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The formation of AFB_1-macromolecular adducts in rats and humans at dietary levels of exposure

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Abstract

The levels of aflatoxin B_1-DNA and aflatoxin B_1-albumin adducts were investigated by accelerator mass spectrometry (AMS) in humans and rats following exposure to a known, dietary relevant amount of carbon-14 labeled aflatoxin B_1 ($[^{14}C]AFB_1$). The aims of the study were to: (a) investigate the dose-dependent formation of DNA and protein adducts at very low doses of AFB_1 (0.16 ng/kg–12.3 μg/kg) in the rat; (b) measure the levels of AFB_1-albumin and AFB_1-DNA adducts at known, relevant exposures in humans (c) study rat to human extrapolations of AFB_1-albumin and DNA adduct levels. The results in the rat showed that both AFB_1-albumin adduct and AFB_1-DNA adduct formation were linear over this wide dose range. The order of adduct formation within the tissues studied was liver > kidney > colon > lung = spleen. Consenting volunteers received 1 μg (~15 ng/kg) of $[^{14}C]AFB_1$ in a capsule approximately ~3.5–7 h prior to undergoing colon surgery. The mean level of human AFB_1-albumin adducts was 38.8 ± 19.55 pg $[^{14}C]AFB_1$/mg albumin/μg AFB_1/kg body weight (b.w.), which was not statistically different to the equivalent dose in the rat (15 ng/kg) 42.29 ± 7.13 pg $[^{14}C]AFB_1$/mg albumin/μg AFB_1/kg b.w. There was evidence to suggest the formation of AFB_1-DNA adducts in the human colon at very low doses. Comparison of the linear regressions of hepatic AFB_1-DNA adduct and AFB_1-albumin adduct levels in rat found them to be statistically similar suggesting that the level of AFB_1-albumin adducts are useful biomarkers for AFB_1 dosimetry and may reflect the DNA adduct levels in the target tissue. $[^{14}C]AFB_1$-DNA and $[^{14}C]AFB_1$-albumin adducts were hydrolysed and analysed by HPLC to confirm that the $[^{14}C]$ measured by AMS was derived from the expected $[^{14}C]AFB_1$ adducts.

Keywords: Aflatoxin; Adducts; Accelerator mass spectroscopy; Low dose; Risk assessment

1. Introduction

Aflatoxin B_1 (AFB_1) is a potent human liver carcinogen (IARC, 1993) formed as a secondary metabolite of moulds *Aspergillus flavus* and *Aspergillus parasticus*. It is found as a food contaminant in a variety of foods including corn, peanuts, soybeans, soy sauce and cereal crops (IARC, 1993, Eaton and Groopman, 1994). Exposure to AFB_1 is a particular problem in parts of Africa and China, where the warm and humid storage conditions of staple crops promotes the formation of high levels of AFB_1.

AFB_1 is metabolised to the highly reactive AFB_1-8,9-epoxide which reacts with DNA to form the AFB_1-N7-guanine adduct (AF-gua) (Essigmann et al., 1977; Croy et al., 1978; Garner and Martin 1977; Lin et al., 1977). The epoxide is unstable and also undergoes hydrolysis to the AFB_1-8,9-dihydrodiol (AF-diol) which reacts with proteins (Wild et al., 1986; Sabbioni et al., 1987).

Abbreviations: LLNL, Lawrence Livermore National Laboratory; AMS, accelerator mass spectrometry; AFB_1, aflatoxin B_1; b.w., body weight; AF-gua, aflatoxin B_1 N7-guanine; AF-diol, aflatoxin B_1-8,9-dihydrodiol; DMD, dimethyldioxirane; S:N ratio, signal to noise ratio; FAPY1, 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydro-pyrimid-5-yl-formamido)-9-hydroxy-AFB_1; FAPY2, 8,9-dihydro-8-(2-amino-6-formamido-4-oxo-3,4-dihydropyrimid-5-amino)9-hydroxy AFB_1.

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Molecular epidemiology studies commonly measure human exposure to AFB_1 as urinary AF-gua adducts or peripheral AFB_1-albumin adducts (Wild et al., 1990; Groopman, 1994, Montesano et al., 1997; Wang et al., 1996). Albumin can be easily extracted from blood and provides a relatively non-intrusive measure of biologically effective dose of AFB_1 in humans (Wild et al., 1996; Montesano et al., 1997).

Assessment of AFB_1 exposure by questionnaire and diet measurements is problematical and potentially unreliable due to the heterogeneous distribution of AFB_1 in foods. Human validation of AFB_1-albumin adducts as surrogate biomarkers for exposure by the administration of known, dietary relevant levels of AFB_1 has not been previously possible due to difficulties in the detection of DNA and protein adducts. Methods used for the measurement of DNA include immunoassay, ^{32}P-post-labelling and LC/GC-MS which have limits of detection in the range of adducts/10^7 to 10^9 nucleotides (Poirier et al., 2000). The recent use of accelerator mass spectrometry (AMS) however, should allow the measurement of adducts 10–100-fold lower, making adduct validation in humans possible. Thus, one of the aims of this study was to administer a known, dietary relevant level of [^{14}C]AFB_1 to humans and to measure the levels of DNA and protein adducts by AMS.

In addition to molecular epidemiology studies in humans, extensive research has also been performed on AFB_1 carcinogenesis and AFB_1-DNA and protein adduct formation in rodents, where extrapolations from rodents to humans have been made. The methods used for the detection of DNA adducts has meant that previous rodent to human extrapolations have made assumptions about differences in AFB_1 exposure, in addition to species differences. In this study it was possible to investigate the levels of adducts at the same, dietary relevant dose in humans and rodents. By performing rat studies over a wide dose range it was possible to establish the linearity of the dose-dependent adduct formation at very low doses.

