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A Double Monoclonal Time-Resolved Immunofluorometric Assay of Carcinoembryonic Antigen in Serum

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A non-isotopic heterogeneous non-competitive immunoassay for carcinoembryonic antigen is described. CEA present in serum binds simultaneously to two monoclonal antibodies specific for different antigenic determinants of CEA. One antibody is immobilized on a solid-phase (microtiter well) and the other is biotinylated. The amount of biotinylated antibody bound is measured on the dried solid-phase by time-resolved fluorometry after adding streptavidin labeled with the europium chelate 4,7-bis(chlorosulfophenyl)-1,10 phenanthroline-2, 9-dicarboxylic acid (BCPDA) in the presence of excess Eu^{3+}. The assay is simple to perform, its characteristics are similar to those of other isotopic and non-isotopic techniques, and it is suitable for routine clinical use.

KEY WORDS: non-isotopic immunoassay; time-resolved fluorescence; tumor markers; europium chelates.

Introduction

Carcinoembryonic antigen (CEA) is a tumor associated glycoprotein first discovered in 1965 by Gold and Freedman (1,2). It is expressed in considerable amounts during embryonic life mainly by the large intestine and postnatally by many epithelial-derived tumors. Serial measurements of CEA in serum have been used extensively in monitoring the recurrence or progression of disease, response to therapy, and follow-up management of patients with carcinoma of the colon, breast, lung, prostate, pancreas and ovaries (3–5). Preoperative CEA levels have been recognized to have prognostic significance in cancer patients, but the test is not recommended as a screening procedure to detect cancer in the general population or to establish the diagnosis of cancer. This is due to the fact that increased CEA levels in serum have also been reported in non-malignant inflammatory disorders of the intestinal tract, lung and liver and in chronic smokers (5–7).

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The original CEA immunoassays (8–10) were based on the principles of radioimmunoassay (competitive-type) and employed polyclonal antibodies. However, recent attempts are concentrated towards the development of a methodology that eliminates the use of radiolabeled tracers, and thus freedom from handling, exposure to, and disposal of radioactivity, and other associated disadvantages of radioactive labels. Moreover, polyclonal antibodies are now considered obsolete for CEA quantitation, and monoclonal antibodies in non-competitive (two-site, sandwich type) assay configurations are now widely used. Recent reports suggest that monoclonal antibody-based assays are more specific, with less cross-reactitivies to non-specific antigens and thus more clinically useful (11,12).

Current non-isotopic CEA immunoassays use a detection system that incorporates enzymes (13,14), luminescent (15,16) or fluorescent probes as labels. Europium chelates have recently been introduced as alternative labels to radioisotopic compounds for development of fluorescence immunoassays. The advantages of europium chelates are summarized in recent reviews (17,18).

In this report we present a new time-resolved immunofluorometric assay for the quantitation of CEA in serum using a europium chelate as label. In the assay, CEA simultaneously reacts with two monoclonal antibodies, one of which is immobilized in a microtiter well and the other is biotinylated. The captured immunocomplex is then quantified with streptavidin labeled with the europium chelate using time-resolved fluorescence. The assay is fast (< 4 h protocol), simple and reliable and can be used for the routine measurement of CEA in serum.

Materials and methods

Instrumentation

For solid-phase time-resolved fluorometric measurements, we used the CyberFluor™ 615 Immunoanalyzer. Radioactivity counting was performed with the LKB Wallace 1275 Minigamma counter.

TABLE 1
Monoclonal Antibodies Used in This Study

Clone Code	Ig-Class	Affinity Constant, L/mol	Epitope
5903	IgG_1	6×10^{10}	A
5904	IgM	4×10^{10}	A
5905	IgG_1	1×10^{11}	A
5906	IgG_1	3×10^{10}	D
5907	IgG_1	1×10^{10}	A
5908	IgG_{2b}	6×10^{10}	A
5909	IgG_1	7×10^{10}	C
5911	IgG_1	2×10^{10}	E
5912[a]	IgG_1	2×10^{10}	F
5913	IgG_1	4×10^{10}	B
5914[b]	IgG_1	1×10^{10}	B

[a]This is the 'coating antibody' used in this study.
[b]This antibody was biotinylated and used for detection.

