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MUTAGENIC ACTIVATION OF 2,4-DIAMINOANISOLE AND 2-AMINOFLUORENE *IN VITRO* BY LIVER AND KIDNEY FRACTIONS FROM AROMATIC HYDROCARBON RESPONSIVE AND NONRESPONSIVE MICE

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Abstract—The mutagenicity of the two carcinogenic arylamines 2,4-diaminoanisole (2,4-DAA) and 2-aminofluorene (AF) was compared using liver and kidney fractions from two aromatic hydrocarbon (3-methylcholanthrene, MC) responsive and two nonresponsive mouse strains. MC pretreatment of mice caused an increase in 2,4-DAA mutagenicity with liver fractions from all four strains; however, much higher increases were seen in the two responsive than in the two nonresponsive strains. Kidney fractions had very low basal 2,4-DAA mutagenic activity. MC treatment led to 14-27-fold increase in 2,4-DAA mutagenicity in the responsive C57BL/6/BOM (B6) strain, but not in any of the other strains. AF mutagenicity was increased with liver fractions from all four mouse strains, but to the greatest extent in the B6 mice. AF showed high basal mutagenic activity with kidney fractions from all four strains, but MC treatment did not cause any increase in AF mutagenicity in any of the strains. Thus, there was a clear difference in the pattern of metabolic activation of the two arylamines 2,4-DAA and AF by liver and kidney fractions in mice, both with respect to constitutive activities and to the response to aromatic hydrocarbons.

Evidence has been presented for a single gene difference between B6† and D2 inbred mice in the induction of a hepatic microsomal monooxygenase activity, AHH, and cytochrome(s) P-448‡ synthesis by pretreatment with the aromatic hydrocarbon MC[1–3]. So-called aromatic hydrocarbon responsiveness involves two or more regulatory genes (termed *Ah-1* and *Ah-2*) which activate and control the structural gene(s) for cytochrome(s) P-448 [2,3]. The gene products of these *Ah* loci are in some way regulatory and it has been suggested that one of the gene products is a cytosolic receptor which can specifically bind aromatic hydrocarbon inducers [4]. The *Ah* loci in responsive mice control the induction of cytochrome(s) P-448 and numerous associated monooxygenase activities, including AHH [3]. However, there are many cytochrome P-450-mediated monooxygenases not associated with the *Ah* loci. Other structural cytochrome P-450 gene loci presumably exist, each of which may respond to a single or multiple stimuli, ultimately leading to the expression of other basal and inducible monooxygenase activities [3].

The activation of the carcinogen 2-acetylaminofluorene to a mutagen in the *Salmonella* test system is associated with aromatic hydrocarbon responsiveness in mice [5]. Whereas AHH activity is expressed as an autosomal dominant trait, 2-acetylaminofluorene mutagenicity *in vitro* appears to be expressed additively. The rate-limiting step of 2-acetylaminofluorene mutagenesis is suggested to be the activation by cytochrome(s) P-448 to the *N*-hydroxy-derivative [5].

We have previously reported on the involvement of cytochrome P-448 in the metabolic activation of the carcinogenic hair-dye component 2,4-DAA to a mutagen in the *Salmonella* test system [6] and to covalently bound intermediates *in vitro* [7] and *in vivo* [8]. It was suggested that activation of 2,4-DAA to a mutagen *in vitro* is associated with aromatic hydrocarbon responsiveness and that the mutagenic intermediate(s) might be the hydroxylamine(s) [6]. We have now studied the mutagenic activation of 2,4-DAA with liver and kidney fractions in two aromatic hydrocarbon responsive and two nonresponsive mouse strains and compared this with the mutagenicity of the carcinogenic arylamine AF.

MATERIALS AND METHODS

Chemicals. 2,4-DAA was obtained from ICN Pharmaceuticals, USA; MC from Ferak, Federal Republic of Germany; benzo [*a*] pyrene from Eastman Kodak, USA; β-naphthoflavone from Aldrich, Federal Republic of Germany; AF, NADP, glucose 6-phosphate and DMSO From Koch-Light, England. Other chemicals were of the best available commercial grades.

