Laser-Excited Immunofluorometric Assay of Prolactin, with Use of Antibodies Coupled to Lanthanide-Labeled Diethyleneetriaminepentaacetic Acid

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We describe an immunofluorometric assay for prolactin based on lanthanide labeling of a monoclonal antibody and measuring time-resolved fluorescence. In this "sandwich"-type assay, the label (Eu\(^{3+}\)) was bound to the second antibody by means of a simple, rapid method involving the anhydride of diethyleneetriaminepentaacetic acid. To measure the photoluminescence of europium (or other lanthanides), we have developed a time-resolved fluorometer with a nitrogen laser as the pulsed excitation source. During the assay, the solid-phase antibody immobilized inside a polystyrene tube is incubated with the plasma sample and the second antibody in a one-step procedure. Results for 67 human plasmas correlated well (r = 0.98) with those by an immunoradiometric method.

Additional Keyphrases: time-resolved immunofluorometric assay · monoclonal antibodies · hormones · europium label · immunoradiometric assay compared

In the past few years, the sensitivity of fluorimmonunoassay techniques has been greatly increased by the use of fluorophors with long excited-state lifetimes. Such fluorophors can be detected in very low concentrations by means of a pulsed excitation source coupled to a time-resolved detection system. With an appropriate delay time until measurement, one can reject the optical noise originating in scattered radiation and in unwanted fluorescence (from sample and cuvette). By thus improving the specificity of the signal, the detection limit of long-decay-time probes is several orders of magnitude less than that of conventional fluorophors.

Because of their fairly long fluorescence lifetime (\(\approx 1\) ms), certain lanthanide metal chelates were suggested (1, 2) as potential labels for nonradioisotopic immunooassay. In 1982-1983, the first time-resolved fluorimmonooassays (TRIMAs) described in the literature were successfully applied to determinations of human choriongonadotropin (3), pancreatic phospholipase A\(_2\) (4), hepatitis B surface antigen (5), and rubella antibodies (6). In each case, europium (Eu\(^{3+}\)) was attached to an antibody via an EDTA derivative. TRIMAs can be performed as immunofluorometric "sandwich"-type assays or as solid-phase competitive techniques (7), according to the size of the analyzed antigen. In both, after an extraction step, europium photoluminescence is induced by excitation with a xenon flashtube and measured by photon counting (8).

More recently, Kuo et al. (9) have described another procedure, in which antigens are labeled with terbium (Tb\(^{3+}\)), for IgG determination. This competitive immunoassay does not require washing and extraction steps. Terbium is directly excited in the incubation medium by a helium-cadmium laser beam mechanically interrupted by a light chopper. Unfortunately, the sensitivity of this technique is limited by the weak efficiency of the chelating agent for inducing terbium luminescence.

An alternative to the use of rare-earth chelates in homogeneous TRIMAs has been developed by Sidki et al. (10). Antigens are labeled with erythrosin, which is easily detectable by time-resolved luminescence spectrometry. This simple "phosphoroimmunoassay" has the potential to become an efficient tool in drug monitoring.

Despite the rapid development of laser technology in the past decade, pulsed lasers have not yet been routinely used in clinical chemistry. Here we describe a laser-excited time-resolved immunofluorometric assay (TRIMAs), based on a simple and efficient antibody-labeling method with diethyleneetriaminepentaacetic acid (DTPA) as the chelating agent of europium. After the assay, a pulsed nitrogen laser is used to excite the europium, which has been dissociated from the second antibody to form a luminescent chelate with a \(\beta\)-diketone (11). We illustrate this method by applying it to the determination of prolactin in human plasma.

Materials and Methods

Instrumentation

To measure concentrations of lanthanide ions with a great sensitivity and a very low detection limit (down to \(10^{-18}\) mol), we have developed a laser-excited time-resolved fluorometer. The principle of the apparatus is shown schematically in Figure 1.

