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NATURE OF INITIAL DAMAGE TO EHRLICH ASCITES CELLS CAUSED BY *PRYMNESIUM PARVUM* TOXIN

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SUMMARY

Initial changes in cellular content of cations, small molecules and macromolecules in Prymnesium-toxin-treated Ehrlich ascites tumor cells were studied using the differential flotation technique. Uptake of $^{42}K^+$ and $^{24}Na^+$ by these cells allowed for determination of K^+ and Na^+ influxes.

The damaged cell membrane showed increased permeability, expressed 1 min after toxin treatment, to Na^+ and K^+, as well as to small molecules up to 300 mol. wt. This impairment of osmotic balance can explain the swelling of cells during the first stage of cytotoxic damage.

INTRODUCTION

Prymnesium extracts have various known biological effects, such as hemolytic[1], cytotoxic[2] and bacteriolytic[3] activities and an action on nerve–muscle preparations[4,5]. In Prymnesium ichthyotoxicity, the primary effect observed in the damaged fish is an injury to the selective permeability of the gills[6]. It seems that damage to the cell membrane is common to all these activities.

For a better understanding of the nature of this damage, it is desirable to study a homogenous cell suspension rather than complicated systems, such as tissues or a whole organism. Ehrlich ascites tumor cells are well known as a suitable experimental system, and considerable information about these cells has accumulated. The cytotoxic activity of Prymnesium extracts on Ehrlich ascites cells includes two stages: the first involving water uptake and swelling, and the second leading to cell lysis. The second stage of swelling and lysis is both pH and temperature dependent, and can be inhibited when the pH is changed from 7.4 to 6.4 or when the temperature is lowered from 37 to 27° (ref. 2). It was postulated that water penetration to the cell, expressed in the swelling at the first stage, is due to the impairment of

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osmotic balance of ions and small molecules as a result of damage to the selective permeability of the cell membrane. We have studied changes in the cellular content of ions and small molecules and in the sodium and potassium fluxes induced by the toxin damage, which allow for speculation as to the nature of the damage caused to the cell membrane at the first stage of damage.

MATERIALS AND METHODS

Prymnesium toxin extracts used in this work were a crude extract (prepared as described by DAFNI AND SHILO[2]), having a specific cytotoxic activity of 12 000 cytotoxic units/ml[7], and the purified toxin B described by ULITZUR AND SHILO[8].

Ehrlich ascites tumor cells were propagated, collected and maintained as previously described[2]. The cells were suspended in Krebs–Ringer phosphate buffer (100 mmoles Na^+, 5 mmoles K^+ and 1.3 mmoles Mg^{2+}) at pH 6.4 to a density of $1.2 \cdot 10^7$–$2.5 \cdot 10^7$ Ehrlich ascites cells per ml.

Measurement of cellular content of cations, small molecules and macromolecules

Ehrlich ascites cell suspensions were incubated with 30 cytotoxic units toxin B per ml at 37°. At various time intervals, 0.2-ml aliquots were centrifuged in the Beckman 152 microfuge for 1.5 min at 10 000 × *g* in polypropylene microfuge tubes, using 0.05 ml phthalate ester (whose density for suspensions in various stages of damage was 1.014, 1.015 or 1.017 g/cm[3]) as separating fluid for differential flotation[9]. This technique allowed complete and rapid separation of cells and supernatant, and quantitative measurement of the changes in the composition of the cell pellet. For this, the microfuge tube tip was cut with a sharp razor blade through the separating fluid close above the pellet. The pellet in the tube tip was then resuspended in bidistilled water, stored in the cold overnight, and recentrifuged (15 min at 27 000 × *g*). Na^+ and K^+ content of the supernatant was measured in the Atomic Absorption spectrophotometer (model 153) with NaCl and KCl (0.01 mmole) serving as standards. Special care was taken throughout the experiment to prevent sodium contamination.

To measure protein, RNA, amino acids and small nucleotides, parallel cell pellet samples were prepared and suspended in distilled water as described above. Amino acids and nucleotides were extracted in cold 5 % trichloroacetic acid and determined by the method of MOORE AND STEIN[10] and of MEJBAUM[11] with leucine (B.D.H.) and ribose (Sigma grade) as standards. RNA was extracted in hot 5 % trichloroacetic acid and determined by the method of MOORE AND STEIN[10] using yeast RNA (Sigma type III) as standard. The residue of the hot trichloroacetic acid extraction was boiled in 1 M NaOH and protein was determined by the method of LOWRY *et al.*[12] using bovine serum albumin (Armour Pharm. Co.) as standard.