Animals. Male B6, D2, BA and AK spf mice weighing 20–25 g (i.e. 6–9 weeks old) were purchased from

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† The following abbreviations are used: B6 mice, mice of the C57BL/6/BOM strain; D2 mice, mice of the DBA/2J/BOM strain; BA mice, mice of the Balbc/A/BOM strain; AK mice, mice of the AKR/A/BOM strain; 2,4-DAA, 2,4-diaminoanisole; AF, 2-aminofluorene; MC, 3-methylcholanthrene; S9 fraction, the 9000*g* supernatant homogenate fraction; AHH, aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase; DMSO, dimethyl sulfoxide.

‡ In this report cytochrome(s) P-448 denotes the species of membrane-bound CO-binding hemoprotein(s) which increases after aromatic hydrocarbon treatment; cytochrome P-450 denotes other microsomal CO-binding hemoproteins.

Bomholtgård Breeding and Research Centre, Denmark. The animals were fed a pelleted laboratory diet and water *ad lib.* and housed in metal cages with birch chip bedding. The animal room was operated under a constant 12 hr light/dark cycle. Treatment of the animals consisted of a single intraperitoneal injection of MC (or in some cases β-naphthoflavone) 80 mg/kg in corn oil 40 hr before killing. The controls received an equivalent volume of corn oil (0.1 ml per 10 g). For each experiment, pooled organs from 10–20 animals per treatment group were used.

Preparation of enzyme fractions. All steps were carried out at 0–4° with cold sterile solutions and sterile equipment. The animals were killed by decapitation. The livers and kidneys were immediately removed and homogenized with a motor driven glass–Teflon homogenizer in 2 vol. ice-cold 1.15 % KCl containing 20mM-Tris-buffer, pH 7.4. The homogenates were centrifuged for 20 min at 9000g, and the supernatants (S9 fractions) were diluted to appropriate protein concentrations after protein determination according to Lowry *et al.* [9] For studies of AHH activity, microsomes were prepared by centrifugation of the S9 fractions at 105 000g for 60 min. The microsomal pellets were resuspended in the KCl–Tris-buffer.

Mutagenesis assay. Mutagenic activity was detected with *Salmonella typhimurium* TA 1538 according to Ames *et al.* [10], using liver or kidney S9 fractions, a NADPH generating cofactor mixture and the test compounds dissolved in DMSO. The test strain was a generous gift of Dr. B. N. Ames, Berkeley, California, USA.

AHH activity. AHH activity was determined according to the method of Nebert and Gelboin [11] using 80 μM benzo [*a*] pyrene and 0.2 mg microsomal protein per ml.

RESULTS

Liver and kidney microsomal AHH activities. Microsomal AHH activities were determined in liver and kidneys from the two aromatic hydrocarbon responsive strains, B6 and BA, and from the two nonresponsive strains, AK and D2 (Table 1). Control levels in liver

