Friedman RM, Kohn LD: Biochem Biophys Res Commun 70:1078, 1976.
27. Morita A, Tsao D, Kim YS: J Biol Chem 255:2549, 1980.
28. Critchley DR, Magnani JL, Fishman PH: J Biol Chem 256:8724, 1981.
29. Lee PM, Ketis NV, Barber KR, Grant CWM: Biochim Biophys Acta 601:302, 1980.
30. Edelman GM: Science 192:218, 1976.
31. Petty HR: Exp Cell Res 128:439, 1980.
32. Edelson PJ, Cohn ZA: J Exp Med 140:1364, 1974.