Direct Measurement of Antigens in Serum by Time-Resolved Fluorimmunoassay

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A long-lived fluorescence label (Tb3+) has been attached to the antigen of interest by using a bifunctional chelating agent 1-(p-benzenediazonium)-EDTA. A nonequilibrium competitive-binding immunoassay protocol, in conjunction with time-resolved detection of the long-lived fluorescence label, allows the antigen to be analyzed directly in samples containing diluted human serum. Results obtained for immunoglobulin G with this simple and rapid procedure correlated well (r = 0.93) with those by a commercially available fluorescence immunoassay method.

The fairly strong natural fluorescence of serum and antiserum samples is one of the main obstacles preventing the replacement of radioimmunoassay (RIA) by fluorescence immunoassay (FIA). Because of this high background, the detection limits for fluorescence-labeled proteins measured in serum samples are typically 100- to 1000-fold higher than in a pure buffer. A way commonly used to overcome this problem is to isolate the labeled protein by using various separation and washing steps that generally make the analysis procedure complex, time-consuming, and difficult to automate. An alternative approach, suggested by Wieder (1) and by Soini and Hemmilä (2), is to label the protein with a long-lived fluorophore and to use time-resolved detection to reject the short-lived fluorescence background from serum. Although this procedure depends heavily on the choice of a suitable fluorescent tag, certain chelates of europium and terbium appear to fulfill the requirements of long lifetime (~1 ms) and high fluorescence yield (1-4). Time-resolved fluorimmunoassay has been used successfully in analysis for human chorionic gonadotropin (5) and human pancreatic phospholipase A2 (6). In both of the studies, an immobilized antigen–antibody complex was reacted to form a "sandwich" with a second antibody that contained a europium-chelate label. The europium ion was then extracted from the bound sandwich complex and was converted to a β-diketone chelate in buffer solution before fluorescence was measured.

We report here the use of a simpler procedure, in which the long-lived fluorescence from a rare-earth chelate is measured directly in serum-containing samples. The terbium label is attached to the protein antigen via the bifunctional chelating agent 1-(p-benzenediazonium)-EDTA. The labeled antigen and an unknown amount of unlabeled antigen are allowed to react with an immobilized antibody in a classical nonequilibrium competitive-binding immunoassay. By measuring the long-lived fluorescence of the labeled antigen remaining free in solution, we can quantitatively determine the concentration of antigen present in the original serum sample. This method is illustrated by application to immunoglobulin G (IgG), a commonly occurring protein that is present in normal human serum at a typical concentration of 12 g/L (80 µmol/L). We chose this antigen because it is readily available and can be measured with well-established fluorescence immunoassay procedures (7).

Materials and Methods

Reagents

Human IgG (lot no. 1-4506) and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO. Immobilized rabbit anti-human IgG "Immunobeads" (lot no. 26103) were from Bio-Rad Laboratories, Richmond, CA, as was the standard fluorescence immunoassay kit for IgG (Immuo-Fluor, lot no. 26276).

Several buffers were used in the fluorimmunoassay. Buffer A contained 10 mM of KH2PO4, 150 mM of NaCl, and 10 µmol of TbCl3 per liter; the pH was adjusted to 7.5 with aqueous NaOH (500 g/L). Buffer B was made by reconstituting 0.2 g of lysophilized bovine serum albumin (BSA) in 20 mL of buffer A. Buffer C was made by adding 1 mL of 10 mM/L TbCl3 solution to 5 mL of buffer A.

Procedures

Preparation of labeled antigen. Diazophenyl-EDTA was synthesized by a modification of the procedure of Sundberg et al. (8) suggested by Meares (9). The compound was stable in concentrated hydrochloric acid at −80 °C for several months. The chelating agent was neutralized to pH 5 before use by sequential addition of aqueous NaOH solutions (5, 1, 0.5, and 0.1 mol/L). The antigen, human IgG (10 mg in 1 mL of buffer C), was added dropwise to 50 µL of neutralized diazophenyl-EDTA (20 mM/L), and the pH was adjusted to 8.5 with dilute NaOH (0.1 mol/L). The coupling reaction was allowed to proceed for approximately 20 h at 0 °C. Under these conditions, four label molecules, on the average, were bound to each antigen. The labeled antigen was then separated from excess reagents and terbium ion by size-exclusion chromatography (Sephadex G-50 column, 18-mL bed volume), monitored at 254 nm with an ultraviolet absorbance detector. The first fraction eluted, faintly yellow in color, corresponded to the labeled IgG. This fraction was collected and the concentration of labeled IgG was determined by using a standard fluorescence immunoassay method. The labeled antigen may be stored at 4 °C for several months without loss of immunological activity.

