PSEUDOMONAS AERUGINOSA TOXINS: EFFECT ON EHRLICH CARCINOMA AND VERO CELLS IN VITRO

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SUMMARY

The main objective of the present investigation was to screen *P. aeruginosa* toxins for *in vitro* activity and investigate biochemical mode of action. Toxins were isolated after concentration of the culture filtrate and separation of the filtrate on Sephadex G-100 column in four fractions. On the basis of primary screening results the dialysate and fractions 3 and 4 effectively inhibited incorporation of [^{14}C]adenine (DNA, RNA) and [^{14}C]valine (protein) into the TCA-insoluble fraction of both Ehrlich ascites carcinoma (EAC) and Vero cells. The toxins also interfered with energy-yielding processes (endogenous respiration). The dialysate decreased the level of ATP in EAC cells after two hours incubation *in vitro*.

KEY WORDS

Pseudomonas aeruginosa toxins, Ehrlich ascites cells, Vero cells, cytotoxicity, respiration, ATP level

INTRODUCTION

Many bacterial toxins affect the functions of mammalian cells by stimulating or inhibiting their growth. Various toxins have different molecular actions on their target cells. The sequence of intracellular events underlying toxin-mediated killing of eukaryotic cells is only partially understood /1/.

Pseudomonas aeruginosa is an increasingly important pathogen, producing a large number of products which have the potential to contribute to pathogenesis /2, 3/. Exotoxin A has been shown to be cytotoxic to a number of cells lines. Pavlovskis and Gordon /4/ have shown inhibition of uptake of [^{14}C]uridine and [^{14}C]amino acids by exotoxin A in the case of Vero cells. Iglewski and Kabat /5/ have reported that *Pseudomonas* exotoxin A inhibits protein synthesis in a rabbit reticulocyte lysate, and Leppla /6/ has shown that it blocks amino acid incorporation in a wheat germ cell-free system. Geokas *et al.* /7/ have reported that in isolated pancreatic acini, exotoxin A inhibits the synthesis and secretion of proteins.

Similarly to diphtheria toxin, exotoxin A inhibits protein synthesis by catalyzing the transfer of the ADP-ribose moiety of oxidized NAD to elongation factor 2. This reaction causes the inactivation of this factor which participates in the elongation process of polypeptide chains /5/. Mohamed and co-workers /8/ have shown inhibition of protein and DNA synthesis by the exotoxin of *P. pseudomallei* in cultured macrophages. The pseudomonal cytotoxin /9, 10/, an acidic protein of approximately 28,000 mol. wt., acts primarily on plasma membranes. The toxin binds to the high affinity binding site on Ehrlich ascites tumor cells /11/ and forms pores with a diameter of about 2 nm, as shown on endothelial cells, rat erythrocytes and Ehrlich cells /12, 13, 14/. Crowell and Lutz /15/ studied binding of the ^{125}I-cytotoxin and the resulting increase of cation permeability in erythrocytes of various mammalian species. Lau and co-workers /1/ have reported that cytotoxin stimulates the release of amylase and protease zymogens with a concomitant increase in membrane permeability and decrease of cellular mRNA levels.

So far, however, little is known about the action of *P. aeruginosa* toxins on biosynthetic and energy-yielding processes in EAC and Vero cells. In the present study we followed the effects of selected toxins on biosynthetic processes, endogenous respiration and level of

ATP. Ehrlich ascites tumor cells have been used extensively as an experimental model for biochemical investigation /16, 17/.

MATERIALS AND METHODS

Ehrlich ascites carcinoma (EAC) cells were maintained and propagated in strain H Swiss albino mice (Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dobra Voda, Czechoslovakia), about 10 weeks old and 20 to 25 g body weight, as described previously /18, 19/. Ascitic plasma was extracted and the incidental layer of erythrocytes was removed /20/. The cells were suspended in Krebs-Ringer phosphate buffer, pH 7.4, without calcium but with ascitic serum (2.5% v/v) and glucose (final concentration, 3.0 mmol/l). The number of cells was adjusted to 5×10^6/ml of medium /19/. All operations were performed at 0-4°C.