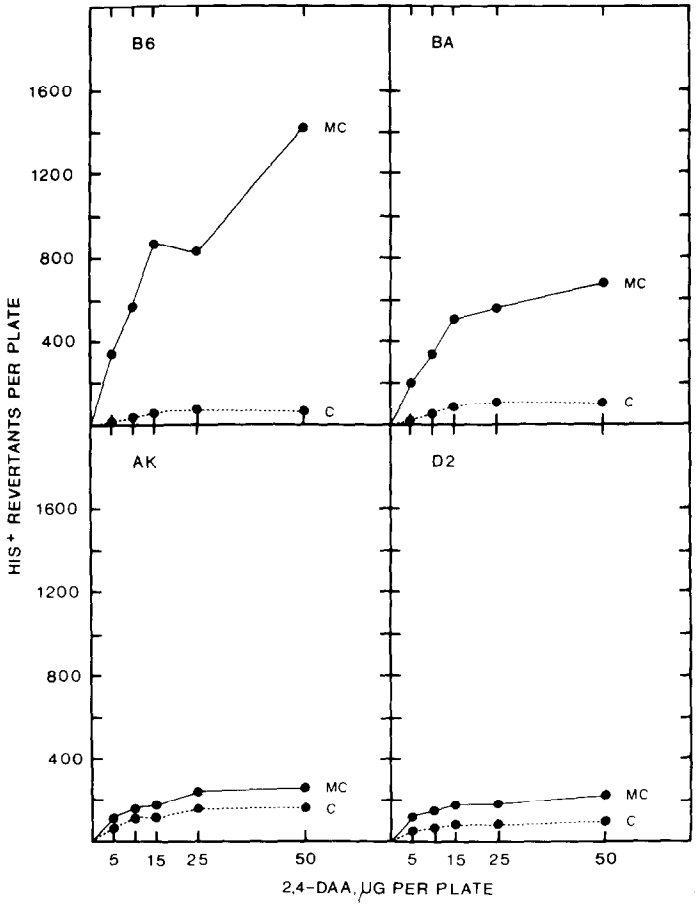

Fig. 1. 2,4-DAA mutagenicity *in vitro* as a function of mutagen concentration with control (C) and MC pretreated (MC) liver S9 fractions from B6, BA, AK and D2 mice. Each plate contained 1 mg of liver S9 protein and from 5 to 50 μg of 2,4-DAA. Values are means of duplicates, spontaneous revertants have been subtracted (range 14–35).

microsomes from the four strains were of similar magnitude. MC treatment led to a 5.2-and 2.7-fold increase in B6 and BA mice, respectively, whereas activity in AK and D2 mice was not increased. Using kidney microsomes, basal levels were much lower than in liver, and only activity in B6 mice was markedly increased (9.7-fold) by prior MC treatment. In these experiments a decrease in AHH activity with kidney fractions from D2 and AK mice was seen after MC treatment.

Activation of 2,4-DAA and AF to mutagens with liver S9 fractions. Using 1 mg S9 protein from control animals per plate, 2,4-DAA maximally gave rise to 100–160 revertants per plate (Fig. 1). MC pretreatment led to increases in mutagenic activity with preparations from all four mouse strains. However, the response was quite different between the hydrocarbon responsive and the nonresponsive strains. Plotting the ratios of the values from MC treated animals over those from control animals, an increase of 12–24 times in B6 mice was seen (Fig. 3). The relative increase using S9 fractions from MC treated BA mice was 5–10 times over control values, whereas the ratios using S9 fractions from AK and D2 mice were only between 1.5 and 2.5. The small but significant increase in 2,4-DAA mutagenicity with liver fractions from MC-treated AK and D2 mice was found in repeated experiments. A similar pattern was seen when experiments were performed with varying protein concentrations of fractions from the four strains and 10 μg 2.4-DAA per plate (data not shown). Similar results were also found using another aromatic hydrocarbon inducer, β-naphthoflavone.

Table 1. Effects of MC pretreatment on microsomal AHH activity in liver and kidneys from B6, BA, AK and D2 mice

Mouse strain	Liver		Kidney	
	Control	MC	Control	MC
B6	586 ± 29	3053 ± 189	41 ± 10	399 ± 17
BA	660 ± 38	1765 ± 14	20 ± 5	34 ± 3
AK	660 ± 72	619 ± 25	56 ± 4	38 ± 3
D2	528 ± 29	537 ± 14	32 ± 1	17 ± 0

