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Monitoring of Individual Human Exposure to Aflatoxins (AF) and N-Nitrosamines (NNO) by Immunoassays

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Highly sensitive immunoassays have been used to quantitate aflatoxins (AF) and N-nitrosamines (NNO) in human body fluids and tissues, respectively. This approach was taken in order to quantitate environmental exposure to these agents at an individual level to facilitate the investigation of their role in the etiology of human cancer. In order to analyse AF in human urine, an immunopurification step has been developed by using AF-specific antibody bound to AH-Sepharose 4B gel in a small (4-ml gel volume) affinity column prior to enzyme-linked immunosorbent assay (ELISA). The ELISA can be used to quantitate aflatoxin B_1 (AFB_1) over the range 0.01 ng/ml to 10 ng/ml and the assay system has been validated by using human urine samples spiked with AFB_1 over this concentration range. In addition, 29 urine samples from the Philippines have been analysed and found to contain a range of levels from zero to 4.25 ng/ml AFB_1 equivalent with a mean of 0.875 ng/ml. This compared with a mean of 0.066 ng/ml AFB_1 equivalent in samples from France.

Radioimmunoassay of O^6-methyldeoxyguanosine (O^6-medG) has been performed on human oesophageal and cardiac stomach mucosal DNA from tissue samples obtained during surgery in Linxian County, People's Republic of China, an area of high risk for both oesophageal and stomach cancer. Using the methodology described and having 1 mg of hydrolyzed DNA allows the detection of approximately 25 fmol O^6medG per mg DNA. Of the 37 tissue samples analyzed from Linxian County, 17 samples had levels of O^6-medG ranging from 15 to 50 fmol/mg DNA, ten showed higher levels up to 160 fmol/mg DNA, and the

Abbreviations used: AF, aflatoxins; NNO N-Nitrosamines; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay; O^6-medG, O^6-methyldeoxyguanosine; O^6-AT, O^6-alkylguanine DNA alkyltransferase; PHC, primary hepatocellular carcinoma; HBV, hepatitis B virus; AFB_1-N7-G, 8,9-dihydro-8(N^7-guanyl)-9-hydroxy aflatoxin B_1; AFB_1-FAPY, 8,9-dihydro-8-(N^7-formyl-2′,5′,6′,-triamino-4′-oxo-N^5-pyrimidyl)-9-hydroxy aflatoxin B_1.

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remaining ten samples were below the limit of detection. For comparison, 12 tissue samples were obtained from hospitals in Europe and all showed levels below 45 fmol O^6-medG/mg DNA with seven below the limit of detection. All tissue samples from Linxian County showed normal levels of O^6-alkylguanine DNA alkyltransferase when compared to levels in other parts of the world.

The approaches described appear promising for assessing the role of AFB_1 in the etiology of human liver cancer and of nitrosamines as possible causative agents in oesophageal or stomach cancer.

Key words: N-nitrosamines, aflatoxins, ELISA, RIA, human environmental exposure monitoring

Epidemiology has been successful in identifying various agents or types of exposures as responsible for the induction of some human cancers. However, this has occurred mainly in situations where the exposure was very high or the type of cancer detected was of unusual occurrence in the general population. The low sensitivity of epidemiological studies is such that is is difficult to ascertain the etiology of human cancer when this is of multifactorial origin or the result of exposure to relatively low levels of carcinogens. In these situations, the determination of an association between the environmental occurrence of certain carcinogens and a given tumour in man appears more and more problematic. The possibility of quantitating a low-level environmental exposure to a particular carcinogen at an individual level appears to be a promising approach for establishing such an association. The considerations outlined above apply particularly to the role of aflatoxin B_1 in the etiology of liver cancer and of N-Nitrosamines (NNO) as causative agents of some human cancers.

With these two groups of carcinogens an increased understanding of their intracellular fate has helped to determine which are the most desirable end-points for measurement of individual human exposure. These may be measurement of the carcinogen itself, or a degradation product or measurement of a specific reaction product in either body fluids or tissues. The development of antibodies highly specific for carcinogens or carcinogen reaction products [1–5] and their application in extremely sensitive immunoassays have provided the necessary methodology for such measurements. In our laboratory, we have been concentrating on the application of immunoassays to quantitate at an individual level (1) aflatoxins (AF) in human body fluids and (2) the presence in human tissues of DNA adducts derived from exposure to NNO. We briefly report here the development of such methodologies and their application.