Vero cells (USOL, Prague, Czechoslovakia) were obtained from a continuous line of 6-7 days old culture (1200 ml Roux bottles were used). Growth medium was MEM (USOL) with addition of 1% $NaHCO_3$, glutamine (250 μg/ml), gentamycin (40 μg/ml), streptomycin sulfate (100 μg/ml) and penicillin (100 μg/ml), enriched with 10% bovine serum (Bioveta, CSFR). The cells were suspended in saline phosphate medium (PBS) with 5 mmol glucose, pH 7.4. The number of cells was adjusted to 2×10^6/ml of buffer /21/.

Materials

P. aeruginosa strain No. 162 was isolated from a patient with clinical diagnosis of diarrhea. The cultivation of this strain was carried out in proteose peptone medium with intensive aeration during 24 h. The culture filtrate was separated on a Sephadex G-100 column /21/. The culture filtrate yielded four toxic fractions (Fig. 1). The substances were dissolved in PBS in the presence of 5 mmol glucose shortly before use. [8-^{14}C]Adenine sulfate (specific activity, 44 mCi/mmol) and [U-^{14}C] valine (specific activity, 175 mCi/mmol) were received from the Institute for Research, Production and Applications of Radioisotopes, Prague, Czechovslovakia. Other chemicals and enzymes necessary for the determination of ATP were purchased from Boehringer (Mannheim, Germany). All other re-

agents were obtained from Sigma Chemical Co. (St. Louis, MO), USA).

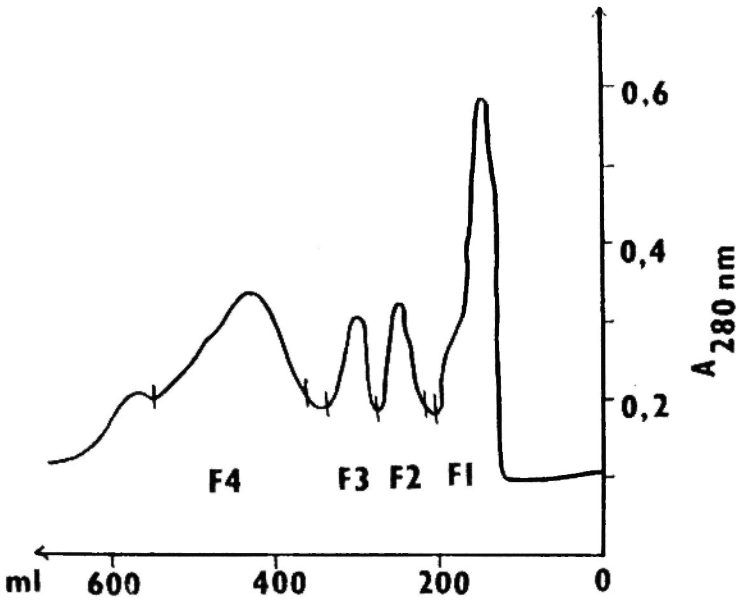

Fig. 1: The separation course of the concentrated culture filtrate (dialysate) of *P. aeruginosa* on a Sephadex G-100 column. M_r fractions: F1 > 100,000; F2 = 40,000; F3 = 25,000; F4 < 10,000.

Primary biochemical screening (cytotoxicity assays)

The procedure used in evaluating the cytotoxic effect of the pseudomonal toxins was similar to that used when testing other metabolic inhibitors /22, 23/. EAC and Vero cells were incubated 1 h in the presence of five selected concentrations of the substance, under defined conditions *in vitro*, and the active synthesis of nucleic acids and proteins was followed. After 1 h of drug exposure, the test-tubes were transferred into an ice bath. [8-^{14}C]Adenine was added to the first series to a final concentration of 0.187 μCi per 1.02 μg and L-[U-^{14}C]valine was added to the second series to a final concentration of 0.165 μCi per 2.64 μg (EAC). In the case of Vero cells, [8-^{14}C]adenine was added to a final concentration of 0.005 μCi

per 4 μg and L-[U-^{14}C]valine to a final concentration of 0.125 μCi per 100 μg. Both series were again incubated for 1 h at 37°C. In control experiments only Krebs-Ringer phosphate medium (EAC) or PBS (Vero) were used. Incorporation was terminated by adding 1 ml of 5% trichloroacetic acid to each test-tube in an ice bath. The samples were centrifuged at 2000 *g*, the precipitate washed with 2x5 ml of cold 2.5% TCA. After adding 6 ml Brayuv scintillation solution (Spolana, Czechoslovakia) to the precipitate, the radioactivity was measured using the liquid scintillation system LS-100C (Beckman, USA).

