A conjugate of the A1 peptide of cholera toxin and the lectin of *Wisteria floribunda* that activates the adenylate cyclase of intact cells

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The active A1 peptide of cholera toxin was linked by a disulphide bond to the lectin of *Wisteria floribunda*. The resulting conjugate activated the adenylate cyclase of intact U937 or K562 cells at the same concentrations as native toxin did, but to a greater extent. Activation was inhibited by *N*-acetyl-D-galactosamine or by antisera to the lectin or peptide. The characteristic lag phase between addition of toxin to cells and activation of cyclase was not found with the conjugate or with free A1 peptide.

Cholera toxin *Adenylate cyclase* *Lectin* *Hybrid* *Conjugate*

1. INTRODUCTION

The active A1 peptide of cholera toxin (review [1]) is transported into the cell following interaction between the B subunits of the toxin and ganglioside GM1 in the outer membrane. It then catalyses the ADP-ribosylation of an intracellular component of the adenylate cyclase complex.

The active component of several other toxins (e.g., diphtheria toxin, ricin) has been introduced into cells by coupling it to a protein not the normal binding component of the toxin but also capable of binding to cells (see [2]). This paper shows that an artificial conjugate of the A1 peptide with the lectin of *Wisteria floribunda* (which binds to non-reducing a-linked *N*-acetyl-D-galactose residues on the outside of many cells [3,4]) could activate the adenylate cyclase of intact cells.

2. EXPERIMENTAL

A1 peptide (a kind gift from Dr J. Tayot of the Institut Merieux) was purified to homogeneity on Sephadex G-75 in 0.1 M glycine–HCl, 6.5 M urea, 5 mM dithiothreitol, pH 3.2 [5]. *W. floribunda* lectin was from E.Y. Laboratories, CA. Both proteins have free sulphydryl groups, and were hybridized by oxidation, essentially as in [6], and the concentration of the products determined from the intensity of staining in polyacrylamide gels.

Cells were kindly grown by Dr Veronica van Heyningen in a modified RPMI1640 medium (Gibco, Scotland). They were centrifuged at 200 × *g* for 3 min, resuspended in 0.1 M sodium phosphate, 0.15 M NaCl (pH 7.0), and 50-μl aliquots (about 10^6 cells) prepared. To these were added 5 μl of solution of toxin, conjugate of peptide; and the mixture was incubated at 20°C, normally for 2 h. One ml of the phosphate buffer was added and the tubes were agitated and centrifuged for 15 s in a Beckman microfuge. After washing the cells once, they were cooled to −70°C for lysis. The lysed cells were incubated with 40 μl assay medium (0.47 mM [2,8-^{3}H]ATP (1.5 MBq/ml, Amersham), 8.4 mM $MgCl_2$, 1.56 mM cyclic AMP, 1.56 mM dithiothreitol, 20 mM phosphocreatine, 1 mg/ml creatine kinase and 47 mM 3-(*N*-morpholino)propanesulphonic acid, adjusted if necessary to pH 8.8 with NaOH) for 45 min at 37°C, and the cyclic AMP produced assayed as in [7].

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3. RESULTS

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate under reducing conditions of the product of the oxidation of A1 peptide and lectin showed small residual amounts of these two proteins and of their dimers together with another protein (M_r ~54000), usually about 80% of the total protein and presumably the desired conjugate. A sample of this conjugate was eluted from a parallel gel and electrophoresed again in the presence of 2-mercaptoethanol: the two monomers were released. Furthermore, transfer of a similar gel by 'blotting' to nitrocellulose followed by treatment of the blot with antisera to each monomer [8] showed that the monomers reacted only with the specific antisera, but the conjugate reacted with both. These experiments showed that a hybrid protein had been prepared. Attempts to purify it were not successful, and the following experiments therefore use the unpurified solution.

3.1. *Activity*

Fig.1 shows the response of intact U937 cells (derived from a histiocytic lymphoma [9]) to various concentrations of toxin, conjugate, and free peptide. The conjugate activated the cyclase at a $\geqslant$100-fold lower concentration than the free peptide did (so that the activity cannot have been due to contaminating free peptide).

The activity of the conjugate (at 10^{-8} M) was inhibited almost totally by preincubation with antisera to the lectin, A1 peptide or toxin or with *N*-acetylgalactosamine (which binds to the lectin binding site [3,4]). Preincubating the cells with ganglioside GM1 did not increase the response to toxin or conjugate, but the action of toxin and conjugate (but not free peptide) was inhibited if these proteins were preincubated with ganglioside. Such inhibition is always found with native toxin [10] and is presumably also found with the conjugate because of the substituted *N*-acetylgalactosamine residue in the ganglioside.

A characteristic of the action of the toxin on whole cells is that there is a lag phase (whose duration varies from about 15 to 90 min depending on the type of cell) between the initial binding to the cell surface and the activation of adenylate cyclase. Fig.2 shows that this was true of the action of toxin on the U937 cells, but not of the conjugate or of the free peptide, which both showed no lag phase.

