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GENETIC EXPRESSION OF AFLATOXIN METABOLISM

EFFECTS OF 3-METHYLCHOLANTHRENE AND β-NAPHTHOFLAVONE ON HEPATIC MICROSOMAL METABOLISM AND MUTAGENIC ACTIVATION OF AFLATOXINS*

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Abstract—The effects of pretreatment with 3-methylcholanthrene (MC) and β-naphthoflavone (βNF) on the hepatic microsome-mediated mutagenesis of aflatoxin B_1 (AFB_1) and benzo[*a*]pyrene, and on the metabolism of aflatoxins B_1 and B_2, were investigated in inbred mouse strains. The inbred strains of mice studied included Ah nonresponsibe strains (DBA/2Ha, AKR/Sn and RF/J), which were also nonresponsive to the induction of the metabolism of AFB_1 to AFM_1 (AFB_1-4-hydroxylase activity), and Ah responsive strains (C57BL/6Ha, ICR/Ha, C3H/St, A/St, Balb/cCr, C57e/Ha and CBA/Pi), which were also responsive to the induction of AFB_1-4-hydroxylase activity. The hepatic microsome-mediated enzyme activities studied included: mutagenic activation of AFB_1 and benzo[a]pyrene in the Ames *Salmonella typhimurium* TA-98 system; metabolism of AFB_1 and AFB_2 to AFM_1 and AFM_2, respectively; and benzo[*a*]pyrene metabolism measured as the formation of fluorescent phenolic metabolites, i.e. aryl hydrocarbon hydroxylase (AHH) activity. Time–course and dose–response studies in C57BL/6Ha mice revealed that the metabolism of aflatoxin B_1/B_2 to aflatoxin M_1/M_2 (AFB_1/B_2-4-hydroxylase activity) was induced by both MC and βNF. In the nonresponsive strains studied, pretreatment with MC or βNF produced essentially little alteration of AFB_1-4-hydroxylase activity or AHH activity or the mutagenic activation of AFB_1 and benzo[*a*]pyrene. On the other hand, AFB_1-4-hydroxylase activity in the responsive strains was induced 4- to 10-fold by MC (60 mg/kg) and 2.5- to 7-fold by βNF (150 mg/kg). Also in the responsive strains, induction of AFB_1-4-hydroxylase activity was strongly associated with (a) the depression of the mutagenic activation of AFB_1, and (b) with the induction of both AHH and the mutagenic activation of benzo[*a*]pyrene. In summary, the results described in this report suggest that: (a) induction of AFB_1-4-hydroxylase activity by MC (or βNF) is associated with the depression of AFB_1 mutagenesis and with the induction of benzo[*a*]pyrene mutagenesis; and (b) induction by MC (or βNF) of AHH activity, AFB_1-4-hydroxylase activity and AFB_2-4-hydroxylase activity is controlled by either the same or closely linked genetic factors.

Microsomal multisubstrate monooxygenase, a membrane-bound inducible enzyme system principally found in the liver, is involved in the metabolism of most drugs, chemical carcinogens and several other chemicals [1]. This monooxygenase can be resolved into three main components, e.g. cytochrome P-450, cytochrome P-450 reductase and the lipid fraction, which on reconstitution metabolizes a number of chemicals [2]. During recent years, biochemical and immunological evidence has accumulated demonstrating the existence of at least six different types of cytochrome P-450s in the rat liver [3]. Various cytochrome P-450s have been identified on the basis of differences in induction and inhibition specificities, spectral characteristics, and mobility on gel electrophoresis [2, 3]. For example, cytochrome P-450 is inducible by pretreatment of animals with phenobarbital, whereas cytochrome P_1-450/448 is inducible by pretreatment of animals with polycyclic aromatic hydrocarbons, e.g. MC‡ and TCDD.

Aflatoxins, a group of structurally related chemicals, are mycotoxins produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus*. Alfatoxins contaminate several human and animal foods and have been implicated in the etiology of human liver disease [4, 5]. Of the four naturally occurring aflatoxins (viz. aflatoxins B_1, B_2, G_1 and G_2), AFB_1 is the most potent hepatocarcinogenic and hepatotoxic agent; aflatoxin G_1 is relatively less active than AFB_1, whereas aflatoxins B_2 and G_2 are relatively inactive [5]. AFB_1 is not active *per se* but requires hepatic microsome-mediated metabolism to reactive derivatives that bind to tissue macromolecules [6–9]. Our recent studies [10] have implicated involvement of at least three different forms of cytochrome P-450 in the metabolism of AFB_1 to various metabolites including aflatoxins M_1, P_1, Q_1 and AFB_1-2,3-oxide which has been identified as the major DNA-binding metabolite formed *in vivo* and *in vitro* [8, 11, 12]. AFB_1-2,3-oxygenase, which converts AFB_1 to

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‡ Abbreviations: MC, 3-methylcholanthrene; βNF, β-naphthoflavone; AHH, aryl hydrocarbon hydroxylase; AFB_1, aflatoxin B_1; AFM_1, aflatoxin M_1; AFB_2, aflatoxin B_2; AFM_2, aflatoxin M_2; BP, benzo[*a*]pyrene; and TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

AFB_1-2,3-oxide, is inducible in rats and in various strains of mice by pretreatment with phenobarbital, whereas, like aryl hydrocarbon hydroxylase, AFB_1/AFB_2-4-hydroxylase, which converts AFB_1/AFB_2 to AFM_1/AFM_2, is (are) inducible by pretreatment of rats and only certain strains of mice with MC and TCDD [10, 13, 14].