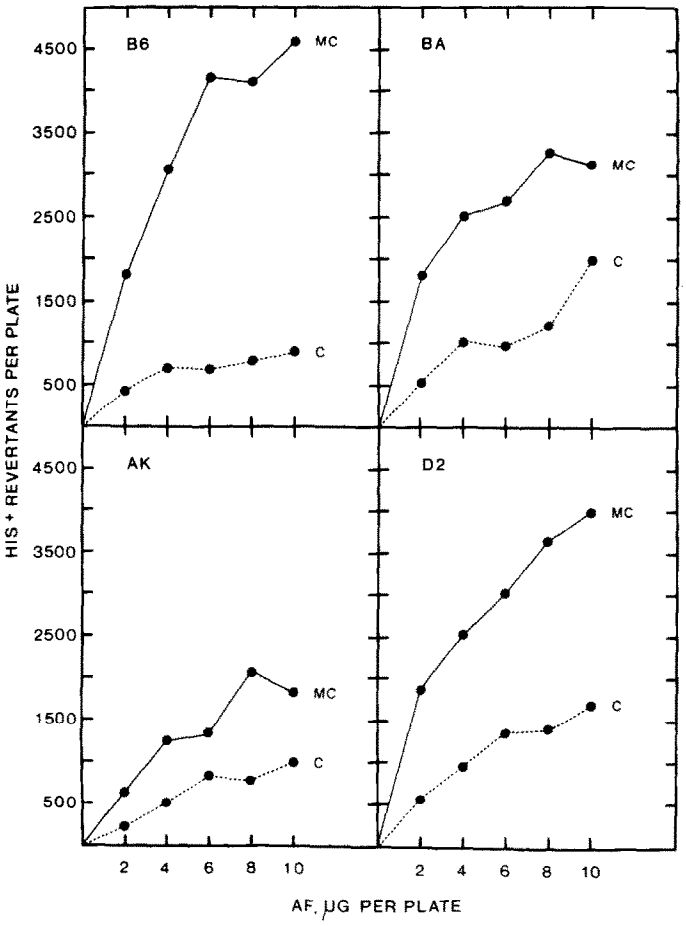

Fig. 2. AF mutagenicity *in vitro* as a function of mutagen concentration with control (C) and MC pretreated (MC) liver S9 fractions from B6, BA, AK and D2 mice. Each plate contained 1 mg of liver S9 protein and from 2 to 10 μg of AF. Values are means of duplicates, spontaneous revertants have been subtracted (range 10–27).

In Figure 2 is shown the mutagenic activation of varying concentrations of AF using 1 mg S9 protein from B6, BA, AK and D2 mouse liver. Basal activities were much higher than with 2,4-DAA, 10 μg per plate giving between 950 and 2300 revertant colonies. As with 2,4-DAA, MC pretreatment caused increases in activity with all four preparations, but the relative increases were not as pronounced. The largest increase was seen with fractions from B6 mice, 4.2–6.1 fold (Fig. 3). The increases in the three other strains were of similar magnitude, BA mice 1.6–3.4-, AK 1.6–2.9- and D2 2.2–3.3-fold, respectively. Similar patterns were seen in experiments using varying amounts of protein and fixed mutagen concentration (data not shown).

Comparing the effects of MC treatment on liver microsomal AHH activity with the effects of mutagenic activation of 2,4-DAA and AF by liver S9 fractions (Fig. 3), there are obvious differences in responses. With both arylamines there was an increase in mutagenic activity with MC treated S9 fractions from the two nonresponsive strains. However, with 2,4-DAA, much larger increases were seen using fractions from the two responsive strains than with fractions from the two nonresponsive strains. With AF, only B6 mice showed larger increases than the AK and D2 mouse strains after MC treatment.

Activation of 2,4-DAA and AF to mutagens with kidney S9 fractions. Using kidney S9 fractions from control mice, very low mutagenic activity was seen with 2,4-DAA (Fig. 4). With fractions from MC pretreated mice, a very large increase was found in the B6 strain (14–27-fold), whereas kidney preparations from the other three strains did not show any increase. This correlated very well with the pattern of AHH activity in kidney microsomes (Table 1). Mutagenic activation of AF with kidney S9 fractions gave quite a different picture (Figs. 5–6). Basal activities were very high in all four mouse strains. Ten μg AF and 1 mg S9 protein per plate gave rise to 3000–6800 revertants per plate. This is even higher than the activities seen with liver fractions. MC treatment did not cause any substantial increase in any of the four mouse strains, either when assayed at varying protein concentrations (Fig. 5) or varying mutagen concentrations (Fig. 6). Clearly, the