Similar experiments showed comparable activity of the conjugate with intact K562 cells ([11], derived from a myeloid leukaemia) although free peptide was inactive with these cells. However, neither the conjugate nor the peptide produced a significant response in an Epstein-Barr virus transformed B-cell line (MST), although these cells did respond weakly to toxin.

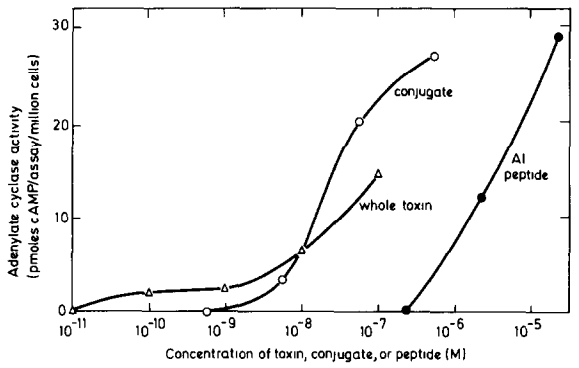

Fig.1. Dose response curves for the activation of adenylate cyclase in U937 cells. Cells were incubated with different concentrations of whole toxin (△), conjugate (○) and A1 peptide (●) and the intracellular activity of adenylate cyclase then measured as described in the text.

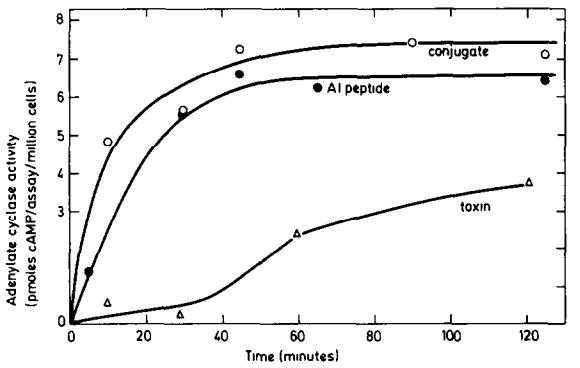

Fig.2. Time course of the activation of adenylate cyclase in U937 cells. Cells were incubated with whole toxin (△, 10^{-7} M), conjugate (○, 6×10^{-7} M) or A1 peptide (●, 2×10^{-5} M) for various times and the intracellular activity of adenylate cyclase was then measured as described in the text.

4. DISCUSSION

The hybrid protein between the A1 peptide of cholera toxin and the lectin of *W. floribunda* activates the adenylate cyclase of U937 cells more effectively than toxin does. This activation cannot be due to contaminating free peptide or native toxin, or to activity with lysed cells, since the hybrid is at least 100-times more active than the peptide even with U937 cells, and 20 μM peptide (the highest concentration achievable) was inactive with the K562 cells. Furthermore both antisera to the lectin and inhibitors of its binding (*N*-acetylgalactosamine) inhibited the action of the hybrid, showing that the lectin component played an essential role.

When native toxin interacts with a cell, the A1 peptide is delivered to the interior following the interaction of the B subunits with the ganglioside. When the conjugate interacts with a cell this delivery is mediated by the interaction of a different protein, presumably with a different receptor. This shows that the entry does not involve any specific interaction between the B subunits and ganglioside: rather, some sort of binding is needed, but it does not matter which. This supports the idea [10] that the entry of A1 may be a comparatively non-specific process involving random crossing of the hydrophobic membrane and argues against theories in which dissolving the B subunits in the membrane play a prominent part. Such theories are, in any event, made less likely by the observation [12] that A1 peptide, but not the B subunits, can dissolve in the membrane. If the main function of the binding of the B subunits to the ganglioside is simply to increase the local concentration of the toxin (and hence of the peptide) at the cell surface, then binding of any ligand to any receptor will do.

It is not clear from these experiments at what stage the disulphide bond linking the peptide and the lectin is reduced. Such a reduction would normally occur quite easily at the redox potential inside the cell and might or might not be enzyme catalysed [13]. A disulphide bond between the A1 and A2 peptides is similarly reduced when native toxin acts on cells. It is also possible that the A1 peptide can protrude through the membrane while remaining bound to lectin outside.

The lack of lag phase with the conjugate may reflect easier release of the A1 peptide from the conjugate than from the toxin. There is evidence (e.g. [14]) that the lag with toxin may be due at least partly to the time taken to generate free A1. If this is true, it would also account for the observed lack of lag with purified A1.

The ability to make this hybrid of cholera toxin suggests that other similar hybrids with binding components of different specificity could probably also be made, and would be found to be active. This raises the possibility of using specific binding protein that would make it possible to produce a conjugate that powerfully activated adenylate cyclase in only one particular organ or type of cell. This could be useful pharmacologically.

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