In vitro Aflatoxin B₁-DNA Binding by Microsomes and Its Modulation by Cytosol: Comparison of Various Mammalian and Avian Livers in Relation to Species Difference in Susceptibility

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Activation and inactivation of aflatoxin B₁ (B₁) by microsomes and cytosol prepared from the liver of various mammalian and avian species were studied in vitro by determining the microsomal activity to bind aflatoxin to calf thymus DNA and the cytosol activity to inhibit the hamster microsome-mediated aflatoxin-DNA binding. The microsomal activity to bind aflatoxin to DNA was higher in day-old duckling and female chicken than in the other species, being similar in the male hamster, male chicken, both sexes of Japanese quail and laying duck, and lower in the male rat and male mastomys than in the other species. The hamster cytosol inhibited the aflatoxin-DNA binding markedly in the presence of glutathione (GSH) but not at all in its absence. In contrast, the avian cytosol showed a similar level of aflatoxin-DNA binding regardless of the presence or absence of GSH, suggesting that the contribution of cytosol glutathione S-transferase (GST) to B₁ detoxification is negligible in the avian species. Nevertheless the cytosol of the avian species such as the Japanese quail and chicken showed apparent inhibitory activity toward aflatoxin-DNA binding. Relative susceptibility of the mammalian and avian species to the toxic and carcinogenic effects of B₁ could be explained by the combined activities of microsomes and cytosol, indicating the importance of the opposite activities of microsomes and cytosol toward aflatoxin-DNA binding in the species difference in susceptibility.

Key words: aflatoxin B₁; liver; DNA-binding; in vitro; cytosol; microsome

Introduction

Aflatoxin B₁ (B₁) is a hepatocarcinogenic and hepatotoxic mycotoxin produced by Aspergillus species. There are large differences in susceptibility to this toxin between animal species. Rats and ducks are relatively susceptible, but hamsters and mice are relatively resistant, to the carcinogenic effect of the toxin[1]. Also, the potency of the acute toxicity is different from species to species as shown by the LD₅₀ value, which ranges from 0.3 mg/kg to more than 10 mg/kg[2].

The species differences in susceptibility to B₁ have been studied in relation to hepatic metabolism of B₁[1,18]. B₁ is oxidized by microsomal P-450 enzymes to less toxic aflatoxin M₁ (M₁), P₁(P₁) and Q₁(Q₁), but the observed differences in this step of B₁ metabolism between species can not account for the species difference in susceptibility[3]. The other oxidation pathway via the P-450 enzymes is formation of the B₁-8, 9-epoxide, which binds covalently to DNA and other cellular macromolecules[4,5]. This covalent binding to cellular components is regarded as an essential step in the action of B₁[6,7], and a good correlation has been observed between the aflatoxin-DNA binding levels in hepatic cells of animals or cultured hepatocytes exposed to B₁ and the species difference in susceptibility[7–10]. On the other hand, species susceptibility could not be correlated with microsomal
enzyme activity to form B1-epoxide, but was rather well correlated with formation of aflatoxin-glutathione (GSH) conjugates via cytosol glutathione S-transferase (GST). However, the importance of cytosol activity has been supported only by observations in some mammalian species and the quail, and therefore whether it is applicable to a wide range of species, including other avian species than the quail, remains uncertain. To obtain insight into this point, we studied the in vitro B1 activation and detoxification by microsomes and cytosol prepared from the liver of various mammalian and avian species.

Materials and Methods

Chemicals.

\[^{3}H(G)\] B1 (sp. act. 15 Ci/mmol) was purchased from Moravec Biochemicals Inc. (Brea, Ca, USA). Before being used, its purity was confirmed to be more than 97% by using TLC. Nonradioactive B1, P1, Q1, M1, aflatoxicol (RO), GSH, nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and calf thymus DNA (sodium salt) were purchased from Sigma Chemical Co. (St Louis, MO, USA). TLC plates of silica gel 60 were purchased from Merck Co. (Darmstadt, Germany). Glucose 6-phosphate (G-6-P) and G-6-P dehydrogenase were purchased from Boehringer Mannheim GmbH (Germany), \( \beta \)-glucuronidase (30 U/g, from bovine liver) was from Serva Feinbiochemica GmbH & Co. (Heidelberg, Germany), and ACSII was from Amersham Corp. (CA, USA).

