Entrance of Cholera Enterotoxin Subunits into Cells

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Quantitative analysis of the staining of cholera enterotoxin on the surface of cells with specific antibodies against each subunit of cholera toxin, using a Fluorescence-Activated Cell Sorter, showed that not only subunit A but also subunit B penetrates the cell membrane. The detection of each subunit inside the cell was facilitated by the use of saponin, an agent which increases membrane permeability.

INTRODUCTION

Studies on the mechanism of the interaction between cholera enterotoxin (CT) and sensitive cells have shown (Gill, 1978; Holmgren, 1978) that subunit B (SB) binds to the surface receptor, ganglioside GM1, after which subunit A (SA) activates adenylate cyclase. This leads to an elevation of intracellular cyclic AMP, which triggers various cytological events.

Some lines of evidence suggest that SA must be either in the cytosol or within the cell membrane in order to activate adenylate cyclase (Bennett & Cuatrecasas, 1977). We have studied the mode of internalization of CT into mouse thymus cells by the use of an immunofluorescence method employing specific antibodies against each subunit of CT. The detection of the intracellular antigen was facilitated by the use of saponin, an agent which increases membrane permeability. Our results indicate that not only subunit A but also subunit B penetrates the cell membrane after attachment of the whole toxin molecule to the cell surface.

METHODS

Cells. Five-week-old mouse (strain ddN) thymus cells were chosen for the experiment, because it has been shown that CT is bound to up to 80% of these cells (Tsuru et al., 1981), and the effect of the toxin could be measured by the increase in intracellular cyclic AMP (Holmgren & Lönroth, 1976).

Antigens. Cholera enterotoxin and its subunits A and B were prepared according to the method described previously (Ohtomo et al., 1976). Each substance produced a separate single band on sodium dodecyl sulphate (1%)-polyacrylamide (7.5%) gel electrophoresis.

Antisera. Specific antibodies to SA and SB were prepared according to the method of Muraoka et al. (1977) with highly purified antigens, and were further purified by affinity chromatography. Evidence for purity of the antibodies to the specific subunits was the fact that no cross-reactivity was observed in immunodiffusion tests or in passive haemagglutination tests with antisera to the highly purified antigens.

Fluorescence staining of cells. Pelleted cells (1 x 10^7) were treated with 0.1 ml purified CT at 1 μg ml^-1 for 10 min at 4°C. After a brief centrifugation and wash at 4°C to remove unbound CT, the cells were incubated at
4 °C, and at appropriate intervals 0.1 ml of either fluorescein isothiocyanate (FITC)-conjugated rabbit anti-SA immunoglobulin (0.5 mg protein ml⁻¹) or FITC-conjugated rabbit anti-SB immunoglobulin (0.5 mg protein ml⁻¹) was added and incubation was continued for 10 min at 4 °C. The cells were then washed and analysed using a fluorescence-activated cell sorter (FACS). Cells labelled with antibodies were viable.

FACS analysis. All preparations were analysed by a FACS-II fluorescence-activated cell sorter (Becton Dickinson Electronics Laboratory, Mountain View, Calif., U.S.A.). The cells were processed at 2000 to 3000 cells s⁻¹ and the intensity of fluorescence was recorded for each individual cell on the pulse-height analyser. The percentage of labelled cells was determined by counting the number of cells emitting fluorescent signals above the background and dividing by the total number of viable cells examined. Light-scattering signals distinguished live from dead cells. The calculation by the FACS analyser was based on the analysis of 10⁴ individual viable cells.

Saponin. Saponin was a generous gift from Dr I. Ohtsuki (Kyushu University, Fukuoka, Japan); its properties were reported previously in detail (Ohtsuki et al., 1978). Saponin (10⁻⁴ g ml⁻¹) was added to 1 × 10⁷ cells and incubated for 15 min at 0 °C. This treatment increased permeability and allowed uniform entry of macromolecular tracers into all cell compartments, with the exception of the contents of secretory and storage granules (Ohtsuki et al., 1978; Seeman, 1967; Seeman et al., 1973).

