Linear dose—response relationship for DNA adducts in rat liver from chronic exposure to aflatoxin B₁

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Male F-344 rats were given [^{3}H]aflatoxin B_1 (AFB$_1$) in the drinking water at three exposure levels (0.02, 0.6, 20 μg/l, resulting in average dose levels of 2.2, 73, 2110 ng/kg per day). After 4, 6 and 8 weeks, DNA was isolated from the livers and analyzed for aflatoxin—DNA adducts. The level of DNA adducts did not increase significantly after 4 weeks, indicating that a steady-state for adduct formation and removal had nearly been reached. At 8 weeks, the adduct levels were 0.91, 32 and 850 nucleotide—aflatoxin adducts per 10^9 nucleotides, i.e. clearly proportional to the dose. At the high dose level, a near 50% tumor incidence would be expected in a 2-year bioassay with F-344 rats while the low dose used is within the range of estimated human dietary exposures to aflatoxin in Western countries. The proportionality seen between exposure and steady-state DNA adduct level is discussed with respect to a linear extrapolation of the tumor risk to low dose.

Introduction

Covalent binding to DNA is an activity common to a large number of chemical carcinogens (1). With the use of radiolabeled carcinogen, DNA adducts are detectable at dose levels that are much lower than those required for a statistically significant increase in tumor incidence in an animal bioassay for carcinogenicity. The shape of the dose—response curve for DNA adduct formation is therefore considered useful information for a biologically based risk extrapolation (2).

Aflatoxins comprise a group of fungal metabolites of which aflatoxin B_1 (AFB$_1$*) is among the most potent carcinogens found in food (3). A relationship between mycotoxin exposure and human liver cancer incidence is suggested by dose-dependent correlations of exposure to aflatoxins and cancer incidence rates in some areas of Africa and Asia (4). While doses of up to 220 ng/kg body wt/day have been reported in these countries, daily exposure to aflatoxin in the USA is estimated to be between 0.25 ng/kg (5) and 3 ng/kg (6).

The mechanism of action of AFB$_1$ involves enzymatic oxidation to an epoxide and subsequent covalent binding to DNA, predominantly at the nitrogen-7 of guanine (7,8). The rates of the processes that govern formation and removal of carcinogen—DNA adducts, e.g. diffusion, chemical and enzymatic reactions, are expected to be proportional to the concentration in the low dose range. Proportionality between exposure dose and DNA adduct level has indeed been reported with single doses of AFB$_1$ (9). With chronic exposure, the level of DNA adduct is a result of continuous formation and removal, and it is the steady-state DNA damage reached after some time that probably determines

*Abbreviations: AFB$_1$, aflatoxin B$_1$.

the lifetime risk of cancer. The data available so far are derived from AFB$_1$ dose levels that would significantly increase tumor incidence (10—12). They do not provide information for low-dose risk extrapolation. In this report, the dose—response relationship for the steady-state level of DNA adducts reached in rat liver DNA at levels of human exposure to AFB$_1$ is analysed.

Materials and methods

Materials

[^{3}H]AFB$_1$ (1 mCi/ml in ethanol) was from Moravek Biochemicals, City of Industry, CA. The radiochemical purity was determined by TLC on silica-gel plates developed with chloroform/acetone, 9:1. Unlabeled AFB$_1$ was from Senn Chemicals, Dielsdorf, Switzerland. Male F-344 rats [CDF(F-344)/CrlBR] were from Charles River Wiga, Sulzfeld, FRG.

The radiochemical purity of [^{3}H]AFB$_1$ decreased from >98% to 70% within 1 week at room temperature in the presence of water. Therefore, the application solutions used for experiments I and II were kept at 4°C and were freshly prepared after 5 days. For experiment III, the drinking water was replaced twice weekly.

DNA binding in rat liver

Experiment I. DNA repair. [^{3}H]AFB$_1$ (batch with a sp. act. of 8 Ci/mmol, radiochemical purity >98%) was diluted with distilled water to a chemical concentration of 100 ng/2 ml. Rats (wt range 221—264 g) were given 2 ml/kg by oral gavage (100 ng/kg) once or 10 times at daily intervals. Groups of two rats were killed 24 h, 3, 7 or 11 days after the last administration and DNA was isolated from the livers according to Sagelsdorff *et al.* (13) The sp. act. of the DNA was determined by spectrophotometry (1 mg/ml = 20 A$_{260 \, nm}$) and liquid scintillation counting. Enzymatic hydrolysis of the DNA to the 3'-nucleotides and separation of the aflatoxin—nucleotide adducts from normal nucleotides by reverse-phase HPLC in a phosphate buffer—methanol gradient was performed according to Sagelsdorff *et al.* (14).