Determination of $^{24}Na^+$ and $^{42}K^+$ uptakes

The influxes of the cations to the Ehrlich ascites cells were determined on the basis of uptake of radioactively labelled ions. For this purpose we used a radioisotope mixture of ^{42}KCl (800 μC/ml) and $^{24}NaCl$ (150 μC/ml) having specific activities of 350 μC $^{42}K^+$ per mg K^+ and 1150 μC $^{24}Na^+$ per mg Na^+ (obtained from the Israel Atomic Energy Commission, Nuclear Research Centre, Nahal Sorek). In the experiment, 2-ml aliquots of an Ehrlich ascites cell suspension (containing $1.16 \cdot 10^7$ cells per

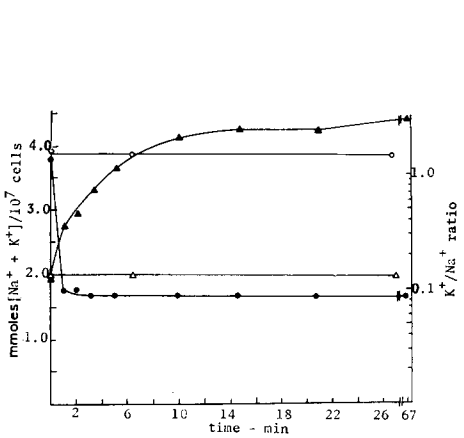
ml in the Krebs–Ringer phosphate buffer at pH 6.4) were incubated in 10-ml flasks at 37° with shaking. After 3 min, 2.5 μl crude toxin per ml was added to the experimental flask (giving a final concentration of 30 cytotoxic units). Isotope-mixture aliquots of 50 μl were added to the experimental flask 1.3 min after toxin addition and to the control flask within 10 min of incubation. Samples (0.1 or 0.2 ml) were removed at various time intervals and treated by the differential flotation method (described above) using separating fluid with a density of 1.015 g/cm^3. The pellets were treated as described by GIBERMAN AND ROSENBERG[13] and their γ-radioactivity measured in a NaI well-type scintillation counter attached to a multichannel analyzer of 100 channels.

RESULTS

Changes in the relative values of K^+ and Na^+ intracellular content

Fig. 1 shows the changes in the ratio of K^+/Na^+ and in the sum total of these cations ($K^+ + Na^+$) inside toxin-treated Ehrlich ascites cells at pH 6.4. The K^+/Na^+ ratio shows a sharp initial decrease (to about one-tenth of control value), and then levels off after the first minute. The sum of the cations ($K^+ + Na^+$) increases sharply during the first minute, continues to increase more gradually during the next nine minutes, and then reaches of plateau. Similar results were obtained in this system at pH 7.4 for the first 10 min of incubation.

Since almost all the potassium ions leak out of the cells during the first minute, it appears that Na^+ continues to penetrate into the damaged cells until osmotic equilibrium is reached.



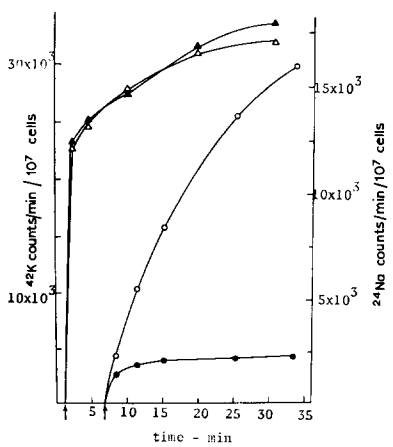


Fig. 1. Changes in cellular content and ratio of K^+ and Na^+ in toxin-treated Ehrlich ascites cells. Experimental techniques are described in the text. K^+/Na^+, ○, $[K^+ + Na^+]$, △; treated cells, black symbols; untreated control, white symbols.

Fig. 2. Kinetics of $^{42}K^+$ and $^{24}Na^+$ uptakes in toxin-treated and control Ehrlich ascites cells. Details of this determination are found in the text. Prymnesium toxin was added to experimental flask at 0 min of incubation time. The arrows indicate when the isotope mixture was added. Experimental cells (toxin-treated): $^{42}K^+$, △, $^{24}Na^+$, ▲; control cells: $^{42}K^+$, ○, $^{24}Na^+$, ●.

K^+ and Na^+ influxes to toxin-treated Ehrlich ascites cells

In order to calculate the cation fluxes into damaged cells, the uptake kinetics of $^{42}K^+$ and $^{24}Na^+$ were measured (Fig. 2). Both curves show two components: an initial rapid rate within the first minute, and a slower continuous rate to the level where isotopic equilibrium is reached. The latter rate most probably represents penetration of the cations through the cell membrane, while the initial rapid rate seems to be a result of adsorption of the ions to the membrane. Therefore, the uptake values obtained in the second interval (from about the first minute to the eighth minute after addition of radioactive cations) were used for calculating influx derivatives of potassium ($\Delta^{42}K^+/\Delta t$) and sodium ($\Delta^{24}Na^+/\Delta t$). The influx values given in Table I were obtained by correlating these derivatives with numbers of Ehrlich ascites cells in the suspension. Because of the irregular shape of treated Ehrlich ascites cells, which makes it difficult to determine their surface area, the influx values are given as flux per cell, and not as flux per unit surface area of the cell membrane.

