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ENZYMATIC AND NON-ENZYMATIC FORMATION OF FREE RADICALS FROM AFLATOXIN B_1

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Formation of free radicals from aflatoxin B_1 was demonstrated in systems of a) NADPH/microsome, b) H_2O_2/peroxidase, c) UV irradiation, d) silver oxide oxidation and e) alkaline dimethyl sulfoxide. In a) to c), radical spots were detected in thin-layer chromatograms. Among several known metabolites of aflatoxin B_1, aflatoxin M_1 was active in generation of free radical. These results suggest a possible role of free radical metabolites in the cytotoxicity of this hepatocarcinogen.

KEY WORDS: Aflatoxin, radicals, microsome, peroxidase, active oxygens.

INTRODUCTION

There is increasing evidence that active oxygen species are involved in the process of chemical carcinogenesis.[1] Potent tumor promoters directly or indirectly induce generation of superoxide, whose inhibition parallels with inhibition of tumorigenesis. Inhibitory action of superoxide dismutase (SOD) was observed not only in two stage carcinogenesis with tumour promoters but also in complete carcinogenesis without tumor promoters.[2]

We previously reported that many potent chemical carcinogens were metabolized into free radicals and potentiated the generation of suproxide or hydrogen peroxide.[3] There was a good correlation among carcinogenicity, free radical formation and active oxygen generation.[4] Carcinogens included are benzo(a)pyrene, 3-methylcholanthrene (MC), 7,12-dimethylbenzo(a)anthracene, 4-dimethylaminoazobenzene, 2-naphthylamine, mytomycin and adriamycin.

Nakae *et al.*, recently reported that killing of rat hepatocytes by aflatoxin B_1 (AFB_1) or dimethylnitrosoamine was prevented by SKF525A, catalase, SOD, mannitol or deferoxamine.[5] The results indicated the important role of active oxygen species in the cytotoxicity of hepatocarcinogens and suggested the possible existence of free radical metabolites. As for dimethylnitrosoamine, a spin trapping method was applied to detect an unstable free radical.[6] As for AFB_1, no free radical metabolite has ever been reported, except a metabolite of emodin, a related mycotoxin.[7] This is the first report on the detection of free radical formed from AFB_1.

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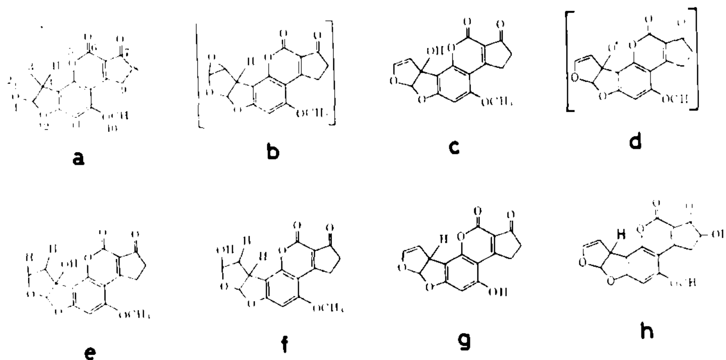

FIGURE 1 Chemical structures of AFB_1 and its metabolites. a, AFB_1; b, aflatoxin 2,3-epoxide; c, AFM_1; d, free radical from AFM_1 (deduced form); e, aflatoxin M_2; f, aflatoxin B_{2a}; g, aflatoxin P_1; h, aflatoxin Q_1.

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MATERIALS AND METHODS

AFB_1 and its metabolites (M_1, M_2, P_1, Q_1 and B_{2a}) were commercially obtained (Sigma Chemical Co, Ltd.) (Figure 1).

