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Identification of cholera toxin-binding sites in the nucleus of intestinal epithelial cells

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Post-embedding immunogold electron microscopy shows several binding sites for cholera toxin in mouse intestinal epithelial cells, particularly in the heterochromatin of the nucleus as well as in the plasma membrane. Anti-ganglioside GM1 antibodies also bound to the nucleus, but did not interfere with the binding of toxin. ^{125}I-labelled toxin bound specifically to a nuclear preparation from rabbit intestinal cells.

Cholera toxin; Immunogold; Heterochromatin; Ganglioside GM1; (Nucleus)

1. INTRODUCTION

Cholera toxin (M_r 84000, for general review see [1]) is composed of one A subunit and five B subunits. The B subunits bind to the outer membrane of cells and, by a mechanism not yet understood, this leads to the entry into the cell of the A1 polypeptide of the A subunit which activates adenylate cyclase by catalysing the ADP-ribosylation of the regulatory G_s protein. The binding of the B subunits is almost exclusively to ganglioside GM1 in the outer membrane. Binding is tight ($K_d = 10^{-9}$ M) and specific: cholera toxin has often been used as a marker for ganglioside GM1.

Previous studies in which the ganglioside GM1 was localized immunocytochemically have used toxin incubated with cells, followed by labelling with peroxidase-conjugated antitoxin [2] or with immunogold [3,4] before embedding. They have shown ganglioside GM1 only on the external membrane.

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In the experiments described in this paper we used the post-embedding immunogold method [5] to investigate binding sites for cholera toxin in epithelial cells of mouse small intestine. Labelling under these conditions shows binding sites (both intra- and extracellular) that are available to the toxin after the tissue had been fixed and sectioned: this does not imply that such sites would be accessible to the toxin in intact cells or in vivo. This technique has been used previously, for example, in investigating the intracellular localization of regulatory polypeptides [6]. We found binding sites for the toxin in the microvilli, in the plasma membrane, and in the heterochromatin of the nucleus.

2. MATERIALS AND METHODS

2.1. *Preparation of tissue for electron microscopy*

Small pieces of freshly excised mouse small intestine were fixed in 1% paraformaldehyde, 0.05% glutaraldehyde, 0.15 M NaCl, 50 mM phosphate buffer, pH 7.4, for 2 h at 4°C, washed in this buffer overnight at 4°C, dehydrated through ethanol; and embedded in hydrophilic resin (3 parts LR gold resin, London Resin Co.; 2 parts glycol methacrylate low acid, and 0.01% benzoin ethyl ester, Polysciences). The resin was polymerized

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by ultra-violet radiation (360 nm) for 24 h at room temperature.

2.2. *Immunolabelling*

Ultrathin sections mounted on collodion-coated nickel grids were incubated with 0.5 M NH_4Cl, in a buffer containing 0.15 M NaCl, 50 mM phosphate, pH 7.0, for 1 h at room temperature to block free aldehyde groups; washed with several changes of buffer, and incubated first for 30 min in 1 mg/ml ovalbumin, and then for 30 min in 850 ng/ml cholera toxin (supplied by Sigma, and used at a concentration chosen experimentally to give specific labelling and a very low background). After several washes in buffer, they were incubated for 1 h in rabbit anti-(cholera toxin) serum [6] (diluted 250 times in the buffer), washed again and finally incubated for 30 min with goat anti-(rabbit IgG) labelled with 15 nm gold (Janssen Pharmaceuticals) diluted fifty times. All incubations were at 37°C. After further washings in buffer and then in water, the grids were counterstained in 2% uranyl acetate and lead citrate, and examined with a Jeol 100CX2 electron microscope.

2.3. *Preparation of a nuclear fraction*

Nuclei were prepared from rabbit intestinal epithelial cells (prepared from gut scrapings) by the method of Bloebel and Potter [8]. A preparation of nuclear envelope with associated heterochromatin was prepared from this by the method of Harris and Milne [9]. It was analysed by polyacrylamide gel electrophoresis in the presence of SDS, and shown to have the expected protein profile [10]. The plasma membrane-marker enzyme sucrase was assayed by the method of Dahlqvist [11].