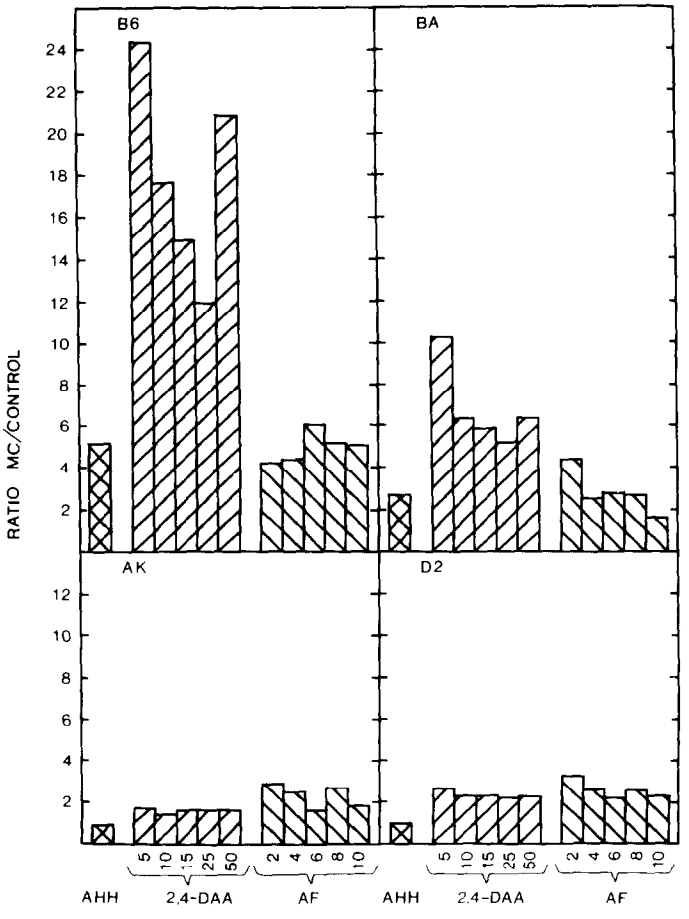

Fig. 3. Ratio of MC-pretreated to control values of liver microsomal AHH activity, and 2,4-DAA and AF mutagenicity with liver S9 fractions from B6, BA, AK and D2 mice. Numbers on abscissa refer to μg mutagen per plate.

mutagenic activation of AF by kidney S9 fractions from B6 mice does not correlate with that of 2,4-DAA or with microsomal AHH activity.

DISCUSSION

AF is believed to be *N*-hydroxylated by microsomal monooxygenases to the corresponding hydroxylamine, which in turn is chemically converted to the nitrenium ion arylating DNA [12]. A similar scheme has been suggested for the activation of 2,4-DAA to a mutagen *in vitro* [6]. Assuming that the enzymatic conversion is the rate limiting step in these reactions, the steady state concentration of the hydroxylamines would presumably be proportional to the rate of mutation reflected in the number of revertants in the *Salmonella* test system. Differences in monooxygenase activities, either because of differences in constitutive levels or as a result of pretreatment with inducers, would therefore be seen as differences in mutagenic activity. We have used mouse strains which show differences in monooxygenase activities after treatment with aromatic hydrocarbons [2] to see whether mutagenic activation of 2,4-DAA and AF follow the same pattern as that of AHH activity.

AHH inducibility in liver microsomes from the responsive B6 and BA strains and the nonresponsive AK and D2 strains follows the expected pattern [1,2], in that responsive strains show 4–6 fold increases, whereas no increases are seen in nonresponsive strains. With kidney microsomes, only B6 mice showed a substantial increase after MC pretreatment. Niwa *et al.* [13] have shown that AHH activity is also induced in extrahepatic tissues such as kidney in the B6 responsive strain. However, Seifried *et al.* [14] claim that the distinction between responsiveness and nonresponsiveness does not apply to extrahepatic tissues.

This study demonstrates differences in activation of the two arylamines 2,4-DAA and AF both when liver and kidney fractions are used as activating systems. 2,4-DAA is converted to a mutagen with mouse liver S9 fractions, and MC treatment causes a much larger increase in the two responsive than the two nonresponsive strains. With kidney fractions, only the strain

