The heavy chain of tetanus toxin can mediate the entry of cytotoxic gelonin into intact cells

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An artificial conjugate of the heavy chain of tetanus toxin linked by a disulphide bond to the impermeant ribosome-inactivating protein gelonin is cytotoxic to intact HT29 cells by inhibiting intracellular protein synthesis. Neither toxin nor gelonin alone has any significant effect. This shows that the heavy chain has the ability to mediate internalization of a protein to which it is bound by a disulphide bond. Thus the normal role of the tetanus toxin heavy chain may be to allow entry of the light chain into a cell.

1. INTRODUCTION

Tetanus toxin is a neurotoxin secreted by *Clostridium tetani*, and is the main causative agent of tetanus, a spastic paralysis of the muscles. Although the mode of action of the toxin is understood at the physiological level – it is retrogradely transported up the motor axon from the neuromuscular junction, then crosses into inhibitory presynaptic neurons where it prevents neurotransmitter release – little is known about its action at the molecular level.

The intact molecule is a single polypeptide chain (M_r 150000) with an internal disulphide bond. Subsequent proteolytic cleavage means that the toxin is usually found as a 100 kDa (heavy or H) chain joined to a 50 kDa (light or L) chain by a single disulphide bond [1,2]. This two-component structure is common to a number of bacterial toxins such as cholera [3] and diphtheria [4]. In the case of these latter toxins, one component binds to the target cell, and is followed by the entry of the other component into the cell where it catalyses a reaction leading to toxicity. A current model [5] suggests that tetanus toxin may work in a similar

manner. Binding of the heavy chain to gangliosides and possibly other receptors on the cell surface is well established [1,2], but it is not known if it has any role during the entry of the light chain into the cell. The 'effector' function of the light chain is even less clear-cut. Ahnert-Hilger et al. [6] have shown inhibition of catecholamine release from permeabilised adrenal chromaffin cells after treatment with the light chain of tetanus toxin alone or with the two-chain form in the presence of a reducing agent. Similar activities of the light chains of tetanus and of botulinum toxins (which are thought to have similar molecular mechanisms) have also been reported in other systems [7,8]. On the other hand, Poulain et al. [9], working on *Aplysia*, reported that both the light and the heavy chains of botulinum toxin need to enter the cell before an effect is observed.

In the present study this question was addressed by the use of a hybrid toxin. The isolated heavy chain of tetanus toxin was conjugated to gelonin, a cell-impermeant analogue of the A-chain of ricin that inhibits ribosomal protein synthesis [9]. Although gelonin itself was minimally toxic to a cultured cell line, the gelonin/heavy-chain conjugate showed considerable toxicity, associated with a reduced level of protein synthesis by the target cells. This demonstrates that the tetanus toxin heavy chain, linked to another polypeptide by a disulphide bond, is capable of mediating the entry into cells of that polypeptide. Once internalised, the polypeptide is then free to act in its usual manner. These observations confirm the binding function of the tetanus toxin heavy chain, and, more importantly, suggest that the heavy chain could also facilitate the entry into cells of another disulphide-linked polypeptide, namely, the light chain of the toxin.

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Abbreviations: DMSO, dimethyl sulphoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium blue; PBS, 10 mM sodium phosphate, 0.9% sodium chloride, pH 7.2; PDP-, pyridyldithiopropionyl-; SDS, sodium dodecylsulphate; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; TCA, trichloroacetic acid

2. MATERIALS AND METHODS

2.1. Isolation of heavy chain

Tetanus toxin and its chains were prepared from a freeze-dried crude sample of an ammonium sulphate precipitate of lysed *Clostridium tetani* cells kindly supplied by Dr N. Fairweather of Wellcome Biotech. The toxin was further purified by ion-exchange chromatography [11], then tryptically cleaved to form the heavy and light chains still joined by a disulphide bridge. This material was then subjected to polyacrylamide gel electrophoresis under reducing conditions and the individual chains subsequently purified by electroelution into 10 mM sodium phosphate buffer, pH 7.4, containing 0.05% (w/v) SDS. (The detergent was included because tetanus H-chain has been shown to bind readily to dialysis membrane and to come out of solution at high concentrations.)

