INHIBITION OF POST-UV IRRADIATION GROWTH IN THE DARK OF *TETRAHYMENA PYRIFORMIS* BY CAFFEINE AND THE ONCOGENIC MYCOTOXIN LUTEOSKYRIN

R.F. MOUTON and P. FROMAGEOT

Service de Biochimie, Département de Biologie, Centre d'Etudes Nucléaires de Saclay, F. 91-Gif-sur-Yvette, France

Received 1 February 1971
Revised version received 15 April 1971

1. Introduction

In the field of environmental life sciences, much attention is now directed toward appraisal of the long term impact of microconcentrations of toxic agents relevant to induction of disease in man. Not all toxic environmental stresses are the end result of man's technology; some arise from a natural sequence of events in the environment through bacterial, viral fungal agents. Thus, for instance, oncogenic molds contaminate rice (*Penicillium islandicum Sopp*) or cereals and peanuts (*Aspergillus flavus*), constituting a Public Health problem for both developing and industrialized countries [1]. The toxic and oncogenic [2–4] properties of *P. islandicum Sopp* prompted the isolation of active components. Luteoskyrin [5, 6], a yellow pigment, is one of them, and its structure has been established [7] as a condensation between two modified octo hydroxy anthraquinones.

Long term feeding tests of luteoskyrin in mice and rats has been reported to provoke liver degeneration and tumours [8–10]. Inhibition of replication has been observed by direct action of luteoskyrin on isolated mammalian cell cultures like rat Changs' liver cells, mice HeLa BB cells in the 10^{-6} M concentration range [11]. 10^{-6} M luteoskyrin inhibits in Ehrlich ascites tumour cells, replication and transcription [12] but induces no modification of energy metabolism, as shown by the aerobic and anaerobic production of lactic acid [13].

In vitro studies have shown binding of luteoskyrin to DNA [14]. A specific complex is formed with purine residues, provided they are unpaired [15, 16]. Thus, the degree of single strandedness of DNA is an important factor for the extent of specific complex formation. This observation prompted us to postulate that the DNA repair function must appear in vivo more sensitive than any other DNA function to the inhibiting action of luteoskyrin, since during repair process following UV irradiation, segments of nucleotides are excised by specific nuclease exposing the complementary single stranded DNA segment to quick interaction with luteoskyrin which could subsequently block the repolymerisation step.

It has been shown that the total length of repaired DNA segment in UV irradiated *Tetrahymena pyriformis* is 5,000 nucleotides [17]. Therefore this organism was chosen for the present investigation.

We compared the growth rate of UV irradiated *Tetrahymena pyriformis* following addition of luteoskyrin and caffeine: caffeine is an effective and specific inhibitor of UV (but not gamma) DNA lesion repair in a variety of organisms [18, 19]. The luteoskyrin and caffeine concentrations used do not affect DNA replication or transcription function in controls, as judged by rate and extent of multiplication. It was found that 2×10^{-7} M luteoskyrin exhibits the same post irradiation growth inhibition as 1×10^{-3} M caffeine, a finding which suggests that luteoskyrin is an efficient anti-repair agent.

2. Materials and methods

An axenic amicronucleate G.L. strain of *Tetrahymena pyriformis* George (Cambridge) was grown in Wiame's culture vessels containing sterile 2% proteose peptone no. 3 Difco. The vessels were maintained at 28° in a water bath with gentle shaking (77 recip./min) and inoculated with 1/10th their volume of 48 hr old preculture. The cells were counted either directly after fixation in 10% formaldehyde using a 0.5 mm depth calibrated Nageotte counting slide or, indirectly by absorbancy measurements at 546 nm, with the proper controls.

The cells were harvested at the beginning of the stationary phase by low speed centrifugation in the cold, washed twice with Na,K-phosphate 10^{-4} M pH 6.5 buffer and suspended in the same buffer under sterile conditions in a Wiame culture vessel maintained as above for proper storage.