Animals

Male Fischer rats (F344/DuCrj, 8 wk), male Syrian Golden hamsters (7 wk) and both sexes of Japanese quail (7–8 wk) were purchased from Japan SLC Inc. (Hamamatsu-shi, Japan), Charles River Japan Inc. (Yokohama-shi, Japan) and Nippon Institute for Biological Science (Kobuchisawa-shi, Japan), respectively. Either sex of White Leghorn chickens (3–4 months), unsexed commercial ducklings (1–3 days) and commercial laying ducks (6 months) were purchased from Funabashi Farm (Funabashi-shi, Japan). Male mastomyses (2.5 months) were bred and maintained in our institute.

Preparation of liver fraction

Animals were killed by decapitation or by bleeding under ether anesthesia, and the livers from 2–5 animals of each species were pooled for preparation of 40% homogenates in ice-cold 0.25 M sucrose. The homogenates were centrifuged at 9,000 g for 15 min (supernatant was designated as S-9 fraction), and then microsomes and cytosol were prepared according to Tarloff et al. The cytosol and S-9 fractions were dialyzed for 20 hrs against 200–500 volumes of 10 mM Na-phosphate buffer (pH 7.4) to remove GSH. The liver fractions were kept at −80°C until used.

Incubation of the liver fraction for B1 metabolism

The incubation medium contained 100 mM phosphate buffer (pH 7.4), 2 mM NADPH, 5.6 mM G-6-P, 0.5 units of G-6-P dehydrogenase, 2 \( \mu \)M B1 containing 50 \( \mu \)Ci of \[^{3}H\] B1, and the liver microsomes equivalent to 1.0 mg protein and/or cytosol equivalent to 0.5 or 2.0 mg protein, respectively, in 1 ml. Calf thymus DNA (0.1 mg/ml) and 5 mM GSH, were included in the medium, when needed. The mixture (1 ml or 0.5 ml) was incubated in air at 37°C for 30 min or 60 min with shaking.

Acid and enzymatic hydrolysates

The S-9 fraction equivalent to 4 mg protein was incubated under the same conditions as in the experiment on B1 metabolism except that it was performed for 60 min in the presence of 100 \( \mu \)M phosphoadenosine–phosphosulfate, 22 mM UDP-glucuronic acid, 5 mM MgCl\(_2\) and 0.27 mM mercaptoethanol. The 60-min incubation mixture was extracted 3 times with equal volumes of chloroform-ethyl acetate (1:1), and the aqueous layer was hydrolyzed with glucuronidase for 1 hr according to Holeski et al. The hydrolyzed mixtures were again extracted 3 times with equal volumes of chloroform-ethyl acetate, and radioactivity of the combined chloroform–ethyl acetate extracts was counted. The remaining portion was acidified with HCl to 0.2 N and incubated at 90°C for 1 hr in sealed tubes. The acid-hydrolyzed mixture was then extracted with chloroform–ethyl acetate in the same manner as described above and the combined extracts were used for determination of radioac-
After incubation of the microsomes and/or cytosol with calf thymus DNA, 0.9 mg DNA was added as a carrier and the incubation mixture was extracted with water-saturated phenol containing hydroxyquinoline (0.1%). After being precipitated with ethanol from the aqueous layer, DNA was washed with ether and then dried in a vacuum desiccator. As the preliminary experiment showed that the amount of DNA recovered by this extraction method did not vary greatly from sample to sample, the levels of B1-DNA binding were expressed on the basis of the amount of DNA contained in the incubation mixture prior to incubation.

