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Enhancement of aflatoxin B_1 cytotoxicity in differentiated rat hepatoma cultures by a prior glucocorticoid treatment of the monolayer

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Summary. The cytotoxic effect of aflatoxin B_1 on cultures of a differentiated rat hepatoma cell line, Faza 967, has been evaluated by scoring the surviving colonies two weeks after briefly exposing the freshly plated cells to the mycotoxin. At the lowest concentration, aflatoxin B_1 exhibits no toxicity, unless the cultures have been pretreated with dexamethasone. HF-1, an hepatoma hybrid cell line exhibiting extinction of the hepatic functions and HF1-4, its subclone, that reexpresses all of these functions, have been compared. A 6hrs exposure to 60ng/ml aflatoxin B_1 is not toxic for HF_1 even after an hormonal treatment, while dexamethasone enhances the effect on HF_1-4. Glucocorticoïds have been shown previously to induce, in the differentiated clones, the hydroxylation of bile acid - a cytochrome P-450-mediated reaction ; in contrast, 3-methylcholanthrene, an inducer of benzopyrene hydroxylase in hepatoma cultures, is without effect on bile acid metabolism and on aflatoxin B_1 cytotoxicity. These results suggest that in the differentiated hepatoma cells, aflatoxin B_1 is converted into a cytotoxic metabolite by a glucocorticoïd-induced monooxygenase belonging to the cytochrome P-450-related group.

The acute toxicity, the mutagenicity and the carcinogenicity of AFB_1 are known to be related to its metabolic activation (1-13). But the nature of the activating system has not yet been settled and some authors have even established a distinction between AFB_1 lethal and mutagen activities towards bacteria and its carcinogenic properties on one hand, and its cytotoxic effect on eukaryotic cells on the other, casting doubt on the need for a metabolic processing in the latter case (1). Since AFB_1 is a major hepatocarcinogen and hepatotoxic compound, the importance of its metabolic conversion should be precisely evaluated in its target cell. In vivo experiments involve many interfering factors ; the simplest approach would be to estimate the requirement for activation in a hepatocyte culture. This study has not been carried out previously with normal liver cultures in an unequivocal way for lack of a system in which the monooxygenases of the tested cells themselves could be, at will, present in an inactive or an activated state at the time of AFB_1 administration. Furthermore, in vitro inhibitors would be useless, since many of these compounds are good hydroxylation inducers when added to the culture medium (14,15). As regards hepatoma cultures, they are indeed practically devoid of any basal monooxygenase activity and exhibit a sharp increase in aryl

AFB_1 = aflatoxin B_1 ; MC = 3-methylcholanthrene ; DMSO = dimethylsulfoxide. BP = benzopyrene ; PB = phenobarbital.

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hydrocarbon (benzopyrene) hydroxylase after the addition of MC, but the only induced monooxygenase belongs to the cytochrome P-448 related group ; until lately, no cytochrome P-450 enzyme had ever been detected in a permanent cell line (16).

We have recently shown that the addition of a glucocorticoïd in physiological amounts to a monolayer culture of a differentiated rat hepatoma, Faza 967, induces the 7α and 6β-hydroxylation of deoxycholate and the 6β-hydroxylation of chenodeoxycholate (17a, b, c). These reactions are catalysed in rat liver by cytochrome P-450-mediated monooxygenases, akin to the drug-metabolizing enzymes (18,19), The in vivo administration of phenobarbital (PB), the classical inducer of cytochrome P-450-mediated systems, increases the bile acid hydroxylation in liver microsomal preparations (20a, 20b), whereas MC, an in vivo inducer of benzopyrene(BP)-hydroxylase is without effect (21). When added to the cultures of H4II EC3, the hepatoma line from which Faza 967 has been derived (22), MC induces BP-hydroxylase activity (14,15), while the addition of dexamethasone is without effect on this cytochrome P-448 system (23).

As regards the conversion of AFB_1 to DNA alkylating compounds, this epoxidation activity is enhanced in vivo by PB administration, while MC produces its depression (24). Taken together, these observations suggest that the glucocorticoïds might induce in the hepatoma cells precisely the AFB_1 activating system. These cultures would thus provide a good model for testing the need for AFB_1 metabolic transformation, for determining the type of the activating system and the nature and relative importance of the resulting metabolites.

