Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 7728–7731, December 1984
Biochemistry

High-affinity monoclonal antibodies for aflatoxins and their application to solid-phase immunoassays

(monoclonal antibodies/affinity chromatography/environmental carcinogens)

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Contributed by Gerald N. Wogan, August 20, 1984

ABSTRACT **Monoclonal antibodies specific for aflatoxin B_1, aflatoxin B_2, aflatoxin M_1, and the major aflatoxin-DNA adducts were obtained following fusion of mouse SP-2 myeloma cells with spleen cells of mice immunized with aflatoxin B_1 covalently bound to bovine gamma globulin. The aflatoxin-modified protein used to immunize mice was produced chemically by activating aflatoxin B_1 to a 2,3-epoxide derivative, which then covalently bound to the protein. One of the monoclonal antibodies isolated (2B11) was found to be a high-affinity IgM antibody with an affinity constant for aflatoxin B_1, aflatoxin B_2, and aflatoxin M_1 of about 1×10^9 liters per mol. In a competitive radioimmunoassay using [3H]aflatoxin B_1, 3 pmol (1 ng) of aflatoxin B_1, aflatoxin B_2, or aflatoxin M_1 caused 50% inhibition with this antibody. The antibody also had significant cross-reactivity for the major aflatoxin-DNA adducts: 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1 and 2,3-dihydro-2-(N^5-formyl-2′,5′,6′-triamino-4′oxo-N^5-pyrimidyl)-3-hydroxyaflatoxin B_1. The antibody was also covalently bound to Sepharose-4B and used in a column-based solid-phase immunosorbent assay system. Aflatoxins added *in vitro* to phosphate buffer, human urine, human serum, or human milk at levels expected to be obtained in human samples acquired from environmentally exposed individuals were quantitatively recovered by applying the mixture to this antibody affinity column purification system. Preliminary studies using urine samples from rats injected with radiolabeled aflatoxin B_1 have also indicated that aflatoxin metabolites can be isolated by these methods. Furthermore, we have found that the monoclonal antibody affinity columns can be regenerated for multiple use. Therefore, the monoclonal antibodies and their application to affinity chromatography represents a useful and rapid technique to purify environmentally occurring levels of this carcinogen and some of its metabolites for quantitative measurements.**

The aflatoxins are highly toxic and carcinogenic compounds, which are consistently found contaminating human food supplies in many areas of the world, and are epidemiologically linked to increased incidences of human liver cancer in Asia and Africa (1–3). Adverse biological consequences resulting from exposure to aflatoxin B_1 (AFB_1) are thought to be related to its ability to be oxidatively metabolized by constitutative cellular enzymes. Of particular importance may be the ability of AFB_1 to form covalently linked adducts with cellular macromolecules, including DNA. It is well established that AFB_1 forms covalent DNA adducts following enzymatic oxidation to a highly reactive 2,3-epoxide, which nucleophilically attacks the N^7 atom of guanine (4–6). The major DNA lesion produced by this reaction, 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1 (AF-N^7-Gua), has been studied as an indicator compound of genotoxic damage to the cellular genetic material (7–9).

A major objective of our work has been the development of noninvasive screening procedures for assessing the exposure of humans to environmentally occurring carcinogens. Useful protocols require an ability to quantify chemical carcinogens and their metabolites, especially DNA adducts, in readily accessible body fluids, such as serum and urine. We have been developing these screening methodologies by using immunoassays with monoclonal antibodies. We previously have reported production of monoclonal antibodies that recognize aflatoxin-DNA derivatives (10, 11). These early antibodies were found to be specific for AFB_1-adducted DNA and in a competitive ELISA were able to detect AFB_1 residues in DNA down to a level of 1 per 1,355,000 nucleotides (11). However, these antibodies did not recognize AFB_1, free aflatoxin-DNA adducts, or any other metabolites. Another laboratory has also reported the production of a monoclonal antibody that recognizes DNA containing 2,3-dihydro-2-(N^5-formyl-2′,5′,6′-triamino-4′-oxo-N^5-pyrimidyl)-3-hydroxyaflatoxin B_1 (AF-FAPyr) in DNA (12). We were encouraged by these preliminary results to continue attempts to produce monoclonal antibodies with properties necessary to detect aflatoxins and their derivatives in environmental samples.

