Development of Aflatoxin B₁-Lysine Adduct Monoclonal Antibody for Human Exposure Studies

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Mouse monoclonal antibodies were developed against a synthetic aflatoxin B₁ (AFB)-lysine-cationized bovine serum albumin conjugate. The isotype of one of these antibodies, IIA4B3, has been classified as immunoglobulin G1(λ). The affinity and specificity of IIA4B3 were further characterized by a competitive radioimmunoassay. The affinities of IIA4B3 for AFB and its associated adducts and metabolites are ranked as follows: AFB-lysine > 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxy-AFB > AFB = 8,9-dihydro-8-(N⁷-guanyl)-9-hydroxy-AFB > aflatoxin M₁ > aflatoxin Q₁. IIA4B3 had about a 10-fold higher affinity for binding to AFB-lysine adduct than to AFB when ³H-AFB-lysine was used as the tracer. The concentration for 50% inhibition for AFB-lysine was 0.610 pmol; that for AFB was 6.85 pmol. IIA4B3 had affinities at least sevenfold and twofold higher than those of 2B11, a previously developed antibody against parent AFB, for the major aflatoxin-DNA adducts 8,9-dihydro-8-(N⁷-guanyl)-9-hydroxy-AFB and 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxy-AFB, respectively. An analytical method based on a competitive radioimmunoassay with IIA4B3 and ³H-AFB-lysine was validated with a limit of detection of 10 fmol of AFB-lysine adduct. The method has been applied to the measurement of AFB-albumin adduct levels in human serum samples collected from the residents of areas at high risk for liver cancer.

Aflatoxins (AF), mainly produced by *Aspergillus flavus* and *A. parasiticus*, are a group of naturally occurring fungal metabolites that have long been recognized as significant environmental contaminants (17, 28). Aflatoxin B₁ (AFB), the most common mycotoxin found in human food and animal feed, is a potent hepatotoxic and genotoxic agent which has been listed as a known human carcinogen (group I) (6, 17, 28, 29). Exposure to dietary AFB is one of the major risk factors in the etiology of human hepatocellular carcinoma in several regions of Africa and Southeast Asia (6, 15, 17, 28, 29, 34). The development and application of highly sensitive and specific methods for detecting AFB and its associated metabolites and macromolecular adducts are critical for identifying individuals at high risk (13).

The molecular biomarkers currently used in human and animal exposure studies are AFB metabolites and AFB macromolecular adducts, such as aflatoxin M₁ (AFM₁) and AFB-N⁷-guanine (AFB-N⁷-Gua), in urine and AFB-albumin adducts in serum (12, 13, 21, 22, 30). The use of AFB-albumin adducts as biomarkers is important because their estimated longer in vivo half-life compared to that of urinary metabolites may reflect integrated exposures over longer time periods (13, 27). From a practical perspective pertinent to epidemiological studies, the measurement of serum AFB-albumin adduct levels offers a rapid, facile approach that can be used to screen very large numbers of people. Data from human exposure studies have also demonstrated that the excretion of urinary AFB-N⁷-

Gua and the formation of AFB-albumin adducts are highly correlated (13).

Three major analytical techniques are currently available for measuring AFB-albumin adduct levels in human blood: enzyme-linked immunosorbent assay (ELISA) (5, 33, 35), radioimmunoassay (RIA) (8, 26, 30), and immunoaffinity chromatography followed by high-performance liquid chromatography (HPLC) with fluorescence detection (24, 30, 33). All of these methods are antibody-based assays; therefore, the results of each assay are influenced by the specificity and the sensitivity of the antibodies used. Since the major AFB-albumin adduct had been identified as the AFB-lysine adduct (23, 25), the development of more specific monoclonal antibodies recognizing this adduct was initiated. In the study reported here, we have produced and characterized a new mouse monoclonal antibody (IIA4B3), developed using a synthetic AFB-lysine-cationized bovine serum albumin (cBSA) conjugate, and have compared its affinity and specificity for AFB and its metabolites and adducts to those of a previously developed monoclonal antibody (2B11) (11). This new antibody has the requisite specificity for use in measuring AFB-lysine adduct levels in human serum samples.