REAGENTS

Chemicals

The europium chelator 4,7 bis(chlorosulfophenyl)-1,10 phenanthroline-2,9-dicarboxylic acid (BDPDA) was synthesized as described in (19). Streptavidin and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, MO 63178. $EuCl_3$ hexahydrate was purchased from Aldrich Chemical Co., Milwaukee, WI 53201. Sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-biotin) was from Pierce Chemical Co., Rockford, IL 61105. All other chemicals were from Sigma. White opaque 12-well microtitration strips were products of Dynatech Labs, Alexandria, VA 22314.

Buffers

The coating buffer was 50 mmol/L Tris solution of pH 7.80. The blocking buffer was a 50 mmol/L sodium phosphate solution of pH 7.4 containing 10 g BSA and 0.5 g sodium azide per liter. The assay buffer was 0.10 mol/L Tris solution, pH 7.80, containing 29.8 g KCl, 10 g BSA and 0.5 g sodium azide per liter. The tracer diluent was a 0.05 mol/L Tris solution, pH 7.80, containing 1 mol NaCl, 40 g BSA and 0.5 g sodium azide per liter. The wash solution was 9 g/L NaCl solution containing 0.5 mL polyoxyethylene sorbitan monolaurate (Tween 20) per liter.

Monoclonal antibodies

A total of 11 murine monoclonal antibodies have been examined in this study. All antibodies were produced by Medix Biochemica, Kauniainen, Finland and purified from ascites fluid by ion-exchange chromatography or affinity (protein A) chromatography. Some characteristics of these antibodies are summarized in Table 1.

Standards

The CEA standards were prepared by dissolving highly purified CEA (from Scripps Labs, San Diego, CA 92103) in a 50 mmol/L Tris buffer, pH 7.50, containing 0.15 mol/L NaCl, 0.05% sodium azide, 6% BSA and 1% bovine globulins. These standards were calibrated using the Tandem-R CEA assay (see below).

SPECIMENS

Human serum samples were provided by Dr. A. Malkin (Sunnybrook Medical Centre, Toronto, Canada). The samples were obtained from individuals who were under investigation for or had confirmed tumors and were stored for no more than 1 week at 4 °C. Lyphocheck immunoassay control serum (human) levels I, II, and III were purchased from Bio-Rad Labs, Clinical Division, Richmond, CA 94801.

PREPARATION STEPS

Preparation of biotin-anti-CEA conjugate

The biotinylation procedure has been described previously (20–22). After dialysis the biotin anti-CEA conjugate (antibody code 5914) was tested at various dilutions to determine the optimal concentration for the assay, and it was stored at 4 °C. Before use, the stock preparation was diluted in the assay buffer to prepare a 5 mg/L solution.

Preparation of the tracer (Streptavidin-thyroglobulin-BCPDA-Eu^{3+})

The preparation of streptavidin-protein conjugates is detailed elsewhere (23). The concentration of streptavidin in the working solution of BCPDA-labeled streptavidin-thyroglobulin conjugate was 0.15 mg/L; that of $EuCl_3$ was 40 μmol/L.

Coating of microtiter wells with anti-CEA antibody

The coating antibody solution was prepared by diluting the monoclonal anti-CEA antibody (Code 5912) in the coating buffer to a concentration of 5 mg/L. We then added 100 μL of the antibody solution per well into wells of microtiter strips and allowed the antibody to adsorb at 4 °C. After overnight incubation, the wells were washed twice with the wash solution, 200 μL of the blocking buffer was added per well and incubated for 1 h at room temperature to block the remaining active sites. If the wells are not used immediately after blocking, they can be stored with the blocking buffer at 4 °C for at least 2 weeks. Before use, the wells were washed twice with the wash solution.

