Time-Resolved Fluoroimmunoassay of Aflatoxins

Paolo Degan,1,2,6 Giorgio Montagnoli,2,6 and Christopher P. Wild4

Preparation of purified and Eu-labeled antibodies specific for aflatoxins is described. Their use is illustrated by a solid-phase competitive time-resolved fluoroimmunoassay, results of which were correlated with those of an enzyme-linked immunosorbent assay based on use of the unmodified antibody to aflatoxin. This procedure is discussed as a quick, sensitive, and reliable immunoassay for use in mycotoxin screening in foodstuffs and body fluids.

Additional Keyphrases: mycotoxins · human and veterinary toxicology · screening · enzyme-linked immunosorbent assay

Aflatoxins are naturally occurring mycotoxins synthesized by Aspergillus flavus and A. parasiticus, which can be found as contaminants in human and animal foodstuffs (see 1 for a review). Because conditions for fungal biosynthesis of aflatoxins are optimal in hot, humid climates, high contamination of staple foods in South Asia and Sub-Saharan Africa has been commonly observed (1). AFB1, and AFG, are the most common aflatoxins found in food, and both are hepatocarcinogenic in a variety of animal species, including primates (1–5). Epidemiological studies have shown a good correlation between the incidence of hepatocellular carcinoma and the degree of food contamination by aflatoxins, implying a causal association when combined with the effect of chronic infection with hepatitis B virus (6–10).

Metabolism of AFB1 by the microsomal mixed-function oxygenase system results in the formation of a number of reduced and oxidized derivatives, including AFBM, AFT, AFQ, and the unstable reactive AFB1,8,9-epoxide. This last metabolite can form adducts with nucleic acids and proteins (1, 11–14). The same metabolites and adducts have been detected in human tissues and body fluids, and give an indication of human environmental exposure to aflatoxins (15, 16 and references therein, 16–18). These sensitive methods (immunoassay, HPLC with fluorescence detection) are aimed at further clarifying aflatoxin exposure in individuals and its role in the etiology of hepatocellular carcinoma (19, 20). Europium-based time-resolved fluorescence immunoassays have been increasingly used in recent years as quick, sensitive, and reproducible microanalytical methods (21–25). Here we report an application of europium ions as labels for anti-aflatoxin antibodies in the immunological determination of aflatoxins. Although not yet validated with biological samples, the assay presented here shows the relevant features of this technique, which we believe can constitute a valid alternative to the methods already in use for aflatoxin quantification, both in foodstuffs and in human and animal body fluids.

Materials and Methods

Reagents. AFB1, AFBM, AFT, AFG, Tween 20 surfactant, and BSA were obtained from Sigma Chemical Co. (St. Louis, MO); EuCl3, diethylenetriamine pentaaacetic acid anhydride, and thenoyltrifluoroacetone from Aldrich Chemie (Steinheim, F.R.G.); methyltriiodylammonium chloride ("Adogen 464") from Serva Feinbiochemica (Heidelberg, F.R.G.); CNBr-activated Sepharose 4 B, Sephadex G-50, and 12-well polystyrene microtiter strips from Pharmacia Fine Chemicals (Uppsala, Sweden); and PEG 4000 from Baker Chemical Co. (Phillipsburg, NJ).

Instrumentation. We measured absorbance with a Uvicord IB (LKB, Bromma, Sweden) and a UVVDEC 510 spectrophotometer (JASCO, Tokyo, Japan) and fluorescence intensity with a JASCO FP 770 spectrofluorometer and an LKB Wallac ARCUS 1230 fluorometer with time resolution. The ELISA plate reader was a Titertek Multiscan Reader (Flow Labs., Puteaux, France) equipped with a 450-nm filter.