Over the past 10 years AMS has been used to analyse samples containing attomole levels of [^{14}C] from laboratory animals and humans administered [^{14}C] labeled carcinogens (Turteltaub et al., 1990; Vogel and Turteltaub, 1994; Frantz et al., 1995; Freeman and Vogel, 1995, Mani et al., 1999; Mauthe et al., 1999; Dingley et al., 1999). In this current study, the sensitivity of AMS was used to investigate the levels of AFB_1-DNA and protein adduct formation at dietary relevant doses by administration of [^{14}C]AFB_1. Although AFB_1 is a potent liver toxin it also effects other tissues, including the lung, kidney and colon, both in rodents and humans (Autrup et al., 1979; Newberne and Roger, 1973, Kolars et al., 1994; Coulombe, 1994; Larsson and Tjalve, 1995). Human liver tissue is rarely removed as part of clinical treatment for liver conditions. Thus in this study the levels of AFB_1-DNA in colon tissue and albumin adducts in blood were investigated in subjects undergoing colon surgery following administration of [^{14}C]AFB_1, with the following aims: (a) to establish the relationships between AFB_1 exposure and AFB_1-DNA and AFB_1-albumin adduct formation over a wide dose range between 0.16 ng/kg and 12.3 μg/kg in the rat, (b) to establish the level of formation of AFB_1-DNA and AFB_1-albumin adducts in humans after a known exposure to [^{14}C]AFB_1, (c) to evaluate the rat model for human exposure to AFB_1 in the form of DNA and albumin adducts.

2. Materials and methods

2.1. Animals

Male Fischer rats (200–250 g) (Harlan UK, Oxon, UK) were housed two animals/cage for the duration of the experiment. Fischer rats were chosen for these studies as they have been reported to be the strain most susceptible to the toxic and carcinogenic effects of AFB_1 (Wild et al., 1996).

2.2. Chemicals

[^{14}C]AFB_1 (S.A. 80 mCi/mmol) was purchased from Moravek Biochemicals, Brea, CA. Unlabeled AFB_1 was purchased from Sigma (Dorset, UK). The lactose and gelatine capsules were obtained from York District Hospital. HPLC grade solvents and analytical grade chemicals were from standard commercial suppliers. [^{14}C]AFB_1 purity was determined to be $>99\%$ by HPLC co-elution with an unlabeled standard and also by scintillation counting where a decay spectrum was obtained.

2.3. Dosing solutions and capsule preparation

The dosing solutions for the rat experiments were prepared 1 day prior to the experiment. The dosing vehicle was a DMSO:saline [1:3 (v/v)] mixture, where volumes were based on a recommended oral gavage dosing volume of 2 ml/kg b.w. The rats received stated doses of undiluted [^{14}C]AFB_1. For human administration, the capsules were prepared by filling a gelatine capsule with lactose and injecting 1μg [^{14}C]AFB_1 in a small volume of ethanol into the capsule. The ethanol was allowed to evaporate and the capsule was sealed. All the capsules were prepared at the same time. The levels of [^{14}C]AFB_1 added to the capsules were checked in triplicate immediately prior to and after capsule preparation by liquid scintillation counting. The capsules were stored in the dark at 4 °C in the Medical Physics Department of York District Hospital until used.

2.4. Synthesis of AFB_1-DNA and AFB_1-albumin standards

The AFB_1-DNA adducts and AFB_1-albumin standards were synthesised *via* a common intermediate, aflatoxin B_1-8,9-epoxide. AFB_1-8,9-epoxide was synthesised from dimethyldioxirane (DMD) using the method described by Baertschi et al. (1988). DMD was synthesised according to the method of Murray and Jeyaraman (1985). Calf thymus DNA was adducted with AFB_1-8,9-epoxide in a 1:1.5 stoichiometric reaction in the dark for 15 min at RT. The DNA adduct standards 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydro-pyrimid-5-yl-formamido)-9-hydroxy-AFB_1 (FAPY1), 8,9-dihydro-8-(2-amino-6-formamido-4-oxo-3,4-dihydropyrimid-5-amino)9-hydroxy AFB_1 (FAPY2) and AF-gua were synthesised from the AFB_1-adducted DNA according to methods described by Hertzog et al., (1982). The AFB_1-lysine standard was synthesised from the AFB_1-8,9-epoxide and *N*-acetyl-lysine using the methods of Chapot and Wild (1991); a further AFB_1-lysine standard was kindly provided by Professor C. Wild, University of Leeds, UK.

2.5. Dosing of rats with $[^{14}C]AFB_1$

Animals were starved overnight prior to the experiment to reproduce the human study. Doses of $[^{14}C]AFB_1$ were administered to $n=4$ animals by oral gavage as follows—0.16 ng/kg to 12.3 μg/kg with doses of 0.16 ng/kg (1.85 Bq), 2.1 ng/kg (20 Bq), 6.2 ng/kg (57.3 Bq), 15 ng/kg (140 Bq), 150 ng/kg (1.4k Bq), 2.54 μg/kg (24.1 kBq) and 12.3 μg/kg (116.9 kBq), the amount of $[^{14}C]$ administered is shown in parentheses. Six hours post-dosing, the animals were placed under terminal anaesthesia, blood was removed via cardiac puncture and following cervical dislocation, the liver, lung, kidney, colon and spleen were dissected. A non-dosed animal was used in each experiment to provide a $[^{14}C]$ control. The tissues were immediately frozen in liquid nitrogen and stored at −70 °C for later DNA extraction. The blood samples were collected into tubes containing EDTA anti-coagulant [L.I.P. (Equipment & Services) Ltd., West Yorkshire, UK].

2.6. Human volunteers

Subjects undergoing GI tract surgery for cancer at the York District Hospital were enrolled into the study. Participation was based on voluntary consent after the aims, objectives and health consequences of the study were comprehensively explained. The study was explained to the subjects initially by a research nurse, and the following day by the consultant surgeon. Subjects were also given an information leaflet explaining the study. Subjects were asked to complete a brief questionnaire about their dietary habits. The study was ethically approved by the York District Hospital Local Research Ethics Committee (YDH LREC) and independently by the Institutional Review Board (IRB) at LLNL, CA, USA. Permission for the administration of $[^{14}C]$ radiolabeled substances was obtained from the Department of Health Administration of Radioactive Substances Advisory Committee (ARSAC) in UK.

