ABSTRACT

Aflatoxin (AF) albumin adducts are found in peripheral blood after exposure to aflatoxin B1 (AFB1) and the measurement of these adducts is potentially a useful tool in the epidemiological study of the role of AFB1 in the etiology of liver cancer. Three complementary approaches to the quantitation of AF-albumin adducts are described: (a) enzyme-linked immunosorbent assay (ELISA) performed directly on intact albumin (direct ELISA); (b) ELISA performed on an albumin hydrolysate (hydrolysis ELISA); (c) high-performance liquid chromatographic fluorescence detection of AF-lysine adduct after albumin hydrolysis and immunofinity purification. These techniques have been validated by direct comparison with rat albumin samples modified to a known extent. Identification limits of ~100, 5.0, and 5.0 pg AF/mg human albumin were determined for the three methods, respectively. Samples obtained from individuals from Thailand, The Gambia, Kenya, and France have been used to validate the measurement of AF-albumin adducts by these three methods. Levels of 7 to 338 pg AF/mg albumin were observed in the former two countries while no adducts were detected in samples from France. The relative properties of the three assays, with special regard to their application in epidemiological studies, are considered. A combination of the hydrolysis ELISA for large scale screening followed by confirmatory analyses in positive samples by high-performance liquid chromatographic fluorescence is suggested as an optimum methodology.

INTRODUCTION

The AFs are a group of potent hepatotoxic and hepatocarcinogenic secondary fungal metabolites which can be widespread in human foods (1). Exposure to aflatoxin has been associated with an increased incidence of PHC in several correlation studies in Kenya, Swaziland, Mozambique, Transkei, Thailand (see ref. 2), and The People's Republic of China (3). In addition, two case-control studies have been reported in The Philippines (4) and Hong Kong (5). The former study showed a higher AF exposure in PHC cases compared to controls, while the latter study, where exposure information was unreliable, revealed no such correlation. The method of AF exposure assessment in the correlation studies was by extrapolation from analysis of food sample contamination and in the case-control studies was by questionnaire. As a result, exposure measurement was relatively crude in all studies. In addition, this data provides no information on the biologically effective dose of AF at the individual level, i.e., the amount of the activated agent that has actually reacted with critical cellular targets, such as DNA, protein or RNA, but only on individual exposure, i.e., the concentration of a particular chemical to which the individual is subjected based on estimates in the food (see ref. 6). This former parameter may be influenced by intake, distribution, metabolic (in)activation and excretion of AF and becomes of particular importance when considering the interaction with other known risk factors for PHC, such as HBV, alcohol, and tobacco (2, 7). Thus, the biologically effective dose of AF for any individual would not be expected to reflect only food intake but also the host associated and environmental variants mentioned above. Such measurements have recently become feasible in the field of molecular and biochemical epidemiology due to developments of sensitive assay methodologies (7-11).

Among the desirable features of a laboratory assay for AF exposure would be that of giving a long-term exposure assessment in a sample obtained noninvasively. Several approaches have been used to measure AF metabolites and/or nucleic acid adducts in human urine, serum, and milk (see 12-14), but in these samples excretion is likely to be rapid and reflect relatively short-term (a few days) exposure. This prompted attempts to find a more stable marker of exposure. It has been demonstrated that AFB1 binds quantitatively in relation to dose to peripheral blood albumin in rats (15-18). Upon repeated exposure, accumulation of binding occurs and the level of albumin binding parallels the binding to liver DNA (16). The major albumin adduct in rats has been characterized as an AF-lysine residue (18). In addition, 1-3% of the administered AF dose was bound to circulating albumin after a single exposure in these studies. This contrasts with the lower level of binding to hemoglobin (19) and lymphocyte DNA (13) in rats. The half-life of albumin in humans is about 20 days and thus AF-albumin appears promising as a marker of AF exposure over a period of weeks/months.