We report here the production of a high affinity IgM monoclonal antibody that recognizes aflatoxin B_1, aflatoxin B_2, aflatoxin M_1, and the major aflatoxin-DNA adducts; AF-N^7-Gua and AF-FAPyr. This antibody was used to construct a reusable antibody affinity chromatographic column capable of isolating aflatoxins from complex biological fluids, such as serum, urine, and milk. Initial applications of this antibody affinity column in development of analytical methods are also discussed.

MATERIALS AND METHODS

Cell Fusion. The myeloma cell line used for fusion, SP-2, was maintained in suspension in Dulbecco's modified Eagle's medium supplemented with 20% (vol/vol) fetal calf serum, glutamine, penicillin, streptomycin and nonessential amino acids (GIBCO). The cell fusion procedures have been described (13).

Preparation of AFB_1-Bovine Gamma Globulin (AFB_1-BGG) Immunogen. BGG (Calbiochem) was dissolved in phosphate-buffered saline (P_i/NaCl) (pH 7.0) at 10 mg/ml. The BGG

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Abbreviations: AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFQ_1, aflatoxin Q_1; AFM_1, aflatoxin M_1; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; AF-N^7-Gua, 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1; AF-FAPyr, 2,3-dihydro-2-(N^5-formyl-2′,5′,6′-triamino-4′-oxo-N^5-pyrimidyl)-3-hydroxyaflatoxin B_1; Me_2SO, dimethyl sulfoxide; BGG, bovine gamma globulin; BSA, bovine serum albumin.

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was derivatized with AFB_1 by using a modified chemical procedure with *m*-chloroperoxybenzoic acid as described by Martin and Garner (6). In a typical reaction, 1 mg of AFB_1 (3.2 μmol) was dissolved in 2 ml of methylene chloride and added to a 5-molar excess of *m*-chloroperoxybenzoic, also dissolved in 2 ml of methylene chloride. BGG in buffer was added to the reaction mixture to produce a 5-molar excess of AFB_1 relative to the protein content. The mixture was vigorously stirred overnight at ambient temperature. The reaction was terminated by centrifuging at 2,000 × *g* for 20 min. The aqueous epiphase containing the modified protein was then extensively dialyzed against P_i/NaCl (pH 7.4). The level of modification of the protein was quantified by absorbance at 362 nm using a molar extinction coefficient of 18,000. In a typical reaction, there were 40–50 AFB_1 residues bound per molecule of BGG.

Preparation of AFB_1-Modified Bovine Serum Albumin (BSA). AFB_1 was coupled to BSA by the procedure described above. AFB_1-modified BSA was synthesized for use in screening procedures for aflatoxin specific antibodies. The average level of binding of aflatoxin B_1 to BSA was 20–30 molecules of aflatoxin per molecule protein.

Immunization of Mice. Female BALB/By CJ mice (Jackson Laboratories), 16 weeks old, were immunized with AFB_1-modified BGG dissolved in P_i/NaCl and emulsified with an equal volume of complete Freund's adjuvant. Two groups of five mice each were immunized by intraperitoneal injection of 37.5 μg of AFB_1-BGG or 12 μg of AFB_1-BGG in 0.2 ml of solution, respectively. At 5 weeks and 9 weeks after the initial injection, all animals received the same amount of antigen emulsified with incomplete Freund's adjuvant. About 10 days after the second injections, serum samples collected by tail bleeding were assayed by ELISA for antibody activity. Three days prior to fusion, each mouse was given a final immunization of the same AFB_1-BGG amount in 0.1 ml of P_i/NaCl injected into the tail vein.