2.7. Human dosing with $[^{14}C]AFB_1$

Subjects received 1 μg $[^{14}C]AFB_1$ (9.84 kBq) in a gelatine capsule by mouth ~3.5–7 h prior to surgery. Blood samples were obtained prior to and during surgery, tissue samples were removed during the surgical procedure. Information was also obtained on any medication that the subjects received which may have effected the metabolism and disposition of $[^{14}C]AFB_1$ (Table 2).

2.8. Isolation and purification of $[^{14}C]AFB_1$-albumin

Albumin was isolated from the blood samples using methods described by Turner et al. (1998), and quantified using a protein assay kit by Pierce & Warriner UK Ltd, (Cheshire, UK). Control and pre-dose blood samples were used in the rat and human analyses respectively, as controls for any extraneous $[^{14}C]$ entering the sample.

2.9. DNA extraction from tissue samples

Frozen tissue samples wrapped in saran wrap and foil were disrupted using a pestle. The disrupted tissue was incubated at 37 °C overnight in a lysis buffer (8 ml/g tissue) (10 mM tris–HCl (pH 8), 4 M urea, 1% (w/v) Triton X-100, 10 mM EDTA, 100 mM NaCl, 10 mM DTT, 0.8 mg/ml proteinase K). The digested tissue was then incubated with RNase A (0.5 mg/ml supernatant) and RNase T_1 (0.5 mg/ml supernatant) for 1 h at room temperature (RT) prior to DNA extraction using Qiagen tip-500 and t-2500 ion exchange columns (Qiagen Ltd., Surrey, UK). The extracted DNA was precipitated with an equal volume of RT propan-2-ol and left overnight at −20 °C. Centrifugation at 3000 g for 90 min resulted in a DNA pellet that was washed in 70% (v/v) ethanol. The pellet was resuspended in 500 μl of H_2O. DNA purity and quantity was determined by UV spectrophotometry at 230, 260 and 280 nm. DNA was considered pure when the 260/230 and 260/280 nm absorbance ratios ranged between 2.0–2.5 and 1.7–1.9 respectively. In order to act as additional controls for contamination of samples by exogenous $[^{14}C]$ during the DNA isolation process, livers from several non-dosed rats were divided into ~0.7–1 g aliquots. DNA from an aliquot of rat liver was extracted with each set of dosed rat or human tissue samples.

2.10. Quantitation of the levels of [^{14}C]AFB_1 in the samples

Levels of [^{14}C]AFB_1 in tissue, DNA and albumin samples were analyzed by either scintillation counting or AMS, Where no [^{14}C] was detected using liquid scintillation counting (LSC), samples were analysed by AMS, as previously described (Vogel, 1992; Freeman et al., 1995).

AMS measures the relative ratios of [^{14}C] to [^{13}C] in graphitised samples. The ratios of [^{14}C]/[^{13}C] generated from AMS analysis were then converted to levels of adducts/10^{12} nucleotides for the DNA samples and pg AFB_1/mg albumin for the albumin samples. A number of different factors were incorporated into the analysis including the % carbon in the sample (where the % carbon in DNA and albumin was 29 and 49% respectively, measured using a C:H:N analyser). In addition, the specific activity of the [^{14}C]AFB_1, the amount of the original sample analysed and the presence or absence of carbon carrier were also considered.

LSC and AMS analyses only provide a quantitative measure of [^{14}C] in the samples analysed. Measurements of DNA and albumin samples from these experiments do not confirm that the [^{14}C] is from [^{14}C]AFB_1 or that the adducts formed are the expected DNA and albumin adducts as previously reported (Essigmann et al., 1977; Croy et al., 1978; Garner and Martin, 1977; Lin et al., 1977; Wild et al., 1986; Sabbioni et al., 1987; Sabbioni, 1990). In order to determine the nature of the [^{14}C] measured in these analyses, HPLC of digested or hydrolysed albumin and DNA respectively was performed. The levels of AFB_1-albumin and AFB_1-DNA adducts analysed by HPLC were below the detection limits of the HPLC UV detector, thus co-injection with the appropriate standards was necessary for characterisation of the adducts.

2.11. In vitro spiking study

To confirm that the data obtained represented true covalent binding and not AFB_1-DNA associations via non-covalent interactions colon samples from undosed patients were lysed and spiked with ^{14}C radiolabelled AFB_1. The DNA was then extracted as described above. The amount of ^{14}C added was based on the highest levels of ^{14}C seen in the lysed tissue samples and multiplied by a factor of 10.

2.12. HPLC analysis of digested and purified [^{14}C]AFB_1-albumin

Digestion of the albumin to its constituent amino acids, clean up and subsequent HPLC analysis, was performed using methods described by Turner et al. (1998). Briefly, aliquots of rat and human albumin following exposure to [^{14}C]AFB_1 were digested with Pronase. The resulting digested protein was purified by C_{18} solid phase extraction (Waters Corp., Watford, UK) and anti-aflatoxin (2E9) immunoaffinity columns (Rhone Diagnostic Technologies, Strathclyde, UK). Rat albumin digests were analysed by HPLC using the same conditions determined for an AFB_1-lysine standard. The HPLC analysis was performed using an Anachem 305 binary pump system with a single wavelength UV detector set to 360 nm where the system was controlled by Gilson 712 software (Anachem Ltd, Luton, UK). The samples were analysed on a 25 cm C_{18} reverse phase Phase-Sep column (Phase Separations Ltd, Deeside, UK) using a methanol and water linear gradient 40% MeOH(v/v) to 100% MeOH over 20 min, the column was reconditioned over the next 25 min.