As a first approach,we decided to compare the cytotoxic effect of the mycotoxin on Faza 967 cells, cultivated in a dexamethasone-containing medium, a MC-containing medium or a control medium. In addition, we compared AFB_1 toxicity, with or without a prior hormonal treatment, towards two hybrid hepatoma clones, one exhibiting extinction of the liver functions and one of its subclone that reexpresses all of these functions.

Material and Methods.

Cell culture. The hepatoma clones were kindly provided by M.C. Weiss. Faza 967 is a differentiated subclone of Fu-5, obtained from H4II EC3, derived from the H-35 hepatoma produced chemically in a male rat by Reuber (25). The hybrid HF1, resulting from the cross of Fao, a differentiated subclone of Faza 967, with H5, a dedifferentiated line derived from Fu5, exhibits extinction of the liver functions. The subclone HF1-4, obtained from HF1 by selection in a glucose-free medium, reexpresses every liver specific trait tested, in particular chenodeoxycholate hydroxylation (17c,d).The cells, thawed from a frozen stock, are cultivated as monolayers in tissue-culture Petri dishes ; they are maintained at 37°C, in a air/CO2 atmosphere (90 : 10 v/v), in a mixture of equal volumes of modified Ham's F12 and of NCTC 109, supplemented with 7.5% heat-decomplemented fetal calf serum, 1g/liter bovine serum albumin, 7.1mg/liter taurine, 100 i.u. penicillin G and 50µg/liter streptomycin sulfate. The medium is changed every two days ; subcultures are carried out with the help of a trypsin/EDTA solution in Ca^{++} and Mg^{++}-free phosphate buffered saline adjusted to pH 7.3.

Chemicals. AFB_1 was purchased from Calbiochem ; a 2mg/ml stock solution in dimethylsulfoxide (DMSO), analytical grade (Merck), was stored in the dark at -25°C and thawed and diluted into the culture medium just before use. All assays involving AFB_1 were carried out in dim light. MC (Sigma) was stored as a 1mg/ml stock solution in DMSO. Dexamethasone phosphate was a gift of Merck, Sharp and Dohme Res. Lab.

I. Cytotoxicity test by plating efficiency, first procedure.

A number of 60mm dishes were seeded with 100, 500 and 1000 cells per plate in 5ml control medium. After an 18 hour attachment period, dexamethasone phosphate (1.2µM final concentration) was added to half of the dishes. After 68 hours of undisturbed further incubation, AFB_1 - or the corresponding volume of DMSO - was added in 1ml control medium at various final concentrations (0.1, 0.25, 0.50, 1.00 and 2.50µg/ml ; 6ml per plate). All tests were run in duplicate. The experimental pattern thus resulted in four groups : group 1 (control medium, DMSO), group 2 (dexamethasone containing medium, DMSO), group 3 (control medium, AFB_1) group 4 (dexamethasone containing medium, AFB_1) . After a 6 hours' exposure, the media were removed and 5ml/plate control or dexamethasone containing medium were added to the corresponding cultures. The plates were left undisturbed for one week. The media were then removed from all dishes and replaced by 5ml of fresh control medium. After five days (the 16th day after plating), the cultures from groups 1 and 3 were fixed and stained (Giemsa) ; 48 h later, the dexamethasone-treated cultures, whose colonies had grown much less and were smaller, were also fixed and stained before counting.

II. Cytotoxicity tests by plating efficiency, second procedure.

The experiment was repeated with two major modifications : 1) dexamethasone concentration was brought down to 0.8µM and the various media, when removed at the end of the 6 hours' exposure to AFB_1, were replaced in all groups by control medium. Thus, the dexamethasone was present for only 74 hours instead of ten days. 2) A lower range of AFB_1 was tested, from 0.01 to 0.1µg/ml.