Detailed studies of the genetics of AFB_1-4-hydroxylase induction would be facilitated if a relatively noncarcinogenic inducer could be used to replace MC and TCDD. Boobis *et al.* [15] have reported that MC and βNF operate via the same mechanism in the induction of aryl hydrocarbon hydroxylase and thus can be used interchangeably. The induction of aryl hydrocarbon hydroxylase in mice is associated with an enhancement in the microsome-mediated mutagenic activation of benzo[*a*]pyrene and its binding to DNA and proteins [16–18]. This pleotropic effect of aryl hydrocarbon hydroxylase induction is believed to be the consequence of increased metabolism of benzo[*a*]pyrene to reactive expoxides and diol-epoxides [19, 20]. Since the induction of aryl hydrocarbon hydroxylase has been reported to be associated with the induction of AFB_1-4-hydroxylase [13] and since metabolism of AFB_1 to AFM_1 constitutes deactivation [21, 22], it was of interest to investigate (a) whether βNF would induce AFB_1-4-hydroxylase in the Ah responsive strains but not in the Ah nonresponsive strains, and (b) the effects of AFB_1-4-hydroxylase induction on the microsome-mediated activation of AFB_1 and benzo[*a*]pyrene.

In the present report, we have sought to investigate the dose–response and time–course effects of MC and βNF in the induction of the metabolism of AFB_1 and AFB_2 to AFM_1 and AFM_2, respectively, in C57BL/6 mice, an Ah responsive strain. A single effective dose—a dose that produced a significant degree of induction—of each of the two inducers was also tested in several Ah nonresponsive and responsive strains of mice for their effects on AFB_1-4-hydroxylase activity, and on the hepatic microsome-mediated mutagenic activation of AFB_1 and benzo[*a*]pyrene in the Ames *Salmonella typhimurium* TA-98 system. The results of these studies are reported here.

MATERIALS AND METHODS

Chemicals. Chemicals were obtained from the following sources: aflatoxins B_1 and B_2 from Calbiochem, San Diego, CA; ^{3}H-labeled aflatoxins B_1 and B_2 from Moraveck Biochemicals, City of Industry, CA; benzo[*a*]pyrene and dimethyl sulfoxide from the Aldrich Chemical Co., Milwaukee, WI; and MC, βNF and NADPH from the Sigma Chemical Co., St. Louis, MO. The rest of the chemicals of the highest available quality were purchased from commercial sources. *Salmonella typhimurium* strain TA-98 was originally obtained from Dr. Bruce Ames, University of California, Berkeley, CA.

Animals. The animal resources division of this Institute provided various inbred strains of mice, except RF/J which was obtained from the Jackson Laboratories, Bar Harbor, ME. Female mice between the ages of 6 and 8 weeks were used in all experiments.

Pretreatment of mice. For each strain of mice, 44 hr prior to being killed four to six mice in each group received a single intraperitoneal injection of MC or βNF dissolved in 0.15 ml corn oil. Control mice received corn oil alone.

Preparation of liver microsomes. Hepatic microsomes were prepared as described earlier [13]. Livers were removed and rinsed with cold 0.9% NaCl and then homogenized in 0.25 M sucrose solution. Liver homogenates were centrifuged at 9,000 *g* for 15 min at 4°. The resulting supernatant fraction was further centrifuged at 105,000 *g* for 75 min to obtain the microsomal pellet. Protein concentration of the microsomal samples was determined by the method of Lowry *et al.* [23]. Metabolism of AFB_1 and AFB_2 to AFM_1 and AFM_2, respectively, was carried out with microsomal preparations isolated from each mouse, whereas for the metabolism of AFB_1 to DNA-binding metabolites pooled microsomes were used.

For the mutagenesis assays, microsomes were isolated under sterile conditions using autoclaved glassware and sterile sucrose solution. Microsomal preparations from four to six mice were pooled and employed in the mutagenesis assays.

Metabolism of aflatoxins B_1 and B_2 to aflatoxins M_1 and M_2. Using hepatic microsomes from individual mice, AFB_1-4-hydroxylase activity, which catalyzes the formation of AFM_1 from AFB_1, was assayed by the previously reported method [13, 14]. Briefly, in a total volume of 250 μl hepatic microsomes (about 1.0 mg protein) were incubated in a buffer mixture with [^{3}H]AFB_1 (0.57 mM) and an NADPH-generating system (prewarmed for 15 min at 37°). At the termination of the 15-min incubation, the reaction was stopped with 200 μl of cold (−20°) methanol, followed by 1 ml of 0.1 M potassium phosphate buffer, pH 6.0. The mixture was extracted with chloroform, and an aliquot of chloroform was evaporated to dryness under nitrogen. The residue was redissolved in ethyl acetate and spotted on a silica gel thin-layer chromatography plate (5 × 20 cm). The plate was developed in a solvent mixture of chloroform–isopropyl alcohol (95 : 5), dried, viewed under ultraviolet light, and the fluorescent band corresponding to AFM_1 demarcated, scraped and counted by scintillation spectrometry. AFB_2-4-hydroxylase activity, which catalyzes the metabolism of AFB_2 to AFM_2, was measured by a similar procedure [14].