COMPARISON METHODS

We used two commercially available immunoassay procedures: (a) Abbott CEA-EIA Monoclonal (Abbott Laboratories, North Chicago, IL 60064); and (b) Tandem-R CEA (Hybritech Inc., San Diego, CA

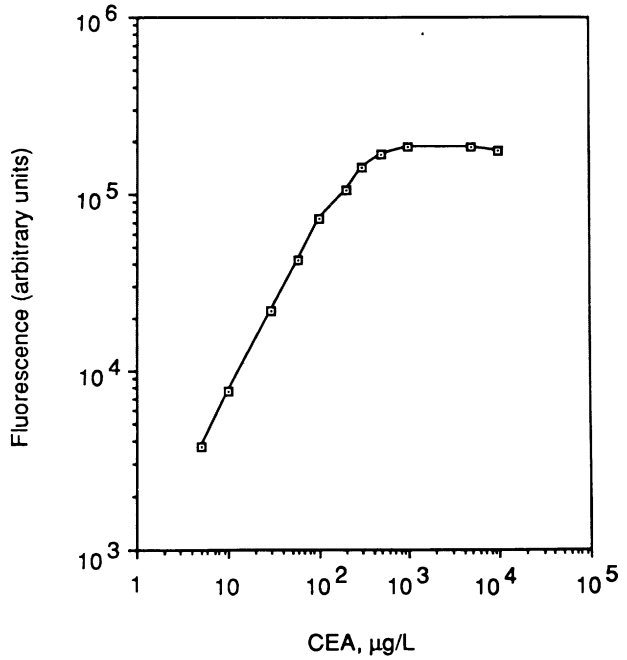

Figure 1—Dose-response curve for the proposed CEA assay.

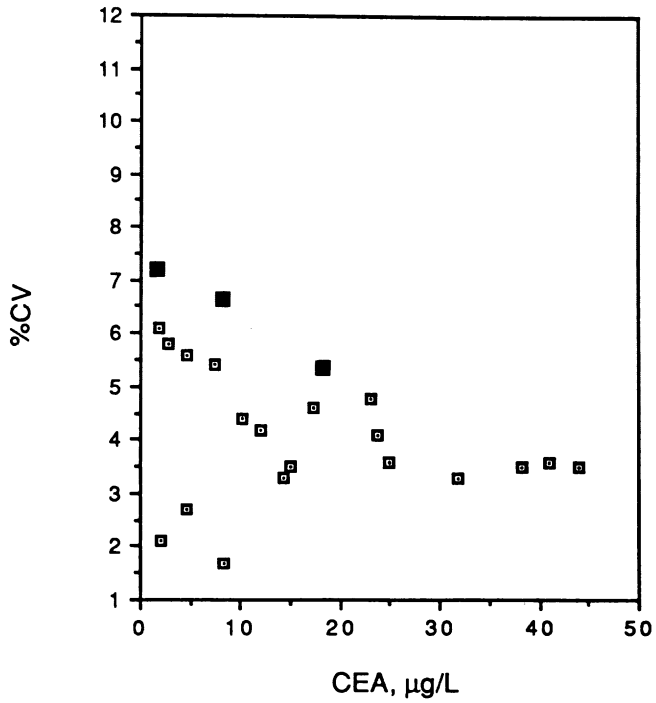

Figure 2—Precision of the proposed CEA assay at various levels of CEA. (⊡) within-run (n = 12 for each CEA level); (■) day-to-day (n = 10).

92121). Procedures recommended by the manufacturers were followed for duplicate measurements of the specimens.

Immunoassay procedure

The assay was performed by adding in duplicate 20 μL of standards, controls or patient serum samples and 100 μL of the biotinylated antibody working solution into the coated wells. The plate was then shaken for 3 h by means of an automated shaking device at room temperature. The reaction mixture was then aspirated and the wells were washed four times with the wash solution using a 12-well aspirating-washing device. One hundred μL of the tracer working solution was then pipetted into each well. After 30-min incubation with shaking at room temperature, the wells were washed as above four times with the wash solution and dried for 10 min with the CyberFluor forced air plate dryer. The fluorescence on the dried solid-phase was measured with the CyberFluor™ 615 Immunoanalyzer which also performed the data reduction.

Results

Detection limit and dynamic range

Figure 1 shows a typical dose-response curve for this assay. The curve is linear up to 100 μg of CEA per liter and there is no significant decrease in signal at concentrations of CEA as high as 20,000 μg/L, indicating that the well-known 'high dose hook effect' is not present at least up to this point. The detection limit of the assay defined as the concentration corresponding to the mean plus three standard deviations of the zero-standard signal, for 12 replicates, was 0.2 μg/L.