**TLC and HPLC analyses**

The chloroform-ethyl acetate (1:1) extracts of the incubation mixture of the microsomes and/or cytosol were applied to TLC plates together with standard B1, M1, P1, Q1 and RO to facilitate identification. The plates were developed with chloroform-acetone-water (88:12:1.5). Portions of the plates, corresponding to the origin, the M1, P1, Q1, RO and B1 regions and the region between B1 and the solvent front,
were scraped into vials for determination of radioactivity. For reverse phase HPLC analysis of \(^{3}\text{H}\) aflatoxin metabolites, a fixed wavelength detector (UV-8020, Tosoh Co., Tokyo, Japan) set at 340 nm was connected to the C18 3 \(\mu\)m, 4.6 mm × 15 cm Chemcosord column (Chemco Co., Osaka, Japan). Chromatographic separation was performed by elution for 5 min with 17.5% ethanol followed by a 17.5-27.5% ethanol linear gradient for 15 min. The mobile phases were buffered with 1 mM sodium acetate, pH 4.5. The column temperature was maintained at 40°C and the flow rate was set at 0.6 ml/min. These HPLC conditions permitted good separation of standard aflatoxins. Successive 0.5 min eluates were collected into vials for radioactivity determination.

**Quantitation of protein**

Protein concentrations of the microsomes, cytosol and S-9 were determined with a spectrophotometer (U-3200, Hitachi Ltd., Tokyo, Japan) using reagent from Bio-Rad Laboratories (CA, USA) and bovine serum albumin as the standard.

**Determination of radioactivity**

Radioactivity was determined in a scintillation counter (TRI-CARB 1500, Packard Japan K. K., Tokyo, Japan) using ACSII (Amersham Corp., 111, USA) as the scintillator.

**Results**

**Microsomal activity to bind aflatoxin to calf thymus DNA (Fig. 1(1), Fig. 2)**

The microsomal activity to bind aflatoxin to DNA was determined by incubating the microsomes with B1 and calf thymus DNA for 30 min. The decreasing order of activity was the day-old duckling, female chicken, female quail, male quail, male hamster, laying duck, male chicken, male rat and male mastomys livers. Day-old duckling liver showed more than twice the activity of the rat liver. The livers of the male hamster and quail of both sexes showed similar activities. The activity of the mastomys liver was very low, being nearly 1/3 of that of the rat.

**Cytosol activity to inhibit aflatoxin-DNA binding (Fig. 1(2), Fig. 3)**

Cytosol activity to inhibit the microsome-mediated aflatoxin-DNA binding was determined by incubating cytosols from various species with the hamster microsomes for 30 min. Cytosols from the hamster and mastomys inhibited the aflatoxin-DNA binding by 56% and 73%, respectively, at the cytosol protein concentration of 2.0 mg/ml in the presence of GSH. In contrast, inhibition was not observed with the hamster cytosol, and was only 20% for the mastomys cytosol, at the same concentration of cytosol protein in the absence of GSH. The difference in inhibition between the cytosol incubated in the presence of GSH and that in the absence of GSH may reflect the GST activity toward the B1-epoxide\(^14\). Consistent with the reported findings on GST activity\(^14\), the activity of the rat cytosol to inhibit the aflatoxin-DNA binding in the presence of GSH was very low compared with that of the hamster cytosol.

The inhibitory activity of the cytosol was also observed in livers of the avian species, although the difference in inhibition between the cytosol incubated in the presence of GSH and that in the...
absence of GSH was very small or zero. The highest activity was noted in the female chicken cytosol, which showed more than 50% inhibition. The female quail and male chicken cytosols showed 30% inhibition, and the duck and male quail cytosols 20–23% inhibition, in the absence of GSH in the incubation mixtures. The duckling cytosol showed the lowest activity among the avian species.