MATERIALS AND METHODS

Materials. ³H-AFB (28 Ci/mmol) was purchased from Moravek (Brea, Calif.), purified by using a Sep-pak C₁₈ cartridge (Waters Corp., Milford, Mass.), and then stored in 100% ethanol at −20°C. Radiolabeled AFB was assessed to be greater than 98% pure by HPLC. Unlabeled AFB, AFM₁, aflatoxin Q₁ (AFQ₁), albumin determination reagent (bromocresol purple), human albumin standards, normal human serum, bovine serum albumin (BSA; fraction V), and horse serum were obtained from Sigma Chemical Co. (St Louis, Mo.). N-α-Acetyl-L-lysine was purchased from Aldrich Chemical Co. (Milwaukee, Wis.). The protein assay dye reagent concentrate and protein standard were purchased from Bio-Rad Laboratories Inc. (Hercules, Calif.). Pronase (70,000 proteolytic units/g of dry

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weight) was obtained from Calbiochem (La Jolla, Calif.). The immunoglobulin M (IgM) monoclonal antibody (2B11) was produced as described previously (11). All other materials and chemicals were commercially available.

Synthesis of AFB-lysine and AFB-DNA adducts. AFB-lysine and ^3H -AFB-lysine were synthesized from the reaction of 8,9-dihydro-8,9-dibromo-AFB or 8,9-dihydro-8,9-dibromo- ^3H -AFB with *N*- α -acetyl-L-lysine and purified by HPLC as described previously (25). AFB-*N*⁷-Gua and 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimidin-5-yl formamido)-9-hydroxy-AFB (AFB-FAPyr) were synthesized by reaction of calf thymus DNA with chemically synthesized AFB-8,9-epoxide, which was prepared by the reaction of AFB with dimethyldioxirane as described by Baertschi et al. (2). The oxidant dimethyldioxirane was synthesized as described by Murray and Jeyaraman (20) and Adam et al. (1). AFB-8,9-epoxide-modified DNA was ethanol precipitated, washed, dried, and resuspended in water. AFB-*N*⁷-Gua was made through hydrolysis of the modified DNA with 0.1 N HCl at 95°C for 15 min and purified by HPLC. AFB-FAPyr was made by incubation of the modified DNA with 0.1 N NaOH at 37°C for 30 min and purified by HPLC as described previously (10).

Preparation of antigen and immunization. AFB-lysine was coupled to cBSA using the Inject SuperCarrier EDC system from Pierce (Rockford, Ill.) and purified by gel filtration. The system utilizes 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)-mediated amide formation as the conjugation reaction, which allows haptens containing carboxyl and amide groups to easily and effectively be coupled to cBSA (3). Ten 6- to 8-week-old female BALB/c mice were administered two intraperitoneal injections of 50 μg of AFB-lysine-cBSA conjugate emulsified with 25 μl of Hunter's TiterMax R-1 adjuvant (CytRx, Norcross, Ga.) at 2-week intervals. Mice were screened by an ELISA (see below) for specific serum antibody induction, and the mice with the best specificity were given an intravenous boost (16 μg) of conjugate 4 days before spleens were removed.

Hybridization. Spleen cells from immunized mice were fused with the nonsecreting murine myeloma cell line Sp2/0 (ATCC CRL 1581) as described by Calteff and Milstein (7) with some modifications. Briefly, about 10^6 Sp2/0 cells and 10^8 splenocytes were mixed in the presence of 50% polyethylene glycol (PEG) 1450 (Sigma P7181) in Hybri-Max DMEM/F-12 (Gibco 11330-024). The cells were then suspended in complete medium containing 20% fetal bovine serum (Gemini 100-107), 1% Pen/Strep (Sigma 0781), and 1% conditioned medium from the Sp2/0 culture. The cell suspension was equally distributed into two 24-well plates (Falcon 3524) and incubated in 5% CO_2 at 37°C. Twenty-four hours later, hypoxanthine-aminopterin-thymidine selection for hybrids was initiated and continued for the next 12 days. Wells were screened by an ELISA for specific antibody, and contents of positive wells were subcultured and cloned by limiting dilution (14).

Screening ELISA. A direct ELISA (14) was used to select specific antibody-producing clones. Immulon 2 ELISA plates (Dynatech, Arlington, Va.) coated with 5 ng of AFB-lysine-cBSA conjugate or cBSA alone per well were blocked with 30 mM phosphate-buffered saline (PBS) containing 1% normal horse serum. Quantitation of binding of specific antibody from culture supernatants was performed through reactions with peroxidase-labeled goat anti-mouse IgG (heavy and light chains; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and IgM (μ chain; Sigma) and with *o*-phenylenediamine (Sigma) as the substrate. The absorbance at 490 nm was measured with a UV max kinetic microplate reader (Molecular Devices, Menlo Park, Calif.).