Enzyme Immunoassays. ELISA was used to determine aflatoxin-specific antibodies in mouse sera and hybridoma media. Procedures used were modifications of previously described protocols (10, 11). Briefly, polyvinyl chloride microtiter plates (Dynatech, Alexandria, VA) containing in each well 50 μl of AFB_1-BSA dissolved in P_i/NaCl at 2 μg/ml were incubated for 2–4 hr at ambient temperature. Control plates containing 50 μl of BSA in P_i/NaCl at 2 μg/ml were treated similarly. Antigen solution was aspirated, and the plates were washed three times with tap water. Solutions containing 0.2% BSA or 0.2% gelatin, type IV (Sigma), both in P_i/NaCl were added, and the plates were incubated for 1 hr at ambient temperature, a procedure designed to limit nonspecific binding of antibodies. The plates were washed in tap water, and 50 μl of mouse sera or hybridoma media was added to each well. To titer the mouse sera, 1:3 serial dilutions in 10% horse serum were prepared over a range from 1:50 to 3:50,000. In the case of hybridoma media, 50-μl aliquots were used without dilution. Plates were incubated for 90 min at 37°C, after which they were thoroughly washed. Specific antibodies bound to the plate were detected by incubating 50 μl per well of a 1:200 dilution of rat anti-mouse κ chain antibody coupled with alkaline phosphatase for 2 hr at ambient temperature. The plates were rewashed and incubated with 100 μl per well of *p*-nitrophenyl phosphate (Sigma) at 1 mg/ml in 0.1 M diethanolamine buffer (pH 9.8). Quantitation of the *p*-nitrophenol production was performed by measuring absorbance at 405 nm with a microtiter plate reader (Dynatech, Alexandria, VA).

The isotypes of the monoclonal antibodies were determined in noncompetitive ELISA by using the mouse immunoglobulin subtype identification kit purchased from Boehringer Mannheim.

Competitive Radioimmunoassay. The relative binding constants of different aflatoxin derivatives were determined by RIA. We routinely used an assay with a 300-μl total volume, 100 μl of which consisted of the [3H]AFB_1 tracer (specific activity, 3.4 Ci/mmol; 1 Ci = 37 GBq) purchased from Moravek Biochemicals (Ueda, CA). The tracer was diluted in 1% normal mouse serum/0.1% BSA in P_i/NaCl to a level of about 20,000 cpm/100 μl. The monoclonal antibody was diluted to a concentration that precipitated 30–50% of the AFB_1 tracer. The antibody was added to the reaction mixture in 100 μl of 10% fetal calf serum in P_i/NaCl. The test sample, consisting of nonradiolabeled AFB_1 or its metabolites, were added to the tube in a 100-μl volume. The reaction mixture was then incubated at ambient temperature for 2 hr, following which the mixture was brought to a volume of 1-ml with P_i/NaCl. An equal volume of ice-cold saturated ammonium sulfate was then added; the sample was mixed and allowed to stand on ice for 15 min. The sample was then centrifuged for 15 min at 2,000 × *g*, and the percentage of inhibition in the reaction was determined by the method of Muller (14).

Isolation and Purification of Monoclonal Antibodies. Hybridomas secreting antibodies of interest were grown as ascites tumor cells in BALB/c mice, which previously had been injected with 0.5 ml of pristane (Aldrich). Ascites fluid collected from these animals were pooled and either used directly in the immunoassays or further purified by saturated ammonium sulfate precipitation and dialysis against P_i/NaCl.

The IgM monoclonal antibody (2B11) was further purified by HPLC with a TSK 4000SW column and isocratic elution at 0.5 ml per min. Proteins were monitored at 280 nm, and IgM and IgG standards purchased from Cappel Laboratories (Cochranville, PA) were used as molecular weight standards. Using this procedure, we were able to isolate up to 1 mg of chromatographically pure 2B11 per HPLC run.

Preparation of Monoclonal Antibody Affinity Column. A monoclonal antibody affinity column was prepared by the procedures described by Pharmacia. The monoclonal antibody (2B11) was dissolved in coupling buffer (0.1 M $NaHCO_3$, pH 8.3/0.5 M NaCl) at 1 mg/ml. This solution was coupled to 2.5 g of cyanogen bromide-activated Sepharose-4B (SIGMA). The resin had been incubated in 8 ml of 1 mM HCl overnight. The unbound sites of the antibody-bound gel were blocked by incubating the gel with 1.0 M ethanolamine (pH 8.5). For the data reported in this paper, a 2-ml bed volume of the affinity gel was used. The flow rate for the separations was about 0.5 ml/min. All procedures were performed at ambient temperature.