Further support has come from a report of the immunological detection of AF in albumin hydrolysates from Chinese subjects (20). A good correlation was seen between AFB1 intake, calculated from food analysis, and the AF adducts in serum albumin. In this study, however, the methodology employed was labor intensive, involving several chromatography steps during sample preparation. We have assessed several alternative approaches for measuring AF-albumin adducts, including a simple, rapid immunoassay either for AF bound to intact albumin or for AF-amino acid residues following albumin hydrolysis. These methods have been validated, by comparison with standard in vivo, modified albumin samples of known AF adduct level, and applied to the measurement of a variety of human samples. The various approaches are critically assessed with regard to their potential application in epidemiological studies.

MATERIALS AND METHODS

Chemicals. Unlabeled AFB1 was from Sigma, St. Louis, MO, while [3H]-AFB1 (20 Ci/mmol) was from Moravek, Brea, CA, and was purified as described previously (16). [14C]-AFB1 (100 mCi/mmol) was kindly provided by Dr. G. E. Neal, MRC, Carshalton, UK.
Human serum used as a control source of albumin was purchased from Sigma, St. Louis, MO, as was human serum albumin (fraction V) and ovalbumin (crude powder). All ELISA reagents were as previously reported (21). Antiserum C was used in all studies (22).

Human Sera. Serum samples from The Gambia were collected from Keneba, West Kiang region, at the MRC clinic from individuals resident in Keneba village. These studies are in collaboration with the Gambia Hepatitis Intervention Study Group, IARC, Fajara, The Gambia. The samples from Thailand were provided by Dr. P. Srivatanakul, and were obtained from Bangkok, Nakornratchasima, and Ubonratchathani, while the samples from Chogoria and Kaloleni in Kenya were provided by Drs. G. W. Lachlan and D. Forman. The serum samples were from apparently healthy volunteers except a few samples from Kenya which were from patients attending hospital for nonliver related disorders. These three series of samples were used in this manuscript to validate the methodologies and form a subset, taken from on-going studies into the role of AF in the etiology of hepatocellular carcinoma, full details of which will be published elsewhere. The sera from France were obtained from volunteers or hospital patients involved in an unrelated study.

Treatment of Rats with [14C]-AFB. Male Wistar rats (220–240 g) were treated with [14C]-AFB, by gavage at doses of 3, 10, 30, 100, 300, and 800 to 1200 µg/kg body weight. Each rat received the same amount of radioactivity (0.13 µCi) but at specific activities of 60.6, 17.7, 6.06, 1.74, 0.62, or 0.18 mCi/mm or, respectively, depending on the dose, i.e., 60.6 mCi/mm for rats receiving 3 µg/kg, [14C]-AFB, was dissolved in a small volume of dichloromethane and then mixed with 0.5 ml olive oil immediately prior to treatment. Rats were housed individually in metabolic cages to allow collection of urine and feces for other studies. All animals were killed and bled 48 h after treatment as described previously (16). Albumin was extracted from aliquots of plasma as described below, with or without the 56°C heating step. Radioactivity was quantitated in aliquots of albumin in 0.5 ml 0.1 N HCl with 10 ml Picofluor scintillation fluid.

Albumin Isolation. Aliquots of plasma or serum (routinely 0.5 ml) were heated at 56°C for 45 min in a water bath to inactivate human immunodeficiency virus. Samples were then cooled on ice for 10 min and saturated ammonium sulphate (750 µl) was added slowly and the mixture vortexed. Precipitated immunoglobulins were removed by centrifugation at 9,000 × g for 15 min at 0°C. The supernatant was removed and 100 µl of 1 M acetic acid added, to adjust to pH 5, and saturated ammonium sulphate (60%) was added slowly and the precipitated albumin. This may be a consequence of a reduced cross-reactivity of partially denatured albumin with the antisera which was raised against AFB1-bovine serum albumin (22).

The yield of albumin from 500 µl of serum or plasma was of the order of 10 µg, representing about 50% of the theoretical yield. We used a high concentration of saturated ammonium sulphate (60%) which caused precipitation of some albumin with the immunoglobulins but resulted in a purer albumin fraction. The precipitation and centrifugation steps were repeated until the supernatant was clear. The precipitate was redissolved in 0.5 ml PBS (pH 7.4) and a 20-µl fraction diluted 1:50 in double distilled water for quantitation by Bradford protein assay. A yield of approximately 10 mg albumin was obtained from 500 µl of serum over a range of 3.3 to 330 pg AFB per 0.1 mg albumin per assay (25 µl). AF-lysine standard was prepared from N-acetyl-L-lysine as described previously (18).