2.2. Preparation of heavy chain/gelonin conjugate

This was achieved by mixing a PDP-derivative of tetanus H-chain with a reduced PDP-derivative of gelonin, based on the methods of Carlsson et al. [12]. A small amount of ^{125}I-labelled gelonin and 0.25 mg unlabelled gelonin in 1 ml borate buffer (50 mM sodium tetraborate, 300 mM NaCl, 0.5% butanol, pH 9) was mixed with 40 μl SPDP (6 mg/ml in DMSO). The reaction was allowed to proceed for 30 min at room temperature and the products dialysed overnight against acetate buffer (100 mM sodium acetate, 100 mM NaCl, 1 mM EDTA, pH 4.5). The dialysed material was then reduced by addition of 2 mg dithiothreitol (DTT). After 30 min at room temperature, excess DTT was removed by passage through a Sephadex G25 column equilibrated with phosphate buffer (100 mM sodium phosphate, 100 mM NaCl, 1 mM EDTA, pH 7.3). PDP-H-chain was prepared in a similar manner using 0.5 mg electroeluted H-chain and 40 μl 20 mM SPDP. In this case no reduction step was included and the excess SPDP was removed on a Sephadex G25 column. PDP-H-chain was mixed with reduced PDP-gelonin for 48 h at room temperature, and the solution concentrated by Amicon filtration before separation on a Sephacryl S200 column equilibrated with borate buffer. Fractions containing the unreacted gelonin were discarded. The remaining material was lyophilised, resuspended in deionised water then dialysed against 10-fold diluted PBS. Finally, the material was lyophilised, then redissolved in RPMI/10% FCS and Millipore-filtered (0.22 μm) before addition to cultured cells.

2.3. Cytotoxicity of conjugates

The cytotoxicity of toxins and derivatives was determined using the human colonic carcinoma cell line HT29. These cells were plated in 200 μl RPMI 1640 medium/10% foetal calf serum at a density of 4 × 10^3 per well in a 96-well culture plate, allowed to reach log-growth stage (36 h), then treated with the test sample (20 μl added) over the following 72 h. Cell viability was then measured using the MTT assay of Carmichael et al. [13]. Briefly, to each well containing 200 ml of medium was added 50 μl of 2 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium blue). The plate was returned to the incubator for a further 3.5 h, after which the medium and MTT were removed (taking care not to dislodge the cells) and the blue formazan reaction product dissolved by adding 100 μl dimethylsulphoxide to each well. The absorbance at 570 nm of each well was measured on an ELISA plate reader; this value was proportional to the number of living cells in the well. Control experiments to establish the ability of this assay to indicate cytotoxicity were carried out using cycloheximide, ricin and diphtheria toxin as the cytotoxic agents.

The effects of toxin and conjugates on cellular protein synthesis were determined according to the method described by Draper and Simon [14]. The medium was removed from the cells (either treated or untreated) and replaced with RPMI deficient in L-leucine. After 2 h, 1 μCi [^{3}H]L-leucine was added and the plates returned to the incubator for 1 h. The cells were next washed twice with 0.05 M phosphate buffer, 0.15 M NaCl, pH 7.2, containing 1 mg/ml unlabelled leucine, then solubilised in this buffer containing 0.05%
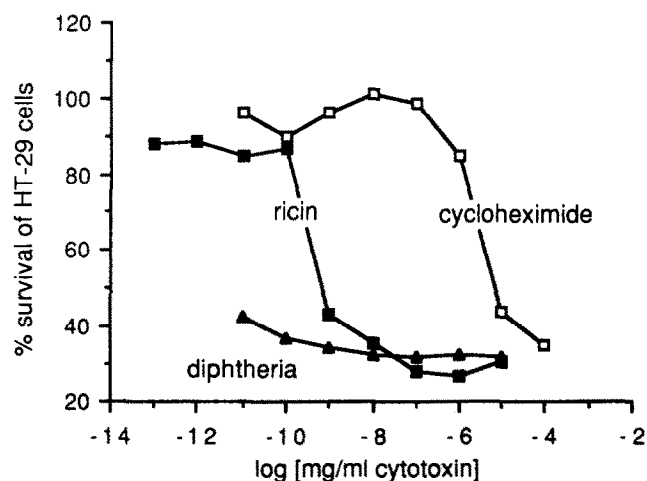

Fig. 1. Effect of various cytotoxins (ricin, diphtheria toxin or cycloheximide) on the viability of intact HT29 cells as measured by the MTT assay as described in the text. 100% = absorbance at 570 nm of formazan product in wells to which no cytotoxic agent was added.

SDS, 1 mg/ml DNase-1, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Aliquots were transferred to numbered Whatman GF/C filters, soaked in 5% trichloroacetic acid for 30 min, then washed twice with 95% ethanol. After drying, the filters were assayed for radioactivity by scintillation counting. Cytotoxicity and protein synthesis assays were performed in triplicate wells.

3. RESULTS AND DISCUSSION

The results of the cytotoxicity experiments are presented in Figs 1–3. Fig. 1 shows the use of the MTT assay to demonstrate that the survival of HT29 cells after a 72 h exposure to each of 3 different cytotoxic agents (ricin, diphtheria toxin, or cycloheximide) was reduced to 35–40% of the control value in a manner dependent on dose over several orders of magnitude from less than nanograms to more than milligrams per
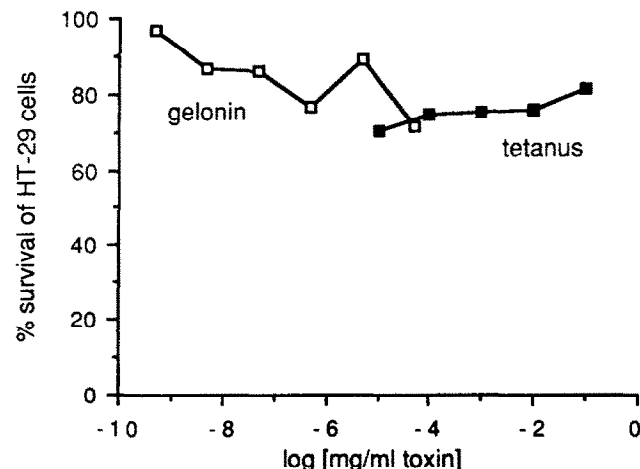