Metabolism of aflatoxin B_1 to DNA-binding metabolites. AFB_1-2,3-oxygenase, which catalyzes the conversion of AFB_1 to the DNA-binding metabolite(s), was assayed by the following method. Livers of three or four mice in a group were pooled for the isolation of the microsomes. The composition of the incubation mixture used to demonstrate DNA binding was as follows: potassium phosphate buffer, pH 7.4, 0.08 M; magnesium chloride, 3.3 mM; EDTA, 0.2 mM; NADPH-generating system containing 0.81 mM NADP, 17 mM DL-isocitrate, and isocitrate dehydrogenase (200 μg protein); native calf thymus DNA, 2 mg; hepatic microsomes (1 mg protein); and [^{3}H]AFB_1 (0.44 mM) in 25 μl of dimethyl sulfoxide

(specific activity, about 2.5 mCi/mmole). The buffer mixture containing the NADPH-generating system was incubated at 37° for 15 min to ensure the presence of an adequate amount of NADPH. The reaction was started with the addition of [^{3}H]AFB_1, and the incubation was carried out for 15 min. The total volume of the incubation mixture was 2.3 ml. At the termination of the incubation, DNA was extracted into the aqueous phase. The DNA was then precipitated, redissolved, and subsequently deproteinized by repeated extraction with a chloroform–isoamyl alcohol (19 : 1) mixture. The final DNA preparation had an $A_{260} : A_{280}$ ratio of 1.9 and contained less than 3% protein or RNA. Details of this method have been reported previously [9].

Aryl hydrocarbon hydroxylase activity. This activity was measured by a previously reported method described earlier [13].

Mutagenesis assay. For assaying the mutagenic activation of AFB_1 and benzo[*a*]pyrene, the Ames Salmonella test system [24, 25] was used with some modifications. The incubation mixture contained 0.1 ml of overnight bacterial culture (TA-98) in nutrient broth and 0.5 ml of microsomal mixture (containing in 1 ml; sodium phosphate buffer, pH 7.4, 100 μmoles; $MgCl_2$, 8 μmoles; KCl, 33 μmoles; NADPH, 0.91 μmoles; and hepatic microsomal protein, 0.10 to 1 mg). To this solution 25 μl carcinogen solution (200 ng AFB_1 or 5 μg benzo[*a*]pyrene) or 25 μl dimethylsulfoxide (control plates) was added, and the mixture was agitated lightly on a vortex. The reaction mixture was incubated with mild shaking at 37° for 5 min, and then 50 μl of menadione solution (108 nmoles) was added to terminate the mutagenic activation of the carcinogen. A solution (2 ml) of top agar, maintained in the molten state at 45°, was then added to the mixture, and the contents were mixed and then overlayed on Vogel Bonner glucose minimal agar plates. Histidine reversions were scored after 48 hr of incubation at 37°. Each sample was done in duplicate, and the results are expressed as the mean values after subtracting the background.

The above conditions employed in the mutagenesis assay are based on results of several initial experiments which demonstrated that 108 nmoles of menadione completely stopped the NADPH-dependent mutagenesis of AFB_1 and benzo[*a*]pyrene, blocked the microsome-mediated binding of AFB_1 to DNA by 75–85%, and allowed greater than 94% bacterial survival at this concentration, i.e. killed less than 6% of the bacteria. In addition, these experiments also demonstrated that the number of histidine revertants formed was linear with the time of incubation up to 5 min for AFB_1 and 15 min for benzo[*a*]pyrene, and the optimal mutagen concentration was 200 ng for AFB_1 and 5 μg for benzo[*a*]pyrene. Therefore, the conditions described above were optimal and reasonable for comparative evaluations.

RESULTS

Dose–response effects of MC and βNF on AFB_1/AFB_2-4-hydroxylase activity. Using various doses of MC (0–200 mg/kg) and βNF (0–400 mg/kg), induction of the metabolism of AFB_1 to AFM_1 and of AFB_2 to AFM_2 were investigated in C57BL/6 mice. MC produced a dose-dependent increase in AFB_1-4-hydroxylase activity (Fig. 1A). Maximal activity occurred at a dose of 40 mg/kg and the activity decreased slowly with the increasing dose of MC such that at a dose of 200 mg/kg the activity was decreased about 30% from the maximal activity. AFB_1-4-hydroxylase activity also increased as a function of βNF dose (Fig. 1B). However, the activity continually increased up to the maximum dose (400 mg/kg) employed.

AFB_2-4-hydroxylase activity also increased as a function of the dose of either inducer (Fig. 2, A and B). While the profile of induction by βNF was qualitatively similar to that obtained for AFB_1-4-hydroxylase (compare Figs. 1B and 2B), the profile of induction by MC was different (compare Figs. 1A and 2A). Maximal induction occurred at a dose of 80–100 mg/kg of MC (Fig. 2A), almost twice the dose required for the induction of AFB_1-4-hydroxylase. Furthermore, the highest dose of MC employed inhibited AFB_2-4-hydroxylase activity much more than it inhibited AFB_1-4-hydroxylase activity; at 200 mg/kg MC, AFB_2-4-hydroxylase activity was decreased 74% from the maximal activity, compared to a decrease of 30% from the maximal activity observed for AFB_1-4-hydroxylase (compared Figs. 1A and 2A).

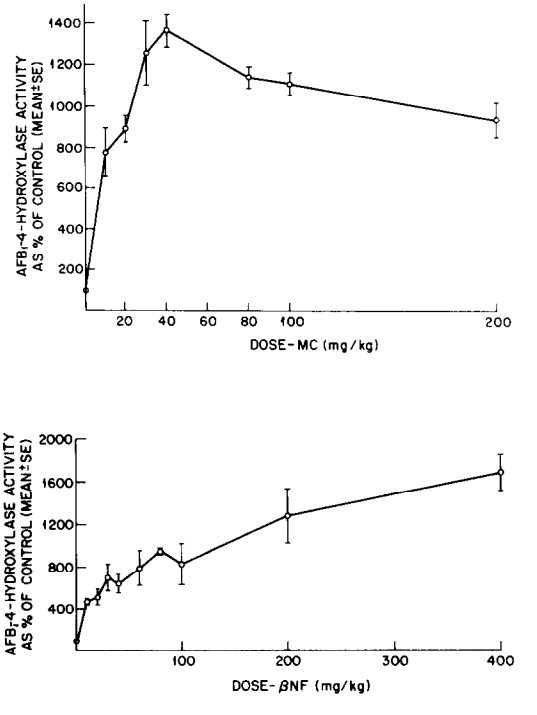

Fig. 1. Dose–response of the induction of the conversion of AFB_1 to AFM_1 by hepatic microsomes of (A) MC- and (B) βNF-treated C57BL/6 mice. Each value shown in this figure and in Figs. 2 and 3 represents a mean (± S.E.) of four or five individual mice. Mice received MC or βNF i.p. 44 hr prior to being killed. Other details are described in the text.