Precision

Intra-assay reproducibility was determined for data on 12 replicate analyses of tri-level commercial serum controls and patient samples in the CEA concentration range of 2.0 to 44.0 μg/L. As shown in Figure 2, within-run CV's were ≤ 6% in all cases. Tri-level commercial controls analyzed over a period of 10 days gave CV's ranging from 4.8 to 7.2% (Figure 2).

Analytical recovery

CEA-supplemented serum samples were prepared by adding known concentrations of exogenous CEA (11 to 40 μg/L) to aliquots of pooled serum samples. Analytical recovery was assessed by analyzing the samples before and after the additions and subtracting the estimated endogenous CEA concentrations. Recovery was in the range of 77–109% (Table 2) with a mean ± SD of 96.5 ± 9.6%.

Dilution linearity

This was assessed by using serum samples with high CEA concentrations. The specimens were di-

TABLE 2
Analytical Recovery of CEA Added to Five Sera

Sample	CEA, μg/L Initially Present	Added	Recovered	% Recovered[a]
1	1.97	11.0	11.8	107
		23.0	20.6	90
		40.0	41.6	104
2	2.64	11.0	9.8	89
		23.0	22.4	97
		40.0	39.9	100
3	4.65	11.0	8.5	77
		23.0	23.8	103
		40.0	38.5	96
4	6.34	11.0	8.8	80
		23.0	21.6	94
		40.0	43.7	109
5	2.06	11.0	10.3	94
		23.0	22.8	99
		40.0	43.5	109

[a] $x = 96.5 \pm 9.6$

luted with the zero standard up to 32-fold and then assayed for CEA. As shown in Table 3, the values for the observed concentrations of CEA were close to the expected values derived from the CEA concentrations of CEA in the undiluted samples. The correlation coefficient for a linear regression between observed and expected values was better than 0.97 for each dilution series.

CORRELATION WITH OTHER METHODS

CEA in 149 clinical samples was assayed by the present method (y) and by a commercial (Abbott) EIA kit (x) (Figure 3a). The regression equation was $y = 0.11 + 0.998\ x$, $r = 0.98$. Another series of 22 samples was compared to a different kit (Hybritech) (Figure 3b). The regression equation was $y = 0.48 + 0.97\ x$, $r = 0.91$.

TABLE 3
Linearity: Results for Dilutions of Sera with High CEA Concentration (μg/L)

Sample		Dilution None	1/2	1/4	1/8	1/16	1/32
1	Expected	—	14.6	7.3	3.7	1.9	0.9
	Measured	29.2	14.1	8.2	4.6	2.6	0.9
2	Expected	—	24.2	12.1	6.0	3.0	1.5
	Measured	48.3	23.6	12.5	8.4	3.1	2.0
3	Expected	—	30.2	15.1	7.6	3.8	1.9
	Measured	60.4	30.0	16.5	8.0	4.2	2.1
4	Expected	—	16.0	8.0	4.0	2.0	1.0
	Measured	32.0	17.0	10.2	5.5	2.8	1.1
5	Expected	—	6.0	3.0	1.5	0.8	0.4
	Measured	12.0	6.0	3.1	1.3	0.7	0.4
6	Expected	—	6.2	3.1	1.6	0.8	0.4
	Measured	12.4	6.8	3.7	1.5	0.8	0.5
7	Expected	—	10.0	5.0	2.5	1.2	0.6
	Measured	19.9	9.7	5.2	2.0	1.2	0.7

Discussion

It is clear from the recent literature reports that monoclonal antibody based, two-site immunometric methodologies for CEA offer better specificity and have lower false positive rates (CEA > 5 μg/L) for smoking subjects and subjects with non-malignant disorders other than cirrhosis (12). The other well-known advantages of monoclonal antibodies also contribute to the more rapid analysis and easier assay protocols in comparison to polyclonal antibody based assays.

