Dimethyl sulphoxide protects cells against polypeptide toxins and poliovirus

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The effect of dimethyl sulphoxide and other cryoprotective compounds on the sensitivity of cells to polypeptide toxins and to poliovirus was tested. In the presence of these compounds, which all affect membrane fluidity, the cells were protected against the toxic proteins and against poliovirus. The large protection obtained was not due to reduced binding and endocytosis of the toxins. Apparently, the cryoprotective compounds interfere with the entry of toxins and of the poliovirus genome across the cell membrane.

The polypeptide toxins diphtheria toxin, modeccin, abrin and ricin are known to act on targets in the cytosol and must therefore be transferred through the cell membrane in order to intoxicate cells (for review, see Olsnes & Sandvig, 1983). These toxins are structurally similar, and they all consist of two polypeptide chains connected by a disulphide bond. One of the chains binds to cell-surface receptors, whereas the other chain enters the cytosol, inhibits protein synthesis and eventually kills the cell. Also, during infection with poliovirus, a macromolecule, the RNA genome, enters the cytosol. In studies of the mechanism of toxin entry into cells, the use of compounds that block toxin entry at different steps have been useful (Draper & Simon, 1980; Sandvig & Olsnes, 1980, 1981, 1982*a,b*). Here we have studied the effect on toxin and virus uptake of Me₂SO and other compounds known to stabilize cellular membranes.

Materials and methods

Abrin, ricin, modeccin, diphtheria toxin and antitoxins against them were prepared as described previously (Olsnes, 1978*a,b*; Sandvig & Olsnes, 1981; Olsnes *et al.*, 1982). ¹²⁵I-labelled toxins were prepared by the Iodogen method (Fraker & Speck, 1978). Purified *Pseudomonas aeruginosa* exotoxin A was generously given by Dr. Stephen H. Leppla [USAMRIID (U.S. Army Medical Research Institute for Infectious Diseases), Frederick, MD, U.S.A.]. A strain (Brun-

ende) of poliovirus type 1 was propagated in HeLa S₃ cells and purified as described by Cole *et al.* (1971). Light-sensitive virus containing Neutral Red was prepared essentially as described by Mandel (1967). Virus titration, virus labelling and analysis of virus-particle alteration by sucrose-gradient centrifugation were performed as previously described (Madshus *et al.*, 1984).

To measure toxin binding, ¹²⁵I-labelled toxins were incubated with cells growing in 24-well disposable trays. In the case of diphtheria toxin, the cells were incubated with labelled toxin in serum-free medium containing 0.1 mg of bovine serum albumin/ml for 4 h at 0°C. The cells were then washed three times in medium, dissolved in 0.1 M-KOH and the radioactivity associated with the cells was measured. ¹²⁵I-abrin, -ricin and -modeccin were incubated with the cells for 1 h at 37°C. Total binding was then measured as above. To measure the rate of endocytosis, medium containing 0.1 M-lactose was added and the incubation was continued for 15 min more. The cells were then washed twice with medium containing lactose, dissolved in 0.1 M-KOH, and the radioactivity was measured.

Mouse L-cells, BHK (baby-hamster kidney) cells, Vero cells and HeLa S₃ cells were maintained in monolayer cultures in minimum essential medium containing 10% (v/v) foetal-calf serum (Gibco, Glasgow, Scotland, U.K.). The sensitivity of cells to toxins and virus under the different conditions here described was tested by measuring protein-synthesis inhibition (Sandvig & Olsnes, 1981). The experiments were carried out in duplicate. The difference between duplicates was less than 10% of the average value.

Abbreviation used: Me₂SO, dimethyl sulphoxide.

Results and discussion

When Vero cells were incubated with 10% (v/v) Me_2SO for 1 h at 37°C and then exposed to toxin, approx. 1000 times more diphtheria toxin was necessary to reduce protein synthesis to half the control value than in the absence of 10% Me_2SO (Fig. 1a), whereas in the case of abrin and ricin the difference was approx. 200-fold (Fig. 1b). When the concentration of Me_2SO was reduced to 5%, there was no protection of the cells (Fig. 1a). Concentrations of Me_2SO between 5 and 10% gave intermediary levels of protection.

Me_2SO at 10% (v/v) also protected Vero cells against modeccin, and it protected mouse L-cells against *Pseudomonas* toxin (results not shown). Preincubation of the toxins with 10% Me_2SO did not reduce their activity when subsequently added to cells in the absence of Me_2SO .

If the 1 h preincubation with Me_2SO was omitted and diphtheria toxin and 10% Me_2SO were added simultaneously to the cells, the protection was reduced by a factor of 10. On the other hand, even 24 h preincubation of cells with 2.5% Me_2SO did not have any protective effect.

The intoxication by diphtheria toxin, modeccin, abrin and ricin involves binding of toxins to cell-surface receptors, internalization by endocytosis and transfer of at least the enzymically active moiety across the cell membrane (for review, see Olsnes & Sandvig, 1983). To test the possibility that the protection observed could be due to inhibition by Me_2SO of toxin binding, studies of the binding of ^{125}I -labelled toxins were carried out as described in the Materials and methods section. As shown in Table 1, the binding of the toxins to the cells in the presence of 10% Me_2SO was reduced to 50–100% of that obtained in the absence of Me_2SO . However, it is clear that this reduction is small compared with the large effect of Me_2SO on the intoxication of the cells shown in Fig. 1.

