# ACTION OF OCHRATOXIN A ON CULTURED HEPATOMA CELLS – REVERSION OF INHIBITION BY PHENYLALANINE

E.-E. CREPPY, A. A. J. LUGNIER, G. BECK, R. RÖSCHENTHALER* and G. DIRHEIMER

*Laboratoire de Toxicologie et de Biologie Moléculaires, Faculté de Pharmacie, Université Louis Pasteur et I.B.M.C. du C.N.R.S., 15 rue Descartes, Strasbourg, France* and **Institut für Mikrobiologie, Universität Münster, Westphalen, GFR*

Received 18 May 1979
Revised version received 8 June 1979

## 1. Introduction

Ochratoxin A (OT-A), a mycotoxin composed of a phenylalanine moiety bound by an $\alpha$-amide bond to a chlorinated dihydroisocoumarin is highly toxic for some microorganisms [1] as well as for higher animals [2] essentially causing liver and kidney damage. It inhibits protein synthesis by competition with phenylalanine in the phenylalanyl-tRNA synthetase catalyzed reaction as has been shown either with bacterial or with yeast systems [1,3,4]; but on animal cells neither this effect nor the effect on nucleic acid synthesis has yet been tested. In this paper we have determined the amount of OT-A necessary to completely inhibit protein synthesis in hepatoma tissue culture cells (HTC) and have also tested its activity on DNA and RNA synthesis. Finally, since OT-A is a competitive inhibitor with respect to phenylalanine, we have tested the capability of this amino acid for reversion of the mycotoxin action.

## 2. Materials and methods

HTC cells were derived from a malignant tumour of rat liver parenchymal cells (Morris hepatoma 7288c) and adapted to growth in cell culture by Thompson et al. [5]. Cells (initial concentration: $2.8 \times 10^5$ cells/ml) were grown in Swim's medium 77 supplemented with 10% calf serum (Lab. Gibco-Flobio France) at 37°C in shaker suspension cultures (30 or 45 ml). Their generation time was approximately 18–28 h, and growth continued for 3 to 4 days, before reaching stationary phase.

For determinations of cell multiplication 1 ml samples were removed and the number of viable cells was determined by Trypan blue dye exclusion in a Neubauer microcytometer. The rate of cell multiplication was defined as the number of viable cells in a treated culture with respect to the initial number of viable cells. We have assumed that a certain concentration of toxin has a cytostatic effect when the number of viable cells remains constant, and that it has a cytotoxic one when this number diminishes.

For determinations of HTC cell protein synthesis the cells were centrifuged at $600 \times g$ for 5 min at 4°C. The pellet was washed 4 times with 10 ml Swim's medium 77. Washed cells were then resuspended in Swim's medium 77, which is 200 $\mu$M in leucine, but without calf serum, to a final concentration of $3 \times 10^5$ cells/ml in shaker suspension culture of 30 ml. It must be emphasized that Swim's medium 77 contains 100 $\mu$M phenylalanine. OT-A and U-[$^3$H]-leucine (14 pmol/30 ml) (C.E.A., Saclay, France: spec. act. 35 Ci/mol) were added simultaneously and the cells were incubated in a shaking waterbath. Samples of 200 $\mu$l were removed and transferred into 1 ml of 1 M NaOH and incubated at 25°C for 30 min. Protein was precipitated by addition of 1 ml of cold 20% trichloroacetic acid. After 60 min at 4°C the contents of each tube were filtered through Whatman GF/C glass filters under vacuum. The filters were washed twice with 5% trichloroacetic acid and once with 95% ethanol and were dried 30 min at 60°C. Radioactivity was counted in an Intertechnique SL32 scintillation counter as described earlier [6].

For cell DNA and RNA synthesis 45 ml HTC cell

suspension cultures, with approx. $3 \times 10^5$ cells/ml, were incubated at 37°C on a hanging bar magnetic stirrer with 1.6 mg OT-A per flask and 0.3 ml [6-$^3$H]-thymidine or [6-$^3$H]uridine (spec. act. 46 Ci/mol). Every 15 min 0.2 ml suspension culture was removed and the reaction was stopped by addition of 1 ml cold 20% trichloroacetic acid. The reaction mixture was filtered through Whatman GF/C glass filters under vacuum. The filters were washed 5 times with 10% cold trichloroacetic acid, twice with 95% ethanol and dried at 60°C for 1 h. Each filter was burned in an Intertechnique 4101 liquid scintillation sample oxidizer and the so obtained $[^3H]H_2O$ samples were collected in vials containing 20 ml of scintillation mixture (dioxane 700 ml, toluene 300 ml, naphthalene 20 g/l and butyl-PBD 7 g/l) and counted. All assays were done in triplicate and only the average values are reported.