TABLE I

K^+ AND Na^+ INFLUXES TO TOXIN TREATED AND UNTREATED EHRLICH ASCITES CELLS

The influxes were calculated on the basis of data given in Fig. 2, assuming that the respective amounts of K^+ and Na^+ in the medium (including radioactive isotopes) are 6.9 mmoles and 100 mmoles. Other details in text.

Ehrlich ascites cells	*Influx (10^{-16} M/cell per min)*	
	K^+	*Na^+*
Treated	7.4 ± 0.4	87 ± 5
Untreated	20.7 ± 1.0	7.8 ± 0.4

Leakage of intracellular molecules

Fig. 3A shows the intracellular contents of amino acids and nucleotides in toxin-treated Ehrlich ascites cells, and Fig. 3B that of protein and RNA. While the amino acids and nucleotides rapidly leak out of the cells, no protein or RNA seem to leak out under these conditions (pH 6.4).

DISCUSSION

Permeability of the Ehrlich ascite cell membrane to K^+ and Na^+ is increased as a result of Prymnesium toxin treatment, as evidenced by changes in cellular cation content and the cation influxes. Since the exchanges of K^+ and Na^+ are completed within 0.5–3.0 min at pH 6.4, as well as at pH 7.4, the primary effects of the toxin do not seem to be pH dependent. The further increase in intracellular sodium content during initial toxin damage might be due to gradient diffusion related to the increased cell volume and the relatively high concentration of Na^+ in the buffer medium. The damage caused by the toxin seems to be nonspecific, permitting free movement of both K^+ and Na^+. The decrease in potassium ion influx to the damaged cells (compared to control cells) (Table I) indicates that this damage allows potassium leakage and impairs active transport.

The nonspecific nature of the toxin damage is further evidenced by the leakage of small molecules (amino acids and nucleotides), whose kinetics resembles that of potassium ion leakage. Macromolecules, such as protein and RNA, do not leak at pH 6.4, while they do leak at the second stage (after 10 min) of toxin damage at pH 7.4 (ref. 2). It would seem therefore that the Prymnesium toxin initially produces holes in the cell membrane through which ions and small molecules of molecular weight up to 300 can pass. The conditions stabilizing the membrane of the swollen. cell and preventing its lysis at pH 6.4 deserve further investigation. The effects of Prymnesium toxin was studied by MORAN AND ILANI[14] on an artificial bileaflet membrane formed between two aqueous phases from a solution of lecithin and cholesterol in methyl oleate. The toxin-activated artificial membrane showed reduced resistence to electric current, and did not discriminate between Na^+, K^+ or Cl^-. It was concluded that the drop in resistence is due to a nonspecific increase in membrane permeability to the ions.

The antibiotic-mediated transport in many different cell systems and synthetic membranes[15–17], caused by valinomycin, nigericin and some related antibiotics, also involves changes in membrane permeability to cations and protons. However, these antibiotics differentially influence the transport of specific cations and protons, presumably through their incorporation in the membrane structure where they serve as specific carriers[18]. The membrane damage caused by Prymnesium toxin, on the other hand, involves nonspecific changes in permeability like that caused by certain

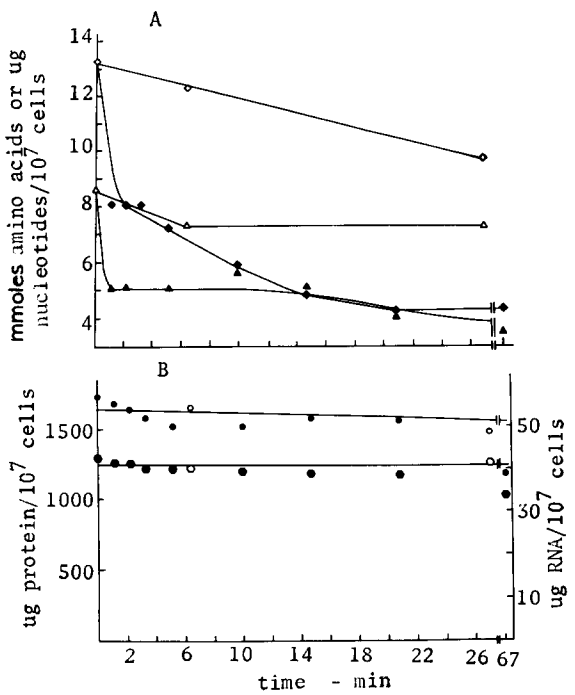


Fig. 3. Changes in cellular contents of amino acids and nucleotides (A), protein and RNA (B). Methods are described in the text. Amino acids, △; nucleotides, ◇; protein, □; RNA, ○; treated cells, black symbols; untreated control, white symbols.

of the polyene antibiotics.[19] It is of interest to compare Prymnesium cytotoxin damage to that of other cytolysins of microbial origin[20], such as the S- and O-streptolysins, the α- and δ-staphylococcal toxins, α-toxin of *Clostridium perfringens*, and subtilisin of *Bacillus subtilis*[21]. In common with Prymnesium toxin, these microbial toxins damage similar types of cells, and their damage involves morphological changes usually expressed in a sequence of swelling, leakage and lysis.

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