The ELISA methodology has been described in detail before (21). The following modifications were made: (a) a preincubation of 100 µl 0.1% ovalbumin, 60 min at room temperature was used prior to adding antibody and samples or standards to the well and (b) the samples or standards followed by the antibody (25 µl/well) were added directly to ELISA wells and were not premixed. All samples were assayed in quadruplicate on at least two separate days and mean inhibition values <20% were considered as negative.

Albumin Hydrolysis Prior to ELISA. Albumin samples (2 mg) were digested with 0.67 µg proteinase K (Boehringer) in a total volume of 0.8 ml PBS (pH 7.4) for 15 h at 37°C. Following hydrolysis, 10 mg of BSA were added to improve precipitation, followed by 2 volumes of cold ethanol. Samples were kept at −20°C for 2 h, centrifuged 1500 × g for 15 min and the supernatant diluted to 6% ethanol in PBS. The samples were then loaded onto an activated Sep-pak C18 cartridge (Waters, Milford, MA), washed with 5 ml water, 5 ml 5% methanol, and the AF eluted with 5 ml 80% methanol. Eluates were dried, reconstituted in 500 µl PBS containing 1% fetal calf serum (to saturate residual protease K activity) and tested in ELISA using AF-lysine standards; 1 fmol (0.456 pg) to 30 fmol per 25 µl.

RESULTS

Albumin Preparation. A heating step was included in the protocol to inactivate the human immunodeficiency virus. In rats treated with [14C]-AFB1, albumin bound radioactivity was quantitated in samples from heated or unheated serum. The results in the two sets of samples were very similar, with no consistent trend in levels following heating, and a correlation coefficient of 0.88 (n = 13) between the two sets of samples. In addition, the heat treatment decreased the inhibition by unmodified albumin. This may be a consequence of a reduced cross-reactivity of partially denatured albumin with the antisera which was raised against AFB1-bovine serum albumin (22).

The yield of albumin from 500 µl of serum or plasma was of the order of 10 µg, representing about 50% of the theoretical yield. We used a high concentration of saturated ammonium sulphate (60%) which caused precipitation of some albumin with the immunoglobulins but resulted in a purer albumin fraction as judged by SDS-polyacrylamide gel electrophoresis (data not shown). Using scaled down-extraction volumes serum or plasma samples of 50 µl upwards have been successfully used with yields of 1 mg albumin per 50 µl of sample.

ELISA for AF-Albumin. The standard inhibitors used for the AF-albumin ELISA were prepared from rat albumin isolated from male BDIV rats treated with 3 mg/kg [14C]-AFB (0.75 µCi) and sacrificed 24 h after treatment. Albumin was isolated by precipitation as described above and modification level calculated as 78 ng AF/mg albumin from BDIV rats treated with 3 mg/kg [14Cl-AFB, (0.75 mCi/mm or), and purchased with promise (70,000 proteolytic units/g wt Calbiochem) using 1 mg enzyme per 3 mg albumin. Digestion was for 12 h at 37°C. Human albumin digests were centrifuged and the supernatants applied to an aflatoxin antibody affinity column (Aflatest-10; Vicam Corp., Medford, MA). This purification was necessary as the use of Sep-pak cartridge alone did not remove all interfering peaks of fluorescence and therefore limited assay sensitivity. After washing the column with 1 ml PBS the retained material was eluted with 1.5 ml methanol 0.1 M phosphate buffer, pH 7.4. The eluate was reduced to ~800 µl in a speed vac and 700 µl were injected onto HPLC. Rat albumin digests were analyzed by HPLC without antibody purification. Peaks chromatographing with AF-lysine were later located onto AF-affinity columns and rechromatographed to confirm the identity of the peaks.

