Effect of Halomethanes on Aflatoxin Induction in Cultures of *Aspergillus parasiticus*

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The addition of tribromo-, tetrabromo- and chloromethanes to cultures of *Aspergillus parasiticus* strongly stimulated aflatoxin biosynthesis. The presence of phenobarbital in the cultures enhanced this effect. Following the addition of ¹⁴CCl₄ to a culture, after 15 d incubation, 8.6% of the total radioactivity was recovered from the mycelium, and 4.7% from the culture filtrate. Some 0.4% of the total radioactivity was found in microsomes, indicating their involvement in CCl₄ activation and biotransformation.

INTRODUCTION

We have suggested that lipoperoxidation plays a role in inducing aflatoxin production (Passi *et al.*, 1984). *In vivo*, the amount of aflatoxin formed in sunflower seeds of different ages inoculated with *Aspergillus parasiticus* was found to be directly related to the peroxide number in their oil content: the higher the peroxide number, the higher the aflatoxin production (Fabbri *et al.*, 1983). *In vitro*, aflatoxin production was highly stimulated by the addition to cultures of *A. parasiticus* or *A. flavus* of: (1) compounds with an epoxide ring [cerulenin and tetrahydrocerulenin (Fanelli *et al.*, 1983*a*), and 9,10-epoxymethylstearate and 9,10:12,13-diepoxy-methylstearate (Fanelli *et al.*, 1983*b*)]; (2) synthetic hydroperoxides (obtained by the reaction of lipoxygenase on linoleic acid) (Fabbri *et al.*, 1983); (3) UV-peroxidated sitosterols and ergosterol (the main sterols of seeds and fungi respectively) (Fanelli *et al.*, 1983*c*); (4) carbon tetrachloride, chloroform and other halomethanes (Fanelli *et al.*, 1984). In the case of carbon tetrachloride we have postulated that stimulation of aflatoxin production by *A. parasiticus* might be due to the peroxidation of lipids of the endoplasmic reticulum by highly reactive radicals formed by the interaction of carbon tetrachloride with the NADPH-cytochrome P-450 system of the fungus. This system has been demonstrated in the microsomes of *A. parasiticus* (Bhatnagar *et al.*, 1982).

In this connection we decided to study the fate of CCl₄ in cultures of *A. parasiticus* supplemented with ¹⁴CCl₄. In addition, dichloro-, trichloro-, tetrachloro-, bromo- and iodomethanes were tested for their effectiveness in inducing aflatoxin production.

METHODS

Fungal cultures. A strain of *Aspergillus parasiticus* Speare (NRRL 2999) that gave high levels of aflatoxins was used. About 10⁶ conidia from 15 d cultures grown on Czapek Dox agar medium (Difco) were incubated in 50 ml synthetic medium (Czapek Dox broth plus 5 mg ZnSO₄·7H₂O l⁻¹ and 1 mg Na₂MoO₄·2H₂O l⁻¹) supplemented with the following. (a) 0.02 to 0.2% (w/v) tetra-, tri- and dihalomethanes purchased from Fluka: CCl₄ (carbon tetrachloride), CHCl₃ (chloroform), CH₂Cl₂ (dichloromethane), CBr₄ (carbon tetrabromide), CHBr₃ (bromoform), CH₂Br₂ (dibromomethane), CI₄ (tetraiodomethane), CHI₃ (iodoform), CH₂I₂ (diiodomethane), BrCCl₃ (trichlorobromomethane) in the presence and absence of phenobarbital (1 mg ml⁻¹; Sigma). (b) 50 µCi ¹⁴CCl₄ (26.9 mCi mmol⁻¹; 995 MBq mmol⁻¹; Amersham) plus 0.2% unlabelled CCl₄.

Cultures were incubated, unshaken, at 30 °C for 15 d. Growth was estimated as dry weight of mycelium, as described by Fanelli *et al.* (1980). The total aflatoxins ($B_1 + B_2 + G_1 + G_2$) were extracted from filtered culture medium and individually analysed by HPLC as previously described (Fanelli *et al.*, 1983a).

Lipid peroxidation. This was measured by the thiobarbituric acid test (Buege & Aust, 1978) on microsomes of each mycelium, prepared as described below. Microsomes were extracted with $CHCl_3/CH_3OH$ (2:1, v/v) and treated with thiobarbituric acid solution. The absorption at 535 nm was measured using a Beckman Acta VI spectrophotometer.