Conversion of B₁ to chloroform-ethyl acetate-extractable metabolites by microsomes

The chloroform-ethyl acetate extract of the 60 min incubation mixture of the liver microsomes was developed on TLC and the distribution of ³H label was determined (Fig 1(3)). As shown in Fig 4, the hamster, mastomys, female chicken and duckling microsomes readily converted B₁ to other metabolites. The Q₁ region from the hamster and the P₁ region from the mastomys were scraped off and extracted with chloroform, and the extracts were subjected to
HPLC for confirmation of the identity of the metabolites. Radioactivity in the Q1 region and that in the P1 region were found mostly at the elution times of standard Q1 (11.5 min) and P1 (14.8 min), respectively, on HPLC, indicating Q1 and P1 formation by the hamster and mastomys microsomes, respectively. For the other animals, a large part of B1 was not converted to other chloroform-ethyl acetate-extractable metabolites. Relatively high radioactivity in the RO region in the avian species could be due to contamination with the cytosol enzyme converting B1 to RO.

Conversion of B1 to chloroform-ethyl acetate-extractable metabolites by cytosol

The cytosol activity to convert B1 to other chloroform-ethyl acetate-extractable metabolites was studied in the same manner as the microsomal conversion, except that cytosol was used instead of microsomes (Fig. 1(4)). Most of the 3H label was found in the B1 and RO regions on TLC (Fig. 5). The cytosol activity to convert B1 to RO showed no correlation with species susceptibility, the cytosol of the avian species being more active than that of the mammalian species (Fig. 5).

In order to ascertain whether the avian cytosol inhibits aflatoxin-DNA binding by converting B1 to other less toxic chloroform-ethyl acetate-extractable metabolites in the presence of the hamster microsomes, B1 metabolism by the cytosols of the duckling and female chicken, which showed quite different activities to inhibit aflatoxin-DNA binding, were examined by incubation for 60 min and developing the chloroform-ethyl acetate extracts of the incubation mixtures on TLC (Fig. 1(5)). Similar patterns of 3H distribution on TLC were observed for the two species (Fig. 6). Furthermore, the propor-
tion of chloroform–ethyl acetate-extractable to 
total $^3$H was nearly the same in the two species; 
35.8% and 35.3% for the female chicken and 
duckling, respectively.

**Formation of acid and glucuronidase hydrolyzable 
metabolites**

Formation of acid- and glucuronidase-hydro-
lyzable metabolites was examined by incuba-
tion of S-9 fractions from various species with 
phosphoadenosine-phosphosulfate, UDP-glucu-
ronic acid and $[^3]$H B1 (Fig. 1(6)). Among 
the livers from various species, the mastomys 
liver converted B1 to glucuronidase-hydrolyz-
able metabolites most efficiently (1.2% of the 
original B1) and the male quail liver did so least 
efficiently (0.29% of the original B1). The duck-
ling liver converted B1 to acid-hydrolyzable me-
tabolites most efficiently (1.1% of the original 
B1) and the mastomys liver did so least efficien-
tly (0.44% of the original B1). The decreasing 
order of total formation of acid- and glucuronid-
ase-hydrolyzable metabolites in the avian spe-
cies was the duckling, female chicken, female 
quail, laying duck, male quail and male chicken, 
showing no clear relationship with the order of 
the cytosol activity to inhibit the microsome-
mediated aflatoxin-DNA binding.

**Discussion**

Consistent with the findings reported by Lot-
likar et al.\(^{14}\), the microosomal activity to bind 
aflatoxin to DNA was higher in the hamster 
than in the rat (Fig. 2), confirming that the spe-
cies difference in susceptibility cannot be attri-
buted only to species difference in microsomal 
activity to form aflatoxin-epoxide\(^1\),\(^3\),\(^4\).

The TLC analysis of the chloroform–ethyl ac-
etate extracts of the microsome incubation showed that the duckling microsomes have relatively high activity to oxidize B1 to more polar chloroform-ethyl acetate-extractable metabolites (Fig. 4). Since the duckling is relatively susceptible to the toxic and carcinogenic effects of B1, our finding suggests that the conversion of B1 by microsomes to other chloroform-ethyl acetate-extractable, less-toxic metabolites, such as M1, P1 and Q1, is not related to the species difference in susceptibility.