Immunobeads. Immunobeads coated with rabbit anti-human IgG antibody were reconstituted in buffer A, and an aliquot corresponding to 2 mg of Immunobeads was added to each sample tube. The sample tubes were centrifuged, decanted, and washed with 200 µL of buffer B. The exposure of the Immunobeads and sample tubes to the inactive protein (BSA) in buffer B appeared to reduce nonspecific binding. A 50-µL aliquot of a serum sample (diluted 600-fold...
in buffer A) or a standard IgG solution (0.03 to 0.8 μmol/L) was added to the solid-phase-bound antibody, and the mixture was incubated for 20 min at room temperature. A 0.15 μmol/L solution of labeled IgG was then added, and the samples were incubated for an additional 70 min at room temperature. The reaction was terminated by centrifugation to separate the immobilized antigen–antibody complex from the supernatant liquid, which was subsequently analyzed by time-resolved laser fluorimetry.

Apparatus

The time-resolved photon-counting fluorimeter is shown schematically in Figure 1. As the excitation source we used a helium–cadmium ion laser (Model 4050B; Liconix, Sunnyvale, CA), with continuous-wave output at 325 nm (5–10 mW). The laser beam was periodically interrupted at 500 Hz by a variable-frequency light chopper (Model 192; EG & G Princeton Applied Research, Princeton, NJ) before passing through the sample cell, which was a quartz cuvette with 1-cm pathlength and 0.2 cm width (Micro Spectro Cell 8495-E20; Thomas Scientific, Philadelphia, PA). A fast photodiode monitored the chopped laser beam and triggered a pulse generator (Model 8010; Berkeley Nucleonics Corp., Berkeley, CA) that opened the gate of the photon counter after a suitable preset delay. Sample fluorescence, collected perpendicular to and coplanar with the excitation beam, was spectrally isolated by an interference filter (550 nm, 40 nm bandwidth) and was then detected by a photomultiplier tube (Centronic Q 4249 BA; Bailey Instrument Co., Saddle Brook, NJ). The output of the photomultiplier was processed by a discriminator, photon counter, and rate meter (Models 438, 871, and 449, respectively; EG & G Ortec, Oak Ridge, TN), and finally was displayed on a stripchart recorder (Model 7100B; Hewlett-Packard, Palo Alto, CA).

The optical system was designed so that the sample was exposed to the laser beam only when the photomultiplier tube was blocked, whereas the photomultiplier tube detected sample fluorescence only when the laser beam was blocked from the cuvette. This timing arrangement, accomplished by two displaced sets of apertures in the light chopper wheel (see Figure 1), prevented saturation of the photomultiplier tube by stray laser light or by short-lived fluorescence from the serum sample. The photodiode and photomultiplier outputs were monitored simultaneously by an oscilloscope (Model 1740A, Hewlett-Packard) in order to optimize the sequence and duration of timing events in the measurement of long-lived fluorescence. The optimized timing sequence for the Tb–EDTA chelate was as follows: 400 μs excitation, 100 μs delay, 1000 μs photon counting, and 500 μs delay and recovery time. Thus, the entire measurement cycle was 2000 μs, half of which involved fluorescence photon counting. For a typical sample measurement, many timing cycles were integrated to yield a total photon counting period of 1 min.

Results and Discussion

Time-resolved fluorimunoassay is a new and promising methodology that combines the sensitivity of fluorescence with the specificity of immunoassay. In this procedure, nonspecific fluorescence is minimized by using a long-lived fluorophore and measuring the label fluorescence after the short-lived background luminescence has decayed. Although this methodology is still in its infancy, its potential for the replacement of RIA methods is clear. Our intent in this work was to demonstrate the simple, rapid measurement of antigens in serum-containing samples rather than to provide an optimized protocol for clinical application.