Experiments with $^{14}CCl_4$. After 15 d incubation in the presence of 50 μCi $^{14}CCl_4$ plus 0.2% unlabelled CCl_4 , each radioactive culture of *A. parasiticus* was filtered on a sintered glass filter under vacuum. The mycelium was washed three times with saline and successively filtered; then it was dried over anhydrous P_2O_5 under vacuum and weighed. The combined filtrates of the washing were added to the culture medium filtrate and the total volume was measured.

Samples of mycelium and filtrate were extracted with $CHCl_3/CH_3OH$ (2:1, v/v). After evaporation of the solvent under nitrogen, a measured sample of each extract was fractionated by TLC according to the method of Passi *et al.* (1981) to separate lipid fractions. The aflatoxins present in the residual $CHCl_3/CH_3OH$ extracts were separated by HPLC on a reverse-phase column (RP 18; Merck) and individually collected.

A sample of the mycelium was homogenized in hypo-osmotic medium (10 mM-Tris/EDTA). The cellular fractions were separated by differential centrifugation: the 'heavy fraction' (which contains nuclei, plasma membrane and some non-disrupted cells) was pelleted at 800 g for 15 min, the mitochondrial fraction at 7000 g for 20 min, and the microsomal fraction at 100000 g for 60 min.

The amounts of radioactivity present in the mycelium and filtrate, in their lipid fractions, in the aflatoxins, and in the pellets and supernatants following the different centrifugations were counted with a Beckman beta counter.

RESULTS

Table 1 shows the percentage recovery of radioactivity in a culture of *A. parasiticus* grown for 15 d in the presence of 50 μCi $^{14}CCl_4$ plus 0.2% unlabelled CCl_4 . The intracellular localization of ^{14}C and the radioactivity present in the lipid fractions and aflatoxins of $CHCl_3/CH_3OH$ extracts from both mycelium and culture filtrate are indicated.

Table 2 shows the growth (dry weight of mycelium) and aflatoxin production of *A. parasiticus* grown for 15 d at 30 °C on synthetic medium supplemented with different halomethanes in the presence or absence of 1 mg phenobarbital ml^{-1} . The halomethanes, because of their high specific gravity and limited water solubility, settled at the bottom of the flasks after their addition to cultures. However, after 12 d incubation they had disappeared from the liquid cultures with the exception of 0.2% and 0.1% Cl_4 and CBr_4 and 0.2% CHI_3 . $BrCCl_3$ (0.1%), $CHBr_3$ (0.2%), CCl_4 (0.2%), CBr_4 (0.02%), in decreasing order, highly stimulated aflatoxin production; the dihalomethanes were less effective; 0.2% and 0.1% concentrations of Cl_4 and CBr_4 were toxic, inhibiting growth. The addition of phenobarbital to cultures enhanced the potency of the above-mentioned compounds to induce aflatoxin output. Thiobarbituric acid tests on lipid extracts of microsomes were always negative. In other words, there was no apparent lipoperoxidation within the microsomes, measured as malondialdehyde production.

DISCUSSION

Our results on the fate of $^{14}CCl_4$ added to cultures of *A. parasiticus* showed that, after 15 d incubation, about 13% of the total radioactivity was recovered from the culture: 8.6% from mycelium and 4.7% from culture filtrate (see Table 1). As regards the intracellular localization of ^{14}C , appreciable amounts of radioactivity were found in microsomes, indicating their possible involvement in CCl_4 activation and biotransformation. About 73% of the ^{14}C found in the mycelium was recovered in $CHCl_3/CH_3OH$ extracts, mainly localized in polar lipids (phospholipids, glycolipids, etc.) which remain at the origin of the TLC plate with the system used for the separation of lipids. Diglycerides, sterols, free fatty acids, triglycerides and sterol esters also contained ^{14}C , indicating that a labelled catabolite, possibly [^{14}C]acetyl-CoA, entered into biosynthesis of these fractions. Aflatoxins B_1 and G_1 , separated on a reverse-phase HPLC column, showed appreciable levels of radioactivity, about three times higher in the filtrate than in the mycelium.

Table 1. Recovery (%) of radioactivity in a culture of *A. parasiticus* 15 d after addition of $^{14}\text{CCl}_4$ plus unlabelled CCl_4

Each result represents the mean of two determinations. Radioactivity is expressed as percentage of the $^{14}\text{CCl}_4$ activity added to the culture. See Methods for details of the fractionation procedures.