Endogenous respiration

Cellular respiration was measured with a Clark-type oxygen electrode (Gilson Medical Electronics, Yellow Springs, USA). Endogenous respiration of Ehrlich ascites carcinoma cells was determined as described previously /24/. Vero cells were harvested from a 4-6 days old culture, washed in a saline phosphate medium, suspended in the same medium, and the number of cells was adjusted to 12x10^6/ml of medium. The toxins were dissolved in saline phosphate medium shortly before use. Rates of oxygen uptake by Vero cells were measured in a thermostatically controlled (37°C) reaction vessel equipped with a stirring device. Oxygen consumption was monitored for approximately 10 min and the linear portion of the oxygen consumption curve was utilized to calculate oxygen consumption rates. The respiratory rate was expressed as nanomoles of oxygen consumed per min and corresponding dry weight of the cells.

ATP determination

ATP level determination was carried out according to the method described in detail in a previous paper /25/.

Protein determination

Protein concentration in the cell suspension was estimated according to the method of Lowry *et al.* /26/.

RESULTS

Cytotoxic activity was assessed as the degree of incorporation inhibition of [^{14}C]adenine and [^{14}C]valine into the TCA-insoluble fraction of both EAC and Vero cells under defined *in vitro* conditions. These conditions ensured an active synthesis of nucleic acid and proteins for at least 2 h /19/. The cells were preincubated for 1 h in the presence of at least four concentrations of the toxins. Labeled precursors were added to the suspension and after incubation for another 1 h the incorporation was stopped by adding TCA.

The results given in Table 1 show that the dialysate and the fractions F3 and F4 (Fig. 1) affected incorporation of [^{14}C]adenine, [^{14}C]valine or both into the TCA-insoluble fraction of EAC cells. The degree of inhibition of the two precursors was different. The dialysate and fraction F3 were the most effective toxins. This is shown not only by the percentage inhibition but also by the IC_{50} values. The dialysate significantly affected processes connected with incorporation of [^{14}C]adenine (compare IC_{50} for adenine = 80 μg/ml), whereas valine incorporation was affected to a lesser extent (IC_{50} valine > 200 μg/ml). Fractions F1 and F2 were less effective. Fraction F3 inhibited in particular the synthesis of nucleic acids, indicated by the incorporation of [^{14}C]adenine; the IC_{50} value of adenine was 140 μg/ml. Incorporation of valine was less affected, which is evident from the IC_{50} value of valine (173 μg/ml). Fraction F4 is interesting because it markedly inhibited incorporation of valine without markedly inhibiting adenine incorporation.

On the basis of our previous results /27, 28/, it is convenient to use the IC_{50} adenine:IC_{50} valine ratio (R); this enables the differentiation of inhibitors into three groups, according to their primary mode of action. The R values are quite different in the three groups, and it is possible to deduce from the R value of a substance whether it affects more markedly either energy metabolism or the synthesis of proteins or nucleic acids. The ratio R for fraction F3 in Ehrilich ascites cells is 0.809. On the basis of our previous results, we conclude that such a ratio is typical for compounds which interfere with the energy-generating system of cells, which have R values in the range 0.55-2.25; the R values for the other two groups are quite different /27, 28/.

Analogous experiments with Vero cells are presented in Table 2. From these findings, it follows that the dialysate and fractions F1, F3 and F4 are the most effective toxins. This is in agreement with the

TABLE 1

Primary biochemical screening of *P. aeruginosa* toxins. The measure of the cytotoxic effect was the degree of inhibition of [^{14}C]adenine (a) and [^{14}C]valine (b) incorporation into the TCA-insoluble fraction of EAC cells after 2 h incubation *in vitro*.