Aflatoxin, a potent hepatocarcinogen in several animal species [6], has been implicated epidemiologically along with hepatitis B viral (HBV) infection, as having a causal role in human primary hepatocellular carcinoma (PHC) [7–9]. In this paper we report data which established a method for measuring the presence of AF in human urine samples and include data from samples obtained from the Philippines, a country with a known presence of AF and associated high incidence of PHC [10]. In addition, we present results showing the detection of O^6-methyldeoxyguanosine (O^6-medG), a promutagenic alkylation adduct from NNO [11], in oesophageal and cardiac stomach DNA from individuals from Linxian County, People's Republic of China. This population has a high incidence of oesophageal and gastric cancer [12] and there is evidence of environmental exposure to NNO [13]. These two studies illustrate the adequate sensitivity and practical feasibility of such an approach for monitoring human environmental exposure.

DETERMINATION OF AFLATOXINS IN HUMAN URINE BY ELISA

The production and characterization of the the rabbit polyclonal antibody used in the immunoassay for AF have been previously reported in detail [14] and the ELISA method used is a modification of that described by Martin et al [15]. The standard inhibition curve for AFB_1 shown in Figure 1 is linear over the range 1.6–1,600 fmol (0.01–10 ng/ml) AFB_1 with a 50% inhibition value of 115 fmol (0.7 ng/ml). For comparison, the sensitivity of the ELISA for the human urinary metabolites AFM_1 [16], AFP_1 [J.D. Groopman personal communication], and the AF-DNA adducts also found in human urine [17] are shown. Although the assay requires 9-, 390-, 43-, and 23-fold more AFM_1, AFP_1, AFB_1-N7-G, and AFB_1-FAPY, respectively than AFB_1 to give 50% inhibition, the assay sensitivity for these compunds is among the highest available [5]. These data illustrate the broad specificity of this antibody, which also included recognition of AFB_1-conjugated compounds [14].

An affinity column was produced by binding the antibody to AH-Sepharose 4B gel (Pharmacia), thus allowing the extraction of AF from urine samples as a method of purification prior to ELISA. The ability of the affinity column to bind AFB_1 was measured by two methods by using [^{3}H]AFB_1 and unlabelled AFB_1. Initially 2 pmol [^{3}H]AFB_1 in 5 ml PBS was loaded onto the mini-column and bound AF eluted with 1 M acetic acid, pH 2.5. The radioactivity in fractions from the column was determined and analysis showed that 89% of radioactivity was bound prior to acid elution. HPLC analysis showed that unbound radioactivity in a breakthrough peak was due to presence of tritiated water. Similar levels of retention were obtained by using