Purification of the anti-aflatoxin antibody. A two-step procedure was used. First, antibodies reacting against the carrier BSA were removed from the primary antiserum by chromatography on BSA–CNBr-activated Sepharose 4B, prepared according to the manufacturer's instructions. We applied 1-mL aliquots of antiserum to a 1 x 3 cm column equilibrated with carbonate buffer (0.1 mol/L pH 8.2) containing 0.5 mol of NaCl per liter. The antiserum was left to react on the column with the bound BSA for 4 h at room temperature, then was eluted in the same buffer. Unbound material was collected, pooled, and concentrated by dialysis against PEG 4000 at 4 °C, to about a third the original value and subsequently dialyzed against carbonate buffer (10 mmol/L pH 8.2) containing 0.5 mol of NaCl per liter. Finally, the sample was applied to a column of AFB1-ovalbumin bound to CNBr-activated Sepharose 4 B, where the antiserum was allowed to react with the immobilized antigen for 2 h at room temperature. The column was extensively washed with the 10 mmol/L carbonate buffer. Specifically bound antibody was eluted with 1 mol/L NaCl in sodium citrate buffer (0.1 mol/L pH 4.5). The pooled fractions were dialyzed against 2.5 mmol/L carbonate buffer, pH 9.3, and finally concentrated by dialysis against PEG to give a concentration of 1 g/L.

Antibody labeling with diethylenetriamine pentaaacetic acid–Eu3+. We followed the method of Krejcarek and Tucker (26), as modified by Bador et al. (27). Add dropwise to 6 mg of diethylenetriamine pentaaacetic acid anhydride a fraction of the purified antibody in carbonate buffer (0.5–1 g/L). Vigorously mix the solution for 1 min, adjust to pH 7,
and then let it react for 1 h at room temperature. Remove excess diethylaminoethyl pentaacetic acid by dialysis in citrate buffer (50 mmol/L, pH 6.0). Add the derivatized antibody to 0.5 mL of a 5 mmol/L solution of EuCl₃ in the citrate buffer, then let further reaction proceed, with mixing, for 1 h at room temperature. Finally, fractionate the resulting mixture on a 2 × 30 cm column of G50 Sephadex, eluting with 50 mmol/L potassium phosphate buffer, pH 8.3, at a flow rate of 1 mL/min. Monitor the absorbance of the eluate at 280 nm. The labeled proteins are eluted in the second peak. In our hands, under the experimental conditions described, an Eu/protein molar ratio of 8 was achieved. After purification, we stored aliquots of the labeled antibody at −20 °C, a temperature at which there is no decrease in reactivity for at least four months.

**Time-resolved fluorimunoassay.** Coat each well in 12-well polystyrene microtiter strips with 6.8 ng of AFB₁-ovalbumin conjugate in 200 μL total volume by incubation for 48 h at room temperature. The plates may then be stored in the dark, in a moist atmosphere, for two months, with no detectable decrease in efficiency. Before use, wash the strips five times with PBS containing 0.5 mL of Tween 20 per liter, and finally with water. To perform the assay, mix 1500-μL aliquots of standard aflatoxin (1, 2, 5, 10, 20, and 50 μg/mL) with 1500 μL of a 0.5 μg/mL solution of Eu-labeled antibody in potassium phosphate buffer (50 mmol/mL, pH 8.3) and incubate for 20 min at room temperature. Pipette 200-μL aliquots of this mixture into each well and allow the immunoreactions to proceed in the dark for 1 h. Wash the plates as described above, and pipette into each well 200 μL of enhancement solution: per liter, 0.1 mol of sodium acetate, pH 4.46, 0.5 mol of NaCl, 1.6 mmol of thenoyltrifluoroacetone, 110.5 μmol of Adogen 464, and 1 mL of Tween 20. (For characterization and performance of this solution see ref. 28.) Shake the strips for 15 min to ensure maximum development of fluorescence. Measure the Eu²⁺ emission in the micellar solution with the fluorometer with time resolution (λₑx 340 nm, λₑm 613 nm, counting time 1 s). In a typical competitive assay (Figure 1) with AFB₁ in the 2 pg/mL–4 ng/mL concentration range, the fluorescence counts (expressed as log counts) will range from 0.7 to 2.6. For comparison, we quantified the same analyte with ELISA as previously described (17).