Reaction mixture (50 ml) contained Tris-HCl, pH 7.5 (50 mM), KCl (1.15%), $MgCl_2$ (3 mM), NADPH (1 mM), AFB_1 (2.5 mg/0.25 ml dimethyl sulfoxide (DMSO)) and liver microsome (20 mg protein) prepared from male Sprague-Dawley rats pretreated with MC or phenobarbital (PB) as previously described.[8] The reaction was started by addition of NADPH and incubation continued for as long as 15 min. After cooling in ice, the mixture was extracted with an equal volume of cold benzene twice, and the extracts were evaporated to dryness at room temperature. The evaporated residue was dissolved in benzene (0.3 ml) or dioxane (0.2 ml) for electron spin resonance (ESR) measurement. Some ESR samples were also checked for the convertibility into free radicals after addition of ceric sulfate (1–2 mg) as a mild oxidizing agent.

Reaction mixture for peroxidase (100 ml) contained Tris-HCl, pH 7.5 (10 mM), H_2O_2 (10 mM), AFB_1 (5 mg/0.5 ml DMSO) and horseradish peroxidase (18 Units/0.4 mg protein, Sigma Chem. Co. Ltd.). Incubation continued for as long as 30 min.

Photoproducts of AFB_1 was obtained by irradiating dioxane solution of AFB_1 (1.0 mg/0.4 ml) with a mercury lamp (UV dose, 0.18 J/cm^2/min) for 60 min. The solution was introduced into a capped quartz cell which was cooled in a water-bath.

Chemical oxidation of AFB_1 by silver oxide was performed according to the modified method of Liehr *et al.*,[9] and evaporated residue of celite filtrate was used for ESR measurement.

Free radical of AFB_1 at alkaline pH was detected for a mixture of 10 μl 1N NaOH and 100 μl of DMSO solution of AFB_1 (1 mg/0.1 ml).

Radical products of AFB_1 were spotted on silica gel plates (20 × 20 cm) and developed with benzene/dioxane (1:1). After checking with a UV lamp, each zone was scraped and the silica powder was introduced into an ESR tube for radical detection. AFB_1 radicals were no longer detected after development with chloroform/methanol (97:3), which was conventionally used for separation of AFB_1 metabolites. ESR spectra were recorded by a JES RE2X spectrometer at room temperature.

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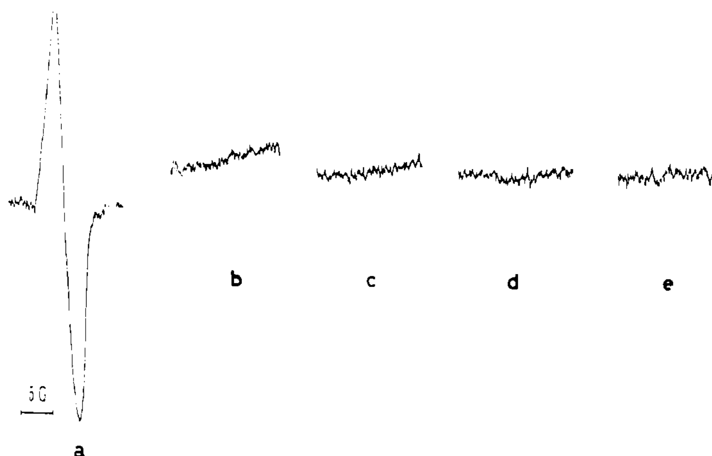

FIGURE 2 ESR spectra of microsome extracts of AFB_1. a, complete system, including both MC- and PB-microsome; b, lacking MC-microsome; c, lacking PB-microsome; d, lacking NADPH; e, lacking AFB_1. Modulation width, 5G. solvents, benzene. The protein concentration of microsomes was 20 mg/50 ml from a to e.

RESULTS

Figure 2 shows a distinct ESR signal observed in the benzene extracts from reaction mixtures containing AFB_1, MC-induced rat microsome, PB-induced rat microscome and NADPH. Omission of any one component from the reaction mixture abolished the signal. The ESR signal obtained showed an asymmetric structure as modulation width was decreased and further evacuation did not improve the situation.