For the study of the reversibility of OT-A action 80 ml of cell suspension ($3 \times 10^5$ cells/ml) in Swim's medium 77 were treated with 90 μM OT-A and 0.5 μCi of [U-$^3$H]leucine. Non-radioactive phenylalanine (50 μM, pH 7.4) was added at different times.

## 3. Results

Figure 1 shows that between an OT-A concentration of 40 μM and 45 μM a cytostatic effect on HTC cells is obtained. Higher concentrations have a cytotoxic effect. At a 90 μM concentration of OT-A the [$^3$H]leucine incorporation into proteins is inhibited after about 30 min and reaches a plateau at 5 h (fig.2A). A second experiment was done in which the cells were preincubated with 90 μM OT-A for 5 h and [$^3$H]leucine was added after this time. No leucine was incorporated into cell proteins indicating that protein synthesis was completely blocked by OT-A after this time (not shown).

The action of OT-A on the synthesis of DNA in HTC cells was followed by the use of [$^3$H]thymidine. No inhibition was found for 5 h (fig.2B). RNA synthesis continued at control rates for 150 min in the presence of OT-A; after that time the [$^3$H]uridine incorporation ceased, suggesting inhibition of RNA synthesis from that time onward (fig.2C).

### 3.1. *Reversibility of the action of OT-A*

Since OT-A acts with respect to phenylalanine as a reversible inhibitor on phenylalanyl-tRNA synthetase it was of interest to test if its action on cell growth and on cell protein synthesis could be reversed by phenylalanine. Preliminary assays were done to test the action of this amino acid on cell protein synthesis. When 25 μM and 50 μM phenylalanine were added to the Swim's medium 77, which already contains 100 μM phenylalanine, the cells incorporated [$^3$H]-leucine into proteins at control rates, whereas when 100 μM phenylalanine is added a slight inhibition was found. We, therefore, add 50 μM of phenylalanine for the subsequent assays.

The inhibition of cellular growth by a 90 μM concentration of OT-A could be completely avoided by the addition of 50 μM of phenylalanine to the cell medium simultaneously with the mycotoxin (fig.3). Under the same conditions no inhibition of incorporation of [$^3$H]leucine into proteins (fig.4A) or [$^3$H]-uridine into RNA was observed.

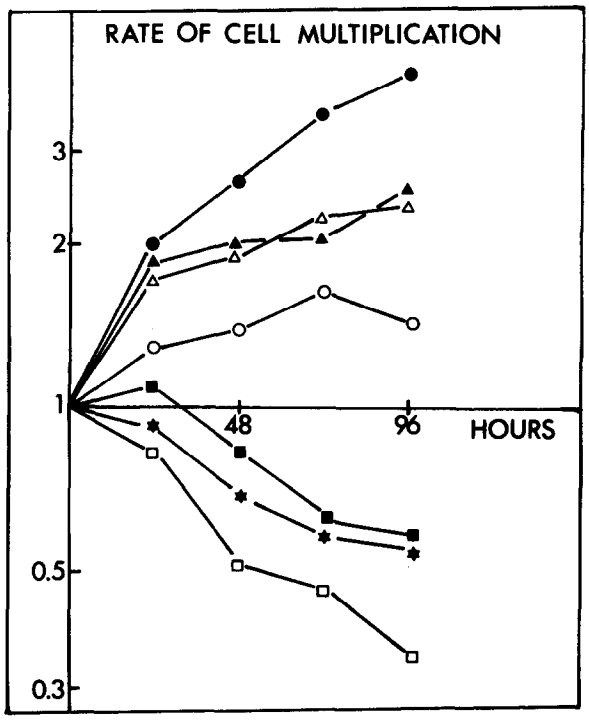
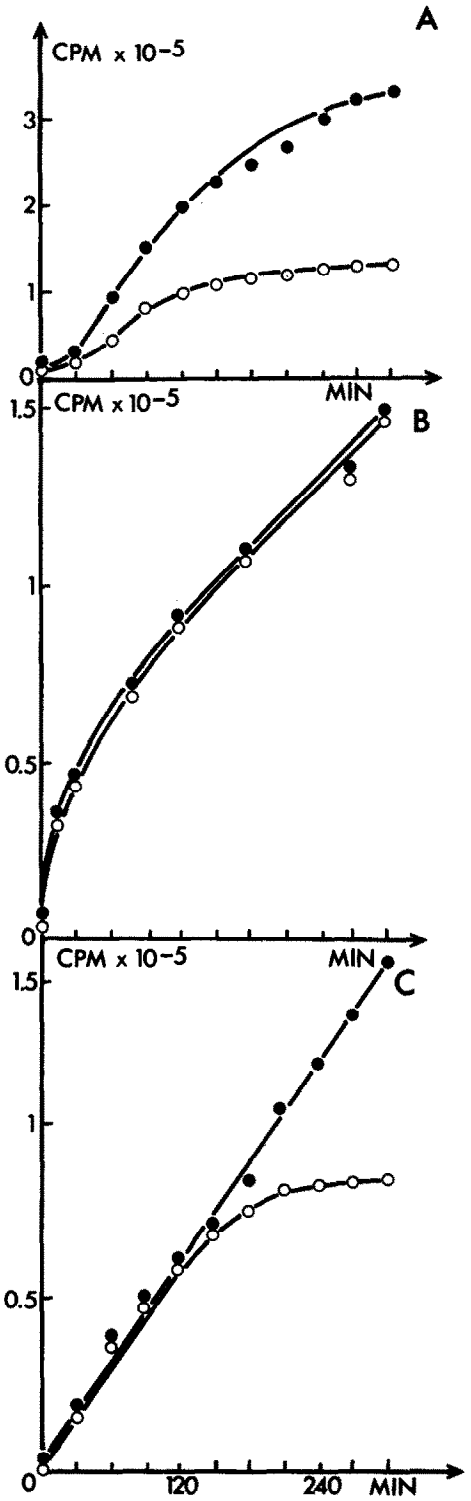