Separation was achieved on a Lichrospher column 100 RP 18 (Merck) using a Kontron spectrofluorometer SFM 23 (excitation 405 nm, emission 470 nm) for detection. A linear 12-min gradient of 96% phosphate buffer (0.02 M, pH 7.2) to 60% methanol was used and the AF-lysine adduct eluted at 12.8 min under these conditions with a detection limit of 20 fmol. Chromatography of AF-lysine adduct from samples with authentic marker compound has been shown in more than one HPLC system (data not shown).
AFB<sub>1</sub>-ALBUMIN ADDUCTS

Fig. 1. Inhibition by unmodified albumin in ELISA. Each point represents an individual sample and in the case of human samples all data points at any one concentration were from separate subjects. Line of best fit is plotted by linear regression. Rat (●); human (○).

Fig. 2. Standard inhibition curves in ELISA for AF-lysine and AF-albumin. Standards prepared as described in “Materials and Methods” (●), AF-lysine; (○), AF-albumin.

min, extracted as described in “Materials and Methods”, was tested using samples from untreated rats and people resident in France, a country in which AF exposure is expected to be low. The data are shown in Fig. 1. Human albumin from a number of individuals gave similar results and a concentration of 4 mg/ml was chosen for future study based on the observation of inhibitions of 15% or less under these conditions. In practice, it was found important to prepare a human serum albumin control, with each batch of samples, from a serum purchased from Sigma. This reference albumin was adjusted to 4 mg/ml and used as the control in ELISA against which samples were then compared. Resulting albumins from human sera from France showed inhibitions of ±15% compared to the reference sample. Thus a limit of 20% inhibition was established as indicative of a positive sample.

Rat albumin consistently showed a greater inhibition than human albumin in ELISA (Fig. 1). As a result, samples were analyzed at 2 mg/ml.

ELISA for AF-Albumin and -Lysine. Standard curves for AF<sub>B1</sub>-albumin and -lysine are shown in Fig. 2. It is of interest that the recognition of the isolated AF-lysine adduct is some 30-fold greater than when the adduct is present in the intact albumin molecule. This increased sensitivity is reflected in the comparison of the two approaches to measure AF<sub>B1</sub> adducts in rat and human sera described below. The AF-lysine adduct is also approximately 6-fold more reactive with antiserum than is AF<sub>B1</sub> itself (~5 fmol AF-lysine versus ~30 fmol AF<sub>B1</sub> for 50% inhibition in ELISA). AF-albumin from rats treated with 3 mg AF<sub>B1</sub>/kg, at an initial modification level of 78 ng AF/mg albumin, was used to prepare standard curves in ELISA in order to have the same adducts in the albumin standard as found in in vivo samples. For example, in vitro AF<sub>B1</sub> modified albumin has a variety of adducts while predominantly AF-lysine appears to occur in vivo (18).

<sup>[14]C</sup>-AFB<sub>1</sub>-Albumin Adducts. A linear relationship was observed between the level of AF bound to albumin and the dose

Fig. 3. Dose response of aflatoxin-albumin adducts in [<sup>[14]C</sup>]AFB<sub>1</sub>-treated rats. Rats were given a single dose of AFB<sub>1</sub>, as described in “Materials and Methods.” Albumin was isolated from the rats killed 48 h after treatment and radioactivity bound per mg albumin was determined. All data points represent individual rats (three rats per dose).

Fig. 4. Aflatoxin-albumin adduct determinations by different methods. Albumin samples isolated from radioactively labeled AFB<sub>1</sub>-treated rats (Fig. 3) were either assayed directly in ELISA (○); by ELISA after proteinase K hydrolysis (●); or by HPLC fluorescence after pronase hydrolysis (●).

of [<sup>[14]C</sup>]AFB<sub>1</sub> administered (Fig. 3). In total, a 400-fold range of doses was used and this material served as a validation for the other assay methodologies (see below). At all doses examined, between 2.4 and 2.8% of the initial dose was bound to albumin at 48 h. This compares well with a previous study in the same strain of rats (16).