Substance		μg/ml							IC_{50} (μg/ml)	R
		0	6.25	12.5	25	50	100	200		
		Incorporation in cpm (percent inhibition in brackets)								
Dialysate	(a)	5219(0)	4824(7.57)	6299(+20.69)	4857(6.96)	3074(41.1)	2230(57.27)	n.d.	80	?
	(b)	5047(0)	5666(+12.3)	6023(+19.3)	4614(8.6)	3494(30.8)	3498(30.7)	3316(34.3)	>200	
F1		8503(0)			8871(+4.3	7711(9.3)	7488(11.9)	7406(12.9)	>200	?
		5830(0)			4836(17.1)	4004(31.3)	4947(15.2)	4572(21.6)	>200	
F2		8503(0)			8659(+1.8)	9400(+10.5)	9224(+8.5)	9325(+9.7)	>200	?
		5830(0)			5851(+0.4)	5162(11.5)	6058(+3.9)	5020(13.9)	>200	
F3		3686(0)			1470(60.1)	1551(57.9)	1911(48.2)	1754(52.4)	140	0.809
		2290(0)			1728(24.5)	1565(31.7)	1808(21.0)	914(60.1)	173	
F4		8503(0)			9617(+13.1)	n.d.	8262(2.8)	6055(28.8)	>200	?
		5830(0)			5348(8.3)	5214(10.6)	2363(59.5)	1429(75.5)	91	

+Stimulation over 100% against control sample. Substances were dissolved in Krebs-Ringer phosphate medium shortly before experiments. n.d. = not determined, R = IC_{50}adenine:IC_{50}valine.

TABLE 2

Primary biochemical screening of *P.aeruginosa* toxins. The measure of the cytotoxic effect was the degree of inhibition of [^{14}C]adenine (a) and [^{14}C]valine (b) incorporation into the TCA-insoluble fraction of Vero cells after 2 h incubation *in vitro*.

Substance		μg/ml						IC_{50} (μg/ml)	R
		0	12.5	25	50	100	200		
		Incorporation in cpm (percent inhibition in brackets)							
Dialysate	(a)	1100(0)	875(30.5)	746(32.2)	749(32.0)	699(36.5)	381(65.4	150	1.13
	(b)	4425(0)	4139(6.5)	3635(17.9)	3442(22.3)	2560(42.0)	1381(68.8)	132	
F1		1600(0)	1556(2.8)	1225(23.5)	1040(35.0)	900(43.8)	756(52.8)	137	0.68
		2417(0)	2905(+20.1)	2894(+19.7)	2433(+0.6)	2017(16.6)	1228(49.2)	200	
F2		930(0)	1111(+19.4)	946(+1.7)	838(9.9)	956(+2.8)	718(27.8)	>200	?
		2120(0)	3685(+73.8)	2117(0.2)	1992(6.1)	2051(3.3)	1047(50.7)	>200	
F3		750(0)	836(+11.4)	746(0.6)	694(7.5)	575(23.4)	293(61.0)	173	0.86
		5450(0)	4039(26.0)	4181(23.3)	3612(33.8)	3431(37.0)	1421(74.0)	135	
F4		1425(0)	1431(+0.4)	1036(27.3)	922(35.3)	672(52.9)	543(61.9)	85	1.0
		4900(0)	4746(3.2)	2889(41.1)	2979(39.2)	2259(53.9)	1149(76.6)	85	
CT		1849(0)	1292(30.2)	1190(35.7)	1105(44.3)	1031(50.3)	1099(50.6)	200	?
		3140(0)	1299(58.7)	1026(67.4)	947(70.0)	880(72.0)	603(80.0)	<12.5	

+stimulation over 100% against control sample. Substances were dissolved in PBS medium shortly before experiments.
R = IC_{50}adenine:IC_{50}valine, CT = cholera toxin.

results presented in Table 1. The dialysate affected incorporation of [^{14}C]adenine and [^{14}C]valine approximately to the same extent as is shown by the IC_{50} values for these precursors. It is interesting that in the case of Vero cells, the synthesis of proteins (Table 2) is the "more sensitive", while in EAC cells it is the synthesis of nucleic acids. Fraction F1 affected incorporation of both precursors into EAC cells to only a small extent. In the case of Vero cells, however, fraction F1 significantly influenced the incorporation of [^{14}C]adenine, and at the highest concentration (200 μg/ml) it also inhibited the incorporation of valine. Fraction F2 did not cause a pronounced inhibition of incorporation of either precursor in both EAC and Vero cells. Fraction F4 showed a similar effect on EAC (Table 1) and Vero cells (Table 2) in the case of inhibition of [^{14}C]adenine. This is clear from the IC_{50} values which are very similar (IC_{50}adenine for EAC = 91 and for Vero cells = 85 μg/ml). Choleragen had more effect on protein synthesis than on biosynthesis of nucleic acids in Vero cells (Table 2). Vero cells are in general "more sensitive" than EAC cells.