**Results and Discussion.**

The rabbit antiserum we used in this study was antiserum C developed by Sizaret et al. in 1982 (29). The antiserum was purified and labeled with the Eu³⁺ chelate as described above. The affinity purification procedure described here, involving the use of a pre-purification step for selection of molecules that primarily recognize protein epitopes, presents some advantage in that it promotes selection of a highly specific population of immunoglobulins directed against the antigen. Furthermore, we used a gentle method of purification to avoid damaging the immunoglobulins, as can occur for instance by use of too-strong conditions for desorption of proteins from the affinity chromatograph, resulting in impaired function of the antibodies. The relatively mild procedure we used did not result in inactivation of the antibody released from the column.

Figure 1 shows a typical standard curve for the assay of AFB₁. The assay can be carried out in the range from 4 pg to 4 ng of AFB₁ per milliliter, with percent inhibition ranging from 15% to 78%, 50% inhibition being at 0.2 ng/mL.

We validated the assay for AFB₁ by comparing its performance with that of the ELISA. Duplicate samples of AFB₁ were analyzed by the two methods. A linear relationship was obtained, and the correlation is represented by the equation (y = present assay, x = ELISA) y = 0.94x + 0.0002; n = 11; r = 0.975. Each point was a mean of five independent determinations, and concentrations of AFB₁ samples were chosen to correspond to the range of sensitivity in both assays: 0.001–2 ng/mL.

Given the broad range of specificity of the antibody to AFB₁ (17, 29, 30), the various aflatoxins and their metabolites (M₁, M₂, G₁, G₂, Q₁, P₁, and their DNA or protein adducts) can be quantified by this method, although the purified Eu-labeled antibody demonstrates a slightly different affinity towards some metabolites with respect to the unlabeled antiserum (Table 1). The method described can, in principle, be applied to other antibodies to aflatoxins, or to antibodies recognizing other mycotoxins, and thus serve as a general approach to immunoassay quantitation.

As reported in Table 1, the affinity of the Eu³⁺-labeled antibody is slightly less than that of the purified antibody (31). This fact, along with the claimed difficulty of comparing assays performed with antibodies labeled with europium ions, because of the different Eu/protein ratios obtained in laboratory procedures, has suggested that the use of a second antibody or of an antibody-binding protein labeled with Eu ions might serve as general probe in this type of assay (32). We investigated the use of a second Eu-labeled antibody (data not shown) and found that such a procedure decreased assay sensitivity while increasing the complexity of the assay.

**Table 1. Specificity of Antibody to AFB₁**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>ELISAᵇ</th>
<th>ELISAᶜ</th>
<th>Present assayᵃ</th>
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</thead>
<tbody>
<tr>
<td>AFB₁</td>
<td>0.7</td>
<td>0.16</td>
<td>0.20</td>
</tr>
<tr>
<td>AFM₁</td>
<td>0.90</td>
<td>0.20</td>
<td>0.24</td>
</tr>
<tr>
<td>AFM₂</td>
<td>1.64</td>
<td>0.37</td>
<td>0.55</td>
</tr>
<tr>
<td>AFG₁</td>
<td>30.6ᵃ</td>
<td>3.05</td>
<td>5.6</td>
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</tbody>
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* Amount of inhibitor required to inhibit antibody binding to solid-phase antigen by 50%. ᵆ Unpurified antibody. ᵇ Purified antibody. ᵈ Purified and modified antibody. ᵉ From RIA data of Sizaret et al. (29).
The present assay allows aflatoxin detection with a simple, quick, and accurate method that improves on the performance of the previous ELISA. Because it is a one-step assay, it is quicker and also less subject to errors induced by a frequent manipulation of samples.

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References