Dimethyl sulphone protects cells against polypeptide toxins and poliovirus

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The effect of dimethyl sulphone 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, 1982a,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, 1978a,b; Sandvig & Olsnes, 1981; Olsnes et al., 1982). ⁱ²⁵I-labelled toxins were prepared by the Iodogen method (Fraker & Speck, 1978). Purified Pseudomonas aeruginosa exotoxin A was generously given by Dr. Stephen H. Leplla [USAMRIID (U.S. Army Medical Research Institute for Infectious Diseases), Frederick, MD, U.S.A.]. A strain (Brunende) of poliovirus type 1 was propagated in HeLa S₁ 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 0.1mg of bovine serum albumin/ml for 4h at 0°C. The cells were then washed three times in medium, dissolved in 0.1M-KOH and the radioactivity associated with the cells was measured. ¹²⁵I-abrin, -ricin and -modeccin were incubated with the cells for 1h at 37°C. Total binding was then measured as above. To measure the rate of endocytosis, medium containing 0.1M-lactose was added and the incubation was continued for 15min more. The cells were then washed twice with medium containing lactose, dissolved in 0.1M-KOH, and the radioactivity was measured.

Mouse L-cells, BHK (baby-hamster kidney) cells, Vero cells and HeLa S₁ 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.
Results and discussion

When Vero cells were incubated with 10% (v/v) Me2SO for 1h 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% Me2SO (Fig. 1a), whereas in the case of abrin and ricin the difference was approx. 200-fold (Fig. 1b). When the concentration of Me2SO was reduced to 5%, there was no protection of the cells (Fig. 1a). Concentrations of Me2SO between 5 and 10% gave intermediary levels of protection.

Me2SO 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% Me2SO did not reduce their activity when subsequently added to cells in the absence of Me2SO.

If the 1h preincubation with Me2SO was omitted and diphtheria toxin and 10% Me2SO were added simultaneously to the cells, the protection was reduced by a factor of 10. On the other hand, even 24h preincubation of cells with 2.5% Me2SO 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 Me2SO of toxin binding, studies of the binding of 125I-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% Me2SO was reduced to 50-100% of that obtained in the absence of Me2SO. However, it is clear that this reduction is small compared with the large effect of Me2SO on the intoxication of the cells shown in Fig. 1.

Conceivably, the presence of Me2SO could interfere with the toxin binding in such a way that bound toxin was unable to initiate intoxication, even after removal of Me2SO. To test this possibility, diphtheria toxin and ricin were added to cells in the absence or presence of Me2SO. After 30min, Me2SO and unbound toxin were removed and the cells were incubated overnight to allow the bound toxin sufficient time to express its

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**Fig. 1. Ability of Me2SO 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, pH7.5 (Sandvig & Olsnes, 1981) Me2SO was added to the concentrations indicated. After 1h at 37°C, increasing amounts of toxins were added and protein synthesis was measured, as described in the Materials and methods section, after 1h (a) or 2h (b) of further incubation. (a): ×, Diphtheria toxin (control); ○, diphtheria toxin +7% (v/v) Me2SO; △, diphtheria toxin +8% (v/v) Me2SO; ●, diphtheria toxin +10% (v/v) Me2SO. (b): ○, abrin; ●, abrin +10% (v/v) Me2SO; △, ricin; ▲, ricin +10% (v/v) Me2SO.
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.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Me2SO (10%)</th>
<th>Total</th>
<th>Lactose-resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modeccin</td>
<td>–</td>
<td>2048 (+203)</td>
<td>736 (+22)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1012 (+93)</td>
<td>563 (+123)</td>
</tr>
<tr>
<td>Abrin</td>
<td>–</td>
<td>4383 (+200)</td>
<td>2358 (+50)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3047 (+13)</td>
<td>1052 (+22)</td>
</tr>
<tr>
<td>Ricin</td>
<td>–</td>
<td>1568 (+62)</td>
<td>679 (+24)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1145 (+68)</td>
<td>401 (+8)</td>
</tr>
<tr>
<td>Diphtheria toxin</td>
<td>–</td>
<td>478 (+50)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>440 (+35)</td>
<td></td>
</tr>
</tbody>
</table>

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): O, Diphtheria toxin; ●, diphtheria toxin + 10% (v/v) Me2SO. (b): ○, Ricin; ●, ricin + 10% (v/v) Me2SO.

Vol. 219
obtained in the presence of Me$_2$SO. It therefore appears that Me$_2$SO 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$_2$SO 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$_2$SO 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$_2$SO also inhibited under these conditions, indicating that, even if Me$_2$SO inhibits acidification, this cannot be the whole explanation for the protection against diphtheria toxin. The results therefore indicate that Me$_2$SO somehow inhibits the penetration of toxin through the cellular membrane.

The ability of low-$M$ 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$_2$SO, induce differentiation in erythroleukaemia cells (Preisler & Lyman, 1975).

To study whether Me$_2$SO inhibits membrane penetration of agents other than protein toxins, we tested the ability of poliovirus to enter cells treated with Me$_2$SO. For this purpose, light-sensitive poliovirus was bound to HeLa $S_2$ cells and then medium containing 10% Me$_2$SO 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](image)

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) ×, none; Δ, 0.5M-formamide; O, 0.7M-formamide; □, 1M-formamide; ▲, 0.5M-dimethylformamide; (b) ×, none; ▽, 0.3M-acetamide; O, 0.7M-acetamide; ▼, 0.3M-dimethylacetamide; (c) ×, none; Δ, 0.7M-urea; ▼, 0.3M-dimethylurea; ▲, 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.

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc. (M)</th>
<th>Sensitivity</th>
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<tbody>
<tr>
<td>Diethylene glycol</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>0.03 (0.01)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.12 (0.055)</td>
</tr>
<tr>
<td></td>
<td>1.4</td>
<td>0.03 (0.018)</td>
</tr>
</tbody>
</table>

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

Cells were incubated with [³⁵S]methionine-labelled virus for 2h 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 45min 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 3min 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 50min at 234000g in a Beckman SW 50.1 rotor, fractions were collected and the radioactivity of 180µl 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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References
