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INHIBITORY EFFECT OF AMMONIUM CHLORIDE AND CHLOROQUINE ON THE ENTRY OF THE TOXIC LECTIN MODECCIN INTO HeLa CELLS

Kirsten Sandvig, Sjur Olsnes and Alexander Pihl
Norsk Hydro's Institute for Cancer Research, Montebello,
Oslo 3, Norway

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SUMMARY: Ammonium chloride and chloroquine protected a variety of cell lines against diphtheria toxin and the toxic lectin modeccin. Experiments where the ability of antibody to neutralize the toxin was measured indicate that in the presence of ammonium chloride and chloroquine, modeccin remains at the cell surface and that the two compounds inhibit the uptake of modeccin into the cytoplasm. A cell line tolerating increased concentrations of modeccin was not protected against modeccin by ammonium chloride or chloroquine, whereas the compounds did protect these cells against diphtheria toxin.

Little is known about the mechanism whereby water soluble macromolecules penetrate the plasma membrane of cells. An interesting clue is offered by the finding that ammonium chloride interferes with the cellular uptake of a variety of macromolecules (1-3). It is of particular interest that cells susceptible to the lethal effect of diphteria toxin are protected by the presence of NH_4^+-concentrations above 4 mM (4), apparently due to inhibition of toxin uptake (5).

Modeccin is a plant toxin which resembles the toxic lectins abrin and ricin (6,7). These proteins consist of two polypeptide chains joined by disulfide bonds and act by inactivating the 60S ribosomal subunits of the ribosomes. Diphtheria toxin which exerts its effect by inactivating elongation factor 2, likewise consists of two functionally different parts (8).

Although little is known about the mechanism whereby the enzymatically active part of the toxins, the A-chains, are transferred across the plasma membrane it is clear from their mechanism of

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action that they must enter the cytoplasmic phase (9). In the present work we have compared the effects of ammonium chloride and chloroquine on the toxic effect of modeccin, abrin, ricin and diphtheria toxin on cells.

MATERIALS AND METHODS

Abrin, ricin and modeccin were prepared as earlier described (7,10,11). Diphtheria toxin was obtained from Connaught Laboratories Ltd., Willbowdale, Ontario, Canada.

Modeccin was labelled with ^{125}I using the lactoperoxidase method (12). HeLa cells were maintained in monolayer culture at 37°C in Gibco Minimum Essential medium as earlier described for BHK cells (13). Modeccin-resistant cells were obtained as described earlier (7). The binding of ^{125}I-labelled modeccin to HeLa cells was studied as earlier described for abrin and ricin (13).

RESULTS AND DISCUSSION

The effect of ammonium chloride and chloroquine on the sensitivity of HeLa cells to various toxins as measured by inhibition of cellular protein synthesis is shown in Table 1. When the cells were incubated with diphtheria toxin in the presence of 10 mM ammonium chloride no toxic effect was observed, in accordance with earlier findings (4,5). Also chloroquine in a concentration of 0.1 mM had a strong protective effect. Due to the toxicity of chloroquine, cells could not be exposed to this compound for prolonged periods of time. It is, clear, however, that chloroquine gave complete protection against a two hour exposure to diphtheria toxin. In the case of modeccin, ammonium chloride and chloroquine also offered some protection. In contrast, ammonium chloride and chloroquine sensitized the cells to abrin and ricin, even though these have a structure similar to that of modeccin.

The first step in the uptake of the toxins is binding to cell surface receptors. Experiments with ^{125}I-labelled modeccin

TABLE I. EFFECT OF NH_4Cl AND CHLOROQUINE ON THE SENSITIVITY OF HeLa CELLS TO VARIOUS TOXINS

Toxin	Toxin concentration (in ng/ml) required for 50% inhibition of protein synthesis			
	Exposure for 20 hours		Exposure for 2 hours[a]	
	No addition	NH_4Cl	No addition	Chloroquine
Diphtheria toxin	4	>10,000	44	>10,000
Modeccin	0.07	1.0	8.5	56
Abrin	0.5	0.1	5.6	1
Ricin	1.8	0.2	25	0.7

HeLa cells in Gibco Minimum Essential Medium were seeded into Costar$_4$ (Cluster Dish 3524) tissue culture trays at a density of 5×10^4 cells/well. On the next day 10 mM ammonium chloride or 0.1 mM chloroquine was added to some of the wells and then after 30 min increasing amounts of toxins were added. After the exposure times indicated the cells were washed twice with Hank's solution and incubated for 2 hours in serum-free Eagle's Minimum Essential medium containing 21 mM Hepes (pH 7.7), 1/10 the usual amount of leucine and 0.05 µCi/ml of [^{14}C]leucine. Incubation time 37°C. Finally, the cells were dissolved in 0.1 M KOH and the trichloroacetic acid precipitable radioactivity was measured as described (14).