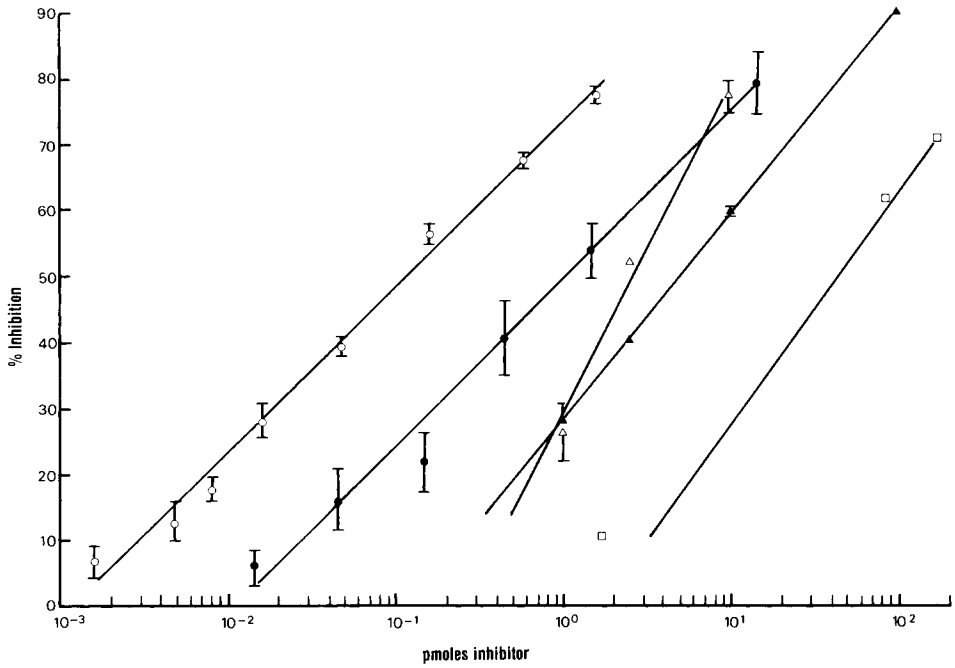

Fig. 1. Standard inhibition curve for (○) AFB_1 in ELISA including cross-reactivity of (●)AFM_1; (□)AFP_1; (△)AFB_1-FAPY; and (▲)AFB_1-N7-G in the assay.

[^{3}H]AFB_1 in human urine samples which were precipitated with ethanol prior to immunopurification.

In order to examine the reproducibility and validity of the assay system, a human urine sample was split into 5-ml aliquots and spiked with AFB_1 or AFM_1 (0.01–10 ng/ml). A good correlation (Fig. 2) was seen between the ELISA determination, following immunopurification on the affinity column, and the quantity of AFB_1 in the spiked sample. In contrast, preliminary data show that recovery of AFM_1 was 6% at 10 ng/ml and 29% at 1 ng/ml AFM_1.

In order to further determine the reproducibility of the assay system, a urine sample obtained from the Philippines, identified as positive for AF contamination, was split into nine fractions of 5 ml which were then processed and assayed individually. The mean value determined was 1.48 $\pm$ 0.27 ng/ml AFB_1 equivalent with a % inhibition range in the ELISA of 48–55.1% (samples diluted 2/5 in PBS for assay).

A preliminary study of 29 urine samples for the Philippines was performed to test the sensitivity of the assay in terms of AF levels present environmentally. Of the 29 samples analysed seven showed inhibition values $<30\%$ in the ELISA with an overall range from zero to 4.25 ng/ml AFB_1 equivalent (see Discussion) and a mean of 0.875 ng/ml. This compared with a mean of 0.066 ng/ml in nine urine samples collected in Lyon, France, with six of those nine giving inhibitions $<30\%$.

PRESENCE OF O^6-METHYLDEOXYGUANOSINE IN HUMAN TISSUE DNA

DNA (2–12 mg) was extracted from tissues obtained from Linxian County, People's Republic of China, and from European areas of low oesophageal cancer and the presence of O^6-medG determined by RIA [18]. The results [19] presented as fmol O^6-medG/mg DNA show (Fig. 3) the presence of the promutagenic lesion O^6-medG in DNA of oesophageal mucosa, cardiac stomach mucosa, and oesophageal tumour tissue from Linxian County. The majority of samples had O^6-medG levels lower than 50 fmol/mg but in ten samples (eight non-tumour oesophagus; two cardiac stomach) levels of 60–160 fmol of O^6-medG/mg DNA were quantitated. In comparison, tissues from Europe showed levels always less than 45 fmol/mg DNA with seven out of 13 below the limit of detection ($<$ 20% inhibition in RIA).

The relatively high levels of O^6-medG detected in the tissues from Linxian County were compared with the levels of O^6-alkylguanine DNA alkyltransferase (O^6AT) present in those tissues (Table I) determined in an in vitro repair assay [20]. The data show that there is no evidence of any deficiency in the population for O^6-AT activity when compared with oesophagus from other parts of the world, nor is there evidence that the repair activity in the oesophagus is lower than other tissues of the gastrointestinal tract.

DISCUSSION

As previously shown in preliminary work by other investigators [21,5], the two studies presented here show that the antibodies available to carcinogens and carcinogen adducts can be used in assays which attain sufficient sensitivity to quantitate environmental levels of target antigens in human tissues and body fluids. Several points, however, in relation to methodology and the choice of end-point to be measured arise from the data we have presented and warrant discussion.