Prior to analysis by HPLC, aliquots of the digested samples were analysed by AMS to determine the levels of radiocarbon in the samples. Four HPLC analyses were performed; a blank run, and digested, purified albumin samples equivalent to 3.5 mg of albumin from the following samples (1) blood obtained prior to exposure to AFB_1, (2) blood drawn approximately 6 h following exposure from the same subject and (3) rat blood obtained 48 h post-dosing. Thirty-second fractions were collected from 0 to 14 min and larger fractions were collected for the remainder of the runs. Each fraction was dried and submitted for AMS analysis.

2.13. HPLC analysis of hydrolysed [^{14}C]AFB_1-DNA

DNA samples from the human and rat studies were hydrolysed using a method described by Hertzog et al. (1982). AFB_1-residues were isolated from hydrolysed DNA using C_{18} solid-phase extraction cartridges. Fractions were dried and reconstituted in 40% MeOH (v/v) prior to HPLC analysis as described above. The identity of [^{14}C] peaks was confirmed by co-injection of AFB_1-diol and the AFB_1-DNA adduct standards FAPY1, FAPY2 and AF-gua. Fractions were collected at 30 s intervals around the retention times of the standards and at longer intervals in other parts of the HPLC run and submitted for AMS analysis.

2.14. Statistics

The albumin- and DNA-adduct data are expressed as mean ± standard deviation as calculated in Microsoft Excel Version 5. The rat data were calculated from $n = 4$ animals per dose, where each DNA or albumin sample was independently extracted, prepared and measured once by AMS.

The mean human albumin adduct level was calculated from $n = 7$ subjects, where one albumin extraction was performed per blood sample and then each albumin sample was independently prepared and measured by

AMS three times. Four DNA extractions were performed on each human tissue sample obtained. Depending upon the yield of DNA, each DNA sample was then prepared and measured up to three times by AMS.

Macromolecular adduct levels are given per unit dose. The regression analyses were performed on log transformed DNA and albumin adduct values vs. log transformed dose of [^{14}C]AFB$_1$/kg b.w. Comparisons of the linear regression analyses were performed using a Student's *t*-test of the regression coefficients. Both the regression analyses and comparison of the regression coefficients were performed in Minitab for Windows Version 10.

3. Results

3.1. Analysis of AFB$_1$-albumin from rats administered [^{14}C]AFB$_1$

The AFB$_1$-albumin adduct level was measured by LSC for the 12.3 μg/kg dose. All other samples were measured by AMS. Due to the loss of the blood samples it was not possible to calculate levels of AFB$_1$-albumin adducts for the 2.54 μg/kg dose. A double log plot of dose against pg [^{14}C]AFB$_1$/ mg albumin shows the formation of AFB$_1$-albumin adducts to be linear with dose in rats (Fig. 1), where the regression and correlation coefficients of the log transformed data are given in Table 1.

3.2. Analysis of AFB$_1$-DNA adducts from rats administered [^{14}C]AFB$_1$

DNA was extracted from the liver, colon, kidney, lung and spleen of each rat from all the experiments. The AFB$_1$-DNA adduct levels in rat liver for the 12.3 μg/kg dose was measured by LSC. All other DNA samples were analysed by AMS. A double log plot showed the adduct levels to be linear with dose. The order of adduct formation within the tissues studied was liver > kidney > colon > lung = spleen over the dose range 6.2 ng/kg to 12.3 μg/kg (Fig. 2). DNA adducts were not detectable below 6.2 ng/kg in organs other than the liver. The regression and correlation coefficients for each line are given in Table 1.

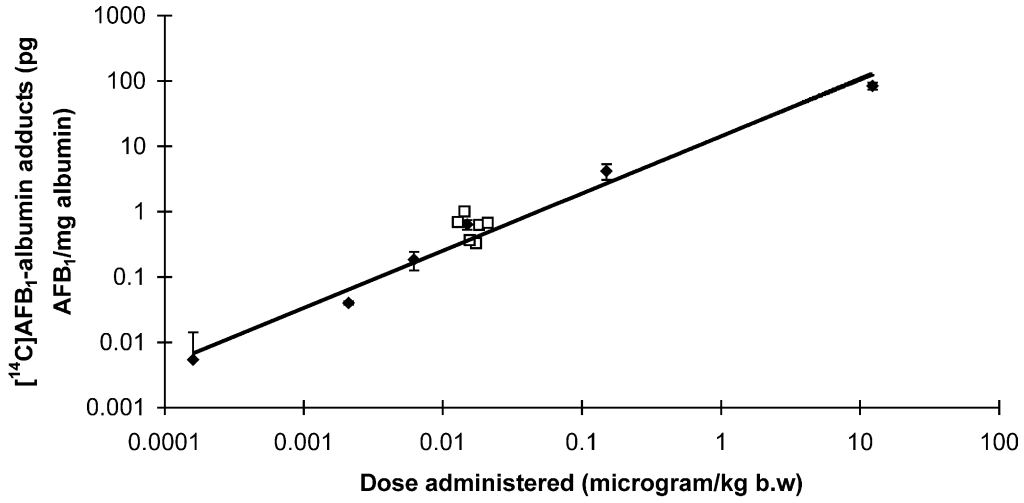

Fig. 1. A graph showing the relationship of aflatoxin B$_1$-albumin adducts with dose in male Fischer rats 6 h post-dose, where the human AFB$_1$-albumin adducts are shown as □.

Table 1
Carcinogen binding indices and regression coefficients of AFB$_1$-DNA and AFB$_1$-albumin adduct analyses in the rat

Tissue	Carcinogen binding indices[a]	Regression coefficient	R^2 value
Albumin	6.83–42.29[a]	0.8759 ± 0.0569	$R^2 = 0.98$
DNA			
Liver	7225–65135	0.9548 ± 0.0647	$R^2 = 0.97$
Kidney	319–1604	0.7950 ± 0.0430	$R^2 = 0.99$
Colon	184–936	0.7651 ± 0.0307	$R^2 = 0.99$
Lung	69–1157	0.6521 ± 0.0996	$R^2 = 0.91$
Spleen	61–755	0.6646 ± 0.0580	$R^2 = 0.97$

[a] An equivalent expression for albumin adducts is given as pg[^{14}C]AFB$_1$/mg albumin/μg AFB$_1$/kg b.w.