[a] Due to the high toxicity of chloroquine the cells were exposed to toxin in the presence of this compound for only 2 hours. Neutralizing amounts of antitoxins were added and the cells were washed twice with Hanks' solution, incubated overnight and treated as above.

showed that ammonium chloride and chloroquine did not inhibit the binding of modeccin to the cells (data not shown).

When cells are incubated with ^{125}I-labelled modeccin at 0°C, the cell-bound toxin can be entirely removed by incubation with lactose. However, at 37°C, with increasing time an increasing fraction of the cell-bound toxin cannot be washed off the cells (lactose resistant toxin). Similar findings have previously been made with abrin and ricin (13,15). The major part of this toxin is apparently taken up by adsorptive pinocytosis. The time-dependent increase in lactose resistant modeccin observed at 37°C was not inhibited by ammonium chloride or

chloroquine (data not shown). Similar findings have been reported for diphtheria toxin (5).

To see if ammonium chloride and chloroquine inhibit the entry of modeccin into the cytoplasm, cells were exposed to the toxin in the presence of these compounds and then they were washed and further incubated without the additions. Under these conditions, cellular protein synthesis was reduced much as when the cells were preincubated with toxin in the absence of ammonium chloride and chloroquine (data not shown). If specific antitoxin was added before removal of ammonium chloride and chloroquine, protein synthesis was inhibited to a lesser extent, showing that ammonium chloride and chloroquine keep modeccin accessible to neutralization by anti-modeccin. The most plausible interpretation of these results is that ammonium chloride and chloroquine inhibit the uptake of cell-bound toxin which can be internalized once chloroquine and ammonium chloride are removed.

The ability of ammonium chloride to protect against modeccin was observed in several different cell lines (BHK cells, L-cells (A 9), "Lewis lung" murine carcinoma cells, CHO cells, Vero cells, human melanoma cells, and human fibroblasts). The only exception found was a HeLa cell line, Mod ^{R}I, which had been selected for resistance to modeccin (7). This cell line possesses about the same number of modeccin receptors on the surface as the parent HeLa cells and the fraction of cell-bound ^{125}I-labelled modeccin which could not be removed with lactose at 37°C, was the same as in the parent HeLa cells. It is shown in Fig. 1 that 10^4 times more modeccin is required to inhibit protein synthesis in Mod R_1 than in the parent HeLa cells. The interesting finding apparent from Fig. 1b

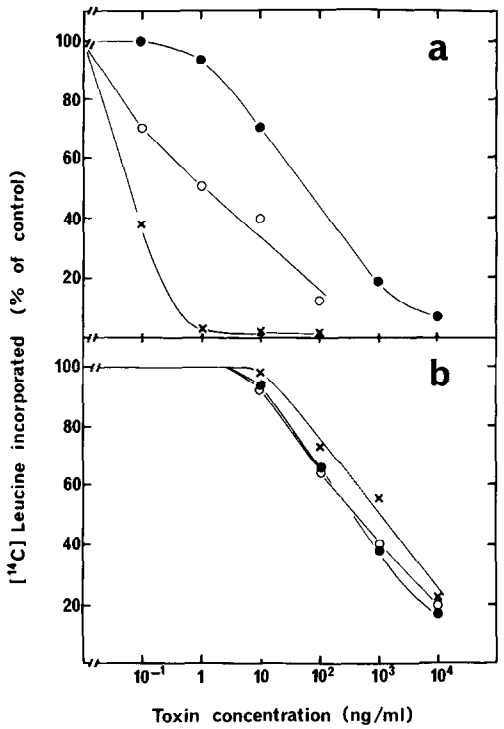

Figure 1: Inability of ammonium chloride to protect Mod ^{R}I cells against the toxic effect of modeccin. HeLa cells (a) and the modeccin resistant variant, Mod $^{R}1$ (b), were grown as in Table 1. Increasing amounts of modeccin were added as indicated and the cells were incubated overnight with and without ammonium chloride present. Finally, the ability of the cells to incorporate [^{14}C]leucine was measured as in Table. 1. (X), no ammonium chloride added; (O), 10 mM ammonium chloride added 30 min before modeccin; (●), 20 mM ammonium chloride added 30 min before modeccin.

is that ammonium chloride did not reduce further the sensitivity of Mod R_1, but rather increased somewhat the sensitivity of these cells to modeccin. The Mod ^{R}I cells are fully sensitive to diphtheria toxin. Interestingly, intoxication of these cells with diphtheria toxin was inhibited by ammonium chloride to the same extent as was the parent HeLa cells (data not shown).