HPLC. All HPLC were performed with a Beckman model 324MP gradient liquid chromatograph equipped with a Beckman model 160 UV detector. For steric-exclusion chromatography of TSK 4000SW column (Beckman) was used at 0.5 ml per min in P_i/NaCl. For the chromatography of aflatoxins, a C_{18} ODS-Ultrasphere reversed-phase column was used with ethanol buffer at pH 5.0, eluted at 1 ml/min.

RESULTS

Hybridomas. Female BALB/By CJ mice were immunized with AFB_1-adducted BGG in complete Freund's adjuvant. Seven of the 10 mice injected were found to produce significant anti-AFB_1 serum titers as measured by noncompetitive ELISA. Spleen cells from five of these mice were fused with SP-2 myeloma cells and a number of monoclonal antibodies, grown as ascites fluid, were obtained. One of these monoclonal antibodies proved to be a high-affinity IgM (2B11), and the specificity and application of this antibody to affinity chromatography are described here.

Characterization of Monoclonal Antibodies. Values for the affinity constant for 2B11 were determined to be 8×10^8 and 1×10^9 liters per mole by Scatchard plot analysis and the

method described by Muller (14), respectively. These values were derived from data obtained by the competitive RIA described above. The specificity of 2B11 in the competitive RIA for AFB_1 and its metabolites, including the major AFB_1-DNA adducts (AF-N^7-Gua and AF-FAPyr), are depicted in Figs. 1 and 2. The 50% inhibition levels for AFB_1, AFB_2, and AFM_1 were found to be 3.0 pmol (1 ng), whereas those for AFG_1, AFG_2, and AFQ_1 were 60.0, 84.0, and 275.0 pmol, respectively. These data clearly indicate that the major epitope for 2B11 recognition of aflatoxin lies in the coumarin ring and the cyclopentenone ring of the aflatoxin molecule.

Fig. 2 depicts the specificity and 50% inhibition in the competitive RIA of 2B11 for AFB_1 and the two major aflatoxin-DNA adducts, AF-FAPyr and AF-N^7-Gua. The 50% inhibition values were 3.0, 24.0, and 89.0 pmol, respectively. Based on the data in Fig. 1, we expected high recognition by the antibody for the two aflatoxin-DNA adducts. In addition, data in Fig. 2 indicate that the antibody is about 4 times more sensitive in detecting (24.0 pmol versus 89.0 pmol) the AF-FAPyr adduct than the AF-N^7-Gua adduct. This finding was unanticipated, since the aflatoxin moiety is identical in these adducts when compared to the parent molecule. These observations suggest that the epitope for the antibody may be partially obstructed in these DNA adducts due to the stereochemistry of covalent binding of aflatoxin to guanine. Experiments (data not shown) using AFB_1-modified DNA and BSA in the competitive RIA determined that the 50% inhibition for these macromolecularly bound aflatoxins were 10.0 and 12.0 pmol in a 300-μl assay, respectively. These data tend to support the suggestions that the epitope recognized by the monoclonal antibody may become sterically hindered when AFB_1 is coupled to a larger molecule.

Solid-Phase Affinity Chromatography with IgM Monoclonal Antibody 2B11. Ascites fluid containing antibody 2B11 was initially fractionated by precipitation with saturated ammonium sulfate, followed by dialysis against P_i/NaCl. The antibody was then further purified by HPLC in a steric-exclusion (molecular weight separation) chromatographic mode, which was used to take advantage of the high molecular weight of the IgM (900,000) in purification of the monoclonal antibody. The purified antibody, having been assayed for activity by noncompetitive RIA, was bound to a solid-phase matrix of Sepharose-4B. The columns made for these experiments contained 1 mg of antibody per ml of column volume.