Measurement of AF-Rat Albumin by Direct ELISA, Hydrolysis ELISA, and Hydrolysis Fluorescence. Albumins from the experiment shown in Fig. 3 were assayed at concentrations of 2 mg/ml in ELISA and were quantitated by comparison with an in vivo modified AF-albumin standard as described in “Materials and Methods.” Data are plotted against the levels determined by <sup>[14]C</sup> radioactivity (Fig. 4). A dose-response relationship could be detected by the ELISA directly on albumin (direct ELISA) although (a) the recovery as compared to <sup>[14]C</sup> data was judged to be 64 ± 10% and (b) the albumins from doses below approximately 30 to 80 µg AFB<sub>1</sub>/kg were not detectable compared to albumins isolated from vehicle treated controls. The sensitivity is at least twofold better in human albumin because
of the lower cross-reactivity of unmodified human albumin, allowing 0.1 mg per assay to be analyzed, compared to 0.05 mg for the rat. The lowest level of modification measurable in rat albumin was around 300 pg AF per mg albumin by direct ELISA.

Following albumin hydrolysis with proteinase K, AF adducts could also be quantitated in a dose-dependent manner (hydrolysis ELISA) (Fig. 4). Using this approach even the albumin from rats treated with 3 µg AF/kg body weight was easily detectable (27–39% inhibition in ELISA). Recovery by this method was determined as 22.7 ± 2.2% by comparison with 14C data and measuring against AF-lysine as a standard in ELISA (see “Discussion”). Although AF-lysine is probably not the only AF containing molecule present after hydrolysis (e.g., AF-peptides may occur), it was observed that a dilution curve of a rat albumin sample in ELISA had a very similar slope to AF-lysine itself, suggesting similar antigenicity of all AF residues after hydrolysis.

Alternatively, AF-lysine adduct was measured by HPLC fluorescence following hydrolysis with pronase and data are also represented in Fig. 4. In this case, AF-lysine in samples from rats treated with 30 pg/kg could be detected and overall recovery compared to 14C data was 5.5 ± 1.3%. Quantitation of the samples from rats treated with 10 µg/kg gave a false-positive response when no antibody affinity purification was used.

Validation of Methods for AF-Albumin Measurement in Human Plasma and Serum. Following the good agreement of data in rat albumin samples between the various techniques we examined a number of human serum samples comparing (a) direct ELISA and (b) hydrolysis ELISA with HPLC fluorescence for AF-lysine. Quantitatively these data are difficult to compare, firstly because the ELISA will measure any AF residue, not just AF-lysine as in the HPLC assay, and secondly, the recoveries between the techniques vary (Table 1). These points are considered in the “Discussion”.

In comparing the direct ELISA with HPLC fluorescence (Table 2) there was a qualitative agreement with five of seven samples. In sample 127, ELISA was positive while no AF-lysine was detected and in sample 164 the reverse situation was observed. These sera were from individuals with no clinically diagnosed liver disease. However, in a further comparison we examined serum albumin from liver cancer patients in which very high inhibitions (up to 75%) were seen by direct ELISA. In these sera, no AF-lysine adducts were detected implying a consistent false-positive signal in direct ELISA. All the albumins isolated from these individuals had a yellow green coloration suggesting a high level of bound bilirubin which may inhibit in ELISA. Thus, the direct ELISA could not be used on sera from liver cancer patients.

In contrast to the above data an excellent correlation was observed (correlation coefficient = 0.97) in 15 albumin samples from individuals from Kenya analyzed by hydrolysis ELISA and HPLC fluorescence (Figs. 5 and 6). Interestingly, all samples below 20 pg AF-lysine/mg albumin by ELISA were negative by HPLC fluorescence. Two samples positive by hydrolysis-ELISA did not contain detectable levels of AF-lysine.

The hydrolysis-ELISA and direct ELISA have been applied to a range of human samples from The Gambia, Thailand, and France and the data using hydrolysis-ELISA are shown (for comparison) in Fig. 7. The prevalence of positive samples has been greater in The Gambia than Thailand and the levels have tended to be higher as well with 13 of 19 Gambian sera showing levels >10 pg AF-lysine per mg albumin and only two of 38 samples from Thailand showing this level. No samples from France have given a positive signal in ELISA.