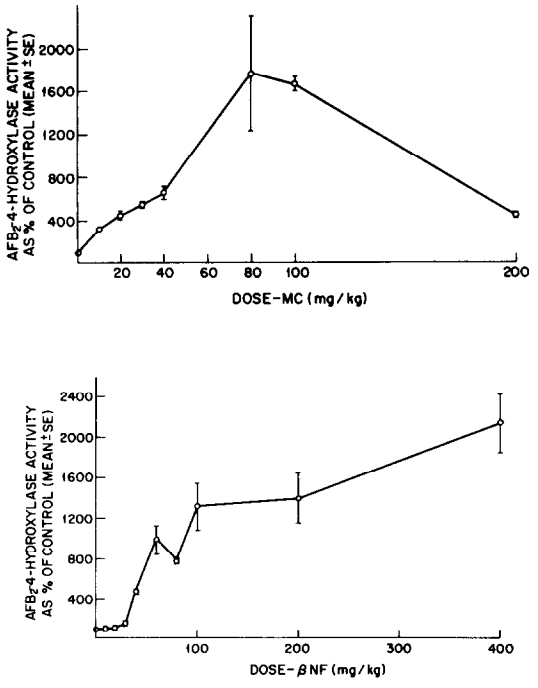

Fig. 2. Dose–response of the induction of the conversion of AFB_2 to AFM_2 by hepatic microsomes of (A) MC- and (B) βNF-treated C57BL/6 mice. Other details are given in the legend of Fig. 1.

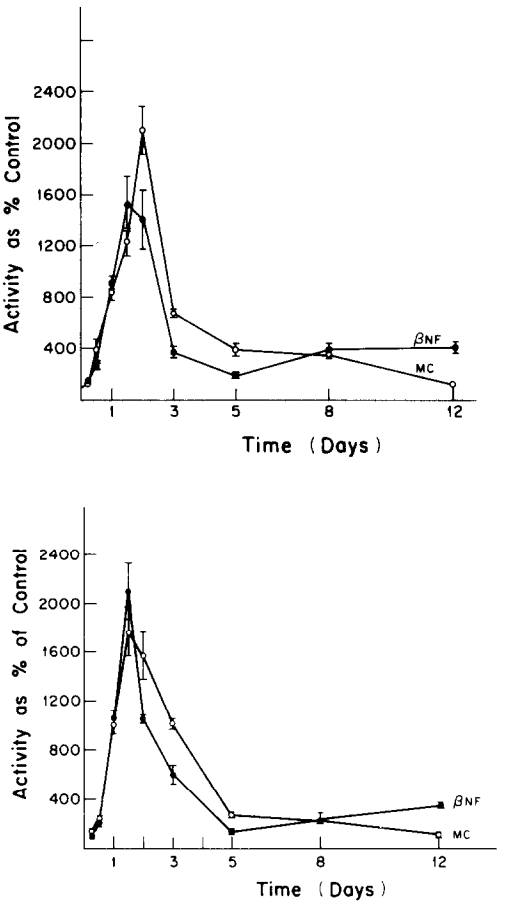

Fig. 3. Time–course of the induction of AFB_1-4-hydroxylase (A) and AFB_2-4-hydroxylase (B) activities in hepatic microsomes from C57BL/6 mice treated i.p. with a single dose of MC (60 mg/kg) or βNF (150 mg/kg).

Time–course effects of MC and βNF pretreatment on AFB_1/AFB_2 4-hydroxylase activity. Time–course of the induction of AFB_1-4-hydroxylase and AFB_2-4-hydroxylase in the C57BL/6 strain was followed over a period of 12 days after the administration of a single effective dose of MC (60 mg/kg) and βNF (150 mg/kg). The results of these investigations are illustrated in Fig. 3.

Following the administration of either MC or βNF, AFB_1-4-hydroxylase activity (Fig. 3A) increased with time, reached a maximum between 36 and 48 hr and then declined significantly by day 5. Thereafter, the activity in hepatic microsomes from MC-treated animals decreased gradually, whereas the activity in microsomes from βNF-treated mice either remained the same or was somewhat higher than the control.

The time–course profiles of AFB_2-4-hydroxylase induction by MC and βNF were similar to those of AFB_1-4-hydroxylase (Fig. 3B). Maximal induction occurred around 36 hr and was similar for both MC and βNF.