Conceivably, the presence of Me_2SO could interfere with the toxin binding in such a way that bound toxin was unable to initiate intoxication, even after removal of Me_2SO . To test this possibility, diphtheria toxin and ricin were added to cells in the absence or presence of Me_2SO . After 30 min, Me_2SO and unbound toxin were removed and the cells were incubated overnight to allow the bound toxin sufficient time to express its

Fig. 1. Ability of Me_2SO to protect cells against diphtheria toxin, abrin and ricin

To Vero cells growing in 24-well disposable trays in Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]-containing medium, pH 7.5 (Sandvig & Olsnes, 1981) Me_2SO was added to the concentrations indicated. After 1 h at 37°C, increasing amounts of toxins were added and protein synthesis was measured, as described in the Materials and methods section, after 1 h (a) or 2 h (b) of further incubation. (a): ×, Diphtheria toxin (control); O, diphtheria toxin + 7% (v/v) Me_2SO ; Δ, diphtheria toxin + 8% (v/v) Me_2SO ; ●, diphtheria toxin + 10% (v/v) Me_2SO . (b): O, abrin; ●, abrin + 10% (v/v) Me_2SO ; Δ, ricin; ▲, ricin + 10% (v/v) Me_2SO .

Table 1. Ability of cells to bind and endocytose ¹²⁵I-labelled toxin in the absence and presence of 10% Me₂SO

When binding was carried out in the presence of Me₂SO, the cells were preincubated with 10% Me₂SO for 1 h before ¹²⁵I-labelled toxin was added. Total binding and lactose-resistant binding were measured as described in the Materials and methods section. The results given are averages for duplicate measurements. The range is given in parenthesis.

Toxin	Me ₂ SO (10%)	Binding (c.p.m.)	
		Total	Lactose-resistant
Modeccin	—	2048 (±203)	736 (±22)
	+	1012 (±93)	563 (±123)
Abrin	—	4383 (±200)	2358 (±50)
	+	3047 (±13)	1052 (±22)
Ricin	—	1568 (±62)	679 (±24)
	+	1145 (±68)	401 (±8)
Diphtheria toxin	—	478 (±50)	
	+	440 (±35)	

maximal toxic effect. Finally, the rate of protein synthesis was measured. As shown in Fig. 2, the cells were intoxicated to essentially the same extent whether or not Me₂SO was present during the binding.

After binding to cell-surface receptors the toxin is taken up by endocytosis. In the case of abrin, ricin and modeccin, which bind to galactose-containing receptors, surface-bound toxin can be washed off the cells with lactose, whereas endocytosed toxin cannot be removed by this procedure (Sandvig & Olsnes, 1979). We therefore measured the extent of endocytosis by assaying the amount of ¹²⁵I-modeccin, -abrin and -ricin that could not be removed from the cells with lactose (lactose-resistant binding). As shown in Table 1, no large differences were found in the presence or absence of Me₂SO. The results showed that Me₂SO did not reduce the rate of endocytosis of these toxins sufficiently to account for the extensive protection

Fig. 2. Ability of toxin bound in the presence of Me₂SO to intoxicate cells after transfer to normal medium. Vero cells growing in 24-well disposable trays were incubated for 1 h at 30°C in the absence and presence of Me₂SO (10%) in HEPES-containing medium, pH 7.5, and then increasing concentrations of toxin were added. After 30 min further incubation, the cells were washed, growth medium was added and, after 18 h additional incubation, protein synthesis was measured as described in the Materials and methods section. (a): ○, Diphtheria toxin; ●, diphtheria toxin + 10% (v/v) Me₂SO. (b): ○, Ricin; ●, ricin + 10% (v/v) Me₂SO.

obtained in the presence of Me_2SO . It therefore appears that Me_2SO inhibits a step in toxin entry occurring after endocytosis has taken place.

Low pH is necessary for entry of diphtheria toxin (Draper & Simon, 1980; Sandvig & Olsnes, 1980, 1981), and there is evidence that diphtheria toxin enters the cytosol from early acidic vesicles (Sandvig & Olsnes, 1981; Sandvig *et al.*, 1984). The presence of Me_2SO reduced the rate of ^{125}I -diphtheria-toxin degradation to one half. This could be due to lack of vesicle acidification. Since diphtheria toxin requires low pH for entry, the possibility existed that the protection by Me_2SO could be due to lack of acidification.

When the pH in the medium is reduced to pH 4.5, surface-bound diphtheria toxin is rapidly transported into the cell, apparently directly through the surface membrane (Sandvig & Olsnes, 1981). Me_2SO also inhibited under these conditions, indicating that, even if Me_2SO inhibits acidification, this cannot be the whole explanation for the protection against diphtheria toxin. The results therefore indicate that Me_2SO somehow inhibits the penetration of toxin through the cellular membrane.