2.4. *Measurement of toxin binding*

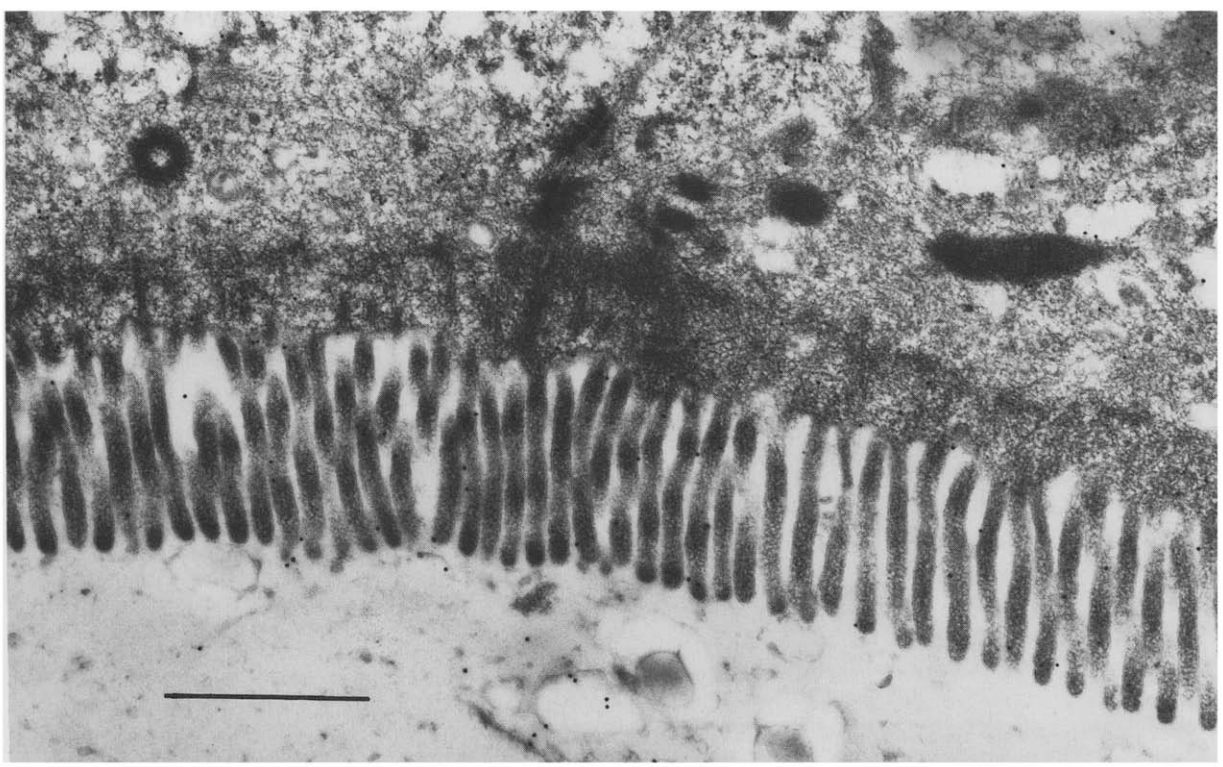
Cholera toxin was iodinated using chloramine-T [12], and used at about 70 GBq/μmol. Its binding to the nuclear preparation was measured essentially as described by Griffiths et al. [13] except that the buffer was 55 mM Tris-HCl, 80 mM NaCl, 10 mM mannose, 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4. 250 μl of the nuclear preparation (400 μg/ml) were used for each triplicate determination. Controls using a forty-fold excess of unlabelled toxin were subtracted from experimental data. All duplicates showed less than 10% variation.

3. RESULTS

3.1. *Post-embedding immunogold electron microscopy*

Some results from the experiments designed to show binding sites for cholera toxin are shown in fig.1. There are two major sites, together with some low levels of binding elsewhere in the cell, e.g. to vesicular structures (perhaps lysosomes and coated vesicles) in the cytoplasm. As would have been predicted from earlier work, there was extensive binding of whole toxin or of isolated subunit

a



B to the plasma membrane and microvilli (as shown in fig.1a). More surprisingly, under the same experimental conditions, there was a heavy distribution of colloidal gold on the heterochromatin of the nuclei (fig.1b). These experiments were done with many different samples, and in all cases the major binding was to the plasma membrane and to the nucleus.