Effects of induction of AFB_1-4-hydroxylase on the mutagenic activation of AFB_1 and benzo[a]pyrene. As discussed earlier, following a single dose of MC or βNF, AFB_1-4-hydroxylase activity responsible for the conversion of AFB_1 to AFM_1 increased with time, reached a peak between 36 and 48 hr and then declined about 80% from the maximal level by day 3 to 5. If the metabolism of AFB_1 to AFM_1 represents detoxification of AFB_1 [21, 22, 27], the induction of AFB_1-4-hydroxylase should result in some depression of the hepatic microsome-mediated mutagenesis of AFB_1. To test this prediction, hepatic microsomes, isolated at various time points following the administration of either MC or βNF to C57BL/6 (AFB_1-4-hydroxylase inducible) and DBA/2 (AFB_1-4-hydroxylase noninducible) mice, were employed to activate AFB_1 and benzo[*a*]pyrene in the bacterial mutagenesis assay. The results are given in Table 1. The hepatic microsome-mediated mutagenic activation of AFB_1 was depressed and that of benzo[*a*]pyrene was enhanced up to 72 hr (day 3) following treatment of C57BL/6 mice with either MC or βNF; however, some of these effects were also observed when hepatic microsomes isolated on day 5 were tested. The activity returned to near control levels in hepatic microsomes isolated from C57BL/6 mice on day 12 following treatment with MC or βNF. On the other hand, both MC and βNF produced essentially little depression and no enhancement, respectively, of the mutagenic activation of AFB_1 and benzo[*a*]pyrene catalyzed by hepatic microsomes isolated on days 1 1/2 (36 hr), 2 and 12 from DBA/2 mice pretreated with either MC or βNF.

The results in Table 1 and those in an earlier preliminary report [27] strongly suggest that induction of AFB_1-4-hydroxylase (and AHH) in the mouse

Table 1. Mutagenic activation of AFB_1 and benzo[*a*]pyrene (BP) catalyzed by hepatic microsomes isolated at different time points following treatment with MC or βNF*

Inbred strain of mice (time after injection)	Treatment	His^+ revertants as % of control AFB_1	His^+ revertants as % of control BP
C57BL/6HA			
12 hr	Corn oil	100	100
	βNF	42	707
	MC	62	985
36 hr	Corn oil	100	100
	βNF	56	972
	MC	76	1036
72 hr	Corn oil	100	100
	βNF	37	332
	MC	51	476
5 days	Corn oil	100	100
	βNF	61	575
	MC	63	662
12 days	Corn oil	100	100
	βNF	66	136
	MC	111	170
DBA/2Ha			
36 hr	Corn oil	100	100
	βNF	90	56
	MC	100	46
48 hr	Corn oil	100	100
	βNF	109	117
	MC	101	160
12 days	Corn oil	100	100
	βNF	112	150
	MC	101	137

* Each value in the table is a mean of the number of mutant colonies observed (background subtracted) on four different plates, of which two received 0.75 mg of microsomal protein/plate and the other two 1 mg of microsomal protein/plate. Mice received a single i.p. injection of MC (60 mg/kg) or βNF (150 mg/kg) 44 hr prior to being killed. Control values (background subtracted) for different microsomal preparations for histidine revertant mutants (colonies/plate) were: C57BL/6, AFB_1, 154–174 colonies; and BP 15–68 colonies; DBA/2Ha, AFB_1, 555–1190; and BP, 12–37.

hepatic microsomes enhances the ability of these microsomes to deactivate AFB_1 and activate benzo[*a*]pyrene in the Ames bacterial mutagenesis test system. To further evaluate this association, hepatic microsomes from three Ah nonresponsive (AFB_1-4-hydroxylase noninducible) strains and seven Ah responsive (AFB_1-4-hydroxylase inducible) strains of mice pretreated with either βNF or MC were used to analyze the mutagenic activation of AFB_1 and benzo[*a*]pyrene in the Ames Salmonella bacterial test system. Each microsomal preparation was tested at various protein concentrations ranging from 0.1 to 1 mg/plate, and the representative mutagenesis data for two nonresponsive (AKR/Sn and DBA/2Ha) and two responsive (ICR/Ha and C57e/Ha) strains of mice are illustrated in Figs. 4–7. As reported previously [27] and as shown here (Figs. 4–7), mutagenic activation of AFB_1 and benzo[*a*]pyrene did not increase proportionately at all microsomal protein concentrations tested. A consistent observation, comparing various mouse strains, was that MC as well as βNF pretreatment of the noninducible strains (DBA/2Ha, AKR/Sn and RF/J) minimally altered the abilities of their hepatic microsomes to activate AFB_1 or benzo[*a*]pyrene, whereas the same pretreatment of the inducible strains (C57BL/6Ha, ICR/Ha, C3H/St, A/St, Balb/cCr, C57e/Ha and CBA/Pi) enhanced the abilities of their hepatic microsomes to deactivate AFB_1 and activate benzo[*a*]pyrene in the Ames bacterial mutagenesis test system. While enhancement of the benzo[*a*]pyrene mutagenesis by pretreatment of the inducible strain with MC (and βNF) was consistently observed at all the microsomal protein concentrations tested, the depression of AFB_1 mutagenesis was observed consistently only when higher control microsomal protein concentrations (0.5 to 1 mg protein/plate) were employed in the mutagenesis assay.

In addition to these observations, considerable interstrain differences in the mutagenic activation of both AFB_1 and benzo[*a*]pyrene were observed. The results of the mutagenesis studies and those of the other studies in which the effects of MC pretreatment of the mice on AHH activity and on the metabolism of AFB_1 to AFM_1 and AFB_1-2,3-oxide (i.e. the active metabolite of AFB_1) were investigated are summarized in Table 2.

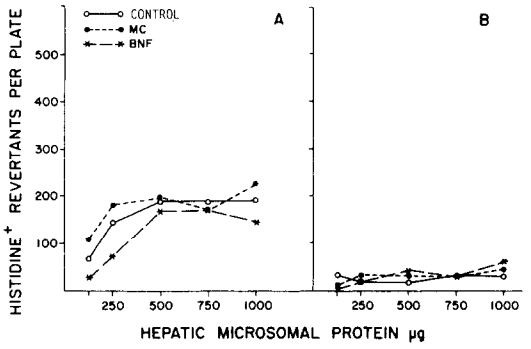

Fig. 4. Effects of βNF and MC pretreatment on the mutagenesis of aflatoxin B_1 (A) and benzo[*a*]pyrene (B), catalyzed by hepatic microsomes from AKR/Sn, an Ah non-responsive strain of mice. Mice received i.p. a single dose of MC (60 mg/kg) or βNF (150 mg/kg) 44 hr prior to being killed. Each point is a mean of duplicate determinations. Details are described in the text.