Isolation and isotype classification of monoclonal antibodies. Selected stable hybridoma clones producing antibody specific for AFB-lysine-cBSA were grown as ascites tumor cells in BALB/c mice which had previously been injected with 0.5 ml of pristane (Aldrich). Ascitic fluid collected from each animal was titrated and further characterized. Isotype classification was performed with an isotyping kit (ISOSTrip; Boehringer) according to the protocol provided by the manufacturer.

Competitive RIA. The affinity of AFB and its metabolites and adducts for monoclonal antibodies IIA4B3 and 2B11 was determined by an RIA as detailed previously (11). Briefly, different concentrations of AFB and derivatives dissolved in 100 μl of phosphate buffer (pH 7.0) were mixed with 100 μl of diluted monoclonal antibody in 10% horse serum-PBS and 100 μl of tracer (^3H -AFB or ^3H -AFB-lysine; 10,000 cpm; approximately 0.4 to 0.5 pmol)–1% normal mouse serum–0.1% BSA in PBS. After 2 h of incubation at ambient temperature, an equal volume of ice-cold saturated ammonium sulfate was added. The samples were mixed, incubated for 15 min, and then centrifuged for 15 min at 9,000 $\times$ g and 4°C. Counts in the supernatant (300 μl) were determined with an LKB 1211 RACKBETA liquid scintillation counter (LKB Instruments, Inc., Gaithersburg, Md.), and the affinity constants were determined by the method of Muller (19).

Measurement of AFB-lysine adduct in human serum samples. Human serum samples were selected from the human serum repository previously established in the Johns Hopkins University School of Hygiene and Public Health, which

stores samples collected from several regions of high liver cancer incidence around the world. The method used for adduct determination was a modification of the previously described antibody 2B11-based method (16). The major modifications were the use of the newly characterized antibody IIA4B3 and synthetic ^3H -AFB-lysine to replace 2B11 and ^3H -AFB in the assay, respectively. The sensitivity and recovery of this modified method were evaluated with normal human serum spiked with graded levels of the synthetic AFB-lysine adduct. Briefly, human serum albumin was concentrated through a Microcon-50 microconcentrator (Amicon, Inc., Beverly, Mass.). The concentrations of albumin and total protein were determined by the bromocresol purple dye binding method (16) and the method of Bradford (4), respectively. Total serum proteins were digested with pronase for 16 to 18 h at 37°C; the digests were extracted with acetone; and the supernatant containing the AFB-lysine adduct was decanted, dried *in vacuo*, and redissolved in PBS for the RIA as described above.

The standard curves for AFB or AFB-lysine adduct in the RIA were determined using a nonlinear regression model described by Gange et al. (9). Non-specific inhibition in the assay was determined by processing of pooled normal human serum standards obtained from Sigma. The average value of the background was subtracted from those of test samples for calculating AFB-lysine adduct levels. The statistical significance of differences between regions was evaluated by analysis of variance and the Student-Newman-Keuls test.

Preparation of immunoaffinity resins. Immunoaffinity resins with IIA4B3 were prepared as previously described (11). Briefly, ascites containing IIA4B3 were precipitated with saturated ammonium sulfate and dialyzed against coupling buffer (0.1 M ammonium carbonate [pH 8.0]). The antibody in coupling buffer was then reacted with swelled cyanogen-activated Sepharose 4-B (Sigma) for 16 h, washed with 0.1 M Tris-HCl (pH 7.2) and then phosphate buffer, and finally resuspended in phosphate buffer (pH 7.0) containing 0.02% thimerosal.

RESULTS

Four of 10 female BALB/c mice injected with AFB-lysine-cBSA conjugate were found to produce significant anti-AFB-lysine-cBSA serum titers, as measured by a direct ELISA. Spleen cells from these mice were fused with Sp2/0 murine myeloma cells, and a number of stable clones were obtained. Three promising clones, determined by titration of the supernatant of their medium by ELISA and RIA, were further grown as ascitic fluid in BALB/c mice. One (IIA4B3) of these monoclonal antibodies, with the highest apparent affinity and specificity, was further characterized. Isotype classification showed that this antibody was IgG1(λ).