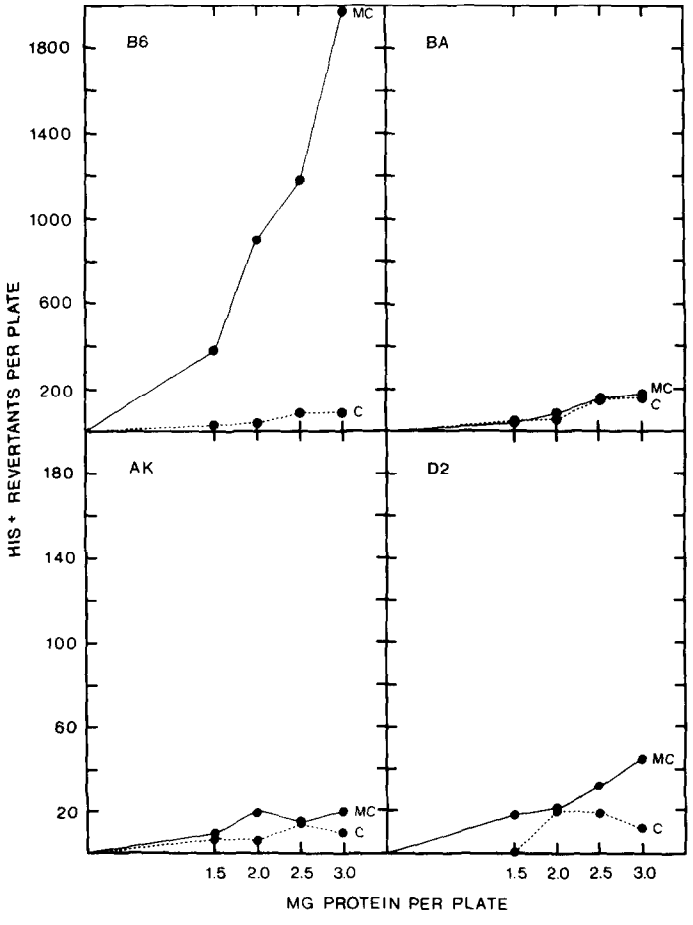

Fig. 4. 2,4-DAA mutagenicity *in vitro* as a function of protein concentration with control (C) and MC pretreated (MC) kidney S9 fractions from B6, BA, AK and D2 mice. Each plate contained 50 μg 2,4-DAA and from 1.5 to 3.0 mg protein. Values are means of duplicates, spontaneous revertants have been subtracted (range 16–42).

which shows marked AHH inducibility by MC, B6, gives a correspondingly large increase in 2,4-DAA mutagenicity. However, MC also causes small increases in 2,4-DAA mutagenicity with liver fractions from AK and D2 mice, in contrast to the lack of effect on AHH activity. Recent evidence indicates that the MC-inducible cytochrome(s) P-448 comprise more than one cytochrome [15,16]. Whereas the induction of two forms of the cytochrome appears to be closely coordinated in the mouse and in the rat [15], two analogous forms seem to be regulated differently in the rabbit [16]. The cytochrome P-448-dependent reaction involved in converting 2,4-DAA to a mutagen is presumably associated with the *Ah* loci, but the monooxygenase responsible for 2,4-DAA activation must be different from that hydroxylating benzo[*a*]pyrene. Evidence suggests that nonresponsive mice have a defective cytosolic receptor for MC [18]. High concentrations of the potent inducer 2,3,7,8-tetrachlorodibenzo-*p*-dioxin leads to induction of AHH activity also in MC nonresponsive strains. An explanation for the present findings would be that the interaction of MC with the defective receptor(s) either does not lead to any increase in monooxygenase activity (using benzo[*a*]pyrene as substrate) or a small increase is seen with other substrates (such as 2,4-DAA).

Conversion of AF to a mutagen by mouse liver S9 fractions is increased in all four strains by MC, with the greatest increase in B6 mice. This was clearly different from the pattern of AHH induction by MC. Using kidney S9 fractions, no marked increases in AF mutagenicity could be found after MC treatment, in contrast to the effect of MC on 2,4-DAA mufagenicity in kidneys from B6 mice. The lack of induction of AF mutagenesis in kidneys corresponds well with the lack of 2-acetylaminofluorene *N*-hydroxylase induction by MC in rabbit kidney [17] and the lack of increase in an electrophoretic band of 54,000 in rabbit kidney microsomes after MC-treatment [16].

In conclusion, a clear distinction can be drawn in the metabolic activation of two carcinogenic arylamines by liver and kidney fractions in mice, both with respect to constitutive activities and to the response to aromatic hydrocarbons.