Initial experiments were performed to determine the ability of the antibody column to bind [3H]AFB_1. In these studies the concentration of [3H]AFB_1 was 1 ng (3 pmol) in 10 ml of P_i/NaCl, a level that would approximate concentrations expected in body fluids of people exposed to contaminated foods. When the sample containing the [3H]AFB_1 was applied to the column, about 10% of the radioactivity washed through in the loading and P_i/NaCl wash phase. (This loss

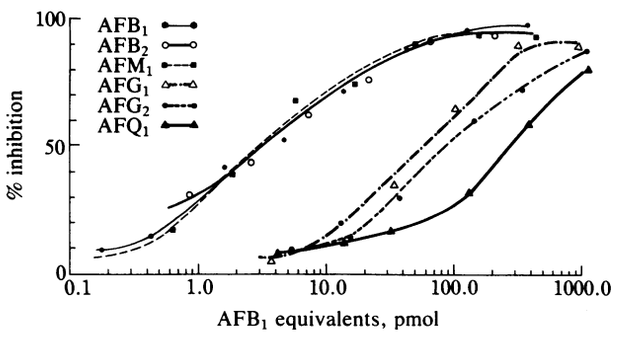


FIG. 1. Competitive RIA using [3H]AFB_1 tracer of aflatoxins.

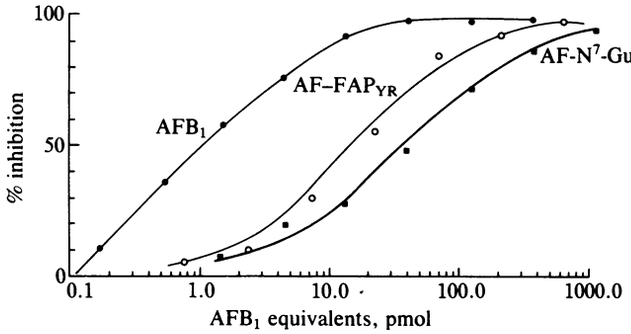


FIG. 2. Competitive RIA using [3H]AFB_1 tracer of AFB_1 and the major aflatoxin DNA adducts.

was attributable to tritium exchange, independently determined by other analytical procedures.) The column was then washed with 2 M potassium thiocyanate, a commonly used elution buffer for antibody affinity chromatography, which eluted <2% of the applied [3H]AFB_1. Sequential washes with phosphate buffer (pH 3.0), diethanolamine buffer (pH 9.9), and phosphate buffer (pH 2.0) removed <1% of the applied [3H]AFB_1. However, quantitative elution of the bound [3H]AFB_1 was achieved by using 50% dimethyl sulfoxide (Me_2SO) in phosphate buffer (pH 7.4) (50% Me_2SO buffer). The antibody column was regenerated by washing the column with P_i/NaCl (pH 7.4); to date, we have used and regenerated this column more than 25 times with no apparent loss of activity.

To determine the optimal Me_2SO concentration for elution of AFB_1, the affinity column prepared as above was eluted sequentially with 1%, 5%, 10%, 20%, 30%, and 40% Me_2SO in P_i/NaCl. Less than 4% of the applied aflatoxin was eluted by all of these Me_2SO solutions, and quantitative recovery was obtained only with 50% Me_2SO. Similar results also were obtained with solutions of dimethylformamide in P_i/NaCl. These properties emphasize the potential for this column for use as a preparative tool in recovery of aflatoxins from complex biological mixtures.

The capacity of the antibody affinity column to bind AFB_1 was determined by radiometric and absorbance techniques and was found to be able to bind 1–1.3 μg of AFB_1 from 10 ml of P_i/NaCl per 1 ml of column bed volume.

***In Vitro* Isolation of AFB_1 from Human Urine, Serum, and Milk.** Freshly collected human urine (10 ml) was centrifuged or filtered through a 0.45-μm filter and spiked with 1 ng of [3H]AFB_1. When urine was applied directly to the affinity column, 60% of the applied AFB_1 failed to bind to the column. However, by using the protocol outlined in Fig. 3, we were able to obtain quantitative binding of the [3H]AFB_1 to the column and recovery in the 50% Me_2SO buffer. Thus, interfering materials in urine can effectively be removed by using a preparative C_{18} Sep-Pak column (Waters Associates) stage in the isolation procedure. In previous studies, we have consistently found that 90–95% of aflatoxins applied to C_{18} Sep-Pak columns can be recovered (15).