Competitive RIA was used to determine the affinity, specificity, and sensitivity of IIA4B3 for recognizing AFB-lysine, AFB, and other AFB metabolites and adducts. The inhibition curves determined by RIA were highly reproducible, with a coefficient of variation of less than 3 to 4%. As shown in Fig. 1A, IIA4B3 had at least a sevenfold higher affinity for AFB-lysine than for AFB when ^3H -AFB was used as the tracer. The rank order of the affinity was as follows: AFB-lysine > AFB-FAPyr > AFB = AFB-*N*⁷-Gua > AFM₁ > AFQ₁. The concentration for 50% inhibition for AFB-lysine was 0.970 pmol; that for AFB was 7.08 pmol.

Since it was possible that the ^3H -AFB tracer used above might be limiting in our studies, further characterization of the specificity of IIA4B3 was performed with ^3H -AFB-lysine as the tracer. As shown in Fig. 1B, IIA4B3 had about a 10-fold higher specificity for binding to AFB-lysine than for binding to AFB when ^3H -AFB-lysine was used as the tracer. The concentration for 50% inhibition of binding to AFB-lysine was 0.610 pmol; that for AFB was 6.85 pmol. The rank order of the affinity under these conditions was as follows: AFB-lysine $\gg$ AFB > AFM₁ = AFB-FAPyr > AFB-*N*⁷-Gua $\gg$ AFQ₁.

Monoclonal antibody 2B11, which was developed against AFB-bovine gamma globulin (11), is the antibody currently used in detecting AFB-albumin adduct in human serum by our

FIG. 1. Inhibition curves for AFB and its metabolites and adducts in a competitive RIA using monoclonal antibody IIA4B3 and ^3H -AFB (A) or ^3H -AFB-lysine (B). The experiments were performed with different concentrations of AFB and its metabolites and adducts dissolved in 100 μl of PBS, IIA4B3 in 100 μl of 10% horse serum, and 100 μl of tracer (^3H -AFB or ^3H -AFB-lysine). Points represent the mean of triplicate determinations (the standard deviation was $\leq 4\%$).

laboratory. The affinities of 2B11 for AFB, AFB-lysine, and other AFB metabolites and adducts were also measured. 2B11 had similar affinities for recognizing AFB, AFB-lysine, and AFM₁ when either ^3H -AFB (Fig. 2A) or ^3H -AFB-lysine (Fig. 2B) was used as the tracer, followed by AFB-FAPyr, AFB-N⁷-Gua, and AFQ₁.

To further compare the sensitivities and specificities of these two antibodies, a series of paired competitive RIAs were carried out using two different tracers. The concentrations for 50% inhibition calculated from these experiments are summarized in Table 1. IIA4B3 was about five-, two-, and sevenfold more sensitive than 2B11 for recognizing AFB-lysine, AFB-

FAPyr, and AFB-N⁷-Gua adducts when ^3H -AFB was used as the tracer. In contrast, 2B11 was about two- and ninefold more sensitive than IIA4B3 for recognizing AFB and AFM₁ under the same conditions. When ^3H -AFB-lysine was used as the tracer, IIA4B3 had similar sensitivities for recognizing AFB as well as AFB metabolites and adducts, with the only exception being AFB-lysine; for AFB-lysine, IIA4B3 was about 12-fold more sensitive than 2B11, based on the concentration for 50% inhibition.

An analytical method based on a competitive RIA using antibody IIA4B3 and synthetic ^3H -AFB-lysine was established by modification of the previous method using antibody 2B11 and ^3H -AFB (30). Sensitivity and recovery were evaluated with

FIG. 2. Inhibition curves for AFB and its metabolites and adducts in a competitive RIA using monoclonal antibody 2B11 and ^3H -AFB (A) or ^3H -AFB-lysine (B). The experiments were performed with different concentrations of AFB and its metabolites and adducts dissolved in 100 μl of PBS, 2B11 in 100 μl of 10% horse serum, and 100 μl of tracer (^3H -AFB or ^3H -AFB-lysine). Points represent the mean of triplicate determinations (the standard deviation was $\leq 3\%$).