Preparation of plasma membranes. Membrane preparations were made by the method of Misra et al. (1975). Thymus cells treated with saponin after CT treatment were suspended in 10 mM-Tris/0.15 M-NaCl (pH 7.4) at 0 °C, and 2 vol. 10 mM-Tris/HCl (pH 7.4) was added slowly while stirring the cell suspension. The mixture was allowed to stand at 0 °C for 5 min, and then 10 mM-Tris/1.5 M-NaCl was added to restore the NaCl molarity to 0.15. The cells were then homogenized in a loosely fitting glass homogenizer with a Teflon rod using 10 to 15 gentle strokes. The homogenate was centrifuged at 300 g for 15 min to remove the cell nuclei. The sediment was resuspended in 10 mM-Tris/0.15 M-NaCl (pH 7.4), homogenized further with five strokes, and centrifuged as before. The two supernatants, free of nuclei, were combined and centrifuged at 4000 g for 20 min to remove mitochondria. After a further centrifugation step (20000 g for 1 h), the pellet was dissolved in 10 mM-Tris/HCl (pH 7.4) mixed with an equal volume of 60% (w/v) sucrose, layered on a 40% sucrose solution, and centrifuged at 4 °C for 15 h at 88000 g. The material at the 30–40% sucrose interface was taken as the plasma membrane fraction. Membrane preparations were treated with FITC-conjugated antisera and subjected to FACS analysis in the same way as intact thymus cells.

RESULTS AND DISCUSSION

In the experiment shown in Fig. 1, thymus cells were treated with CT, and FITC-conjugated anti-SA was added to samples taken 10 min, 1 h and 4 h after the addition of toxin. At 4 h the intensity pattern was quite similar to the control not treated with CT [Figs 1(1) and 1(4)]. A comparison of the scatter profile of the total cell population (A) with that of the fluorescent cells (B) is shown in Fig. 2(a) (anti-SA-labelled cells) and Fig. 2(b) (anti-SB-labelled cells). At 10 min after CT treatment 90% of the total cells were stained with anti-SA and 95% with anti-SB. The percentage of fluorescent cells declined with time, reaching about 18% for SA and 20% for SB at 4 h [Figs 2(a2) and 2(b2)].

The fate of CT following cell binding was examined by treating the cells with saponin, which facilitates the entrance of FITC-conjugated antibodies through the cell membrane (Nakane & Pierce, 1967): 4 h after toxin treatment, 10⁷ cells were treated with 10⁻⁴ g saponin ml⁻¹ for 15 min in an ice bath, and then incubated with FITC-conjugated anti-SA or anti-SB for 10 min at 4 °C. As shown in Fig. 3(a), fluorescence was associated with cells which had been treated with toxin followed by saponin but not with the cells which had not been treated with toxin before saponin treatment. When the cells were treated with saponin immediately after CT treatment, 95% were stained with FITC-conjugated anti-SB (Fig. 3a). When the cells were treated with saponin 4 h after CT treatment, only about 50% were stained (Fig. 3 b). Control cells showed no such fluorescence. Another experiment with FITC-conjugated anti-SA resulted in a similar pattern (Fig. 3c). The shift of the light-scattering pattern of the saponin-treated cells from that of the normal thymus cells (line A in Fig. 2) to the predominance of smaller cells (line A in Fig. 3) may be attributable to cell shrinkage following treatment with saponin. Cell membranes fractionated from the thymus cells treated with saponin at appropriate time intervals after CT treatment were stained with anti-SB as shown in Fig. 4. As seen in Fig. 4(1), 90% of the cell membranes were positively stained with

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Fig. 1. Fluorescence distribution of CT-treated thymus cells labelled with anti-SA antiserum: (1) control cells without CT treatment (FITC-conjugated anti-SA immunoglobulin only); (2) SA binding 10 min after CT treatment; (3) SA binding 1 h after CT treatment; (4) SA binding 4 h after CT treatment.