The importance of cytosol GST toward the epoxide in B1 detoxification has been well established in several mammalian species. Furthermore, the GST activity is well correlated with the species differences in susceptibility of the rat, mouse, hamster, guinea pig and quail. In this study, we determined the cytosol activity to inhibit the microsome-mediated aflatoxin-DNA binding in the presence or absence of GSH. In accordance with the reported findings that the hamster cytosol has a relatively high GST activity, the hamster cytosol inhibited the aflatoxin-DNA binding markedly in the presence of GSH but not at all in its absence (Fig. 3). In contrast to such a large difference between the B1-DNA binding level in the presence of GSH and that in its absence, the avian cytosols showed similar levels of B1-DNA binding regardless of the presence or absence of GSH (Fig. 3). In contrast to such a large difference between the B1-DNA binding level in the presence of GSH and that in its absence, the avian cytosols showed similar levels of B1-DNA binding regardless of the presence or absence of GSH. This would exclude the possibility that the species difference in the cytosol activity to inhibit the aflatoxin-DNA binding does not reflect the species difference in cytosol reduction of B1 to RO. Furthermore, the experiment on conversion of B1 to other chloroform-ethyl acetate-extractable metabolites by the duckling and female chicken cytosols in the presence of the hamster microsomes showed that the difference in cytosolic activity to inhibit aflatoxin-DNA binding does not reflect the difference in metabolism of B1 to less toxic chloroform-ethyl acetate-extractable metabolites. Also, the lack of a marked difference in the proportion of acid-hydrolyzable and glucuronizable fractions of the incubation mixture between the avian species suggests little contribution of the metabolic pathways forming sulfate and glucuronide conjugates.

The relative susceptibility to the toxic effect of B1 can be interpreted in terms of the balance of the microsomal activity to bind aflatoxin to DNA and the cytosol activity to inhibit it. The cytosol activity was very low in the rat, duck and duckling, but the microsomal activity was higher in the duckling and duck than in the rat, being consistent with the relative susceptibility of these three species. Also, similar susceptibility of the quail, chicken and rat is explained by the observation that the cytosol activity was higher in the quail and chicken than in the rat, although these avian species showed higher microsomal activity than the rat. As has been proposed by others, the difference in susceptibility between the rat and hamster can be explained by very high activity of cytosol to inhibit aflatoxin-DNA binding in the hamster compared with the rat, in spite of higher microsomal activity in the hamster than in the rat (Fig. 2).

The rat and duck are susceptible, but the hamster is resistant, to the carcinogenic effect of B1. Both immature and mature ducks are susceptible, but the comparative susceptibility of the rat and duck is unclear. Whether the other species used in this study have susceptibility to the carcinogenic effect is unknown. Such species variation in susceptibility to the carcinogenic effect of B1 can also be explained by the microsomal and cytosolic activities for aflatoxin-DNA binding, as in the interpretation of the species susceptibility to the toxic effect of B1.

Many factors, such as extrahepatic metabolism, gastrointestinal absorption and urinary
excretion of B₁ may influence the species susceptibility to B₁ at the whole-body level. Nevertheless, the combined activities of the liver microsomes and cytosol toward aflatoxin-DNA binding in various species were roughly consistent with the relative susceptibility of the species, indicating that these opposite activities are major determinants of the difference in susceptibility between species, including avian species, although the mechanism of inhibition of aflatoxin-DNA binding by the avian cytosol remains unknown.

The mastomys liver showed characteristic B₁ metabolism. The microsomal activity to oxidize B₁ to other free metabolites, especially to P₁, was very high, as has been observed in the mouse, but the activity to form aflatoxin-DNA was very low, differing from the mouse. The activity of the mastomys cytosol to inhibit aflatoxin-DNA binding was as high as that of the hamster cytosol. All these characteristics seem to be favorable for decreasing the biological potency of B₁. The toxic and carcinogenic effects of B₁ are unknown at present, but this animal could be a good model for studying the action of aflatoxin.

References