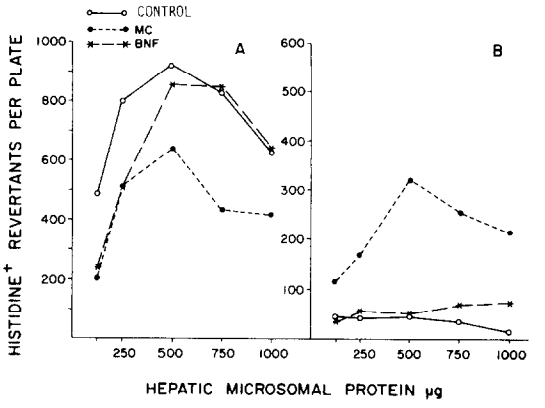

Fig. 6. Effects of βNF and MC pretreatment on the mutagenesis of aflatoxin B_1 (A) and benzo[*a*]pyrene (B) catalyzed by hepatic microsomes from ICR/Ha, an Ah responsive strain of mice. Mice received i.p. a single dose of MC (60 mg/kg) or βNF (150 mg/kg) 44 hr prior to being killed. Each point is a mean of duplicate determinations. Details are described in the text.

In the noninducible strains (DBA/2Ha, AKR/Sn and RF/J), MC failed to induce either AHH activity or AFB_1-4-hydroxylase activity more than 1.5-fold. Similarly, this pretreatment had relatively little effect on the mutagenic activation of either AFB_1 or benzo[*a*]pyrene. Metabolic activation of AFB_1 measured as the formation of DNA-binding metabolite(s), which is essentially believed to be AFB_1-2,3-oxide [8], was, however, depressed by about 35% in DBA/2Ha and AKR/Sn but not in RF/J.

Compared to these results, in the inducible strains (C57BL/6Ha, ICR/Ha, C3H/St, A/St, Balb/cCr, C57e/Ha and CBA/Pi) MC induced AHH activity by 2.7- to 5.7-fold and AFB_1-4-hydroxylase activity by 3.6- to 10.1-fold. In all these strains, MC depressed AFB_1-2,3-oxide formation by 23–36% except in ICR/Ha which was depressed by only 11%. Mutagenic activation of AFB_1 was also depressed in all strains; the depression by MC ranged from a slight depression (5–10% in C3H/St) to about 45% in Balb/cCr. In contrast to this, the mutagenic activation of benzo[*a*]pyrene, except in A/St which among the tested strains was the least inducible for AHH, was enhanced by βNF from 200% in C3H/St to 970% in C57BL/6Ha, and by MC from 300% in C57e/Ha to 1650% in CBA/Pi. C57e/Ha, an Ah responsive strain, is a coat color mutation (black to brown) that has occurred recently in the C57BL/6Ha stock at this Institute.

DISCUSSION

MC and βNF as inducers of AFB_1/AFB_2-4-hydroxylase activity. Boobis *et al.*, [15] compared MC and βNF as inducers of hepatic AHH in

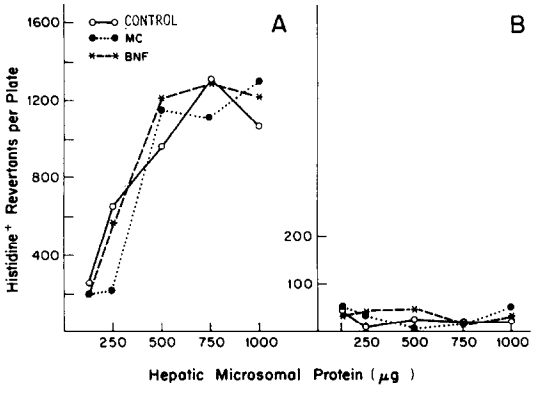

Fig. 5. Effects of βNF and MC pretreatment on the mutagenesis of aflatoxin B_1 (A) and benzo[*a*]pyrene (B) catalyzed by hepatic microsomes from DBA/2Ha, an Ah non-responsive strain of mice. Mice received i.p. a single dose of MC (60 mg/kg) or βNF (150 mg/kg) 48 hr prior to being killed. Each point is a mean of duplicate determinations. Details are described in the text.

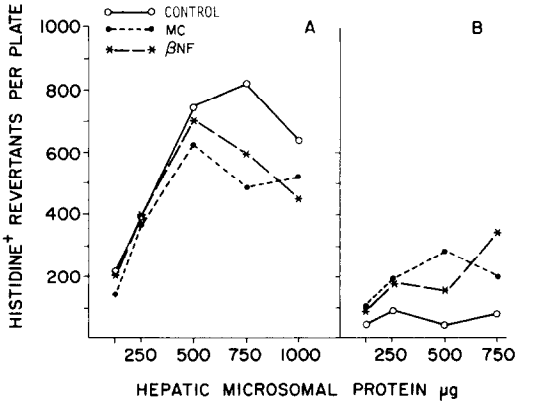

Fig. 7. Effects of βNF and MC pretreatment on the mutagenesis of aflatoxin B_1 (A) and benzo[*a*]pyrene (B) catalyzed by hepatic microsomes from C57e/Ha, an Ah responsive strain of mice. Mice received i.p. a single dose of MC (60 mg/kg) or βNF (150 mg/kg) 44 hr prior to being killed. Each point is a mean of duplicate determinations. Details are described in the text.