III. Mass culture cytotoxicity test.

Five 100 mm plates were seeded with 5.10^6 cells each and the culture maintained in control medium until the plates were about 2/3 full. Three dishes were then supplemented with dexamethasone phosphate (0.8µM final concentration). After 90 hours' incubation, a medium renewal (10ml per plate) and four hours further incubation, each plate was supplemented with 10µg AFB_1 in 20µl DMSO.

IV. Comparative AFB_1 cytotoxicity test on hybrid clones differing in the expression of liver differentiation.

500 cells of both HF_1 and HF_1-4 were plated in 40 100mm dishes in control medium supplemented with extra amounts of hypoxanthine and of thymidine (15,6mg/liter and 44,4mg/liter instead of 2,0mg and 5,7mg respectively). The experience was carried out as in II, except that an unique concentration of AFB_1 (60ng/ml) was tested. The plates were scored after ten days.

V. MC pretreatment test.

a) Preliminary test. Faza 967 cultures were treated for 72 hrs with either MC (2µM), a mixture of MC (2µM) and dexamethasone phosphate (0.8µM) or an equivalent amount of DMSO. The monolayers were then trypsinised and 2.10^5 cells were plated per 100mm dish in control medium. The next day, the inducers were added again for 24 hrs and the cells exposed for 6 hrs to 40ng/ml AFB_1. The medium was replaced by control medium and the cells were scored one week later.

b) Plating efficiency test. 500 Faza 967 cells per 60 mm plate were treated for 72 hrs with either dexamethasone phosphate (0.8µM), MC (2.0µM), or the corresponding amount of DMSO. AFB_1 (40ng/ml final concentration) was then added for 6hrs and the experiment carried out as in II. (10 plates per group).

Table 1 Plating efficiency (%) of hepatoma Faza 967 cells after seeding 100, 500 or 1000 cells per 60mm plate. dexamethasone(1.2μM) from the 18 hour after plating till the 8th day after AFB_1 exposure

Seeded per plate	100				500				1000	
AFB_1 μg/ml *	Control DMSO 1	Dexamethasone DMSO 2	control AFB_1 3	dex AFB_1 4	control DMSO 1	Dexamethasone DMSO 2	control AFB_1 3	dex AFB_1 4	control AFB_1 3	dexamethasone AFB_1 4
0	53	26	-	-	47	22	-	-	-	-
	50	19	-	-	49	25	-	-	-	-
0.10	48	20	33	0	40	31	39	0	30	1.5
	48	34	34	0	40	-	35	0	17	0.5
0.25	48	28	26	0	45	22	30	0	29	0.2
	67	32	29	0	50	24	25	0	26	0
0.50	44	21	16	0	41	21	21	0	-	0
	41	24	19	0	43	27	17	0	-	0
1.00	44	25	0	0	48	25	12	0	12	0
	43	33	3	0	46	22	15	0	12	0
2.50	56	-	1	0	45	27	2	0	3	0
	65	22	1	0	46	24	1	0	2	0

* or the corresponding volume of DMSO

RESULTS*

I. Table I shows the results of the test carried out according to the first procedure. a) The dexamethasone treatment by itself decreased the plating efficiency from 48% to 25%. The glucocorticoid-treated cells grew more slowly, as shown by the presence of a majority of small colonies. b) The glucocorticoid pretreatment dramatically enhanced the cytotoxicity of AFB_1 : no colony survived out of 500 plated cells in group 4, even at 0.1µg/ml, the lowest AFB_1 concentration tested. In contrast, the plating efficiency in the group 3 was only reduced from 46% to 37 and 19% when the cells had been exposed, without a glucocorticoid pretreatment, to 0.1 and 0.5µg/ml AFB_1 respectively. A plating efficiency of 1-2% was still obtained at 2.5µg/ml AFB_1 when 1000 cells/plate had been seeded.