The values of the ratios IC_{50}adenine:IC_{50}valine for Vero cells are in the range of 0.68-1.13. This ratio is comparable with that obtained for EAC cells with fraction F3 (Table 1). It indicates that the mechanism affecting biosynthetic processes is very similar in both types of cells.

Biosynthesis of macromolecules, indicated by incorporation of [^{14}C]adenine and [^{14}C]valine to the corresponding macromolecules of EAC and Vero cells is an energy requiring process. In the next experiment, we followed the effects of *P. aeruginosa* toxins on endogenous respiration of both tumor and normal cells. As shown in Table 3, the dialysate inhibited endogenous respiration of EAC cells in a concentration dependent manner. On the other hand, fraction F1 at the lowest concentrations does not affect oxygen consumption and at the highest concentrations causes a relatively low inhibition of endogenous respiration. The other fractions did not show a marked effect on oxygen consumption of EAC cells.

Similar results were also obtained in Vero cells (Table 4). The most effective inhibitor was the dialysate, which significantly inhibited oxygen consumption, at lower concentrations than in the case of EAC cells (Table 3). This observation is in agreement with previous findings (Tables 1 and 2). Fraction F1 is effective only at higher concentrations than in EAC cells. The other fractions did not affect oxygen consumption of Vero cells.

TABLE 3
Effect of *P. aeruginosa* toxins on endogenous respiration of EAC cells

Substances	μg/ml					
	0	50	100	150	200	250
	nmoles O_2/min/16 mg dry weight					
Dialysate	48.9	48.9	31.9	15.0	n.d.	n.d.
F1	48.9	48.9	48.9	48.9	42.3	42.3
F2	48.9	48.9	48.9	48.9	48.9	46.0
F3	48.9	48.9	48.9	45.1	n.d.	n.d.
F4	48.9	48.9	48.9	48.9	48.9	47.9

Toxins were dissolved in Krebs-Ringer phosphate medium shortly before experiments.
F1-F4 = fractions; n.d. = not determined

TABLE 4
Effect of *P.aeruginosa* toxins on endogenous respiration of Vero cells

Substances	μg/ml							
	0	12.5	25	37.5	50	62.5	125	250
	nmoles O_2/min/10 mg dry weight							
Dialysate	29.1	25.4	12.4	5.4	n.d.	n.d.	n.d.	n.d.
F1	30.9	n.d.	n.d.	n.d.	n.d.	27.3	21.8	10.9
F2	27.3	n.d.	n.d.	n.d.	n.d.	27.3	27.3	27.3
F3	20.0	n.d.	n.d.	n.d.	n.d.	20.0	20.0	20.0
F4	25.4	n.d.	n.d.	n.d.	n.d.	20.0	20.0	20.0

Toxins were dissolved in phosphate buffer saline medium shortly before experiments.
F1-F4 = fractions, n.d. = not determined

TABLE 5
Effect of *P. aeruginosa* toxins on ATP level in EAC cells after 2 h incubation *in vitro*

Substance	μmol/l			
	0	25	50	100
	nmol ATP/mg			
Dialysate	5.47(0)	4.86(11.1)	4.26(22.1)	3.85(29.6)
Fraction (F1)	5.47(0)	5.68(+3.8)	n.d.	5.27(3.7)

The numbers in parentheses represent percentage of inhibition or stimulation (+).
Toxins were dissolved in Krebs-Ringer phosphate medium shortly before experiments.
n.d. = not determined

To obtain direct evidence of interference of *P. aeruginosa* toxins with energy metabolism, the ATP level in EAC cells was followed after 2 h incubation. Only two toxins were tested, i.e., the dialysate and fraction F1. As shown in Table 5, the dialysate decreased the ATP level in EAC cells in proportion to its concentration. Fraction F1 did not influence the ATP level to any marked degree at the concentrations tested. This is not surprising because fraction F1 was not very effective in EAC cells (Tables 1 and 3).