The excitation source consists of a nitrogen laser (Model LA 04; ISA Jobin-Yvon, Longjumeau, France), which emits at 337.1 nm and has a pulse duration of 8 ns, a repetition rate of 50 Hz, and a maximum peak power of 400 kW. The undesirable nonlaser wavelengths are eliminated by mounting an interference filter (337 nm, 10 nm full-width at half-maximum, 30% peak transmission) in front of the sample compartment. The sample cells are cylindrical, round-bottom polystyrene tubes with a 300-\(\mu\)L useful volume and a 6.5-mm optical pathlength. To select the main emission band of the fluorophor, we use a combination of a sharp-cut filter and an interference band-pass filter (for europium: 613 nm, 10 nm full-width at half-maximum, 52% peak transmission). This selective system, well suited for measuring low intensities, may be easily replaced by a grating monochromator for spectroscopic studies.

Emission from the specimen is detected by a side-window photomultiplier tube (Model R9283; Hamamatsu, Hama-

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5 Nonstandard abbreviations: TRIMAs, time-resolved fluorimmonooassay; TRIMAs, time-resolved immunofluorometric assay; DTPA, diethyleneetriaminepentaacetic acid.

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Reagents

DTPA anhydride and human gamma globulin (Cohn Fraction II) were purchased from Sigma Chemical Co., St. Louis, MO 63178. Tri-n-octylphosphine oxide was obtained from Merck AG, Darmstadt, F.R.G. Europium chloride (EuCl₃) was from Aldrich-Chemie-SARL, Strasbourg, France. β-Naphthyltrifluoroacetone was synthesized as described by Reid and Calvin (22).

Prolactin standards, polylysine tubes coated with the first monoclonal antibody to human prolactin (antibody 1), and the second monoclonal antibody (antibody 2) for europium labeling were kindly donated by Biömérieux, Charbonnières les Bains, France. All these reagents were included in the immunoradiometric assay kit from Biömérieux (126; Prolactine Costria; cat. no. 6 682 0).

The assay buffer used for the one-step procedure is a Tris HCl solution (50 mmol/L, pH 7.75) containing 9 g of NaCl, 5 g of bovine serum albumin, 0.5 g of Na₂EDTA, 0.5 g of gamma globulin, 25 μmol of EDTA, and 0.1 g of Tween 40 polyoxyethylene (40) sorbitan monolaurate surfactant per liter.

The washing solution used for rinsing the assay tubes was prepared by dissolving 0.22 g of Tween 20 polyoxyethylene (20) sorbitan monolaurate surfactant in 1 L of doubly distilled water; the pH was adjusted to 8.0 by adding Tris.

The extraction solution for fluorescence measurements of europium was modified from that of Hemmilä et al. (11): the solution contained 10 μmol of β-naphthoyltrifluoroacetone, 40 μmol of tri-n-octylphosphine oxide, and 0.1 mL of Triton X-100 per liter of doubly distilled water; the pH was adjusted to 3.5 with citric acid.

Procedures

Preparation of labeled antibody. To attach Eu³⁺ ions to antibodies simply and efficiently, we have modified the method previously reported by Krejcírek and Tucker (19) and applied by others (14-17) for radioactive labeling of various proteins. We add 200 μL of antibody 2 solution (about 10 mg/mL) to 1 mg of DTPA anhydride (200-fold molar excess of anhydride to protein), adjust the pH to 7.0 with dilute NaOH, and vortex-mix for 1 min. After incubating the solution for 5 min at room temperature, we separated the coupled antibody from free DTPA by two consecutive dialyses: the first for 4 h at 4 °C against 1 L of citrate buffer (50 mmol/L, pH 6.0), the second against another liter of citrate buffer for 24 h at 4 °C.

To label the antibody, we add 100 μL of a 33 mmol/L solution of europium chloride in citrate buffer (50 mmol/L, pH 6.0) to the purified solution and incubate for 30 min at room temperature with gentle agitation. The europium-labeled antibody is then purified by size-exclusion chromatography on a 22 × 1.5 cm column of Sephadex G 50 (Pharmacia, Uppeala, Sweden).