II. With the second procedure. a)The limited 0.8µM dexamethasone treatment had only a slight effect on the plating efficiency(a decrease from $75.8^{\pm}$ 10.3 and $73.2^{\pm}4.6\%$ in group 1 to $65.0^{\pm}8.1$ and $66.4^{\pm}4.6\%$ in group 2, with 100 and 500 plated cells/dish respectively). b) The cytotoxicity of AFB_1 was induced by the glucocorticoid pretreatment. In the absence of the hormonal activation, AFB_1 was not cytotoxic from 0.01 to 0.1µg/ml : group 1 and 3 exhibited the same plating efficiency ($75.8^{\pm}10.3$ and $73.2^{\pm}4.6\%$ in group 1, $72.5^{\pm}5.3$ and $73.8^{\pm}2.6\%$ in group 3).In contrast, an AFB_1 exposure preceeded by a glucocorticoid treatment decreased the plating efficiency progressively from the lowest AFB_1 concentration to the highest (Fig.1).

The size distribution of the surviving colonies provided further details that a cell numeration, although more precise, would not have brought to light (Fig. 2). a) The short dexamethasone treatment slightly increased the relative number of small colonies as compared to the control group. b) AFB_1 exposure without glucocorticoid pretreatment had no significant effect on the size distribution of the colonies. c) AFB_1 exposure after glucocorticoid treatment markedly modified the distribution pattern : the large colonies disappeared and the relative proportion of the small colonies increased progressively at the expense of the medium-size class. A clear effect of the glucocorticoid pretreatment on the size distribution was already visible at the lowest AFB_1 concentration (0.01µg/ml or $3.2.10^{-8}$M).

III. AFB_1 cytotoxic effect on mass monolayer cultures.

No difference was detected between the two groups until after 10 hrs of AFB_1 exposure. At 20 hrs, half the glucocorticoïd pretreated cells had round up and detached from the dishes, while hardly any dead cells could be observed in the control plates, although both groups were still exposed to AFB_1. Moreover, in the

* The DMSO had no visible effect at the tested concentrations. This was checked in every experimental group.

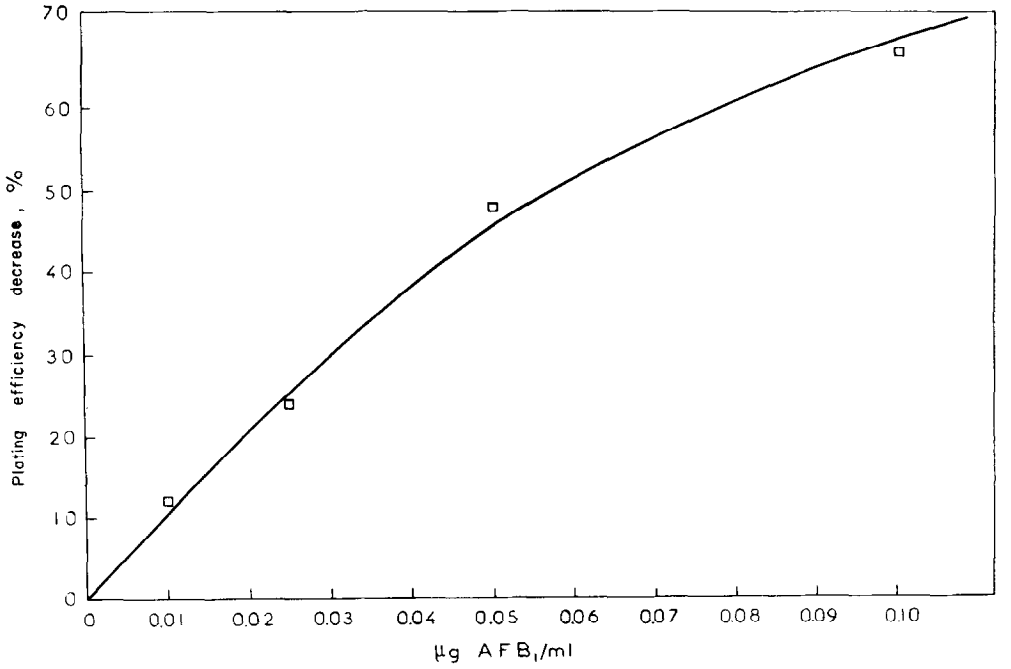

Figure 1 Effect of AFB_1 on plating efficiency after a dexamethasone pretreatment (2nd. procedure). The number of surviving colonies was normalized to the mean number scored in the dexamethasone-treated group exposed to DMSO without AFB_1. There was no decrease in the number of surviving colonies in the group exposed to AFB_1 without a dexamethasone pretreatment (normalized to the mean number of surviving colonies in the corresponding DMSO-exposed control group). DMSO was without effect.