Controls were carried out to show that the observed nuclear binding was specific for toxin, and not, for example, to non-specific binding of the antibodies used in the immunogold labelling.

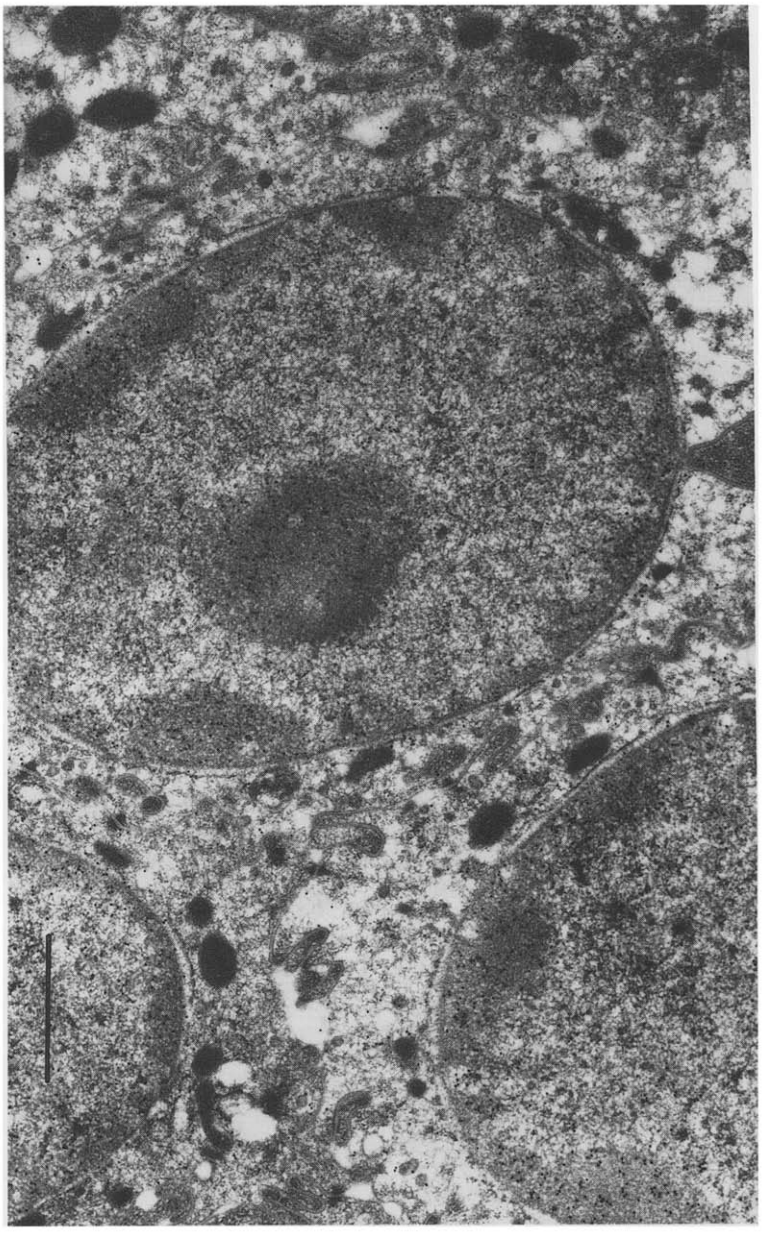


Fig.1. Electron micrographs of sectioned mouse intestinal tissue treated post-embedding with cholera toxin and immunogold. (a) Microvilli treated with 850 ng/ml whole toxin; (b) nuclei from the basal end of adjacent epithelial cells treated with 1 μg/ml subunit B. Bars are 1 μm long.

No gold was observed to bind to the nucleus or to the microvilli when (i) the rabbit antitoxin had been preadsorbed with toxin; (ii) cholera toxin was omitted; (iii) rabbit antitoxin was omitted; and (iv) the incubation was done with gold-labelled second antibody alone without toxin or second antibody.

Essentially identical binding was observed following similar experiments with rat intestinal cells.