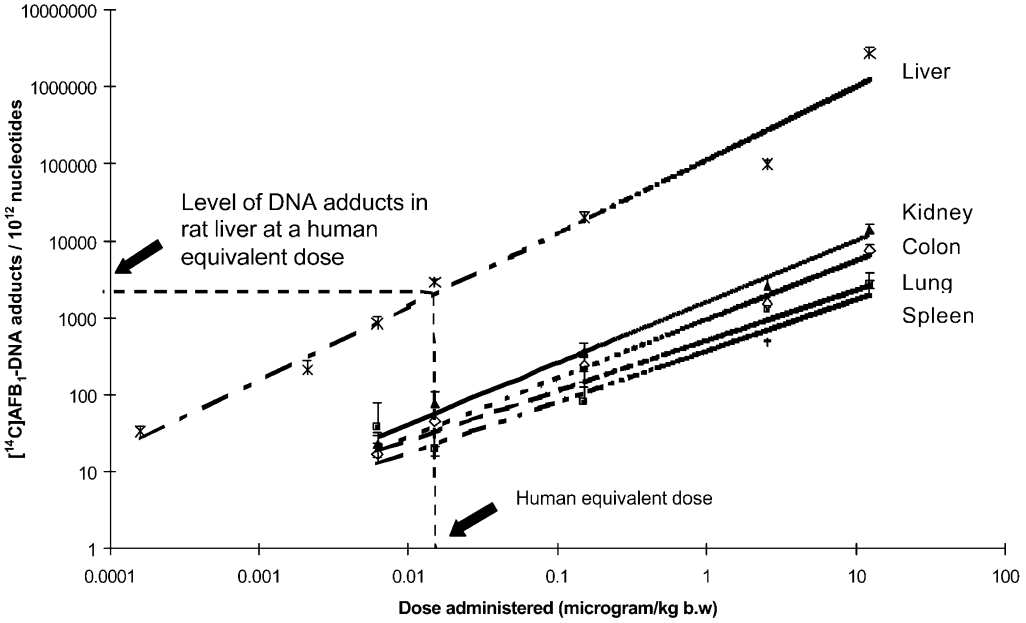

Fig. 2. A graph showing the relationship of aflatoxin B_1-DNA adducts in the liver, colon, kidney, lungs and spleen with dose in male Fischer rats 6 h post-dose. The level of adducts found in rat liver at the human equivalent dose is indicated by →.

Table 2
Brief details about the subjects participating in the study

Subject No.	Sex	Age	Time of surgery (post-capsule)	Weight (kg)	Location of tumour	Medication
1	M	73	4 h 45 m	71.1	Colon	Quinine sulphate, ciprofloxin, ferrocontin
2	M	73	6 h	57.8	Colon	Ferrous sulphate
3	F	81	4 h	55.0	Anal canal	Amyloid, frusomide, aspirin, folic acid, thyroxine
4	M	52	3 h 30 m	78.0	Colon	Nifedipine, prednisolone, ferrous sulphate
5	F	78	6 h 35 m	64.0	Sigmoid	None
6	M	61	5 h	70.0	Colon	None
7	F	63	6 h 10 m	47.6	Sigmoid	Warfarin, enalapril, unspecified diuretic, digoxin

3.3. Analysis of AFB_1-albumin from humans administered [^{14}C]AFB_1

Albumin was extracted from the pre-dose and surgery plasma or serum samples. Table 2 gives brief details about the subjects participating in the study and the times at which the post-dose blood samples were obtained. No detectable levels of [^{14}C] were observed by LSC, thus all the samples were analysed by AMS. The values of AFB_1-albumin adducts are shown in Fig. 1 for comparison to the levels of AFB_1-albumin adducts observed in the rat.

3.4. Analysis of AFB_1-DNA from humans administered [^{14}C]AFB_1

The [^{14}C] in the DNA samples from humans administered [^{14}C]AFB_1 was slightly higher than the level of [^{14}C] measured in control rat DNA (i.e. the assay 'noise') Using a signal:noise ratio of 2 as the detection limit, "" ""[^{14}C]AFB_1-DNA adducts were only detectable in samples from two subjects. The adduct values were 13.4 ± 2.5 and 17.6 ± 3.5 adducts/10^{12} nucleotides respectively ($n=2$ AMS measurements/subject). AMS analysis of whole human colon tissue prior to DNA extraction was also performed. With the exception of one subject (135.2 and 213.6 fmol [^{14}C]AFB_1/g normal and tumour tissue respectively), the values of [^{14}C] in the tissue were 50.5 ± 6.7 fmol [^{14}C]AFB_1/g normal tissue and 41.8 ± 17.0 fmol [^{14}C]AFB_1/g tumour tissue.

3.5. In vitro spiking experiment

To ensure that the [^{14}C] measurements associated with the DNA resulted from covalent binding, an experiment was conducted in which [^{14}C]AFB_1 was added to lysed human colon tissue samples. The lysed spiked sample was then put through the DNA purification procedure

and the resulting purified DNA analysed by AMS. No increase above the background [^{14}C] level was seen demonstrating that the purification process removed all adventitious [^{14}C]. Any increase in this background level for patient samples could therefore be ascribed to covalent DNA adducts.

3.6. HPLC analysis of hydrolysed [^{14}C]AFB$_1$-albumin adducts

Aliquots of albumin from subject pre-dose and post-dose blood samples, in addition to albumin from a rat experiment, were digested as described. Rat albumin from a 1.2 μg/kg, 48 h dosing experiment was used for these studies as there was insufficient albumin remaining from the 6 h studies. The levels of adducts in the 1.2 μg/kg experiment were found to be 13.63±2.21 pg [^{14}C]/mg albumin.