glucocorticoïd + AFB_1 cultures, the nucleoli in the majority of the cells were small, sometimes reduced to a punctiform organelle, whereas in the nuclei of the cells exposed to AFB_1 in control medium without an hormonal pretreatment, the nucleoli remained large, sometimes irregular and nearly as conspicuous as in normal dexamethasone-supplemented cells. In some glucocorticoïd-treated cells exposed to AFB_1, no nucleoli whatsoever could be detected under phase contrast. This was probably the optically visible expression of the previously reported effect of AFB_1 on hepatocyte nucleolus structure, described in EM studies conducted on livers of AFB_1 treated rats (26). This comparative experiment was repeated with the same results.

IV. Comparison of a dedifferentiated clone with its differentiated subclone.

60 ng/ml AFB_1 were not toxic for the clone HF1 exhibiting extinction, even after a glucocorticoïd treatment, while, in the cultures of the reexpressing

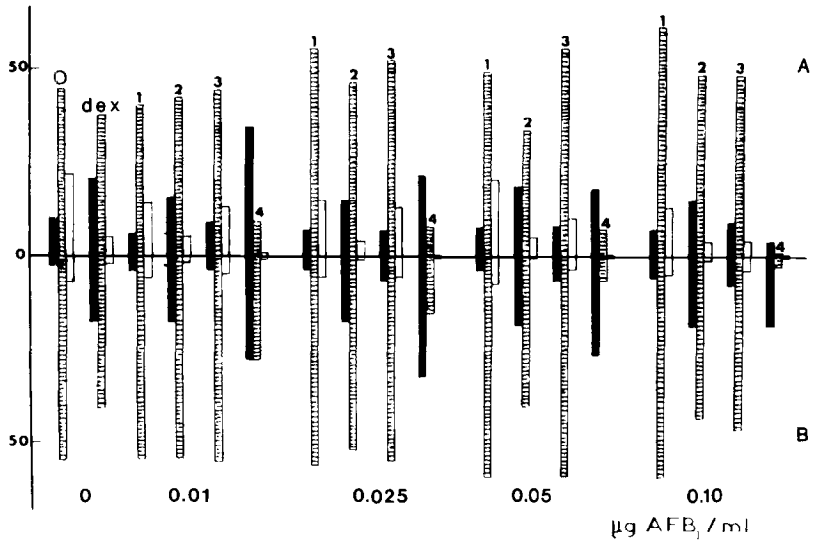

Figure 2 Effect of various treatments on the size distribution of surviving colonies (2d. procedure). Frequency (%) of small (black), medium (striped) and large (white) colonies in the following groups : control medium (0), control medium + dexamethasone treatment (dex), control medium + DMSO exposure (1), dexamethasone treatment + DMSO exposure (2), control medium + AFB_1 in DMSO (3), dexamethasone treatment + AFB_1 in DMSO (4).

A = 500 cells/dish were plated.
B = 100 cells/dish were plated.

subclone HF1-4, the cytotoxicity of AFB_1, high in the absence of a pretreatment, was still higher after a dexamethasone treatment (Table 2).*

V. Comparison of 3-methylcholanthrene and dexamethasone pretreatments on AFB_1 cytotoxicity.

a) The preliminary test showed that a brief AFB_1 exposure after a MC treatment did not reduce the number of cells obtained in one week from the initial 2.10^5 cells/dish, in contrast with the result in the (dexamethasone + MC) group, where a 50% decrease was scored. b) Table 3 gives the results of the plating efficiency assay. The only group demonstrating an AFB_1 cytotoxicity was the dexamethasone treated-one. 40ng/ml AFB_1 were not toxic when administrated after MC or without any prior treatment.