The protected state induced by ammonium chloride apparently requires some time to develop, and, once achieved, it is not rapidly reversible. As shown in Fig. 2 cells preincubated with ammonium chloride for only 1 minute and then washed were not

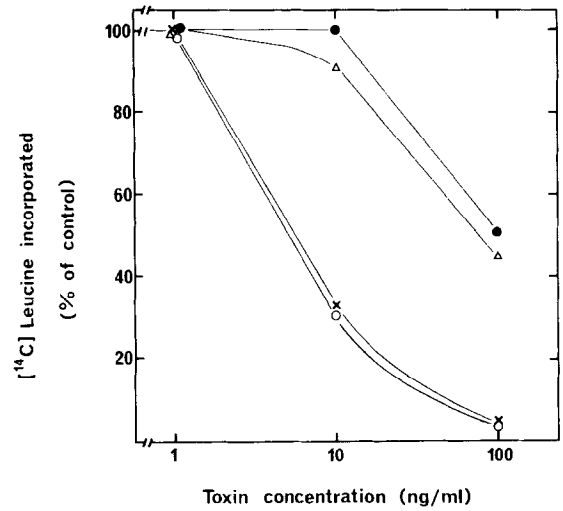

Figure 2: Effect of preincubation of HeLa cells with ammonium chloride on their sensitivity to modeccin. HeLa cells were grown in tissue culture trays as in Table 1 in the absence and presence of ammonium chloride (20 mM). The cells were washed twice in Hanks' solution and fresh medium was added. In some cases 20 mM NH_4Cl was added as well. After 1 minute or 1 hour increasing amounts of modeccin were added, and the cells were incubated for one hour more. The cells were then washed twice in Hanks' solution and medium containing neutralizing amounts of anti-modeccin was added. The cells were incubated at 37°C overnight and their ability to incorporate [^{14}C] leucine was measured as in Table 1. (X), no ammonium chloride added; (O), ammonium chloride present for one minute, and then removed before the addition of modeccin; (△), ammonium chloride present for one hour, and then removed before addition of modeccin; (●), ammonium chloride present one hour before modeccin as well as during the incubation with modeccin.

protected against a subsequent exposure for 1 hour to modeccin. However, cells preincubated with ammonium chloride for 1 hour and then washed were protected almost as well as when ammonium chloride was present during the exposure of the cells to the toxin.

Ammonium chloride and chloroquine have been found to inhibit protein degradation in the lysosomes (16,17). If proteolytic cleavage of modeccin and diphtheria toxin in the lysosomes is necessary to allow the enzymatically active moiety to be released into the cytoplasm, the protective effect of ammonium chloride and chloroquine could conceivably be due to inhibition

of lysosomal activation of the toxins. Ammonium chloride and chloroquine were indeed found to inhibit the degradation of labelled toxins by cells (data not shown). However, it seems unlikely that this is the reason for the protective effect of ammonium chloride and chloroquine. Thus, inhibitors of lysosomal protein degradation, like leupeptin and chymostatin, did not inhibit the toxic effect of diphtheria toxin and modeccin. Furthermore, ammonium chloride and chloroquine keep the toxin accessible to antitoxin. The possibility that toxin taken into lysosomes may still be accessible to and neutralized by antitoxin taken up by pinocytosis is unlikely since preincubation of cells with antitoxin does not protect them from subsequently added toxin (data not shown). The most likely interpretation seems to be that ammonium chloride and chloroquine somehow interfere with the penetration of the toxins through the membrane.

The fact that ammonium chloride and chloroquine do not reduce measurably the transfer of bound toxin to a lactose resistant state supports our earlier suggestion that pinocytosis is not a rate-limiting step in the entry of toxins into the cytoplasm (13) and may not be involved at all in this process. Apparently, the effect of ammonium chloride and chloroquine observed here is due to an effect on the toxin receptors proper, or, on other membrane components involved in the penetration of toxin through the cell membrane.

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