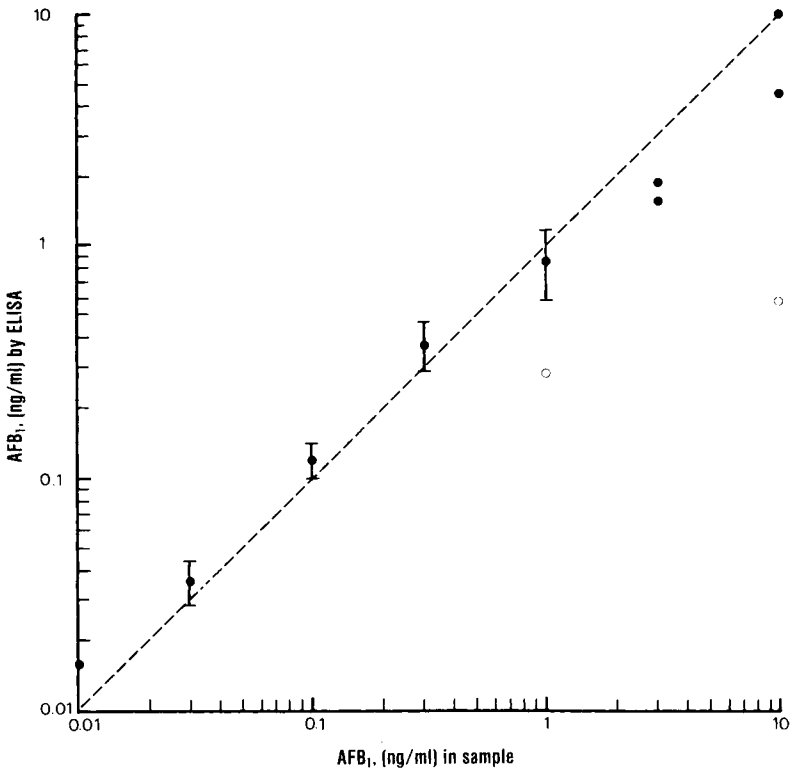

Fig. 2. Correlation study of known quantities of (●)AFB_1 or (○)AFM_1 spiked into human urine samples and measured by ELISA following immunopurification. Each point represents the mean ±SE of three or four individually processed samples except where data points are given as single points, in which case each point represents one determination.

Clearly for routine screening of exposure (eg, field studies) it is necessary to assay in a non-invasive way and to this end urine samples, and blood, are prime candidates. The initial problem we encountered in the AF study however is a common one, ie, nonspecific interference in the ELISA by urinary constituents. This has been overcome as described here, and similarly by Groopman et al [22], by the use of an immunopurification step with an antibody affinity column which specifically extracts AF-related components. Having thus removed nonspecific factors, however, one is still likely to be faced with a number of different AF products in the assay sample due to the complex metabolism and excretion of the carcinogen. For example AFB_1 [23], AFM_1 [16], AF-DNA adducts [17], and AFP_1 [J.D. Groopman, personal communication] have been identified in human urine whilst some AF water-soluble conjugates have been identified in primate [24] and may be present in man. Examination of the antibodies available to AF [5] shows that the production of an antibody with the extreme specificity obtained for O^6-alkylguanine adducts [18,25,26] has not been achieved. In addition, these antibodies have not been fully characterized in terms of their cross-reactivity with water-soluble AF conjugates which (1) may be present in urine samples and (2) may be recognized by many of the antibodies which are

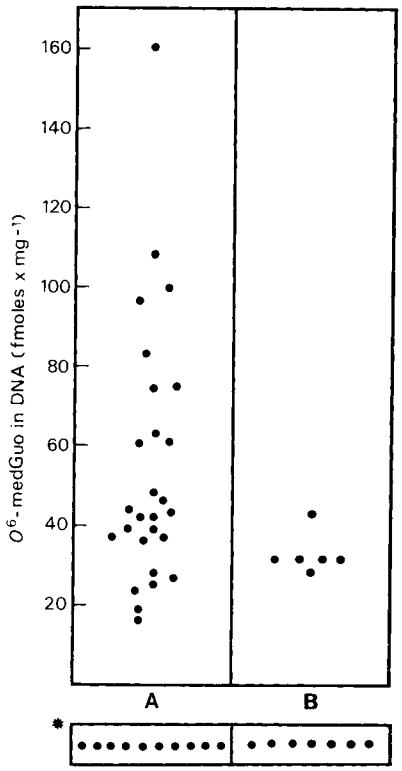

Fig. 3. O^6-methyldeoxyguanosine content of DNA in human oesophageal and cardiac stomach tissue samples, and in samples of oesophageal tumour tissue fron Linxian County, People's Republic of China (A), and Europe (B) measured by competitive radioimmunoassay. Inhibition values of less than 20% were considered negative and are listed in the boxes marked with an asterisk [from 19].

raised to AF conjugates. It follows therefore that the measurement made by immunoassay with the methods described above will be an integration of the absolute quantity of, and the antibody affinity for, each of the AF components present.