Human serum (10 ml) or human milk (10 ml) spiked with [3H]AFB_1 (1 ng) were applied directly to the antibody column without prior treatment, and in both instances quantitative binding followed by the recovery into 50% Me_2SO were observed. Therefore, with both serum and milk, a preparative step using the C_{18} Sep-Pak column was not required for the quantitative recovery of the carcinogen.

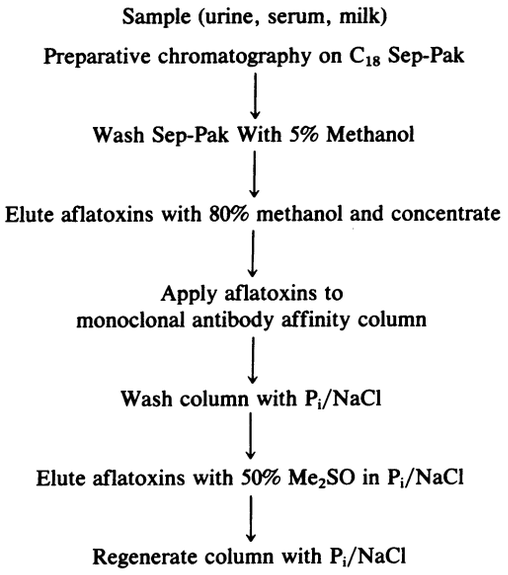
Antibody Affinity Chromatography of AF-N^7-Gua. Initial experiments were performed to determine the recovery of [3H]AF-N^7-Gua (10 ng) spiked into P_i/NaCl. In these experiments we found that quantitative recover of the aflatoxin-DNA adduct applied to the column could be achieved also without any preparative cleanup step. Further studies are

underway to determine the ability to recover these adducts from human serum and urine.

Antibody Affinity Chromatography of *in Vivo* Aflatoxin Rat Urine. Two adult male rats were each injected with 1 mg of [^{14}C]AFB_1 per kg of body weight and their urine was collected for 20 hr, at which time 10–12% of the ^{14}C of the initial dose had been excreted into the urine. Aliquots (100 μl) of each urine containing 290 and 310 ng of aflatoxin, respectively, were diluted with 1.9 ml of P_i/NaCl and applied to the antibody affinity column, and 65% of the applied ^{14}C became bound to the antibody matrix. This material was eluted from the column with 50% Me_2SO buffer and analyzed by reversed-phase HPLC. Preliminary analysis of this fraction revealed that AFM_1 was the major metabolite isolated by the antibody affinity column.

These findings indicate that the protocol outlined in the following flow chart will be a useful preparative technique to isolate and purify aflatoxin B_1 and its metabolites from complex biological samples obtained from environmentally exposed populations. The samples obtained can then be quantitatively analyzed by methods such as HPLC and immunoassay techniques.

Outline of aflatoxin isolation scheme



DISCUSSION

Over the last 5 years, we have produced various monoclonal antibodies that recognize aflatoxins. These antibodies can be used in conjunction with other chemical analytic techniques as noninvasive screening methodologies to monitor human exposure to these environmentally occurring carcinogens. Such screening methods will require the ability to quantify aflatoxin and its metabolites, including DNA adducts, in readily accessible compartments, such as serum and urine. We have reported a chemical protocol to quantify AF-N^7-Gua in urine (15), based upon the isolation of aflatoxin DNA adducts from human urine by preparative and analytical liquid chromatography. Radiometric labeling techniques were used to achieve a limit of detectability in spiked samples of 1 pg of AF-N^7-Gua per ml of urine. However, we found that when attempting to apply these methods to *in vivo* urine samples, nonspecific interfering materials present in urine sample often prevented the attainment of this level of sensitivity. Therefore, we have undertaken efforts to combine chemical analytic procedures with a monoclonal antibody affinity chromatography column.