The AMS results showed an absence of [^{14}C] in the blank run and the pre-dose sample, and co-retention of the [^{14}C] in the rat and human post-dose samples. The [^{14}C] co-retained with an AFB$_1$-lysine standard run under the same HPLC conditions. Fig. 3 shows the levels of [^{14}C] detected in the HPLC fractions of post-dose samples from the rat and human albumin digests respectively with an HPLC trace showing the co-retention of an AFB$_1$-lysine standard. Previous HPLC analyses of AFB$_1$-lysine have been performed using aqueous mobile phases containing buffer or ion pair reagents. These components were omitted from these analyses due to their interference with AMS sample preparation. The lack of buffer or ion pair reagent may account for the broad standard peak observed in Fig. 3.

3.7. HPLC analysis of hydrolysed [^{14}C]AFB$_1$-DNA adducts

DNA samples from normal and tumour human colon tissue and DNA from rat liver were used to establish that the [^{14}C] in the DNA was due to [^{14}C]AFB$_1$-DNA adducts. Initially an HPLC run of synthesized standards of FAPY1, FAPY2, AF-diol and AF-gua was performed, fractions collected and analysed by AMS. Each of the treated samples were run with a co-injection of the above standards. The retention times of the standards varied slightly from run to run but had approximate retention times of 8.8, 11, 14 and 19 min respectively.

The data from the HPLC fractions were analysed in a similar way to the human DNA samples. An average value of background [^{14}C] levels in the HPLC fractions was calculated from areas where no AFB$_1$ standards

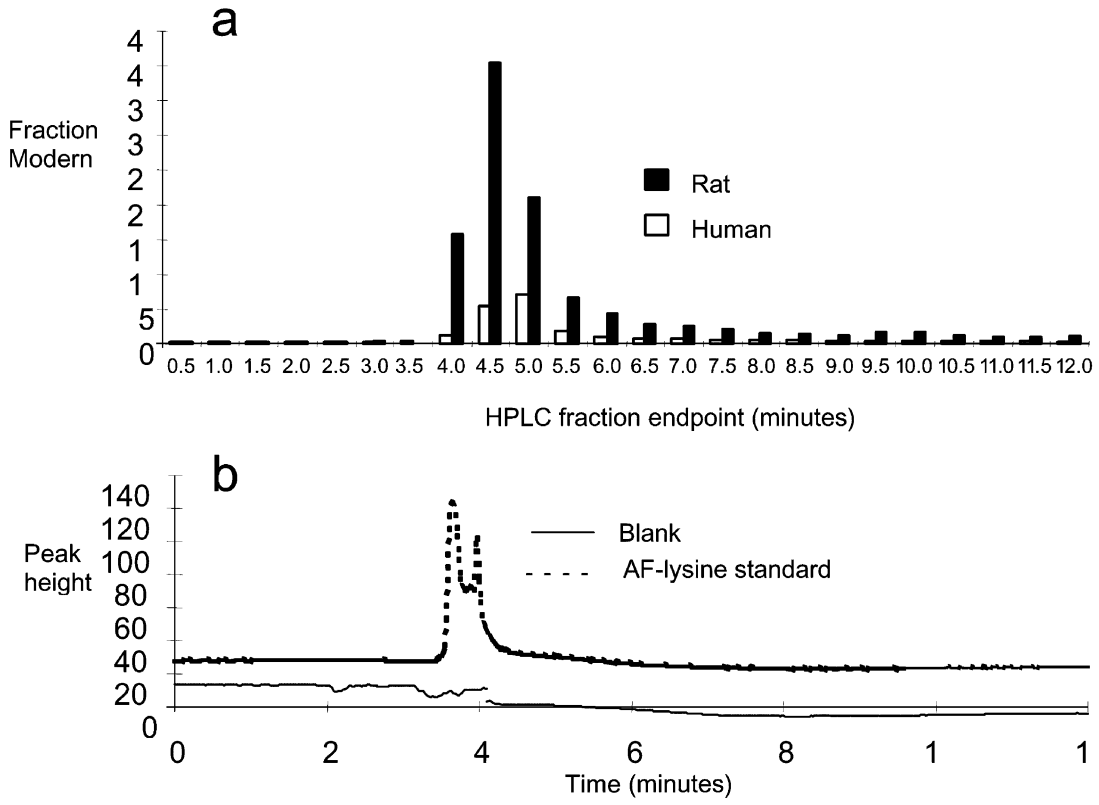

Fig. 3. HPLC analysis of digested rat and human albumin following administration of [^{14}C]AFB$_1$ showing (a) the [^{14}C] content in the HPLC fractions and (b) the HPLC trace using fluorescence detection of an AF-lysine standard. Data are expressed, as fraction modern, where modern is a unit referring to 13.56 milli-dpm, 97.8 amol or 6.11 fCi of ^{14}C per mg of carbon.