At the present time this screening approach permits the assessment of a population for a positive AF exposure, and presumably increases the sensitivity of analysis by measuring a number of AF components together, but care is needed in making population comparisons unless it can be demonstrated that the relative quantities of the various human urinary AF metabolites are constant. This becomes even more important when inter-study comparisons are made where different antibodies have been used and this may explain the large reported differences in AF levels in, for example, Gambia and the People's Republic of China [see 5].

We present the data on the Philippines urine samples as ng/ml AFB_1 equivalent due to the fact that the antibody recognises at least AFM_1 and AFB_1 (Fig. 2) and indeed initial HPLC fractionation of some of the Philippines urine samples, after the immunopurification step, suggests that a major proportion of the ELISA inhibition we measured was by unidentified water-soluble AF conjugates [Wild, unpublished data]. However, because of the lack of characterization of these products we are as

TABLE I. O^6-Methylguanine DNA Alkyltransferase Activity in Human Tissues

Tissue	No. of samples	fmol O^6-meG removed/mg protein		
		Mean	Range	Reference
Linxian County, PRC[b]				
Oesophagus-normal	8	190	87–246	
Oesophagus-tumour	5	326	122–447	[19]
Stomach	15	199	81–257	
Other studies				
Oesophagus	3	217	184–283	[34]
Stomach	5	200	145–250[a]	[35]
	14	730	360–1000	[36]
	7	460	180–1190	[37]
Small intestine	12	210	18–740[a]	[35]
Colon	10	261	135–413	[36]
	10	140	29–280[a]	[35]
	4	350	60–550	[37]
Rectum	3	360	220–470	[37]

[a]Data derived from Figure 2, reference 35.
[b]People's Republic of China.

yet unable to say if they are a major urinary AF component in absolute quantities. The identification of such products and detailed analysis of their cross-reactivity with our antibodies should allow the optimisation of an ELISA for their measurement and a concomitant increase in sensitivity.

In contrast to the somewhat incomplete picture of AF metabolism in vivo, the characterization of the metabolism and reactivity of NNO is much better understood [27]. We have detected the presence of O^6-medG in DNA from oesophageal tissue obtained through surgery from a population in Linxian County at high risk of oesophageal cancer. This observation is particularly significant in view of the pro-mutagenic nature of this lesion, which is considered as having an important role in the multistage process of carcinogenesis [11]. Because of this point, the approach described here represents a valuable integration of laboratory and epidemiological studies for the identification of causes of human cancer. The immunovisualisation of such adducts described recently [28] could eventually permit their determination at a cellular and macromolecular level.

In view of the efficient O^6-AT repair process for O^6-medG [29], which is present in normal quantities in these tissue samples (Table I), our measurement reflects an integrated response of reaction and repair of this lesion in the DNA at the target site. The O^6-medG lesion is present in the DNA despite a large excess of repair protein and it is important to emphasize that our data will be an underestimation of total exposure. The levels determined therefore probably reflect either a persistent fraction of a fairly recent exposure or the accumulation of adducts in regions of DNA which are repaired less efficiently. The latter may include DNA which is in a conformation resistant to repair [30] or DNA in a subpopulation of cells with an O^6-AT repair deficiency such as that seen in some human tumour cell lines in culture [31]. The latter deficiency would be masked by our examination of activity of total tissue protein extracts for O^6-AT activity.

In parallel to these studies, it may be desirable to quantitate a more stable alkylation adduct, where the dose-response relationship would not be complicated by

efficient adduct repair. Experimental studies in rats have shown [32] that another promutagenic lesion, O^4-ethylthymidine, although formed initially in quantities three- to fourfold less that O^6-ethyldeoxyguanosine, accumulates to levels 50-fold higher after 11-wk chronic administration of diethylnitrosamine. This lesion may therefore also be an appropriate end-point for exposure monitoring being both relatively persistent and implicated in tumorigenesis [33].

In conclusion, the methodologies described to assess human exposure to AF or NNO appear a very promising tool to be used in epidemiological studies aiming at the determination of the role of these carcinogens in the etiology of human cancer.

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