* The hormone had a decreasing effect by itself on the plating efficiency in both groups, even stronger in HF1 than in HF1-4. Some glucocorticoïd receptors would thus still be present in the dedifferentiated cells. AFB_1 seems markedly more toxic towards the pseudotetraploïd differentiated hybrid HF1-4 than towards the hyperdiploïd Faza 967 from which Fao, one of HF1's parent, has evolved. This could be apparent and a consequence of the relatively slower growth rate of HF_{1-4}. However, the induction effect of dexamethasone on chenodeoxycholate hydroxylation has indeed been shown to be stronger, on a per cell basis, in the case of HF1-4 than in the case of Faza 967 and of Fao (17c). The corticoïds in the fetal calf serum might explain the toxicity of AFB_1 in the absence of a dexamethasone treatment and be specially effective in the large cells of the hybrid.

Table 2 Comparison of the cytotoxic effect of AFB_1 on clones HF_1 and HF_{1-4}.

	Control	Dexamethasone 0.8µM	Control + AFB_1(60ng/ml)	Dexamethasone + AFB_1 (60ng/ml)
clone HF_1	48 ± 4.2	37 ± 3.3	50 ± 3.6	36 ± 1.3
clone HF_{1-4}	65 ± 3.0	58 ± 3.6	16 ± 1.8	5 ± 1.8

(plating efficiency with or without a prior glucocorticoïd treatment after plating 500 cells per plate)

Table 3 Comparison of the AFB_1 cytotoxic effect on Faza 967 cells after a pretreatment with dexamethasone or MC or without any pretreatment.

Pretreatment	AFB_1 concentration ng/ml 0	40
none	62 ± 4.6	67 ± 3.7
Dexamethasone 0.8µM	65 ± 4.9	12.2 ± 4.8
3-Methylcholanthrene 2.0µM	71.2 ± 3.9	72 ± 3.2

(plating efficiency after plating 500 cells/plate)

DISCUSSION AND CONCLUSION

These experiments show that AFB_1 cytotoxicity in differentiated hepatoma cultures is markedly enhanced by a glucocorticoïd pretreatment. Indeed, at the lowest concentrations, AFB_1 toxicity is entirely dependent on a prior exposure to glucocorticoïd. The hormone thus seems to induce the metabolism of the mycotoxin to an active compound. The direct proof of AFB_1 activation will come probably from the analysis (now in progress) of the metabolites obtained in the two experimental groups. Our results would thus provide a clear-cut instance of hormonal activation of a procarcinogen in a permanent cell line.

These results were first obtained with the differentiated clone Faza 967. A comparison of the closely-related hybrid HF_1 with one of its subclone HF_{1-4} has shown that, in hepatoma cultures, the cytotoxicity of AFB_1 and its hormonal enhancement are linked to the expression of liver-specific traits, in particular

the glucocorticoïd induction of bile acid hydroxylation, a cytochrome P-450 mediated reaction. In contrast, MC, known to induce the cytochrome P-448 benzopyrene hydroxylase in the hepatoma line from which the tested clones have evolved (14,15), is without any effect on bile acid metabolism in vivo (21) and in Faza 967 cultures (17c) and is unable to enhance AFB_1 cytotoxicity. Conversely, dexamethasone does not induce benzopyrene-hydroxylase in hepatoma cultures (23). These observations strongly suggest that the glucocorticoïd induces in these hepatoma lines a cytochrome P-450 mediated monooxygenase which converts AFB_1 to a cytotoxic metabolite.

The influence of various factors on AB_1 activation to cytotoxic and mutagenic compounds could be studied in these hepatoma monolayers which offer the experimental convenience of a cloned cell line, while still expressing many specific differentiation criteria found in normal liver in vivo (22). The presence in the same cell of both the activating enzyme and the target of the active metabolite(s) should confer a high sensitivity to experiments carried in this system. (Our figures compare favorably with those obtained from the DNA repair tests conducted by Williams in primary hepatocytes cultures (27) and from the cytotoxicity assays in the cocultivation experiments of Langenbach et al.) (28). Furthermore, the glucocorticoïd treatment now includes cytochrome P-450 monooxygenases within the range of activating enzymes that can be induced at will in a permanent cell line. This should increase the versatility of hepatoma cultures as a pharmacological tool.

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