Irradiation was performed with a Philips UV lamp delivering 15 ergs/mm^2/sec, mostly at 253.7 nm as measured by the Latarget dosimeter. Immediately after irradiation in sterile gently agitated pyrex Petri dish, the suspension of *Tetrahymena pyriformis* was

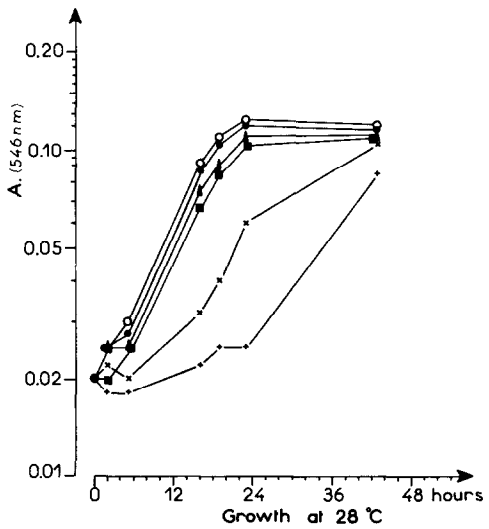

Fig. 1. Growth inhibition of *Tetrahymena pyriformis* with increasing concentrations of luteoskyrin added immediately after the inoculum ○—○ control; ●—● 2.06 × 10^{-7} M luteoskyrin; ▲—▲ 6.18 × 10^{-7} M luteoskyrin; ■—■ 1.03 × 10^{-6} M luteoskyrin; x—x 2.06 × 10^{-6} M luteoskyrin; +—+ 4.12 × 10^{-6} M luteoskyrin.

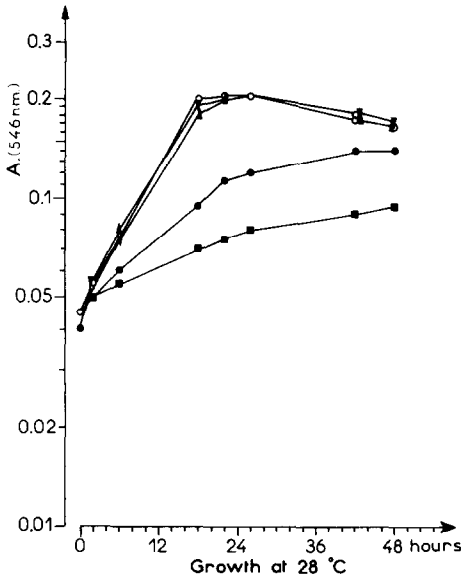

Fig. 2. Growth inhibition of *Tetrahymena pyriformis* with increasing concentrations of caffeine added immediately after the inoculum ○—○ control; ▼—▼ 0.52 × 10^{-3} M caffeine; ▲—▲ 1.03 × 10^{-3} M caffeine; ●—● 2.06 × 10^{-3} M caffeine; ■—■ 4.12 × 10^{-3} M caffeine.

added to the growth medium with or without the anti-repair agents. The room was kept dark or under a faint yellow light to avoid interference from photoreactivation repair.

Purified DNA polymerase I was extracted from *E. coli*. Calf thymus DNA was used as template as described [20]. The inhibitors were added to the template and the enzyme and the reaction started by addition of the deoxynucleoside triphosphates (label ^{3}H-dATP) and the salt mixture containing 10^{-2} M $MgCl_2$.

3. Results and discussion

Figs. 1 and 2 indicate the effects of various concentrations of luteoskyrin and caffeine on the multiplication of *Tetrahymena pyriformis* which appear quite sensitive to the drugs. Lowering their concentration to 2.06 × 10^{-7} M or 1 × 10^{-3} M, respectively, prevented visible alteration of the growth rate or of the morphology of the organism.

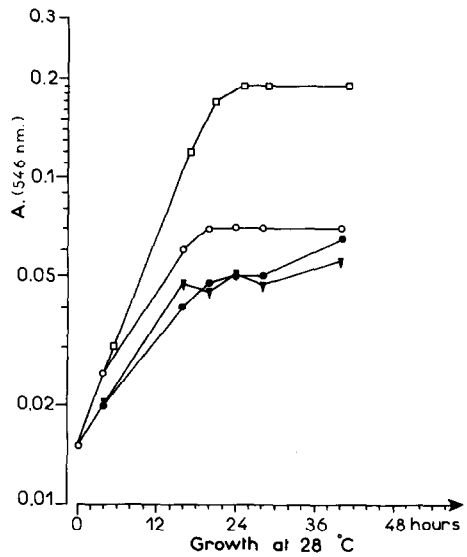

Fig. 3. Inhibition of post-UV irradiation growth pattern of *Tetrahymena pyriformis* by subtoxic concentrations of luteoskyrin or caffeine added immediately after the irradiated (450 ergs/mm^2 UV, 253, 7 nm, 15 ergs/mm^2/sec) inoculum. □—□ non irradiated control; ○—○ irradiated control; ●—● irradiated inoculum + 10^{-3} M caffeine in growth medium; ▼—▼ irradiated inoculum + 2.06 × 10^{-7} M luteoskyrin in growth medium.