Table 2. Effects of MC and βNF treatment of mice on the hepatic microsome-mediated mutagenic activation and metabolism of AFB_1 and benzo[*a*]pyrene*

Inbred strains of mice	Treatment	AFB_1: Histidine$^+$ revertants as (% of control)	BP: Histidine$^+$ revertants as (% of control)	MC pretreatment: AFB_1-2,3 oxygenase (% of control)	MC pretreatment: AFB_1-4-hydroxylase (MC/Control)	MC pretreatment: AHH (MC/Control)
Ah-Nonresponsive						
DBA/2Ha	Corn oil	100	100	100		
	βNF	109	117		1.37	1.06
	MC	101	160	68		
AKR/Sn	Corn oil	100	100	100		
	βNF	82	109		1.32	1.13
	MC	105	140	65		
RF/J	Corn oil	100	100	100		
	βNF	124	138		1.4	1.00†
	MC	106	83	102		
Ah-Responsive						
C57BL/6Ha	Corn oil	100	100	100		
	βNF	56	972		7.8	4.10
	MC	76	1036	66		
ICR/Ha	Corn oil	100	100	100		
	βNF	101	313		8.0	4.01
	MC	58	1034	89		
C3H/St	Corn oil	100	100	100		
	βNF	95	197		10.1	5.70
	MC	90	356	64		
A/St	Corn oil	100	100	100		
	βNF	52	81		4.1	2.65
	MC	89	130	66		
Balb/cCr	Corn oil	100	100	100		
	βNF	90	233		3.6	2.90†
	MC	54	740	73		
C57e/Ha	Corn oil	100	100	100		
	βNF	72	451		ND‡	3.50
	MC	69	323	61		
CBA/Pi	Corn oil	100	100	100		
	βNF	68	642		4.2	3.40†
	MC	77	1650	77		

* Each value for mutagenesis is a mean of the number of mutant colonies (background subtracted) observed on four different plates, of which two received 0.75 mg of microsomal protein/plate and the other two 1 mg of microsomal protein/plate. For AHH activity and the metabolism of AFB_1 to AFM_1, four or five mice of each strain were individually tested; pooled microsomes from four to six mice were tested in duplicate for the formation of the DNA-binding metabolites of AFB_1 (AFB_1-2,3-oxygenase activity). Mice received a single i.p injection of MC (60 mg/kg) or βNF (150 mg/kg) 44 hr prior to being killed. Other details are described in the text. Mutant colonies/plate in control mice of different strains ranged as follows: AFB_1, 192–1075/plate; BP, 12–40/plate. AFB_1-2,3-oxygenase, measured as pmoles AFB_1 bound per mg DNA per 15 min, ranged from 2148 to 8520 in control mice of different strains. AFB_1-4-hydroxylase, measured as pmoles AFM_1 formed per mg of microsomal protein per 15 min, ranged from 178 ± 39 to 3433 ± 946 in control mice of different strains. AHH activity, measured as pmole equivalents of 3-hydroxybenzo[*a*]pyrene formed per mg microsomal protein per min, ranged from 194 ± 23 to 731 ± 131 in control mice of different strains.

† Reported previously in Refs. 13, 18 and 19.

‡ Not determined.

C57BL/6N mice and reported that, following a single dose of βNF or MC (80 mg/kg), AHH activity increased with time, reached an optimum around 36–48 hr, and then remained unaltered for up to 7 days in the case of MC, but declined rapidly reaching control level on day 3 in the case of βNF. Although these studies were extended up to 9 days, the MC-induced activity declined only about 25% between days 7 and 9. The differences in the time–response profiles for MC and βNF were attributed to pharmacokinetic differences between the two inducers. The data were interpreted to suggest that βNF was eliminated from the body much faster than MC which remained in the fat tissue and served as a continued source of stimulus for AHH induction.

As shown in Table 3, we have found that βNF, like MC, induces AFB_1-4-hydroxylase in Ah responsive but not in Ah nonresponsive strains of mice. In these studies, because a single time point and unequal doses of MC and βNF were used, it is not

Table 3. Comparison of the induction of the metabolism of aflatoxin B_1 to aflatoxin M_1 with Ah responsiveness of different inbred strains of mice*

Inbred strains	Aflatoxin B_1-4-hydroxylase (M_1)		Aryl hydrocarbon hydroxylase activity
	βNF/Control	MC/Control	MC/Control
Ah nonresponsive			
DBA/2Ha	1.43	1.37	1.06
AKR/Sn	1.32	1.32	1.13
Ah responsive			
C57BL/6Ha	6.8	7.8	4.10
ICR/Ha	4.9	8.0	4.01
C3H/St	5.4	10.1	5.70
A/St	2.5	4.1	2.65

* Mice received a single i.p. injection of either MC (60 mg/kg) or βNF (150 mg/kg); 44 hr later the mice were killed and hepatic microsomes were isolated and analyzed for AHH activity and the metabolism of AFB_1 to AFM_1. Control mice received the vehicle only. For the measurement of each activity, four or five mice of each strain were analyzed individually.