**DISCUSSION**

When considering the measurement of AF exposure in retrospective and prospective epidemiological studies into the role of AF in the etiology of PHC, AF-albumin adduct analysis appears to be a most promising approach. A number of methods to measure these adducts are considered in this paper. Several properties are required to make a laboratory assay suitable for integration into epidemiological studies, primarily sensitivity (low false negatives), specificity (low false positives), reproducibility, long-term exposure information, quantitative measure of target organ damage, noninvasive sampling, large sample capacity, inexpensive, easy to perform. It is important to discuss the data we present within this context.

Experimental data in rats demonstrate a dose-related increase in binding of AFB, to peripheral blood albumin and/or liver DNA even at very low doses (16, 18, 23) and a fairly constant relationship between the two parameters after single or multiple doses (16). This reflection of target organ DNA damage, while of potential value, must be further examined in experimental conditions which reflect the situation in humans, for example, where liver damage is being induced by HBV, alcohol, or nutritional deficiency. In fact, for example, a recent report (24) in rats suggests that the two parameters may be affected differently by ethanol. Interestingly, data from Groopman et al. (25) have shown a strong correlation, in Chinese subjects, of urinary excretion of AF-7-guanine (DNA/RNA) adduct and AF-albumin adducts in peripheral blood.

The sensitivity of the methods for human albumin analysis are summarized in Table 1 and in the case of HPLC fluorescence depends not only on the absolute sensitivity of the method but also the amount of sample available. The detection limit refers to the actual level measured and must be considered together with the percentage recovery in order to obtain the “real” level required to be present in a sample for detection to be possible, e.g., for fluorescence 5 pg AF-lysine/mg albumin is quantitated but based on a 5.5% recovery (Table 1) then, 90.9 pg/mg would be the level in the original sample. Hydrolysis of the albumin, followed by ELISA is clearly more sensitive than ELISA directly on albumin. Using human albumin rather than rat, the direct ELISA can detect ~100 pg AF per mg albumin due to the lower cross-reactivity with unmodified human serum albumin compared to rat (Fig. 1). This higher inhibition is likely to be due to the cross-reactivity of the intact rat albumin

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**Table 1 Summary of different approaches to measuring AF-albumin in human serum or plasma**

<table>
<thead>
<tr>
<th>Method</th>
<th>Absolute detection limit (pg AF lysine)</th>
<th>Quantity albumin used (mg)</th>
<th>Detection limit (pg AF-lysine mg albumin)</th>
<th>% recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct ELISA</td>
<td>NA</td>
<td>0.1</td>
<td>~100</td>
<td>64 ± 10</td>
</tr>
<tr>
<td>Hydrolysis ELISA</td>
<td>0.5</td>
<td>1.0–2.0*</td>
<td>5</td>
<td>23 ± 2</td>
</tr>
<tr>
<td>Hydrolysis fluorescence</td>
<td>10</td>
<td>2.0 (20)</td>
<td>5 (0.5)</td>
<td>5.5 ± 1.3</td>
</tr>
</tbody>
</table>

*As compared to [14C]-AFB, data.

* 0.1 mg analyzed per ELISA well after hydrolysis of 1.0–2.0 mg.

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Table 2. Analysis of AF-albumin adducts in human sera by direct ELISA and hydrolysis/HPLC fluorescence

<table>
<thead>
<tr>
<th>Sample</th>
<th>ELISA (pg AF/mg albumin)</th>
<th>HPLC fluorescence (pg AF-lysine/mg albumin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0 (-)</td>
<td>0 (-)</td>
</tr>
<tr>
<td>126</td>
<td>175 (+)</td>
<td>7.7 (+)</td>
</tr>
<tr>
<td>127</td>
<td>541 (+)</td>
<td>0 (-)</td>
</tr>
<tr>
<td>143</td>
<td>0 (-)</td>
<td>0 (-)</td>
</tr>
<tr>
<td>159</td>
<td>175 (+)</td>
<td>5.7 (+)</td>
</tr>
<tr>
<td>164</td>
<td>0 (-)</td>
<td>9.0 (+)</td>
</tr>
<tr>
<td>175</td>
<td>670 (+)</td>
<td>17.5 (+)</td>
</tr>
</tbody>
</table>

* Parentheses, qualitative data.