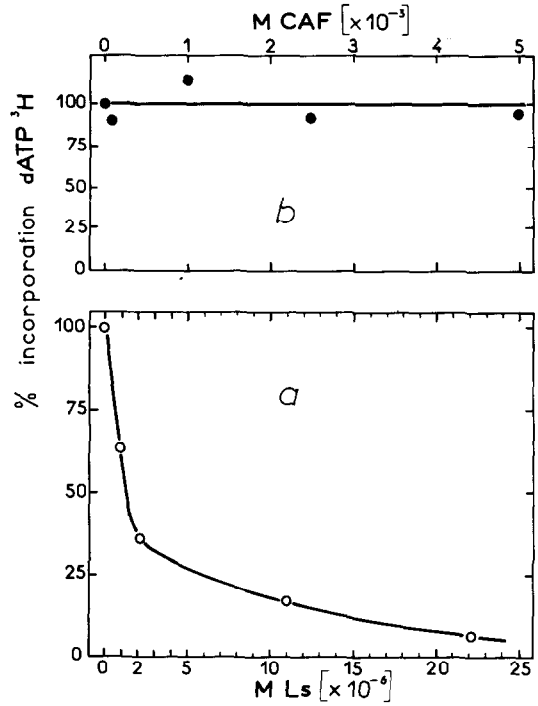

Fig. 4. Comparison of action of (a) luteoskyrin, (b) caffeine on nicked-DNA repolymerisation in vitro. The incubation mixture (0.3 ml) contained 45 nmoles of thymus DNA; 15 poly d(AT) units enzyme; 20 μmoles of glycine buffer pH 9.2; 2 μmoles of $MgCl_2$; 0.3 μmole of 2-mercaptoethanol; 10 nmoles each of dTTP, dCTP, dGTP and ^{3}H-dATP. Luteoskyrin and caffeine are added after the enzyme. After 30 min at 37°, DNA is removed by acid precipitation with 5% TCA at 0°.

Exposure of *Tetrahymena pyriformis* suspensions to 450 ergs/mm^2 UV light increased the delay of multiplication and decreased the generation number [21]. Luteoskyrin or caffeine, added after this treatment, became toxic at the concentrations found non-inhibitory in control cells as shown in fig. 3.

The similar inhibition of post UV irradiation growth pattern by luteoskyrin and caffeine supports the hypothesis that the oncogenic pigment interferes in vivo with the repair process.

As it was expected from the binding characteristics that the probable anti-repair activity of luteoskyrin would affect preferentially the late stage where single stranded DNA is copied, direct evidence for the influence of the mycotoxin on the repair polymerisation step was sought.

The in vitro system using nicked calf thymus DNA indicated (fig. 4) that luteoskyrin inhibited the activity of *E. coli* DNA polymerase in agreement with earlier observation [22]. Caffeine did not interfere, as expected, because it inhibits only the early stage of UV lesion repair at the level of the specific endo and exonuclease [23, 24]. Although the template and the polymerase used are not those of *Tetrahymena pyriformis*, the results obtained with luteoskyrin in vitro very likely account for its post irradiation effect in vivo, namely a DNA repair inhibition.

Random inhibition of spontaneous repair processes by low concentrations of luteoskyrin may promote the formation of permanently altered DNA after replication, leading to defective enzyme or control systems after long term exposure to the drug [25]. Such a mechanism could explain the formation of unusually long chromosomes and other chromosomal aberrations in Erlich tumour cells cultivated for several months in the presence of luteoskyrin [12] and its oncogenic effect.

A parallel could be drawn between cells where the repair processes are inhibited and those defective in the repair endonuclease. Examples of the latter case have recently been found in man [26–28], in which UV irradiation induces skin tumours, xeroderma pigmentosum.