Experiment II: DNA adduct level as a function of oral dose. [^{3}H]AFB$_1$ (batch 8 Ci/mmol) was administered by oral gavage (once or 10 times at daily intervals) at six dose levels, spanning five orders of magnitude from 1 ng/kg to 100 μg/kg. The body wts of the rats ranged between 182 and 220 g. Application solutions
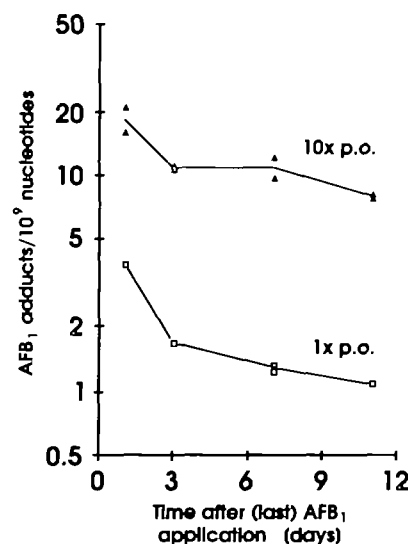

Fig. 1. Aflatoxin—DNA adduct levels in the liver of male F-344 rats, at various times after oral gavage of a single dose or of 10 daily doses of 100 ng/kg tritiated AFB$_1$. Semi-log plot. Two rats per group; one symbol shown when individual difference is smaller than the symbol size.

for dose levels 1, 10 and 100 ng/kg were prepared by appropriate dilution of the stock solution with distilled water (see experiment I). For dose levels 1, 10 and 100 μg/kg, unlabeled AFB_1 in ethanol was used instead of water. Previous experiments have shown that the DNA-binding potency of AFB_1 is not affected by pretreatment for 10 days at 4% (w/v) ethanol in the drinking water or by simultaneous oral administration of up to 4 g/kg ethanol (15). DNA was isolated from the livers 24 h after the last dose and analysed for adducts as described above.

Experiment III. DNA adduct level after chronic exposure in the drinking water. [³H]AFB₁ (batch 20 Ci/mmol) was administered in the drinking water for 4, 6 and 8 weeks at three dose levels to groups of three animals. The drinking water contained 2840 d.p.m./ml as AFB_1 at all dose levels. The chemical concentrations of AFB_1 (20 ng/l, 600 ng/l and 20 μg/l) were adjusted by appropriate addition of unlabeled AFB_1 to the intermediate and the high dose levels. The animal wts ranged between 113 and 159 g (mean = 135 g) at the beginning of the dosing and were 210−264 (mean = 245 g), 246−295 (mean = 273 g) and 279−305 (mean = 294 g) at 28, 43 and 56 days respectively. The weight gain was not different between the groups. The individual dose was calculated on the basis of the water consumption (range 16−22 ml/rat/day) and was averaged over the entire treatment period. Averaged over all animals in the 56 day group, dose levels of 2.2, 73, 2110 ng/kg/day resulted. At the end of the exposure period the animals were killed and liver DNA was analysed as described above (experiment I).

Results

DNA radioactivity

Nucleotide analyses showed that the normal nucleotides were not radiolabeled. All radioactivity eluted without detectable optical density, nearly all of it at elution times known for the lipophilic aflatoxin−nucleotide adducts (16). The sp. act. of the DNA samples was therefore converted to adduct levels on the basis of the sp. act. of the [³H]AFB₁ used and of an average mol. wt of 309 daltons for a DNA nucleotide.

DNA repair

The rate of removal of DNA adducts was investigated in order to define appropriate periods of exposure to reach near steady-state conditions for the level of DNA adducts. Figure 1 shows a semi-log plot of the level of DNA adducts measured in the liver at 1, 3, 7 and 11 days after one single oral dose of 100 ng/kg tritiated AFB_1 to male F-344 rats (1× p.o.), and after the last of 10 oral doses given at daily intervals (10× p.o.). The loss of DNA adducts was faster initially. Later, the adducts

Fig. 2. Aflatoxin−DNA adduct levels in male F-344 rat liver, as a function of dose (x-axis) and after different periods of exposure (different curves) to [³H]AFB₁. Double-log plot. Solid lines connecting three and more points are linear regressions. DW, administration in the drinking water. Standard deviations seen only if larger than the symbol size.

disappeared only slowly, with a half-life of 2−3 weeks. Under the assumption that all processes follow first-order kinetics and following general pharmacokinetic principles, a theoretical value of 93.75% of the limiting (steady-state) adduct value would have to be reached after four elimination half-lives. On the basis of the repair rates determined above, a near steady-state level of DNA adducts is therefore expected to be reached within ~8 weeks.