To determine the conjugation yield (number of Eu³⁺ per molecule of antibody 2), we measure the antibody concentration by Lowry's method for protein (18) and the europium concentration by time-resolved fluorometry at 613 nm. Under the above conditions, we obtain an average ratio of 1.1 Eu³⁺ ion per molecule of antibody, with negligible effect on antibody affinity.

Collection of patients' specimens. Blood samples were collected by venipuncture from ostensibly normal patients (seven men and 13 women, ages 20 to 45 years), from patients with pituitary prolactinomas (two men and six women), and from various patients undergoing a prolactin-stimulation test with thyrotropin-releasing hormone. Tubes containing lithium heparin were used for collection. After centrifugation, plasmas were removed and stored in aliquots at −20 °C to avoid successive thaws.

Time-resolved immunofluorometric assay. Transfer 50 μL of standard or unknown plasma sample into the coated tubes in duplicate, and add 250 μL (0.8 pmol) of Eu³⁺-labeled antibody 2 in assay buffer. Incubate for 3 h at room temperature with continuous shaking, then aspirate and discard the contents of each tube. Wash each tube four times with 500 μL of washing solution, drain, then add 300 μL of extraction solution, which dissociates the europium bound to the solid phase and allows the fluorescent chelate to form. Agitate the sample for 30 min at room temperature, then measure fluorescence by counting photons with the time-resolved fluorometer (delay time, 200 μs; counting time, 1 ms per cycle; integration over 500 cycles).

Results

Optimization of europium detection. In this immunofluorometric assay, europium, which is initially bound to the second antibody via the chelating agent DTPA, cannot be directly excited on the solid phase. After washing the assay tubes, one must displace the Eu³⁺ ions from DTPA to a luminescent chelate in a micellar phase. The highest intensity is obtained with β-naphthoyltrifluoroacetone mixed
with a synergistic agent (tri-n-octylphosphine oxide) in Triton X-100 micelles, according to the method proposed by Hemmilä et al. (11) and used in the Delta system (Dissociation Enhanced Lanthanide Fluorescence Immunoassay; LKB-Wallac, Turku, Finland).

Under these chelating conditions, the luminescence lifetime of europium was determined to be $\tau = 430 \mu s$ at 613 nm. Figure 2 shows the decay curves of both europium and optical noise at 613 nm, and the increase of the signal-to-noise ratio as a function of the delay time. As is obvious from these curves, a delay time of several tens of microseconds is required for a total rejection of the optical noise, which is principally luminescence from the polystyrene cuvette. In integration mode, we achieved the optimal signal-to-noise ratio by using a 200-μs delay time followed by 1000-μs gate time. Such a time-resolved detection provided a background rate of 104 (SD 2) counts/s for distilled water and 96 (SD 3) counts/s for a plasma blank. If the detection limit is the amount of label that yields a signal exceeding the background by 3 SD, as little as $3 \cdot 10^{-18}$ mol of Eu$^{3+}$ can be detected in each cuvette (sample size, 300 μL).

Precision of the label analysis was evaluated by testing 12 replicates for various concentrations of europium in the extraction solution. The CVs of the intensities in separated tubes were 0.6%, 0.9%, and 6.0% for $10^{-10}$, $10^{-11}$, and $10^{-13}$ mol/L, respectively.

Calibration curves of prolactin. Figure 3 shows a typical dose–response curve for human prolactin. The fluorescence intensity, corrected from the zero sample signal, is nearly linear over a wide concentration range of standards. Assuming that the adsorption of dissociated Eu$^{3+}$ ions is negligible, owing to the presence of EDTA in the assay buffer, the europium signal from nonspecific binding of antibody 2 to the solid phase can be calculated by subtracting the counting rate for the blank from the counting rate for the zero sample. This net zero signal is 625-fold lower than the fluorescence intensity of the highest standard.

The minimum detection limit of prolactin in the assay, defined as the concentration at which the net intensity differs significantly from that for the zero sample, was calculated from 10 replicate measurements at zero dose. With 99% confidence, we estimated the detection limit of prolactin to be 0.18 ng/mL in plasma.