The ability of low- M_r compounds to protect cells during freezing, to induce differentiation and to stabilize membranes is related to the basicity of the compounds (Preisler *et al.*, 1976). Thus dimethylformamide was found to be more efficient in this regard than formamide, dimethylacetamide was more efficient than acetamide, and dimethylurea was better than urea. When we tested the ability of these compounds to protect against ricin, we found the same order of efficiency (Fig. 3). As shown in Table 2, diethylene glycol and glycerol also protect against ricin. Both these compounds, in common with Me_2SO , induce differentiation in erythroleukaemia cells (Preisler & Lyman, 1975).

To study whether Me_2SO inhibits membrane penetration of agents other than protein toxins, we tested the ability of poliovirus to enter cells treated with Me_2SO . For this purpose, light-sensitive poliovirus was bound to HeLa S_3 cells and then medium containing 10% Me_2SO was added. The cells were incubated at 37°C for 20 min in the dark and then virus that had not entered the cells was inactivated by exposure to light. Virus RNA that has reached the cytosol is not inactivated under these conditions (Mandel, 1967). Finally the cells

Fig. 3. Ability of different cryoprotective compounds to protect cells against ricin

Vero cells growing in 24-well disposable trays were incubated for 1 h at 30°C in a Hepes-containing medium with and without the indicated compounds. Increasing concentrations of ricin were then added, and, after 3 h further incubation, protein synthesis was measured during a 15 min interval as described in the Materials and methods section. The additions were: (a) $\times$, none; Δ , 0.5M-formamide; $\circ$, 0.7M-formamide; $\square$, 1M-formamide; $\blacktriangle$, 0.5M-dimethylformamide; (b) $\times$, none; ∇ , 0.3M-acetamide; $\circ$, 0.7M-acetamide; $\blacktriangledown$, 0.3M-dimethylacetamide; (c) $\times$, none; Δ , 0.7M-urea; $\blacktriangledown$, 0.3M-dimethylurea; $\blacktriangle$, 0.5M-dimethylurea.

were transferred to normal medium and incubated overnight. As shown in Fig. 4, Me₂SO completely protected the cells from the virus, even when the cells were exposed to medium at pH 5.5, which otherwise induces a rapid entry of virus RNA into the cytosol, apparently directly from the cell surface (Madshus *et al.*, 1984).

We have previously shown that conditions which inhibit conversion of virus into subviral particles also inhibit the infection by virus (Madshus *et al.*, 1984). As shown in Fig. 5, Me₂SO inhibited the alteration of virus at pH 7.5. When the cells were exposed to medium containing Me₂SO at pH 5.5, virus alteration did take place, but no infection occurred (Fig. 4). This shows that virus alteration is not necessarily followed by entry of the genome into the cytosol.

Fig. 4. Ability of Me₂SO to protect cells against poliovirus. Increasing amounts of Neutral Red-containing virus were added to HeLa S₃ cells in the dark. The virus was allowed to bind to the cells for 2 h at 0°C. Then the cells were washed twice, prewarmed medium with and without Me₂SO and with pH as indicated was added, and the cells were incubated at 37°C for 20 min. The cells were then exposed to light, transferred to normal medium and incubated overnight. The next day the rate of [³H]leucine incorporation was measured. The additions were: ■, 10% Me₂SO, pH 5.5; □, 10% Me₂SO, pH 7.5; ○, control, pH 5.5; ●, control, pH 7.5.

Lyman *et al.* (1976) reported that Me₂SO decreases membrane fluidity and increases the phase-transition temperature of phospholipid membranes. They suggested that this is due to

Table 2. Ability of chemical inducers of differentiation to protect against ricin

Inhibition of protein synthesis was measured as in Fig. 2. The sensitivity is given as the ratio between the toxin concentrations required to decrease protein synthesis to 50% of the control value in the absence and presence of the compounds indicated. The values in parentheses are s.d. values between different independent experiments.		
Compound	Concn. (M)	Sensitivity
Diethylene glycol	0.5	1
	0.7	0.03 (0.01)
Glycerol	0.3	1
	1.0	0.12 (0.055)
	1.4	0.03 (0.018)

Fig. 5. Sucrose-gradient centrifugation of [³⁵S]methionine-labelled virus

Cells were incubated with [³⁵S]methionine-labelled virus for 2 h at 0°C and then washed to remove unbound virus. Medium containing Hepes (Sandvig & Olsnes, 1981), with and without 10% (v/v) Me₂SO, and with pH as indicated, was added. After incubation of the cells for 45 min at 37°C, the pH was adjusted to 7.5 and 0.5% Triton X-100 was added. The nuclei were removed by centrifugation for 3 min in an Eppendorf 3200 centrifuge. The supernatants were treated with 0.2% (w/v) sodium dodecyl sulphate and layered on top of 15–30% (w/v) sucrose gradients. After centrifugation for 50 min at 234000g in a Beckman SW 50.1 rotor, fractions were collected and the radioactivity of 180 μl aliquots was measured. V indicates intact virus.

ionic interactions with acidic phospholipids. The results here presented indicate that these membrane changes somehow interfere with the penetration of macromolecules through the membrane.

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