We have chosen the rare-earth ion Tb³⁺ as a long-lived fluorescence label (τ = 1.01 ms), which is attached to the antigen of interest via the bifunctional chelating agent 1-(p-benzenediazonium)-EDTA. Long-lived fluorescence is brought about indirectly by excitation of the aromatic moiety in the chelating agent [250–350 nm (10)], followed by rapid intramolecular energy transfer to the lanthanide ion. The Tb–EDTA chelate fluoresces between 480 and 630 nm, with maximum emission intensity at 550 nm (10). Although this chelate is quite stable in aqueous solution at room temperature [log K = 17.7 (11)], displacement by other strongly bound metal ions such as Cu²⁺ (log K = 18.8) and Fe³⁺ (log K = 25.1) may be a potential chemical interference. Disproportionation of the Tb–EDTA chelate was diminished by minimizing contact with metal surfaces, and by adding excess Tb³⁺ ion to the buffer solutions. Free terbium ion itself has a low molar absorptivity at 325 nm, the excitation wavelength used here, and did not contribute substantially to the long-lived fluorescence signal. In addition, we found that the long-lived fluorescence of the Tb–EDTA chelate was relatively insensitive to common quenching agents, such as dissolved oxygen (0–2.2 mmol/L) and chloride ion (0.2–1 mol/L), and to changes in sample temperature (0–37 °C).

Figure 2 shows the detection limit and linear dynamic range for the Tb–EDTA chelate measured by time-resolved laser fluorimetry. This calibration curve was obtained by subtracting the background photon counting level from the response for various concentrations of Tb–EDTA chelate in standard buffer and serum-containing samples. The background signal for a standard phosphate buffer solution was 400 counts per minute (cpm) with a standard deviation of 20 cpm, whereas that for human serum diluted 600-fold in buffer B was 700 cpm, with a standard deviation of 30 cpm. The detection limit was defined here as the concentration of Tb–EDTA label that yielded a signal exceeding the background by four standard deviations. For both standard buffer and serum-containing samples the minimum detect-
able concentration was $3 \times 10^{-10}$ mol/L, which corresponded to $6 \times 10^{-14}$ mol of labeled antigen per sample tube. As demonstrated in Figure 2, the linear dynamic range covered at least five orders of magnitude, which may be a useful feature for many biomedical and clinical applications.

Our analytical procedure was based on a nonequilibrium competitive-binding immunoassay, in which the antibody was exposed first to the unlabeled antigen (human IgG), then to a known amount of labeled antigen, and the reaction was interrupted before completion. We used this procedure because it increased the sensitivity of the assay method at low concentrations of unlabeled antigen (12). The IgG capacity of 2 mg of the Immuno-beads is stated by the manufacturer to be $1.2 \mu g$, and the known amount of labeled IgG was $2.3 \mu g$ per sample tube. Under these conditions, the unknown amount of unlabeled IgG could range between 0.4 and 11.6 $\mu g$ per sample tube. Because the concentration of IgG in normal human serum ranges between 5 and 14 g/L, we diluted the sample 600-fold so that the concentration of unlabeled antigen was within the most sensitive region of the sigmoid curve shown in Figure 3. Within-run variability was approximately 5% in this region, and the form of the standard curve did not differ appreciably from run to run.

Using human serum samples obtained from a local blood bank, we made a double-blind comparison of the present time-resolved fluroimmunoassay and a commercially available sandwich fluroimmunoassay (Immuno-Fluor). Figure 4 shows the results of 43 measurements acquired in five separate assays. The two methods correlated well ($r = 0.93$), with no apparent systematic deviation as a function of antigen concentration. The slope and intercept of the correlation curve were determined by linear regression to be 0.94 and 0.85 g/L, respectively. The small non-zero intercept may represent different amounts of nonspecific binding in the two immunoassays or variations in response to the four different types of IgG present in human serum.

In conclusion: this study demonstrates that a long-lived fluorescent label in conjunction with time-resolved photon counting detection can be used to reject background interference from other short-lived fluorescent species present in biological matrices. Although the serum samples were heavily diluted for the immunoassay of IgG, efficient background rejection was achieved for more concentrated serum samples, diluted as little as 20-fold. This background rejection removes the need for complicated, time-consuming washing and separation steps, and significantly shortens analysis time (to about 90 min). The limits of detection demonstrated here ($3 \times 10^{-10}$ mol/L) are more than adequate for many commonly occurring proteins; however, further gains are necessary before this method can be applied routinely. At present, the sensitivity may be limited by the efficiency of energy conversion by the 1-(p-benzenediazonium)-EDTA chelating agent. This chelate is likely to be a poor choice when compared with the more efficient $\beta$-diketone complexes of rare-earth ions (5, 6, 13). If better suited long-lived labels can be developed, then time-resolved...
fluoroimmunoassay shows much promise for becoming an important alternative to other immunoassay procedures.

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References
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