TABLE 1. Comparison of competitive inhibition for AFB and its metabolites and adducts with two different monoclonal antibodies and tracers

Compound	IC ₅₀ (pmol) ^a with the following tracer and antibody:			
	³ H-AFB		³ H-AFB-lysine	
	IIA4B3	2B11	IIA4B3	2B11
AFB-lysine	0.97 ± 0.04	5.25 ± 0.07	0.61 ± 0.03	7.65 ± 0.07
AFB	7.08 ± 0.10	3.33 ± 0.07	6.85 ± 0.07	8.45 ± 0.10
AFM ₁	51.70 ± 1.71	5.80 ± 0.18	10.62 ± 0.81	5.76 ± 0.12
AFQ ₁	62.20 ± 3.61	305.50 ± 8.26	430.40 ± 9.45	660.20 ± 4.62
AFB-FAPyr	4.45 ± 0.19	9.15 ± 0.25	11.00 ± 0.89	11.94 ± 0.11
AFB-N ⁷ -Gua	8.42 ± 0.04	57.80 ± 3.93	32.20 ± 1.62	32.40 ± 1.72

^a IC₅₀, concentration for 50% inhibition. Data are given as the mean and standard deviation.

normal human serum spiked with various concentrations of AFB-lysine adduct. Since the amount of serum protein digests in samples was previously found to influence the results of the RIA (30), the assay was evaluated by using both 1 mg and 2 mg of total protein digests. As shown in Table 2, the assay could detect as little as 5 fmol of spiked AFB-lysine, based on the percentage of inhibition in the presence of either 1 or 2 mg of total protein digests. The functional limitation of the assay was about 10 fmol, determined by subtraction of the background value. There were no statistical differences in recoveries for the two assays with different amounts of serum protein digests (Table 3). The assay of 1 mg of total protein digests showed a slightly higher recovery, a result which may indicate an increased sensitivity for the background value, even though the control value based on nonspiked normal human serum had already been subtracted. The assay of 2 mg of total protein digests, which was the optimum amount of protein digests used for the 2B11-based method, showed recoveries ranging from 92.4 to 97.4% for various concentrations of AFB-lysine adduct; this range of recovery was acceptable for analysis. Therefore, both 1 mg and 2 mg of total protein digests were suitable for measuring AFB-lysine adduct in human serum samples.

A total of 37 human serum samples from our serum repository, where more than 8,000 human serum samples collected from worldwide epidemiological studies are stored, were randomly selected and analyzed by both the new antibody method

and the previous method using antibody 2B11. These data shown in Fig. 3 reveal a statistically significant relationship ($P < 0.001$) between these two methods, with a correlation coefficient of 0.86. Thus, while there are some quantitative differences between the two assays, the data for the new antibody assay clearly demonstrate the specific recognition of AFB-lysine adduct.

As indicated in Table 4, another 77 human serum samples from three different regions of the world were analyzed by the new antibody method. Although all of these samples had detectable levels of AFB-lysine adduct, a statistically significant difference was found among these regions by an analysis of variance ($P < 0.01$). The samples from Guangxi, China, had a significantly higher level (0.198 pmol/mg of albumin; $P < 0.01$) of AFB-lysine adduct than samples from other regions. The samples from The Gambia, West Africa, also had higher levels (0.142 pmol/mg of albumin; $P < 0.05$ or 0.01) of AFB-lysine adduct than samples from Qidong, China, over two consecutive years.

Immunoaffinity resins were prepared by coupling IIA4B3 to cyanogen-activated Sepharose 4-B. The binding capacity of the resins was tested with AFB as well as AFB metabolites and adducts. One milliliter of IIA4B3 affinity resin could bind up to 500 ng of AFB-lysine, AFB-N⁷-Gua, AFB-FAPyr, and AFB-mercapturic acid and up to 400 ng of AFB and AFM₁, with individual recoveries of greater than 90%.

DISCUSSION

In this study, a monoclonal antibody (IIA4B3) specific for AFB-lysine adduct was developed, identified, and characterized. IIA4B3 has a much higher affinity for AFB-lysine adduct

TABLE 2. Sensitivity of a competitive RIA^a for detection of AFB-lysine adduct used to spike normal human serum

AFB-lysine (pmol)	% Inhibition ^b in an assay with the following amt of digest:	
	1 mg ^c	2 mg ^d
0	5.2 ± 1.0	4.8 ± 0.9
0.005	7.2 ± 0.4	8.4 ± 0.8
0.015	11.9 ± 1.3	11.2 ± 0.4
0.050	16.8 ± 0.5	14.4 ± 1.3
0.100	22.0 ± 2.9 ^e	16.2 ± 0.5
0.500	61.3 ± 7.3 ^e	45.8 ± 2.1
1.000	78.3 ± 0.9 ^e	68.8 ± 1.0
5.000	100.8 ± 0.7 ^e	87.5 ± 1.1

^a With monoclonal antibody IIA4B3 and ³H-AFB-lysine as the tracer.