Fig. 2. Scatter profiles of CT-treated thymus cells labelled with anti-subunit antisera: A, scatter distribution of total cells; B, scatter distribution of fluorescein-labelled cells. (a1) Anti-SA binding 10 min after CT treatment; (a2) anti-SA binding 4 h after CT treatment. (b1) Anti-SB binding 10 min after CT treatment; (b2) anti-SB binding 4 h after CT treatment.

anti-SB 10 min after CT treatment. However, the percentage of fluorescent membranes decreased with time reaching about 5% for SB at 4 h [Figs 4(2) and 4(3)].

It has been reported that cyclic AMP accumulation in mouse thymocytes observed 30 min after toxin binding is the result of the activation of adenylate cyclase (Zenser & Metzger, 1974). That is, SA is presumed either to have penetrated the cell membrane or to be residing within the membrane (Bennett & Cuatrecasas, 1975) by this time. In our experiments, SA had disappeared from the cell surface by 4 h after CT binding [Fig. 2(a2)]. Taking the role of SA into consideration, it is very probable that SA entered the cell. Our data strongly suggest that SB also entered the cell in parallel with SA [Fig. 2(b2)]. The possibility of total release of SB from the surface receptor was excluded by an experiment which showed that the surface immunofluorescence remained unchanged for at least 4 h when the cells were treated with FITC-bound anti-SB immediately after SB binding.
Cholera enterotoxin may undergo adsorptive pinocytosis after binding to the receptor (Silverstein et al., 1977). In mouse thymus cells, however, it was observed that very little pinocytosis occurred when CT binding was followed by an immediate labelling with either...
anti-SA or anti-SB in the cold (data not shown). Those investigators who reported a patchy distribution of the receptors have usually treated the cells at low temperature, in order to minimize the possibility of pinocytosis of the fluorescent antiserum itself (De Petris & Raff, 1972; Loor et al., 1972; Taylor et al., 1971). Another possibility is that in the absence of antibody, CT molecules were pinocytosed rapidly, but even in this case, the expression of the activity might have to await the penetration of SA through the vesicle membrane. It is also possible that both CT subunits are internalized by penetration through the cell membrane in mouse thymus cells.

Saponin increases cell membrane permeability by interacting with cholesterol in membranes to form cholesterol–saponin micelles. It has been successfully used to promote macromolecular tracer penetration of the plasma membrane and even the intracellular membranes of living and fixed whole cells (Ohtsuki et al., 1978; Seeman, 1967; Seeman et al., 1973). The results shown in Fig. 3 are consistent with the conclusion that FITC-conjugated anti-SA or anti-SB can pass the cell membrane through the pores made by saponin, allowing the detection of the internalized antigen. About a third of all the SB that had disappeared from the cell surface (Fig. 2b) was thus found inside the cell 4 h after adsorption. Labelling of SA resulted in a similar pattern (Fig. 2a).

A possible criticism of this interpretation is that saponin treatment could cause release or exposure of membrane-bound CT. This can, however, be excluded by the results obtained with isolated membranes (Fig. 4). These showed that after brief exposure to CT, membrane-bound CT was detectable by fluorescence labelling in membranes prepared from saponin treated cells; this labelling decreased progressively up to 4 h after treatment with CT. These findings are consistent with the initial binding and subsequent internalization of CT in thymus cells.

In experiments with saponin-treated whole cells it should be noted that a relatively low percentage of fluorescein-stained cells (50% of the total cells treated with saponin at 4 h after CT binding) may be attributable to a less efficient antigen detection by the direct immunofluorescence method employed.

Our results indicate the possible penetration of both subunits of CT through the mouse thymus cell membrane. The simultaneous entrance of SA and SB may imply that SB facilitates the passage of SA through the hydrophobic cell membrane. In view of the time required for cyclic AMP accumulation, our present data may indicate that SA interacts with adenylate cyclase before its complete entrance into the cell.

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REFERENCES


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