A combination of MC- and PB-induced microsomes may constitute a favorable oxidation condition for forming AFB_1 radicals, because protein concentration of microsome was kept constant in the experiment in Figure 2. Benzene extracts with either MC-induced microsome or PB-induced microsome contained metabolites which could be oxidized into free radical form with supplements of ceric sulfate. Control microsome extracts did not exhibit such an ESR signal on addition of ceric sulfate.

Similar ESR signal was detected in the benzene extracts from reaction mixtures containing AFB_1, horseradish peroxidase and hydrogen peroxide (Figure 3b). The signal was distinct when the evaporated extracts were dissolved in dioxane instead of benzene for ESR measurement. Complete hyperfine structure of this radical was also unsuccessful, disturbed by asymmetric signal change.

When dioxane solution of AFB_1 was irradiated by a mercury lamp, a free radical was detected (Figure 3d). The *g*-value and linewidth of this radical is similar to the one obtained by enzymatic reactions. Asymmetric signal structure was also observed in this case. During photoirradiation, the dioxane solution turned pale yellow. Oxidation products of AFB_1 by silver oxide gave a small ESR signal in dioxane (Figure 3e).

On addition of sodium hydroxide, DMSO solution of aflatoxin B_1 gave a symmetrical ESR signal, accompanying a yellow color change (Figure 3f). In this case the

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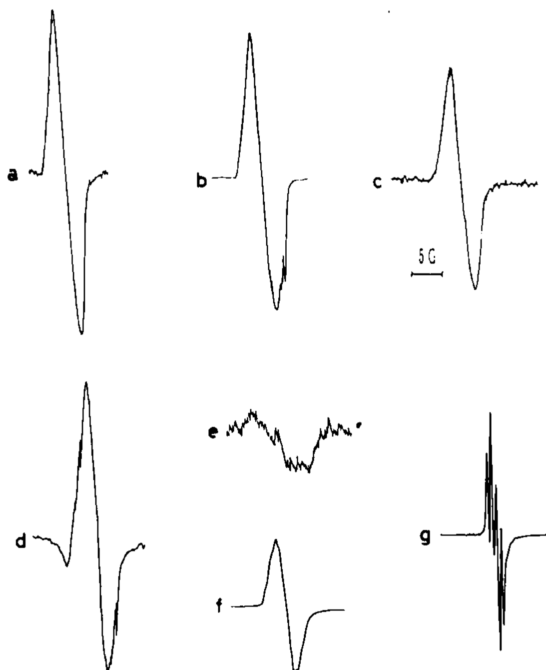

FIGURE 3 ESR spectra of enzymatically and non-enzymatically activated AFB_1. a, microsome extracts; b, peroxidase extracts; c, AFM_1; d, photoirradiation products; e, silver oxide oxidation products; f, g, alkaline DMSO. Modulation widths used were 5G except *g* (0.05G). Solvents used were dioxane except a(benzene), c(benzene/dioxane) and f, g(DMSO). G values and linewidths were: a (2.007, 5G), b (2.006, 5G), c (2.003, 5G), d (2.006, 5G), e (2.007, 9G), f (2.004, 5G).

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hyperfine structure was clearly obtained. This radical is close in *g*-value and linewidth to those mentioned above.

Purification of AFB_1 radicals was attempted by thin-layer chromatography (TLC). Free radicals were detected in restricted fractions of the plates (Figure 4). Peroxidase metabolites and photoirradiation products gave similar but not identical spots of free radical, which were positioned close to, but distinct from, unmetabolized AFB_1. Such fraction was also shared by microsome metabolites, which however contained additional radical fractions. Free radicals of AFB_1 in alkaline DMSO may be different from oxidized AFB_1 radicals because chromatographic separation was not successful in this case.