^b Data are given as the mean and standard deviation ($n = 6$).

^c Digest equivalent to 1 mg of total serum protein.

^d Digest equivalent to 2 mg of total serum protein.

^e The value was statistically significantly different ($P < 0.05$) from the value for the 2-mg sample.

TABLE 3. Accuracy of a competitive RIA^a for detection of AFB-lysine adduct in normal human serum

AFB-lysine (pmol)	% Recovery ^b in an assay with the following amt of digest:	
	1 mg ^c	2 mg ^d
0.10	106.7 ± 8.4 (99.5–109.2)	97.4 ± 9.8 (86.4–110.3)
0.50	104.5 ± 7.8 (98.2–106.5)	96.2 ± 3.4 (94.1–102.2)
1.00	100.3 ± 4.7 (96.4–107.8)	92.4 ± 4.4 (88.6–98.2)
5.00	106.5 ± 6.4 (99.1–111.9)	94.8 ± 4.2 (90.1–100.3)

^a With monoclonal antibody IIA4B3 and ³H-AFB-lysine as the tracer.

^b Data are given as the mean and standard deviation (range) ($n = 6$).

^c Digest equivalent to 1 mg of total serum protein.

^d Digest equivalent to 2 mg of total serum protein.

FIG. 3. Regression and correlation analysis of AFB-lysine adduct in human serum samples detected by IIA4B3- and 2B11-based RIA methods. The samples were processed for albumin, digested, and concentrated. Two milligrams of albumin digest was analyzed by the RIAs, and the levels of AFB-lysine adduct were determined by use of a standard curve calibrated with different concentrations of purified AFB-lysine adduct. MAb, monoclonal antibody.

($\sim 8 \times 10^9$ liters/mol) than for AFB and its metabolites and adducts, as demonstrated in Fig. 1 and Table 1. To our knowledge, this is the first report of the development of a specific monoclonal antibody for AFB-lysine adduct. Previous studies either used a polyclonal antiserum-based ELISA (5, 33) or a monoclonal antibody 2B11-based RIA (8, 26, 30) to detect AFB-albumin adducts in large quantities of human serum samples collected in epidemiological studies. The availability of IIA4B3 will improve the specificity and sensitivity for AFB-lysine adduct measurements in future human studies.

Monoclonal antibody 2B11 has been widely used in AFB biomarker studies because it recognizes AFB and its metabolites and adducts (11, 30). Its ability to recognize AFB-lysine adduct prompted the earlier development of an RIA-based method to measure total AFB-albumin adducts in human serum samples (8, 26, 30). In this study, we compared the affinities and specificities of monoclonal antibodies IIA4B3 and 2B11 for recognizing AFB and its metabolites and adducts. IIA4B3 was about five-, two-, and sevenfold more sensitive than 2B11 for recognizing AFB-lysine, AFB-FAPyr, and AFB- N^7 -Gua adducts, respectively; 2B11 was about two- and ninefold more sensitive than IIA4B3 for recognizing AFB and AFM₁, respectively, under the same conditions (Fig. 2 and Table 1). The marked difference in affinity between the two antibodies was attributable to the different antigens used for raising these two antibodies in mice. 2B11 was raised against AFB-bovine gamma globulin (11), whereas IIA4B3 was developed against AFB-lysine-cBSA; 2B11 is an IgM antibody, whereas IIA4B3 is an IgG1(λ) antibody. The difference in affinity suggests that different antigenic determinants or epitopes may be recognized by these two antibodies. IIA4B3 was about 12-fold more sensitive than 2B11 for recognition of AFB-lysine when ^3H -AFB-lysine was used as the tracer (Table 1). However, these antibodies had similar sensitivities for recognizing AFB and other AFB metabolites and adducts under the same conditions. These results further confirmed that IIA4B3 is an AFB-lysine adduct-specific antibody.