Acknowledgements

Acknowledgement is made of advice from Dr. A. Sentenac for DNA primed polymerase assays and Dr. J.C. Bouhet and P. Pham Van for preparation of pure luteoskyrin. We wish to thank Miss R. Charret, Cellular Biology 4, Mrs F. Iftode and G. Fryd-Versavel, Zoology 2, Faculté des Sciences (91) Orsay, for supplying us with axenic strain of *Tetrahymena pyriformis* and Dr. E. Reich, The Rockefeller University, New York, for a gift of pure DNA polymerase I. Part of this work was supported by the Institut de la Santé et de la Recherche Médicale (INSERM).

References

[1] G.N. Wogan, Mycotoxins in Foodstuffs, MIT Symposium 1964 (MIT Press, Cambridge, Mass.).
[2] Y. Kobayashi, K. Uraguchi, F. Sakai, T. Tatsuno, M. Tsukioka, Y. Sakai, T. Sato, M. Miyake, M. Saito, M. Enomoto, T. Shikata and T. Ishiko, Proc. Japan Acad. 34 (1958) 639.
[3] Y. Kobayashi, K. Uraguchi, F. Sakai, T. Tatsuno, M. Tsukioka, Y. Noguchi, H. Tsunoda, M. Miyake, M. Saito, M. Enomoto, T. Shikata and T. Ishiko, Proc. Japan Acad. 35 (1959) 501.
[4] M. Enomoto, Acta Pathol. Japan 9 (1959) 189.
[5] S. Shibata and I. Kitagava, Pharm. Bull. Tokyo 4 (1956) 309.
[6] A. Platel, Y. Ueno and P. Fromageot, Bull. Soc. Chim. Biol. 50 (1968) 678.
[7] N. Kobayashi, Y. Jitaka and S. Shibata, Acta Crystallog. B 26 (1970) 188.
[8] M. Miyake, M. Saito, M. Enomoto, T. Shikata, T. Ishiko, K.Uraguchi, F. Sakai, T. Tatsuno, M. Tsukioka and Y. Sakai, Acta Pathol. Japan 10 (1960) 75.
[9] K. Uraguchi, T. Tatsuno, F. Sakai, M. Tsukioka, Y. Sakai, O. Yonemitsu, H. Ito, M. Miyake, M. Saito, M. Enomoto, T. Shikata and T. Ishiro, Japan J. Exptl. Med. 31 (1961) 19.
[10] K. Uraguchi, F. Sakai, M. Tsukioka, Y. Noguchi, T. Tatsuno, N. Saito, M. Enomoto, T. Ishiko, T. Shikata and M. Miyake, Japan J. Exptl. Med. 31 (1961) 435.
[11] M. Umeda, Acta Pathol. Japan 14 (1964) 375.
[12] D.O. Schachtschabel, F. Zilliken, M. Saito and G.E. Foley, Exptl. Cell Res. 57 (1969) 19.
[13] Y. Ueno, I. Ueno, K. Ito and T. Tatsuno, Experienta 23 (1967) 1001.
[14] Y. Ueno, A. Platel and P. Fromageot, Biochim. Biophys. Acta 134 (1967) 27.
[15] Y. Ohba and P. Fromageot, European J. Biochem. 1 (1967) 147.
[16] Y. Ohba and P. Fromageot, European J. Biochem. 6 (1968) 98.
[17] C.F. Brunk and P.C. Hanawalt, Radiation Res. 38 (1969) 285.
[18] K. Shimada and Y. Takagi, Biochim. Biophys. Acta 145 (1967) 763.
[19] R. Mouton and O. Tremeau, Intern. J. Radiation Biol. 15 (1969) 369.
[20] C.C. Richardson, C.L. Schildkraut, N.V. Aposhian and A. Kornberg, J. Biol. Chem. 239 (1964) 222.
[21] E. Christensen and A.C. Giese, J. Gen. Physiol. 39 (1956) 513.
[22] A. Sentenac, (1965) unpublished data.
[23] D. Roulland-Dussoix, Mutation Res. 4 (1967) 241.
[24] L. Grossman, J.C. Kaplan, S.R. Kushner and I. Mahler, Cold Spring Harbor Symp. Quant. Biol. 33 (1968) 229.
[25] W. Kuhlmann, H.G. Fromme, E.M. Heege and W. Ostering, Cancer Res. 28 (1968) 2375.
[26] J.E. Cleaver, Intern. J. Radiation Biol. 18 (1970) 557.
[27] R.B. Setlow, J.D. Regan, J. German and W.L. Carrier, Proc. Natl. Acad. Sci. U.S. 64 (1969) 1035.
[28] E.G. Jung, Nature 228 (1970) 361.