Table 2. Growth, and aflatoxin production in culture filtrates, of *A. parasiticus* grown for 15 d at 30 °C on synthetic medium supplemented with different halomethanes in the presence or absence of phenobarbital

Halomethane added to medium	Halomethane concn (%)	Dry wt of mycelium (mg per 50 ml)*	Aflatoxin concn (µg per 50 ml)†	
			Cultures without phenobarbital	Cultures plus phenobarbital
None	—	235.9 ± 29.6	4.5 ± 3.2	10.9 ± 6.0
CCl_4	0.2	346.6 ± 22.7	118.7 ± 16.4	229.3 ± 33.1§
CHCl_3	0.2	228.3 ± 17.9	37.4 ± 6.5	51.3 ± 8.8§
CH_2Cl_2	0.2	221.9 ± 23.7	9.3 ± 4.8	12.2 ± 6.7
CBr_4	0.02‡	222.7 ± 15.6	92.6 ± 20.3	166.5 ± 33.1§
CHBr_3	0.2	224.2 ± 11.0	136.5 ± 12.8	258.4 ± 19.2§
CH_2Br_2	0.2	215.2 ± 9.5	20.1 ± 4.3	33.6 ± 5.2§
CI_4	0.02‡	183.5 ± 20.1	8.6 ± 3.1	11.2 ± 5.3
CHI_3	0.2	198.8 ± 11.7	36.8 ± 4.1	52.6 ± 3.1§
CH_2I_2	0.2	208.1 ± 15.6	7.8 ± 2.6	9.9 ± 2.9
BrCCl_3	0.1	226.6 ± 18.4	156.4 ± 17.2	282.6 ± 21.6§

* Each value represents the mean ± SEM of ten different experiments. No significant differences in mycelial dry weight were found between cultures with and without phenobarbital.

† Each value represents the mean ± SEM of five different experiments.

‡ Concentrations of 0.1 and 0.2% of both CBr_4 and CI_4 were toxic for fungal growth.

§ Significantly different (Student's test for unpaired data) from the values of corresponding cultures without phenobarbital ($P < 0.005$).

We did not analyse the fate of the $^{14}\text{CCl}_4$ not recovered in the cultures. However we think, by analogy with experiments with mammals, that $^{14}\text{CCl}_4$ was probably dispersed into the air during the 15 d incubation, either unmetabolized or biotransformed to $^{14}\text{CO}_2$, $^{14}\text{CHCl}_3$ (the expected product of removal of hydrogen by $^{14}\dot{\text{C}}\text{Cl}_3$) or $^{14}\text{CCl}_3$ – $^{14}\text{CCl}_3$ (the expected dimerization product of $^{14}\dot{\text{C}}\text{Cl}_3$ radicals) (Fowler, 1969). The primary physicochemical factor in the cleavage of a halocarbon to a free radical is the potential for activation to a free radical

species, which increases with both increasing halogenation and decreasing electronegativity of the halogen atoms. Therefore CBr_4 is more likely than CCl_4 to be activated to a free radical because bromine is less electronegative than chlorine, and CCl_4 is more easily activated to a free radical than CHCl_3 because of the greater number of halogen atoms. Our results with iodomethanes are not in line with the above theory. At 0.2% and 0.1% concentrations, both CI_4 and CBr_4 were extremely toxic for the fungus, but 0.02% CI_4 , unlike CBr_4 , did not significantly enhance aflatoxin output. CI_4 is practically insoluble in water but hydrolyses slowly forming iodine and CHI_3 . Furthermore, 0.2% CHI_3 was much less potent than 0.2% CHBr_3 in producing aflatoxin (36.8 ± 4.1 against 136.5 ± 12.8 μg per 50 ml). Probably CI_4 and CHI_3 , because of their limited water and lipid solubility, cannot act by a free radical mechanism in cultures of *A. parasiticus*.

The addition of phenobarbital (1 mg ml^{-1}) to the cultures containing halomethanes significantly enhanced the effect of the halomethanes in inducing aflatoxin production. According to Orrenius & Ernster (1964), phenobarbital increases synthesis of proteins and phospholipids by the endoplasmic reticulum, and the components of the NADPH-cytochrome P-450 system are all induced to stimulate the metabolism of CCl_4 and other drugs. In conclusion we suggest that the induction of aflatoxin biosynthesis in *A. parasiticus* by tribromo-, tetrabromo- and chloromethanes might be due to highly reactive free radicals, and/or other toxic intermediates formed by the interaction of halomethanes with the NADPH-cytochrome P-450 system, attacking neighbouring components in the endoplasmic reticulum and causing, among other things, lipoperoxidation of unsaturated lipids.