TABLE 4. Levels of AFB-lysine adduct in human serum samples

Source of samples	Yr collected	No. of samples	pmol of AFB-lysine adduct/mg of albumin ^a
Guangxi, China	1984	17	0.198 ± 0.076 (0.009–0.329) ^b
The Gambia	1989	20	0.142 ± 0.034 (0.084–0.228) ^c
Qidong, China	1993	20	0.098 ± 0.017 (0.065–0.142)
Qidong, China	1994	20	0.108 ± 0.016 (0.083–0.147)

^a Data are given as the mean and standard deviation.

^b The *P* value was <0.01 for comparisons with samples from The Gambia and Qidong, China.

^c The *P* values were <0.01 and <0.05 for comparisons with the 1993 and 1994 samples from Qidong, China, respectively.

The chemistry of the AFB-lysine adduct has been unequivocally established through nuclear magnetic resonance and other structural studies (23, 25). This adduct has a completely modified furo-furan ring structure relative to AFB and the other metabolites shown in Table 1. Thus, some of the data used to understand the epitope of this new antibody have revealed some surprising results. Using the data obtained with ^3H -AFB as a tracer, it is apparent that monoclonal antibody 2B11 shows equal recognition for AFB and AFM₁, suggesting that the hydroxy group at the C-10 position of AFM₁ does not affect the binding of 2B11. In contrast, antibody IIA4B3 has about a sevenfold lower ability to bind AFM₁ than to bind AFB, indicating that the epitope of this antibody is affected by the chemistry of the furo-furan region of the AF ring structure. Both 2B11 and IIA4B3 have diminished abilities to bind AFQ₁, a metabolite containing a hydroxy group in the cyclopentenone (C-3 position) of the AF parent structure. While this information suggests that both antibodies are sensitive to modification in the cyclopentenone ring, AFQ₁ does have a bathochromic shift in its UV spectrum relative to AFB; this information indicates that hydroxylation of the cyclopentenone ring induces an electronic restructuring of the molecule, resulting in less recognition by these antibodies. The major surprise in this study was the significant recognition of the major AF-DNA adducts by monoclonal antibody IIA4B3 (Table 1). Since a relatively common structure between the lysine and DNA adducts is the nitrogen ring structure at the junction of the AF and lysine or guanine molecules, it appears that the epitope of this new antibody lies within this common chemistry. Thus, these findings indicate that the stereochemistry of these different adducts shares a number of common determinants that can be used to develop more antibodies recognizing these mechanistically important adducts. These findings also suggest that IIA4B3 may have potential for the analysis of AFB-DNA adducts. IIA4B3 immunoaffinity resins were produced and are available for future studies.

An analytical method based on a competitive RIA with IIA4B3 and ^3H -AFB-lysine was established and validated in this study. The method has been applied to 114 human serum samples collected from the residents of three regions with a high risk for AFB exposure and hepatocellular carcinoma (Fig. 3 and Table 4). The results generated here demonstrate that exposure to AFB was very significant in these populations, as indicated by the serum samples containing AFB-lysine adduct. The levels of AFB-lysine adduct measured in human serum samples collected from the residents of The Gambia were

comparable to the levels previously reported (32). The highest average level of AFB-lysine adduct was found in the samples collected from Fusui, Guangxi, China, a result which was consistent with previous reports (8, 24). It should be mentioned that these Fusui samples were collected more than a decade ago, indicating that AFB-lysine adduct is stable enough to serve as a molecular biomarker in samples frozen for a long time period. The stability of AFB-lysine adduct was also reported with human serum samples from Thailand (33). The average levels of AFB-lysine adduct were similar among samples collected from Qidong, China, over two consecutive years, suggesting that exposure to AFB was comparable over time in the studied population; however, great individual variations existed, as shown by the wide range of detectable AFB-lysine adduct levels.

Since the measurement of AFB-lysine adduct is becoming more common in human dosimetry studies, the need to standardize the available methods to directly compare assay values across different studies and techniques is urgent. Once standardization occurs, questions such as the daily level of exposure to AF that results in a specific serum albumin adduct level may be calculated in a reliable manner. Data generated from a comparison of two monoclonal antibodies (Fig. 3) can serve this purpose. The two assays are significantly correlated, suggesting that data obtained from these two antibody-based assays can be interconverted for the purpose of risk assessment in the future. Finally, the newly developed antibody IIA4B3-based RIA and immunoaffinity resins have been successfully applied to recent human chemoprevention trials against AFB exposure (18, 31).

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