Fig. 5. Correlation between AF-albumin adducts in human sera analyzed by hydrolysis ELISA and HPLC fluorescence. Albumin samples (2 mg) were analyzed by both methods as described in “Materials and Methods.” Each point represents a sample from a single individual from Kenya (see “Materials and Methods” for details). For hydrolysis ELISA quantitation was made against AF-lysine standard curve (Fig. 2).

Fig. 6. HPLC fluorescence analysis of a human serum sample. A 2-mg albumin sample obtained from a Kenyan individual (see “Materials and Methods” for details) was hydrolyzed and purified by antibody affinity chromatography. The sample was analyzed by reversed-phase chromatography (see “Materials and Methods”). The AF-lysine peak (12.8 min) was quantitated as 40 fmol by comparison of integration of peak area with authentic standard. This sample showed a level of adduct twofold higher than the defined detection limit of 20 fmol under these conditions.

and while at low levels, e.g., 2 mg, it is less sensitive than hydrolysis ELISA, it is equally sensitive if 20 mg of sample are used (Table 1). In addition, it has the advantage of being highly specific.

A direct comparison of the Thailand and The Gambia data (Fig. 7) with those of Gan et al. (20) is difficult because of the different antibodies being used to quantitate and the fact that we used an AF-lysine standard for ELISA, whereas Gan et al. (20) used an AFB1 standard for radioimmunoassay. Nevertheless, the results for positive samples are of exactly the same order of magnitude (10-350 pg AF/mg albumin) in the two studies and the recovery as judged using [14C]-AFB1-treated rats was also similar in both studies (approximately 25%). A more direct comparison by HPLC fluorescence of AF-lysine has shown a similar level of adduct in the Kenyan and Gambian samples (Table 2 and Fig. 5) and some of the Chinese samples reported previously by Gan et al. (20).

In terms of specificity, the direct ELISA is potentially the most susceptible to nonspecific inhibition because the specificity of the assay relies completely on the antibody. As the antibody was produced against AFB1-BSA, the possible recognition of other albumin modifications cannot be discounted. Around the limit of detection for the direct ELISA for the rat samples (Fig. 4) a greater variability in results was observed and at least in one human sample (Table 2) the presence of AF-lysine, as determined by HPLC fluorescence, was not detected by the direct ELISA. At 0.1 mg albumin per ELISA well we have not seen positive sera from healthy individuals in France, a population of low AF exposure, although one sample from The Gambia (no. 127) was significantly positive in ELISA but not by HPLC fluorescence after hydrolysis. The use of ELISA following albumin hydrolysis is a significant improvement in this respect because the purification on Sep-pak C18 cartridge

G. Sabbioni, G. N. Wogan, and J. D. Groopman, unpublished data.
removed cross-reacting material while the hydrolysis also eliminates any cross-reactivity with intact albumin itself. The quantitation by fluorescence is still more specific in comprising of an HPLC step and demanding particular fluorescent properties. In practice, it is possible to combine the methods using hydrolysis ELISA an an initial screen followed by hydrolysis/HPLC fluorescence to confirm positive samples, at least for AF-lysine. An excellent correlation was seen by these latter two methods (Fig. 5) and the observation of the AF-lysine adduct in human sera (Figs. 5 and 6) is the first report of the presence of a specific AF-albumin adduct in human samples, although the unequivocal identification of the adduct would require further evidence by, for example, mass spectrometry using perhaps sera pooled from several individuals to provide sufficient sample.