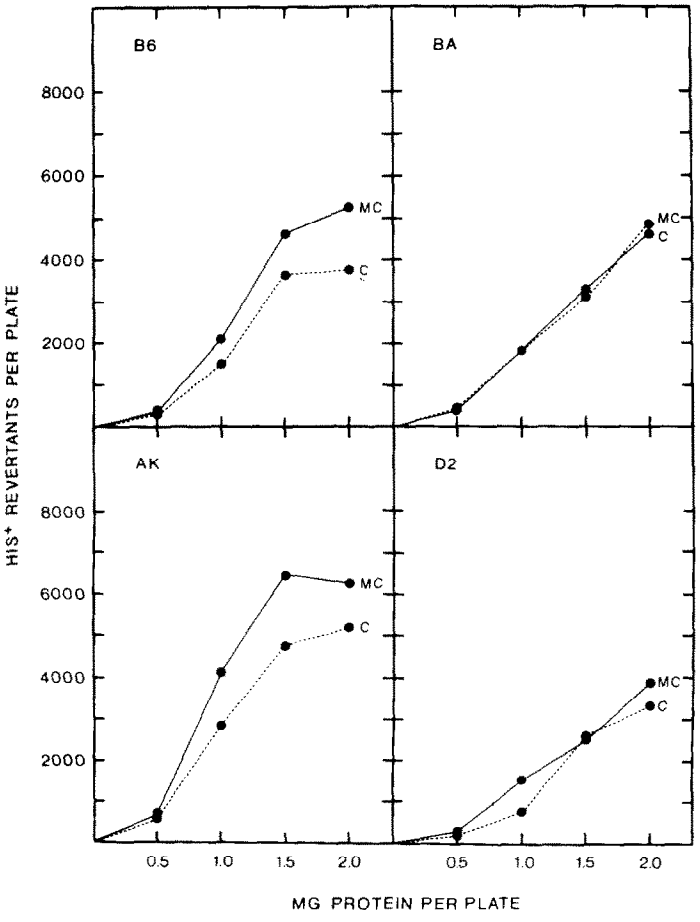

Fig. 5. AF mutagenicity *in vitro* as a function of protein concentration with control (C) and MC-pretreated (MC) kidney S9 fractions from B6, BA, AK and D2 mice. Each plate contained 2 μg AF and from 0.5 to 2.0 mg protein. Values are means of duplicates, spontaneous revertants have been subtracted (range 16–42).

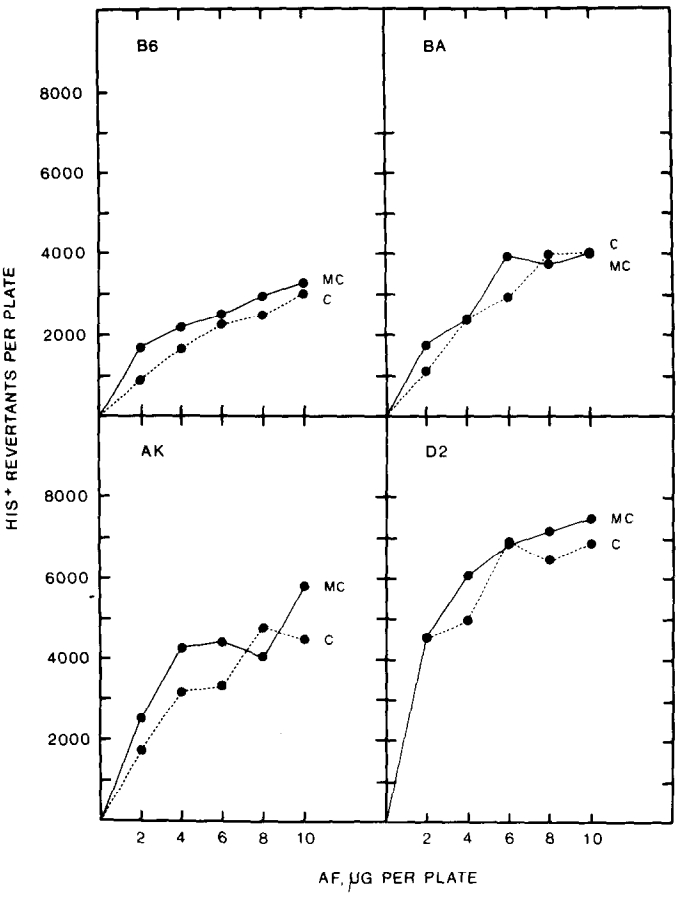

Fig. 6. AF mutagenicity *in vitro* as a function of mutagen concentration with control (C) and MC pretreated (MC) kidney S9 fractions from B6, BA, AK and D2 mice. Each plate contained 1 mg protein and from 2 to 10 μg AF. Values are means of duplicates, spontaneous revertants have been subtracted (range 11–29).

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