possible to say whether the 1.8-fold higher potency of MC was due to any differences in pharmacokinetic parameters between the two inducers. Time–course studies in C57BL/6 also demonstrated that both βNF and MC are potent inducers of AFB_1-4-hydroxylase in the C57BL/6 strain. Both βNF and MC induced AFB_2-4-hydroxylase also, and the time–course profiles were qualitatively similar to those obtained for the induction of AFB_1-4-hydroxylase. In our opinion, the significant difference between the profiles reported here for AFB_1 (AFB_2)-4-hydroxylase(s) and those reported by Boobis *et al.* [15] for AHH in the C57BL/6N strain concerns the effect of MC. In their studies, aryl hydrocarbon hydroxylase activity induced by MC stayed at maximally elevated levels for up to 7 days and on day 9 decreased only 25% from the maximal level, whereas in the present studies AFB_1 (AFB_2)-4-hydroxylase activities induced by MC (or βNF) decreased rapidly after reaching the maximal, and on day 5 the activities were reduced by about 80% from the maximum. However, it is not discernible from these studies whether these differences between aryl hydrocarbon hydroxylase and AFB_1 (and AFB_2)-4-hydroxylases are due to differences in the half-lives of the two (or more) induced cytochrome P-450s which catalyze these reactions (i.e. if different cytochrome P-450s are involved), to differences in the genetic regulation of the two (or more) induction processes, or to some unknown differences between C57BL/6N used by Boobis *et al* [15] and C57BL/6 used by us. Our earlier data [10, 13] have suggested that, most likely, MC-induced cytochrome P-450 (448/P_1-450) which is responsible for the preferential metabolism of AFB_1 to AFM_1 is different from that responsible for aryl hydrocarbon hydroxylase activity. Dose–response profiles obtained here for the induction of AFB_1-4-hydroxylase and AFB_2-4-hydroxylase further support this suggestion and possibly also indicate differences in the regulation of the induction of AFB_1-4-hydroxylase and AFB_2-4-hydroxylase. While in the studies of Boobis *et al* [15] aryl hydrocarbon hydroxylase activity increased at least up to a dose of 320 mg/kg MC, in the present studies AFB_1-4-hydroxylase induction was maximal at a dose of 40 mg/kg and the activity decreased with the increasing dose and at a 200 mg/kg dose reached a level which was about 30% below the maximal level. The inhibitory effects of higher doses of MC were more pronounced in the case of AFB_2-4-hydroxylase, which attained maximal induction at 80 mg/kg and which at 200 mg/kg was reduced by 75% from the maximum.

Relationship between the induction of AFB_1-4-hydroxylase and the mutagenic activation of AFB_1 and benzo[a]pyrene. In the present report, we have extended our preliminary investigations [27] to study the effects of pretreatment of mice with MC and βNF on the hepatic microsome-mediated mutagenic activation and DNA-binding of AFB_1 and have compared the results with the effects of these treatments on the mutagenic activation of benzo[*a*]pyrene. In these investigations, hepatic microsomes derived from several inbred strains of mice (DBA/2Ha, AKR/Sn, RF/J, C57BL/6Ha, ICR/Ha, C3H/St, A/St, Balb/cCr, C57e/Ha and CBA/Pi) were used.

Considerable interstrain variability was observed in the levels of AFB_1-2,3-oxygenase activity in hepatic microsomal preparations derived from either control or MC-induced mice of the ten different strains. In addition to 4- to 5-fold interstrain variability in AFB_1-2,3-oxygenase activity in hepatic microsomes from control or MC-treated mice, MC depressed AFB_1-2,3-oxygenase activity up to 40% in Ah responsive and nonresponsive (except RF/J) strains.

In addition to the observation that AFB_1 is a more potent mutagen in the TA-98 system than benzo[*a*]pyrene (Figs. 4–7), the data in this report suggest that the depression of AFB_1 mutagenesis by MC is more strongly associated with the induction of AFB_1-4-hydroxylase activity than with the depression of AFB_1-2,3-oxygenase activity. This interpretation is supported by the observation that while AFB_1-2,3-oxygenase activity was depressed by MC in both AFB_1-4-hydroxylase inducible (Ah responsive) and noninducible (Ah nonresponsive) strains, mutagenic activation of AFB_1 was depressed only in

the responsive strains. Thus, the present investigations suggest that, for mutagenesis to decrease, metabolism of AFB_1 should proceed at an accelerated rate through a detoxification pathway, e.g. formation of AFM_1 which is <5% as active as AFB_1, either as a mutagen or as a DNA-binding agent with or without further metabolism [21, 22].

Both βNF and MC had relatively little effect on the mutagenic activation of either benzo[*a*]pyrene or AFB_1 catalyzed by hepatic microsomes from three different AFB_1-4-hydroxylase noninducible strains. However, in contrast to these results, an inverse relationship was observed between benzo[*a*]pyrene and AFB_1 mutagenesis when hepatic microsomes from βNF- or MC-treated mice of AFB_1-4-hydroxylase-inducible strains were used. Pretreatment with either chemical decreased microsome-mediated mutagenesis of AFB_1 but enhanced several-fold the mutagenesis of benzo[*a*]pyrene. This inverse relationship was further confirmed in time–course studies in which hepatic microsomes obtained at different timepoints, up to 12 days, following the administration of MC or βNF to C57BL/6 mice, were employed.

The negative association between the effects of MC (and βNF) on benzo[*a*]pyrene and AFB_1 mutagenesis, and the positive association between aryl hydrocarbon hydroxylase induction and AFB_1-4-hydroxylase induction, suggest that the detoxification of AFB_1 via the formation of AFM_1 and the activation of benzo[*a*]pyrene via the formation most likely of benzo[*a*]pyrene-7,8-diol epoxides are controlled either by the same or closely linked genetic factors. Since several human foods, e.g. broccoli, cabbage and cauliflower [28], have been shown to induce AHH activity, it would be interesting to test the effect of these foods on the toxicity and carcinogenicity of AFB_1. Indeed, a very recent report [26], confirming our earlier predictions [13, 27], has demonstrated protection of AFB_1 carcinogenesis in rats fed on cabbage. Similar studies should form the basis for future epidemiologic investigations designed to elucidate the role of such foods in the prophylaxis of liver cancer believed to be associated with the consumption of aflatoxin-contaminated foods in several countries.

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