The precise conditions of albumin hydrolysis will be an important consideration in the reproducibility and interlaboratory comparisons of the two methods employing this step. In rat albumin (Table 1) the hydrolysis ELISA showed 23 ± 2% and the hydrolysis/HPLC fluorescence 5.5 ± 1.3% recovery of total albumin bound AF. However, this is partially explained by the fact that the fluorescence method is measuring only the AF-lysine adduct and not other hydrolysis products, the latter of which, in contrast, would be measured by the antibody approach. Adducts other than the AF-lysine produced by hydrolysis, while still recognized by the antibody, may however give less inhibition in ELISA than this major adduct. Interestingly, an albumin hydrolysate from a rat treated with 30 μg AFB1/kg, when diluted serially for ELISA, had an almost identical slope to AF-lysine itself, suggesting that any adducts present (such as AF in short peptides) react with the antibody to a similar extent as AF-lysine. Recent experiments showed that AF-lysine is stable during hydrolysis, if the high activity pronase of Calbiochem (110,000 proteolytic units/g, which is no longer commercially available) is avoided. Albumin of rats was hydrolyzed with different ratios (1/1, 3/1, 10/1) of albumin/pronase (Calbiochem, 70,000 proteolytic units/g) and for different periods (2, 5, 8, 12, 24, and 48 h). It has been found that the pronase/albumin ratio of 3/1 gave the largest yield of AF-lysine after a 12-h digestion although hydrolysis up to 24 h did not significantly affect the yield of AF-lysine. It was noticed that by purifying the serum albumin by affinity chromatography with cibacron blue (20) there is a major loss of AF-lysine. The decay product has a 3 min shorter retention time on HPLC than AF-lysine (see Fig. 6) and is also recognized by the AF-lysine antibody. Therefore, it is very important to keep the same protocol during the purification in different studies, otherwise the HPLC fluorescence data might not be directly comparable.

It should also be stressed that in humans exposure will also involve at least AFG1 and AFM1, which are present along with AFB1 in food crops (AFG1) and milk (AFM1) and can form covalent adducts with DNA (1) and therefore presumably albumin. The ELISA approaches, using an antibody which recognizes these AFs (21, 22), would measure such adducts, whereas the more specific HPLC fluorescence technique has only been applied to AF-lysine. This could partially account for the fact that the ELISA, for example, detected higher levels of adduct compared to fluorescence and that some samples positive by ELISA were negative by fluorescence (Fig. 5).

Serum or plasma in quantities of 50–100 μl can be used to obtain sufficient albumin (1–2 mg) to perform each of the assays described here. However, if two assays were to be combined, then 200 μl would be required. This amount could still be obtained from a fingerprick blood sample. This would permit the AF-albumin component of a study to be performed on a sample also used, for example, for HBV status determination.

The ease of performance, large sample capacity, and cost are components which are met by the ELISA of AF hydrolyzed albumin. For example, no chromatography is involved, isolation of albumin by precipitation is acceptable (see above) and the only specialized equipment required is a centrifuge, Seppak C18 cartridges and an ELISA plate-reader. This compares with the relatively poorer sensitivity and specificity of the direct ELISA. The HPLC step for fluorescence makes the routine analyses of several hundred samples more difficult but is a powerful approach to confirming the positive samples identified by the hydrolysis ELISA. In situations such as in Thailand (Fig. 7), where only 13% of the samples were positive, this would be a preferred combination of methods.

Based on the half-life of albumin of about 20 days, and assuming that, as in the rat, aflatoxin binding does not alter the half-life of albumin (16, 18), the measurement of AF adducts would give a measure of exposure for the past weeks/months with an accumulation of adducts to a level 30-fold higher than that induced by a single exposure (18). In respect to epidemiological studies this probably is insufficient duration to be of value in case-control studies but would be appropriate for correlation and cohort studies, especially if repeated blood samples were obtained. In addition this methodology could be useful in rapidly identifying the occurrence and extent of human diseases attributable to the intake of mycotoxin(s)-contaminated food and in providing critical data for the elucidation of the etiology of these diseases. Other approaches to monitoring AF, using metabolites or nucleic acid adducts in urine, have been fully discussed elsewhere (12–14). The major limitation of this latter approach is the fact that the measurement probably reflects exposure over the previous 24–48 h. However, (a) the excreted adduct, AF-7-guanine, should give direct information on genetic damage occurring in the liver (25) and (b) the ability to examine the metabolite profile in the urine may give information regarding an individual’s metabolism of AF which would be of additional value in studies of the interaction of AF with other environmental factors (e.g., HBV, liver fluke) in the etiology of liver cancer.

ACKNOWLEDGMENTS

The authors thank Elspeth Perez and Pascale Collard-Bianchi for typing the manuscript.

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