DISCUSSION

The values from biochemical screening represent the first fundamental information about the cytotoxic activity of pseudomonal toxins. The data obtained in a relatively short time indicate whether the tested substance has any cytostatic activity at all, and perhaps also indicate a possible mode of action (R). We have previously reported a rapid radiometric *in vitro* technique of primary screening for anticancer substances /27, 28/. This method, which measures the drug-induced inhibition of [^{14}C]adenine and [^{14}C]valine incorporation, is relatively simple, reliable and sensitive.

From the results of Tables 1 and 2, it can be seen that the dialysate and fractions F3 and F4 have the most toxic effect in both EAC and Vero cells, and also fraction F1 in Vero cells. Though the degree of inhibition of incorporation of the two precursors is different, the mode of action is likely to be similar, indicated not only by the IC_{50} values but mainly by the ratio IC_{50}adenine:IC_{50}valine (R). As has been noted, these ratios are in the range of 0.68 to 1.13, which are typical for substances interfering with energy metabolism /27/.

In order to elucidate at least partly the mechanism of inhibition of incorporation of the [^{14}C]precursors, the effect of some *P. aeruginosa* toxins on endogenous respiration of both cell types (Tables 3 and 4) and ATP level (Table 5) was followed. From the results presented above (Tables 3-5), it follows that the inhibition of the [^{14}C]precursors' incorporation could be the result of interference with the formation or utilization of ATP.

In each case the ratio values R as well as the results in Tables 3 and 4 and ATP level (Table 5) confirm our suggestion that the observed inhibition of incorporation of both precursors could be the result of the dialysate's interference with energy-producing pro-

cesses. This is also the case to a certain extent for F1 and F2 in Vero cells. Differences in the effects on EAC and Vero cells can be explained by the nature of these cells (cancerous and normal) and also by the structure of their cytoplasmic membranes. Fractions F1 and F2 were relatively weakly effective in the case of EAC cells, shown by the results in Tables 1 and 3. While the fraction F3 inhibited both [^{14}C]adenine and [^{14}C]valine incorporation, in EAC cells it had very little effect on endogenous respiration. This means that the inhibition cannot be explained by interference with the formation or utilization of energy; fraction F3 interferes with processes more directly connected with the incorporation of [^{14}C]precursors. The F4 fraction (EAC cells) is similar.

In the Vero cells fraction F4 significantly interferes with incorporation of both [^{14}C]adenine and [^{14}C]valine. The IC_{50} values are the same, i.e., 85 μg/ml. This fraction is the most effective on Vero cells. F4 at the highest concentrations also decreases oxygen consumption in Vero cells (Table 4). The dialysate also significantly decreases oxygen consumption in Vero cells but at lower concentrations. It is not possible to explain the differences between the dialysate and the F4 fraction from these findings. The F4 fraction has the lowest molecular weight among the tested fractions (Fig. 1). We can suppose that the F4 fraction has a membrane effect which is more active in the case of Vero cells. This cytolytic effect would be responsible not only for inhibition of oxygen consumption in the cells, but also for inhibition of biosynthetic processes. Moyer *et al.* /29/ observed that Shiga toxin purified from *Shigella dysenteriae* was directly cytotoxic to human intestinal cells. It inhibited protein and DNA synthesis, changed permeability of membranes and caused death of cells. Recently, Prior *et al.* /30/ studied pseudomonal exotoxin which kills eukaryotic cells possessing specific toxin receptors. The toxin enters the cell via receptor-mediated endocytosis and is translocated into the cytosol where it catalyzes the ADP ribosylation of elongation factor 2 which leads to cell death.

Although in the case of many anti-neoplastic agents, attention has been focused upon their effects on DNA, RNA and protein synthesis, work by Farber /31/ and others indicates that the inability to synthesize ATP in a cell leads to multiple secondary derangements in cellular metabolism.

Further work is necessary to investigate the exact mechanism(s) of action of *P. aeruginosa* toxins.

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