The fact that we found no evidence of lipoperoxidation by application of the thiobarbituric test to lipid extracts from microsomes of *A. parasiticus* in the cultures supplemented with halomethanes would appear to contradict this conclusion. However, there may be a large difference between mammals and *A. parasiticus* in the rate of metabolism of halomethanes. Following administration to mammals, halomethanes concentrate very rapidly (within a few minutes) in the liver with early striking alterations of functional activities and chemical composition of the endoplasmic reticulum (Reynolds & Moslen, 1980).

An example of an informative chemical change is the rapid, but transient, lipoperoxidation of unsaturated fatty acids of the endoplasmic reticulum. Lipoperoxides and their breakdown products rapidly lead to cell damage and death especially if protective enzymes and antioxidants are lacking. In our cultures, halomethanes settled at the bottom of the flasks and only small amounts, depending upon their water solubility, could come into contact with the cells of *A. parasiticus*. Therefore, the process of lipoperoxidation may be very slow and difficult to evaluate. The newly formed lipoperoxides might be transformed into compounds that do not react with thiobarbituric acid, of which aflatoxin may be one. On the other hand, by-products of lipoperoxidation, such as malondialdehyde and other aldehydes might be inactivated by reacting with $-\text{SH}$ and $-\text{NH}_2$ groups of amino acids, so that reactions with thiobarbituric acid would not take place.

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REFERENCES

- BHATNAGAR, R. K., AHMAD, S., KOHLI, K. K., MUKERJI, K. G. & VENKITASUBRAMANIAN, T. A. (1982). Induction of polysubstrate monooxygenase and aflatoxin production by phenobarbital in *Aspergillus parasiticus*. *Biochemical and Biophysical Research Communications* **104**, 1287-1292.
- BUEGE, J. A. & AUST, S. D. (1978). Microsomal lipid peroxidation. *Methods in Enzymology* **51**, 301-310.
- FABBRI, A. A., FANELLI, C., PANFILI, G., PASSI, S. & FASELLA, P. (1983). Lipoperoxidation and aflatoxin biosynthesis by *Aspergillus parasiticus* and *A. flavus*. *Journal of General Microbiology* **129**, 3447-3452.
- FANELLI, C., FABBRI, A. A. & PASSI, S. (1980). Growth requirements and lipid metabolism of *Aspergillus flavus*. *Transactions of the British Mycological Society* **75**, 371-375.
- FANELLI, C., FABBRI, A. A., FINOTTI, E., PANFILI, G. & PASSI, S. (1983a). Cerulenin and tetrahydrocerulenin: stimulating factors of aflatoxin biosynthesis. *Transactions of the British Mycological Society* **81**, 201-204.
- FANELLI, C., FABBRI, A. A., FINOTTI, E. & PASSI, S. (1983b). Stimulation of aflatoxin biosynthesis by lipophilic epoxides. *Journal of General Microbiology* **129**, 1721-1723.
- FANELLI, C., FABBRI, A. A., PASSI, S. & FASELLA, P.

- (1983c). Control mechanisms of aflatoxin production. *International Mycotoxin Symposium, Sydney, August 12-15, 1983*, Abst. P9.
- FANELLI, C., FABBRI, A. A., FINOTTI, E., FASELLA, P. & PASSI, S. (1984). Free radicals and aflatoxin biosynthesis. *Experientia* **40**, 191-193.
- FOWLER, J. S. L. (1969). Carbon tetrachloride metabolism in the rabbit. *British Journal of Pharmacology* **37**, 733-737.
- ORRENIUS, S. & ERNSTER, L. (1964). Phenobarbital-induced synthesis of the oxidative demethylating enzymes of rat liver microsomes. *Biochemical and Biophysical Research Communications* **16**, 60-64.
- PASSI, S., ROTHSCCHILD-BOROS, M. C., FASELLA, P., NAZZARO-PORRO, M. & WHITEHOUSE, D. (1981). An application of high performance liquid chromatography to analysis of lipids in archeological samples. *Journal of Lipid Research* **22**, 778-784.
- PASSI, S., NAZZARO-PORRO, M., FANELLI, C., FABBRI, A. A. & FASELLA, P. (1984). Role of lipoperoxidation in aflatoxin production. *Applied Microbiology and Biotechnology* **19**, 186-190.
- REYNOLDS, E. S. & MOSLEN, M. T. (1980). Free radical damage in liver. In *Free Radicals in Biology*, vol. IV, pp. 49-94. Edited by A. A. Pryor. New York: Academic Press.