Fig.1. Rate of HTC cell multiplication in presence of different concentrations of OT-A. ●——● control; △——△ OT-A 13 μM; ▲——▲ OT-A 27 μM; ○——○ OT-A 40 μM; ■——■ OT-A 45 μM; ★——★ OT-A 60 μM; □——□ OT-A 90 μM.



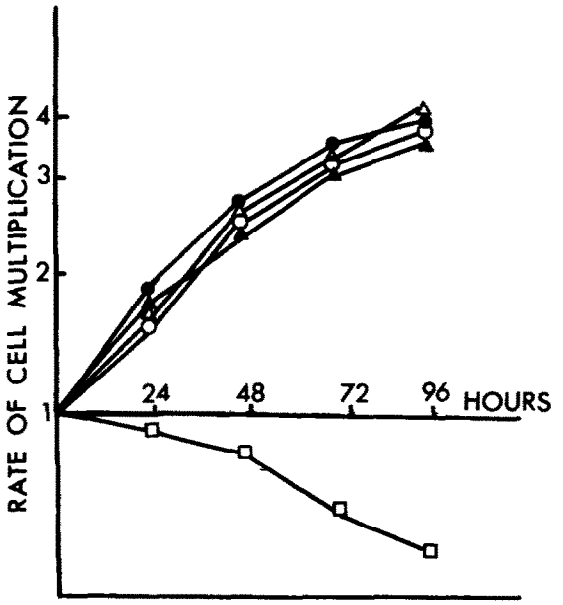




Fig.3. Impairment of the inhibition of OT-A on HTC cell multiplication by added phenylalanine. ●——● control; △——△ control + 50 μM Phe; ▲——▲ control + 100 μM Phe; ○——○ OT-A 90 μM + 50 μM Phe; □——□ OT-A 90 μM. *Remark:* All indicated phenylalanine concentrations represent phenylalanine added to Swim's 77 medium which already contains 100 μM phenylalanine.

If however the phenylalanine was added to the cells after 60 min or 120 min of incubation with the toxin, the protein synthesis started again, but after a lag time of 2 h (fig.4B,C).

A similar response was observed with RNA synthesis.

## 4. Discussion

The cytostatic and cytotoxic effect of OT-A on HTC cells parallels an inhibition of protein synthesis starting 30 min after addition of a concentration of 90 μM of OT-A. 5 h after addition no more protein synthesis takes place.