Nucleotide analyses were consistent with reports in the literature (16,17): the initially formed, late-eluting adduct (putatively assigned to be the AFB₁-guanyl-7 adduct) disappears relatively rapidly, by repair and by chemical modifications; opening of the imidazole ring of guanine results in adducts that appear to be more refractory to DNA repair (16,17).

DNA adduct levels as a function of dose and period of exposure

The levels of DNA adducts are shown in Figure 2 as a function of dose (x-axis). The different curves illustrate that DNA adducts accumulate with increasing periods of exposure, 1× → 10× → 28 days → 43 days → 56 days of treatment. The increase is no longer significant, however, between 28 and 43 days or between 43 and 56 days. This indicates that near steady-state had been reached, as predicted on the basis of the half-lives of the adducts. At 8 weeks, the adduct levels averaged 0.91 ± 0.19, 32 ± 4 and 850 ± 11 aflatoxin−nucleotide adducts/10^9 nucleotides, at the three dose levels—2.2, 73, 2110 ng/kg/day.

The accumulation of adducts with time was substantial: 24 h after a 10-day treatment, the adduct level was 6 times higher than 24 h after a single administration. After 56 days of continuous exposure, the adduct level was 16 times the single-dose value.

The level of DNA adducts was proportional to dose under all treatment conditions. At 8 weeks, for instance, the adduct levels were in a ratio of 1:35:934, while the dose levels were in 1:33:959. Proportionality is also illustrated in the double-log plot of Figure 2 by the slope of 1.0 for all regression lines. Two high-dose data points did not follow this rule: (i) after 10 oral doses of 100 μg/kg/day, the DNA adduct level was no longer proportional to the dose. This could be due to a saturation of the enzymatic activation of AFB₁ at this highest dose level. (ii) The value seen with the 28 day high-dose treatment is also too low. This result was due to one animal that showed an unexplained low adduct value.

Discussion

Our data show that the level of aflatoxin−DNA adducts in rat liver is proportional to dose under all exposure conditions used. The investigated dose range covered five orders of magnitude and included both tumorigenic dose levels and levels of dietary human exposure.

The high dose level used in the drinking water experiment (2.1 μg/kg/day) would have resulted in a significant increase in liver tumors in male F-344 rats. A 50 p.p.b. AFB₁ diet for 1 year (approximate daily dose 2.5 μg/kg) gave rise to a 40% incidence of hepatocellular carcinoma within 2 years (18). It is tempting, therefore, to relate the level of the steady-state DNA damage to a tumor incidence observed after long-term treatment, and to investigate the question of whether the data can be extrapolated to other species. The 56 day cumulative DNA damage at this dose level was 850 adducts/10^9 nucleotides in rat liver DNA. Dashwood and co-workers (19) have found in the trout that an adduct level of 1040/10^9 nucleotides after 2 weeks resulted in a 50% liver tumor incidence within 1 year. In humans, preliminary data on DNA adduct levels are also available. In liver
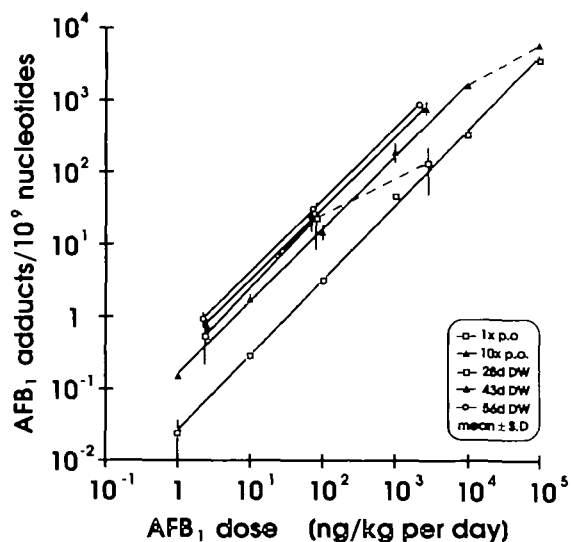
samples from patients undergoing surgery for liver cancer in Taiwan, aflatoxin−DNA adducts were determined with immunological techniques. Adduct levels in the range 1200−1700 per 10⁹ nucleotides were found in normal liver tissue (20). These values are of the same order as those seen in rats and trout at high tumor risk. Eventually, comparisons between experimental and human data can be made on the basis of adduct data.

Proportionality between exposure dose and DNA adduct levels has been shown here but the question remains whether proportionality also holds between DNA damage and tumor incidence. This hypothesis cannot be answered experimentally in the low dose range. In the high dose range, the correlation of hepatic tumor incidence in the trout with the level of aflatoxin−DNA adducts does not appear to deviate from linearity (21). The combined data therefore support the idea that a linear extrapolation of the tumor risk to low dose is appropriate for the induction of liver tumors by AFB₁.

Acknowledgement

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