3.2. *Are the binding sites ganglioside GM1?*

Several experiments were performed in order to find out whether the binding sites in the nuclei were ganglioside GM1. For example, toxin that had been preincubated with a 2×10^5-fold molar excess of ganglioside failed to bind, presumably because the binding site on the toxin was no longer available (although conceivably because of transmitted conformational change to a different binding site). Binding experiments done not with toxin but with a polyclonal anti-GM1 antibody preparation (kindly given to us by Dr N. Gregson of Guy's Hospital, London) gave very similar results to those found with toxin, suggesting a similar distribution of ganglioside and toxin-binding sites. However, preincubation of the post-embedded tissue with this antibody did not inhibit subsequent binding of toxin, nor did preincubation with toxin inhibit binding of antibody.

3.3. *Biochemical measurement of binding*

In order to verify that there are toxin-binding sites present in the nucleus, direct binding experiments were performed using ^{125}I-labelled toxin and a preparation of nuclear envelope from rabbit intestine.

Fig.2 shows a measurement of the binding of ^{125}I-labelled toxin to this preparation: the results are corrected for non-specific binding. Analysis of these data shows that they are compatible with a model in which there are about 4×10^{12} high-affinity binding sites (K_d about 2 nM) per mg protein, and about 6×10^{13} per mg of lower affinity (K_d about 70 nM).

Preliminary binding experiments using nuclear proteins adsorbed to microtitre plates gave similar results, and binding was abolished when the toxin was preincubated with ganglioside GM1. In order to establish the degree of contamination of the nuclear preparation with plasma membrane, it was

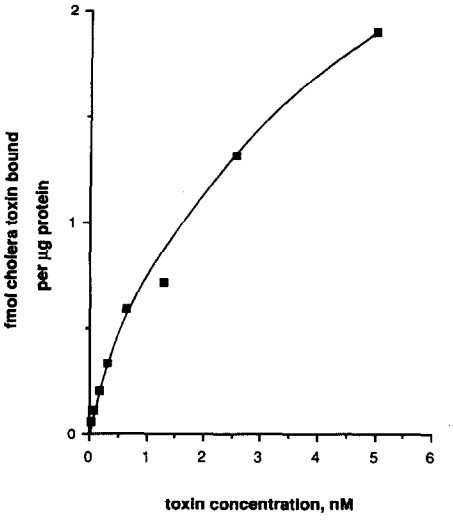


Fig.2. Binding of ^{125}I-labelled whole cholera toxin to a nuclear preparation from rabbit intestinal cells, corrected for non-specific binding.

assayed for sucrase (a marker enzyme for the plasma membrane). The activity suggested that contamination could not have been more than 3% of the protein in the preparation; quite inadequate to account for the toxin binding. Efforts to identify a specific binding protein by 'Western blot' analysis of the membrane preparation were not successful.

4. DISCUSSION

The experiments reported in this paper showed potential binding sites for cholera toxin associated with the nucleus. It seems likely that these sites are ganglioside GM1, the only receptor for the toxin that has been positively identified. This conclusion is supported by our observation that the binding is inhibited when toxin is preincubated with ganglioside and that the distribution in the cell of sites that bind anti-GM1 antibodies parallels that of toxin-binding sites. It is true that these antibodies did not interfere with the binding of toxin, but that could be easily explained if, as is probable, the affinity of the antibody for GM1 is lower than that of the toxin, or if the anti-GM1 does not bind to all the possible sites.

It is still possible, however, that the binding site is not a ganglioside, but, for example, a glycopro-

tein with a similar structure. Evidence from cell fractionation experiments, e.g. [14] has shown gangliosides in some intracellular membranes, but has usually been interpreted as showing no sign of gangliosides in the nucleus. On the other hand, binding of cholera toxin is probably the most sensitive available probe for ganglioside GM1.

Our experiments do not imply that these nuclear-binding sites can be reached by the toxin when it is working in the intact cell or in vivo, nor do they in themselves suggest any physiological role for binding to the nucleus.

Experiments on internalization of peroxidase-conjugated or ^{125}I-labelled toxin into intact cells have shown the toxin to be associated with a number of intracellular organelles, e.g. the Golgi complex [15] and other intracellular vesicles [16]. There has been no evidence for any particular binding to the nucleus. There is evidence, however, that gangliosides can be associated with cell growth and differentiation and so perhaps with gene expression in the nucleus. For example, Spiegel and Fishman [17] have shown that the interaction of the B subunit of cholera toxin with ganglioside GM1 can act as a bimodal growth regulator of cells, acting synergistically with epidermal growth factor.

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