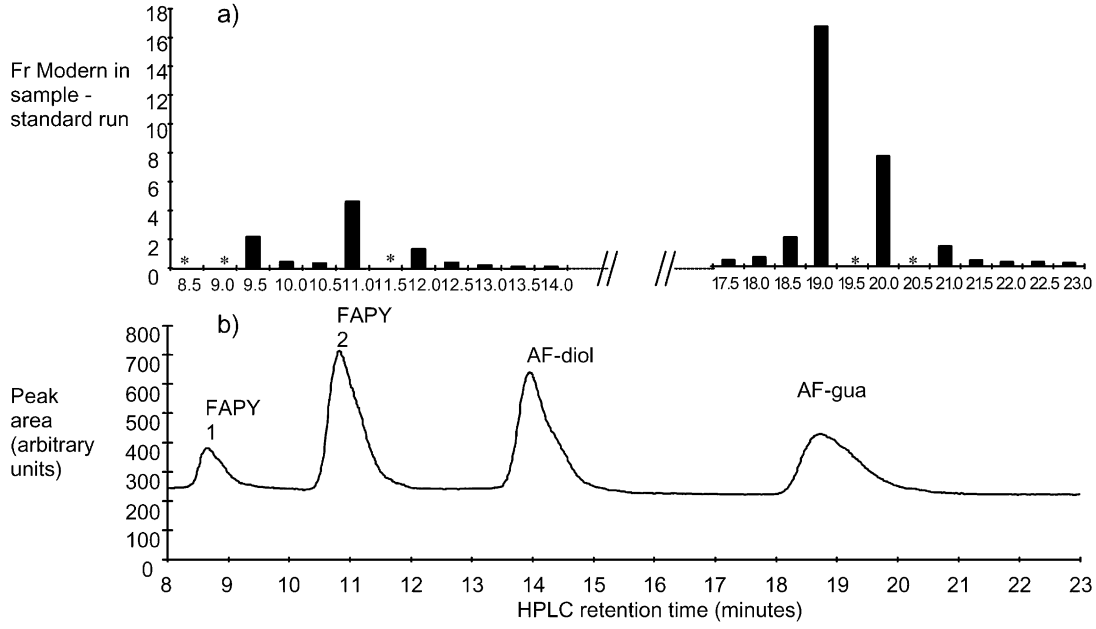

Fig. 4. HPLC analysis of hydrolysed rat liver DNA following administration of [^{14}C]AFB$_1$ (a) [^{14}C] content of HPLC fractions where * indicates the loss of the fraction during AMS sample preparation or measurement, (b) UV chromatogram at 360 nm of the co-injected AFB$_1$-DNA adduct standards.

eluted. This value was attributed as the "noise" of the assay. S:N ratios were then calculated for each fraction in every run. A S:N threshold of 2 was used to determine [^{14}C] levels above background. Analysis of the standard run showed that two of the AFB$_1$ standard fractions had S:N ratios approaching 2 (S:N 1.7 for 10.5-11 minute fraction and S:N 1.5 for 18–18.5 min fraction; corresponding to the elution of FAPY2 and AF-gua respectively).

HPLC fractions from the hydrolysed rat liver DNA from 150 ng/kg [^{14}C]AFB$_1$ experiment contained high levels of [^{14}C], where typical S:N ratios for the fractions co-eluting with AFB$_1$ standards were >15. Fig. 4 shows a histogram from the AMS analysis of HPLC fractions together with the UV chromatogram around the retention times of the co-injected standards. In order to give an indication of the true levels of [^{14}C]AFB$_1$ adducts in the rat, the AMS values from the standard run have been subtracted.

Human DNA from normal colon tissue from more than one patient was combined before DNA hydrolysis and isolation of the adducted bases. One HPLC analysis was performed on this sample, where standards of the AF-diol, FAPY1, FAPY2 and AF-gua were co-injected. Elevated levels of [^{14}C] were detected in the HPLC fractions corresponding to the retention times of FAPY1 (S:N = 3.52), FAPY2 (S:N = 2.49) and AF-gua (S:N = 2.44). Higher than background levels of [^{14}C] were also found in the 22–22.5 min fraction (S:N = 2.38). Due to insufficient DNA, it was not possible to perform this analysis more than once. HPLC analysis of human DNA from colon tumour tissue found S:N ratios in all the fractions to be <2.

4. Discussion

4.1. Analysis of [^{14}C]AFB$_1$ albumin and DNA adducts in the rat

This study reports the linear formation of AFB$_1$-albumin and AFB$_1$-DNA adducts over an 80,000-fold dose range of AFB$_1$ administered to male Fischer rats. The levels of adducts were determined by scintillation counting and AMS; AFB$_1$-albumin adducts were found in the range 6.83–42.29 pg [^{14}C]AFB$_1$/mg albumin/μg AFB$_1$/kg b.w. AFB$_1$-DNA adduct levels were adjusted for dose administered and b.w. by calculating carcinogen binding indices (CBI). CBI were suggested by Lutz (1986) to compare and classify types of carcinogen, where CBI = (mmol chemical bound/mol DNA nucleotide)/(mmol chemical administered/kg b.w.). The CBI for each tissue are given in Table 1 together with the linear regression coefficients for AFB$_1$-albumin and AFB$_1$-DNA adduct analyses. Comparison of the regression coefficients show the levels of DNA adducts were significantly different between all the tissues ($P<0.005$), except the lung and spleen which were not significantly different ($P=0.187$).

There have been a number of previous reports of the linear dose response relationships of hepatic AFB$_1$-DNA adduct formation (Wild et al., 1986; Buss et al.,

1990; Appleton et al., 1982; Phillips et al., 1999). Our studies in the rat show that there is a linear dose response relationship of AFB_1-DNA adducts from 6.2 ng/kg to 12.3 μg/kg in the kidney, lung, colon and spleen as well as the liver. These studies also confirmed the observations of Wild et al. (1986) that there is a linear dose-response relationship of AFB_1-albumin adducts. In addition, the data in the rat reported here suggests that there is no threshold dose for AFB_1-DNA adduct and AFB_1-albumin adduct formation at very low doses.

4.2. Human colon AFB_1-DNA adducts

Reliable AMS data for [^{14}C]AFB_1-DNA adduct formation in the human colon was obtained for two of the seven subjects (13.36 ± 2.52 and 17.64 ± 3.50 [^{14}C]AFB_1adducts/10^{12} nucleotides respectively) following the administration of 1 μg [^{14}C]AFB_1. At the conclusion of this programme a further three patients were dosed with [^{14}C]AFB_1. No increase in [^{14}C]-content was found above background levels. Due to the low levels of radiocarbon it is necessary to obtain a large number of measurements per sample to obtain reproducible data. This was not possible due to the amount of tissue available.

Characterisation of the [^{14}C]AFB_1-DNA adducts in the human colon was only possible by combining DNA samples from a number of subjects. There was only sufficient DNA to perform one HPLC analysis for normal and tumour tissue respectively. There was some evidence for formation of the ring opened AFB_1 adducts FAPY1 and FAPY2 and also of the non-ring opened AF-gua in normal colon tissue, although the levels of [^{14}C] were only slightly greater than the S:N threshold. [^{14}C] was also found to elute a few minutes after the AF-gua standard. Due to the low levels of [^{14}C] present in these samples it was not possible to speculate further on any possible significance of this. This data suggests that low levels of DNA adducts are formed and also that [^{14}C] was present in the colon tissue from which the DNA was extracted. Our data supports the formation of AFB_1-DNA adducts in the human colon as found in vitro by Autrup et al., (1979) and Kolars et al. (1994).