Commercially obtained metabolites of AFB_1, M_1, M_2, P_1, Q_1 and B_{2a} were dissolved in benzene/dioxane (1:1) mixture (10 μg/0.2 ml) and checked for convertibility into free radicals. Only aflatoxin M_1 (AFM_1) solution gave a positive result (Figure 3c) while others remained inert even after addition of ceric sulfate. The free radical of AFM_1 does not derive from the impurity but from the autoxidation product of AFM_1 itself. This is supported by the evidence that the second round of TLC developed the radical fraction from the main fluorescent spot which was separated from the original radical fraction in the first round of TLC. The hyperfine structure of AFM_1 was also found to be asymmetric, although much less asymmetric than that of microsome extracts.

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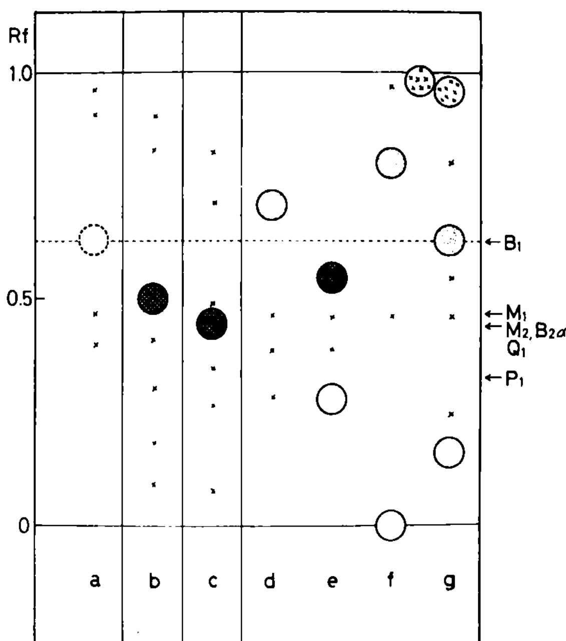

FIGURE 4 Thin-layer chromatograms of AFB_1 radicals. a, alkaline DMSO; b, photoirradiation products; c, peroxidase extracts; d, MC-microsome extracts, finally treated with ceric sulfate; e, PB-microsome extracts, with ceric sulfate; f, MC- and PB-microsome extracts; g, MC- and PB-microsome extracts, with ceric sulfate. Chromatograms were developed with benzene/dioxane (1:1) on silica. Crosses denote fluorescent spots, circles represent radical spots. Relative radical intensities were expressed by shading circles. In a, only a faint radical was detected in AFB_1 fraction. Rf values of AFB_1 and its metabolites are shown on the right. Rf value of AFM_1 radical was 0.40–0.45.

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DISCUSSION

The present investigation unambiguously demonstrated that free radicals were formed enzymatically and non-enzymatically from AFB_1, a potent hepatocarcinogen. Several similar fluorescent spots were detectable among thin-layer chromatograms of microsome extracts, peroxidase extracts and photoirradiated products, indicating the common oxidation pathway of AFB_1. These three systems also share free radical spots with similar Rf values, which are close to those of hydroxylated AFB_1 metabolites. Among several metabolites, AFM_1 was the only one which was convertible into a free radical. Although AFM_1 radical was the most plausible candidate of AFB_1 radical, precise identification was hampered by difficulty in analyzing the hyperfine structures of the spectra. Asymmetry observed might be partly diminished by further purification of radical metabolites.

Nakae *et al.* reported the generation of hydrogen peroxide in AFB_1/microsome system which was prevented by SKF 525A(5). This result is consistent with formation of free radicals in the present investigation. Preliminary experiment suggested the generation of active oxygens from AFM_1 as detected by a spin trapping method with dimethyl-pyrroline-N-oxide. Considering that AFM_1 and AFB_1 have the same cytotoxic activity as well as comparable carcinogenic activity,[10] free radicals could be

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involved in both biological processes. The epoxide of AFB_1 could be the most potent initiator[10] while other metabolites including free radicals might participate in the promotion and progression of hepatocarcinogenesis.

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