Precision. The reproducibility of this prolactin TRIFMA was studied by assaying eight replicates of plasma specimens at various concentrations. The within-assay CVs at 4, 8, 35, and 100 ng/mL were 3.1, 5.6, 6.0, and 4.6%, respectively.

Interference. Tests for possible effects of hemolysis and icterus showed that only strongly hemolyzed plasmas induced a significant decrease of the results; the presence of hemoglobin at low concentration or of bilirubin were without undesirable effect.

Correlation with a conventional immunoradiometric method. Using the laser-excited TRIFMA, we measured prolactin in 67 patients' plasmas in which prolactin had already been determined by an immunoradiometric assay procedure involving the same antibody system (antibody 1 and antibody 2) and the same standards. The samples were selected to include a wide range of concentrations including normal (men, 2 to 14 ng/mL; women, 4 to 22 ng/mL) and pathological values. As Figure 4 shows, the correlation between the two immunometric assays was good ($r = 0.98$), although the results were slightly lower by the TRIFMA method.

Discussion

Several TRIFMA based on the use of lanthanide labels have
been already reported (3–7, 9, 19). The present method, however, is a new approach in this promising field, in terms of both instrumentation (use of a pulsed laser source) and antibody labeling.

The use of a nitrogen laser in the TRIFMA offers several notable advantages over discharge lamps. The first is the high power of its output (photon flux) at the wavelength (337.1 nm) that corresponds to the maximum excitation of the β-naphthyltrifluoroacetone chelate of europium. In conjunction with monochromaticity, the good spatial definition of the beam minimizes stray radiation that would contribute to the optical noise. Moreover, a nitrogen laser operates with pulse widths of roughly 10 ns; this good temporal resolution affords an additional degree of selectivity in time-resolved detection of fluorophors with long excited-state lifetimes. All these features enable the production of very intense specific signals (great sensitivity) without a concomitant increase of background. Under these conditions, the limiting factor of the antigen-detection limit in TRIFMA is not the label detectability but only the nonspecific binding of the labeled antibody. Another advantage of a nitrogen laser is its great stability from pulse to pulse; consequently, no complicated optical reference system is required to maintain convenient reproducibility of the fluorescent signal. Finally, the power of the laser output may be easily adjusted to the required excitation according to the analytical situation. For example, these prolactin measurements never involved use of the maximum laser power. Despite continuing improvements, however, pulsed ultraviolet-wavelength lasers still have two drawbacks for clinical use: cost and size.

The preceding lanthanide-labeling methods applied to time-resolved fluoroimmunoassays were generally modifications of the technique reported by Sundberg et al. (20), who used derivatives of phenyl EDTA. Although these methods provide efficient labeling, they involve complicated, time-consuming syntheses. The coupling and labeling procedure we used had previously been well studied for use in attaching radiolabels (14–17). We considered that this approach would provide a simple, direct, and rapid route for labeling monoclonal antibodies, since DTPA anhydride is a commercially available reagent. Under the particular conditions reported here, we were able to incorporate an average of 1 Eu³⁺ per molecule of antibody. As is clearly shown by our results, such a molar ratio is quite sufficient for the prolactin immunoassay, which does not require great sensitivity. However, by modifying the procedure (greater excess of DTPA anhydride and longer incubation) we obtained a 7:1 molar ratio of label to antibody. Of course, such a high incorporation may affect the immunological activity of the antibody, which is why the gain in specific counting is not proportional to the labeling ratio. For example, increasing the europium incorporation from 1 mol/mol to 7 mol/mol, enhanced the assay sensitivity (net signal per concentration unit of prolactin) by only threefold.

The present results rely on both the excellent quality of the monoclonal antibody system and the specificity and reproducibility of the fluorescent signal detection. Because laser-excited TRIFMA provides a means of measuring prolactin with a sensitivity greater than that of the corresponding radioactive method, we conclude that this type of immunoassay has the potential to be easily extended to most of the polypeptides and proteins.

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References


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