The development of a reusable monoclonal antibody affinity chromatographic column first required the production of high-affinity monoclonal antibodies recognizing aflatoxins. Our initial endeavors to produce monoclonal antibodies that recognize aflatoxins culminated in the production of five antibodies, which were found to be specific for AFB_1-adducted DNA and, in a competitive ELISA, showed a limit of detectability of one AFB_1 residue per 1,355,000 nucleotides (10, 11). Since these antibodies did not cross-react with the aflatoxin derivatives expected to be found in human urine and serum samples, we continued attempts to produce high-affinity monoclonal antibodies with the desired properties. One of these, an IgM (2B11) that was found to have a particularly high affinity towards the aflatoxins (16), was used in the studies described in this report. In the competitive RIA, a 50% inhibition value of about 3 pmol is routinely obtained with this antibody, and there is a lower limit of detectability of about 300 fmol for AFB_1, AFB_2 and AFM_1. Furthermore, we expect that by using more sensitive competitive ELISA/USERIA (ultrasensitive enzyme-linked RIA) techniques, the ultimate sensitivity with this antibody in detecting aflatoxins will be further enhanced (10, 11). We also have found that when this antibody is bound to a solid-phase matrix, a reusable column can be prepared that selectively isolates aflatoxins from complex mixtures, such as urine, serum, and milk. Isolates from this column chromatographed by HPLC show very little contamination by nonaflatoxin derivatives. We are now in the process of validating this technique by using urine samples isolated from rats injected with radiolabeled aflatoxin B_1.

This research was supported in part by Grant 5 PO1 ES 00597 from the National Institutes of Health and Grant 1529-C-1 from the American Cancer Society, Massachusetts Division.

1. Busby, W. F. & Wogan, G. N. (1979) in *Food Borne Infections and Intoxications*, eds. Reimann, H. P. & Bryan, F. L. (Academic, New York), 2nd Ed., pp. 519–610.
2. Wogan, G. N. (1973) in *Methods in Cancer Research*, ed. Busch, H. (Academic, New York), Vol. 7, pp. 309–344.
3. Stoloff, L. (1976) in *Mycotoxins and Other Fungal Related Food Problems*, ed. Rodricks, J. V. (Am. Chem. Soc., Washington, DC), pp. 23–50.
4. Essigmann, J. M., Croy, R. G., Nadzan, A. M., Busby, W. F., Reinhold, V. N., Buchi, G. & Wogan, G. N. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1870–1874.
5. Lin, J. K., Miller, J. A. & Miller, E. C. (1977) *Cancer Res.* **37**, 4430–4438.
6. Martin, C. N. & Garner, R. C. (1977) *Nature (London)* **267**, 863–865.
7. Croy, R. G. & Wogan, G. N. (1981) *Cancer Res.* **41**, 197–203.
8. Hertzog, P. J., Lindsay-Smith, J. R. & Garner, R. C. (1980) *Carcinogenesis* **1**, 787–794.
9. Wang, T. C. & Cerutti, P. (1980) *Biochemistry* **19**, 1692–1698.
10. Haugen, A., Groopman, J. D., Hsu, I. C., Goodrich, G. R., Wogan, G. N. & Harris, C. C. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4124–4127.
11. Groopman, J. D., Haugen, A., Goodrich, G. R., Wogan, G. N. & Harris, C. C. (1982) *Cancer Res.* **42**, 3120–3124.
12. Hertzog, P. J., Lindsay-Smith, J. R. & Garner, R. C. (1982) *Carcinogenesis* **3**, 825–828.
13. Marshak-Rothstein, A., Fink, P., Gridley, T., Raulet, D. H., Bevan, M. J. & Gefter, M. L. (1979) *J. Immunol.* **122**, 2491–2497.
14. Muller, R. (1980) *J. Immunol. Methods* **34**, 345–352.
15. Donahue, P. R., Essigmann, J. M. & Wogan, G. N. (1982) in *Indicators of Genotoxic Exposure*, Banbury Report 13 (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY), pp. 221–229.
16. Groopman, J. D., Trudel, L. J., Marshak-Rothstein, A. & Wogan, G. N. (1984) *Proc. Am. Assoc. Cancer Res.* **25**, 98.