Fig.2. Inhibition of protein (A), DNA (B) and RNA (C) synthesis by OT-A. ●——● control; ○——○ OT-A 90 μM

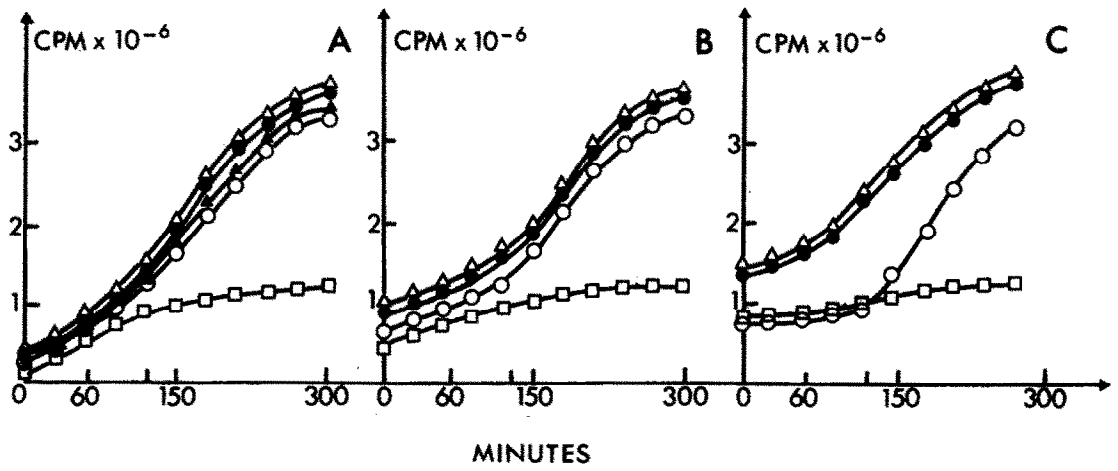



Fig.4. HTC protein synthesis. Reversibility of the OT-A inhibition by phenylalanine. (A) Phenylalanine added at zero time. (B) Phenylalanine added after 60 min of incubation. (C) Phenylalanine added after 120 min of incubation. ●——● control; △——△ control with 50 μM phenylalanine; ▲——▲ control with 100 μM phenylalanine; ○——○ OT-A 90 μM + 50 μM phenylalanine; □——□ OT-A 90 μM. Same remark as in fig.3.

The action of OT-A on RNA synthesis in HTC cells is delayed and begins only after treatment for 150 min. After 5 h no more RNA synthesis takes place. Therefore the effect of OT-A on RNA synthesis is probably a consequence of the effect on protein synthesis. No inhibition of DNA synthesis takes place into this time range.

As we have shown, OT-A-induced inhibition of protein synthesis can be completely prevented if phenylalanine is present simultaneously in the cell culture medium at a concentration of 50 μM. This effect has never been observed before in mammalian cells. The reversal of inhibition could be interpreted as an impairement of entry of OT-A into the cells by a competition with some cell receptor for phenylalanine. But even if phenylalanine is added to the cells 2 h after OT-A (at a time when OT-A has almost completely blocked cell protein synthesis, fig.2A), its action can be reversed. This reversal takes place after a lag time which probably represents the time necessary for phenylalanine to reach a concentration inside the cell which is high enough to compete effectively with the mycotoxin.

It should be of considerable interest, to find out if in OT-A poisoned animals phenylalanine will behave as an antidote. Experiments in this direction are in progress.

## Acknowledgements

This work was supported by grants from the 'Institut National de la Santé et de la Recherche Médicale' (Contrat no. 76.10.613) and from NATO (Research Grant no. 1749). We wish to thank Dr Sam Barranco for reviewing the manuscript.

## References

[1] Konrad, I. and Röschenthaler, R. (1977) FEBS Lett. 83, 341–347.
[2] Harwig, J. (1974) Ochratoxin A and related metabolites, in: I.F.H. Purchase, pp. 345–367, Amsterdam, Oxford, New York.
[3] Bunge, I., Dirheimer, G. and Röschenthaler, R. (1978) Biochem. Biophys. Res. Commun. 83, 398–405.
[4] Creppy, E.-E., Lugnier, A. A. J., Fasiolo, F., Keller, K., Röschenthaler, R. and Dirheimer, G. (1979) Chem. Biol. Interactions 24, 257–261.
[5] Thompson, E. B., Tomkins, G. M. and Curran, J. F. (1966) Proc. Natl. Acad. Sci. USA 56, 296–303.
[6] Lugnier, A. A. J., Le Meur, M.-A., Gerlinger, P. and Dirheimer, G. (1976) Eur. J. Toxicol. 6, 323–333.