Fig. 2. Effect of tetanus toxin and of gelonin on the viability of intact HT29 cells. 100% is as described in the legend to Fig. 1.
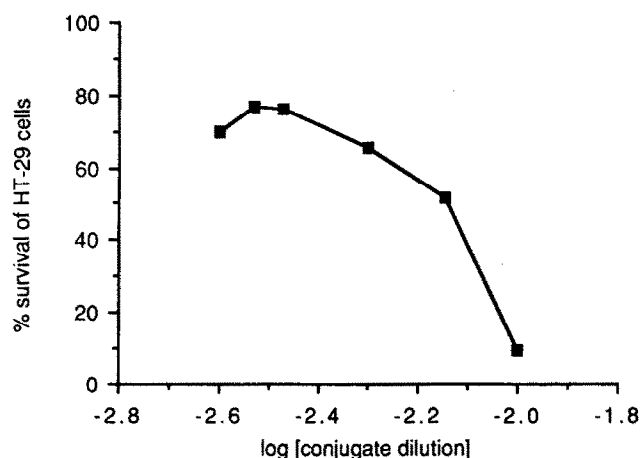
Fig. 3. Effect of the gelonin/heavy-chain conjugate on the viability of intact HT29 cells. 100% is as described in the legend to Fig. 1.
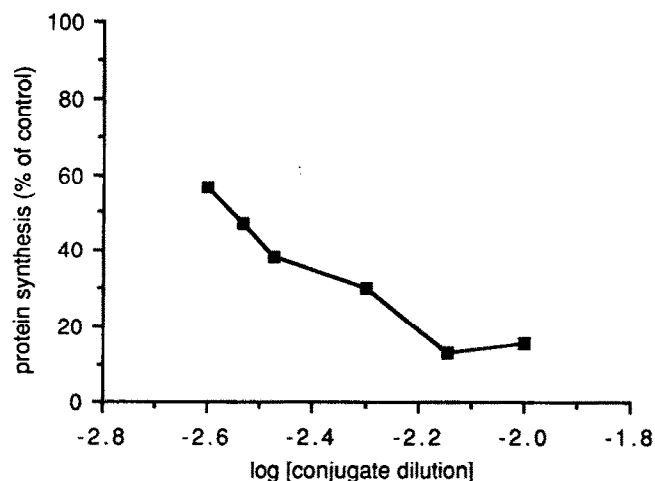

Fig. 4. Effect of the gelonin/heavy-chain conjugate on protein synthesis in HT29 cells as described in the text. 100% = TCA-precipitable counts from wells to which no conjugate was added.

ml. Fig. 2 illustrates the effects of treatment of the cells with a solution of whole tetanus toxin. This toxin solution did kill about 20% of the cells, but since the concentrations used were much higher than those of Fig. 1, and there was no apparent dependency on dose, it seems likely that cell death was not due to the toxin molecule itself. Similarly, as also shown in Fig. 2, gelonin was only weakly cytotoxic and showed little dose-dependence.

In contrast, when various dilutions of the gelonin/heavy-chain conjugate were tested, cell survival ranged from 78% down to approximately 15% in a dose-dependent fashion, as shown in Fig. 3. This cytotoxicity was associated with a dose-dependent inhibition of protein synthesis to less than 20% of the control value (Fig. 4). The absolute concentration of conjugate used in these experiments could not be determined because the amounts involved were below the sensitivity threshold of the protein assay. A crude estimate of the maximum amount present, based on silver or Coomassie blue staining of polyacrylamide gels, shows that the concentration of the undiluted conjugate must have been less than 0.4 μg/ml, implying that the highest concentration tested in the cytotoxicity assay was less than 8 ng/ml. Control experiments using RPMI/FCS or sodium phosphate buffer, without any putative cytotoxin, indicated that addition of these diluents to the cultures did not cause cell death (data not shown).

These results indicate that the heavy chain of tetanus toxin, as part of a disulphide-linked hybrid toxin, was able to facilitate the internalization of the non-tetanus component of the hybrid. The internalized species was then able to bring about cell death by inhibition of cellular protein synthesis. Since neither gelonin (with intact cells) nor tetanus toxin (with intact or permeabilized cells) can have this activity, we suggest

that the hybrid gelonin/heavy-chain conjugate can mimic the native two-chain tetanus toxin molecule, and that our results support the model of toxin action in which the heavy chain in some way allows entry into the cell of the light chain. Once inside, the light chain can then proceed to interfere with normal synaptic transmission.

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