In comparison to the problems encountered with measurement of the human colon DNA adducts, levels of [^{14}C]AFB_1-DNA adducts in rat colon were accurately detected by AMS, where levels of 45.0 ± 11.3 adducts/10^{12} nucleotides were determined at 15 ng/kg (equivalent to the dose administered to humans). Thus the level of [^{14}C]AFB_1-DNA adducts formed in the rat colon appear to be higher than those formed in the human colon at the same dose. Comparison of the rat and human samples was made by adjusting the data by dose/b.w., inclusion of further interspecies scaling adjustments would improve the accuracy of this analysis.

4.3. Human AFB_1-albumin adducts

The mean level of AFB_1-albumin adducts detected in humans in this study was 38.0 ± 19.6 pg AFB_1/mg albumin/μg AFB_1/kg b.w., where the levels of AFB_1-albumin adducts found in the rat at a similar level of exposure (15 ng/kg b.w.) were comparable (42.3 ± 7.1 pg AFB_1/mg albumin/μg AFB_1/kg b.w). The levels of AFB_1-albumin adducts reported by Wild et al. (1996) were 0.51 pg AFB_1-lys equiv./mg alb/μg AFB_1/kg bw in the Fischer rat, where the levels in subjects from The Gambia were three times higher than this. Thus the levels of AFB_1-albumin adducts found in this study were much higher than the levels previously reported (Wild et al., 1996) this could be attributed to differences in the uptake, distribution and metabolism of AFB_1 and study design.

In this study data was obtained on the levels of [^{14}C]AFB_1-albumin adducts following exposure to a known amount (1 μg) of [^{14}C]AFB_1. This data was compared to previously reported studies where adduct levels were calculated based on a number of assumptions. Gan et al. (1988) extrapolated levels of AFB_1-albumin adducts found in humans to estimate levels of AFB_1 exposure, making assumptions on body weight, total albumin levels,% binding to albumin and degradation of AFB_1-albumin adduct due to sample processing. Values of 14–23 ng adduct/μg AFB_1 exposure were obtained (or expressed as% binding values of 1.4–2.3% of the dose). Applying the same assumptions and calculations on the AFB_1-albumin adduct levels, the levels of adducts detected/μg exposure in this study were 42.9–131.7 ng/μg AFB_1. Thus the levels of AFB_1 detected/μg exposure were higher in our study, where the exposure of AFB_1 was known. The discrepancies between the levels of human AFB_1-albumin adducts determined in this study and the levels obtained by Gan et al. (1988) and Wild et al. (1996) respectively may be due to the assumptions made in the calculations; and the general ill health, advanced age and the preparative procedures necessary of the participants of this study prior to colon surgery. Preparative procedures included abstention from food for 12 h prior to surgery; and the general anaesthetics, muscle relaxants and analgesics administered during surgery. These were in addition to other medication the subjects may have been receiving (see Table 2). Ethnic differences in the subjects studied may have also played a role in the differences in adduct formation as a consequence of differences in metabolic capability.

The application of protein adducts in molecular epidemiology is extensively discussed by Skipper and Tannenbaum (1990). They suggest that there are two reasons for looking at protein adducts; those of exposure and risk assessment respectively. The assessment of risk using protein adducts as surrogates for DNA

adducts needs to be investigated on a compound and target organ basis. In order to address this, DNA: protein adduct ratios have been calculated. Wild et al. (1986) found the ratio of hepatic DNA to albumin adduct formation for AFB_1 was constant over the dose range administered where ratios were found to be 3.7, 3.2 and 3.4 at acute doses of 10, 100 and 200 μg AFB_1/kg b.w. respectively. DNA:albumin ratios were calculated for all the organs in this study. The values range from 2.80 ± 0.48 at 12.3 μg/kg and 5.00 ± 1.66 at 2.1 ng/kg for the liver. The trend of increasing hepatic ratio with decreasing dose was investigated by regression analysis. Regression of the log DNA:albumin ratio vs. log dose found a statistically significant correlation ($P > 0.05$, $R^2 = 0.77$) over a 80,000-fold dose range.

To explore the relationship between the level of formation of DNA and albumin adducts further, the significance of the regression coefficients in the rat analyses was calculated. The hepatic AFB_1-DNA adduct and the AFB_1-albumin adduct level regressions were found to be significantly different ($P > 0.005$). It was not possible to perform similar comparisons on the human data due to the lack of quantitative AFB_1-DNA adduct levels in the human colon.

Thus from the results of this study it can be concluded that there is a linear relationship between known exposure to AFB_1 and AFB_1-albumin adduct formation in rats in a dose range of 0.16 ng/kg–12.3 μg/kg, and that the protein adduct levels in the rat are similar to humans. It has also been shown that AFB_1-albumin adduct formation is a good measure of AFB_1 exposure, as levels of adducts observed at known human exposures are similar to those from which the exposure data has been obtained by extrapolation. However, the level of formation of hepatic AFB_1-DNA and AFB_1-albumin adducts with dose was found to be significantly different in the rat suggesting that the formation of AFB_1-albumin adduct levels in humans may not reflect events in the target tissues. It is clear that in comparison with rats, humans form fewer DNA adducts in the colon per unit dose. Thus using the rat as a model for predicting human carcinogenesis may not always be ideal. The data are consistent with the literature where AFB_1 is classically regarded as a hepato-carcinogen, however, the identification of in vivo AFB_1 DNA adducts in the colon is a novel finding and supports previous in vitro studies (Autrup et al., 1979; Kolars et al., 1994). The results from the human study are at the limit of detection of the AMS technique.

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