CEA is known to have about 5–6 antigenic determinants, two of which are peptides (24) and four of which are carbohydrates (25). In our study, we had 11 different monoclonal antibodies available which were grouped in 6 classes (A to F, Table 1). This grouping was based on the ability of the antibodies to form 'pairs' in immunometric type assays where one antibody is immobilized on a solid-support (capture antibody) and the other is labeled (detection antibody). We used all possible combinations of workable pairs to devise 'prototype' CEA assays. Antibodies grouped in the same class (Table 1) cannot form pairs because, presumably, they react with the same epitope. Antibodies forming pairs were either immobilized or biotinylated. We previously used a similar selection strategy to devise a highly sensitive assay for thyrotropin in serum (20). We chose a number of criteria to isolate a preferred pair of antibodies for the CEA assays. In summary, the criteria were as follows: (a) In terms of assay sensitivity, the best pair was the one that gave the steepest dose-response curve and the lowest background signal. (b) In terms of specificity, we selected the pair that most closely correlated with two well-established methodologies, namely, the Abbott and Hybritech kits. However, it should be noted that CEA is heterogeneous and there is variability mainly in the CEA carbohydrate epitopes between patients (25). Thus, variable results can be expected and have been observed between kits that use different monoclonal antibodies as immunoreactants (25).

The detection limit achieved (0.2 μg/L) compares favourably with current non-isotopic CEA assays. We did not observe the 'high-dose hook effect' even at CEA concentrations as high as 20,000 μg/L. The dynamic range of 0–100 μg/L also compares favourably with current techniques, most of which have their highest standard at 60 μg/L. Precision, linearity and recovery data are satisfactory.

The unique feature of the present assay is that it integrates a new time-resolved fluorescence detection method with a 'sandwich' type immunoassay using the biotin-streptavidin system as a link. The biotin-streptavidin system has a number of advan-

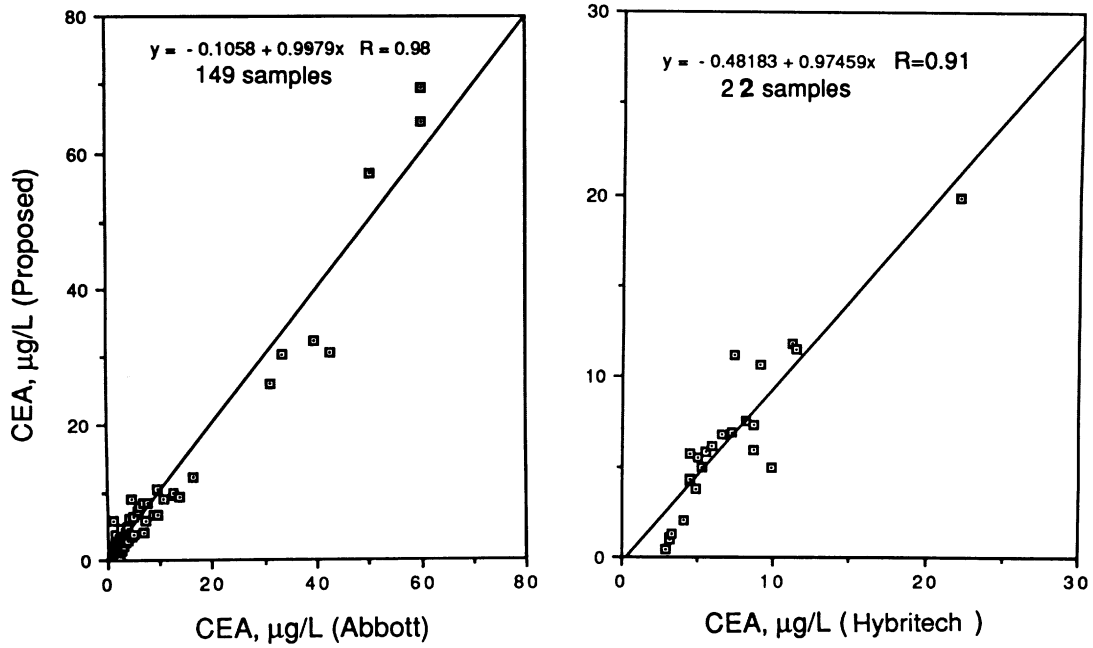

Figure 3—Comparison of the proposed method with two widely used immunoassay procedures; Abbott (left) and Hybritech (right).

tages outlined elsewhere (18). In a modification of this system (23) we have improved the amplification introduced by a factor of at least 5-fold after linking streptavidin to a carrier protein, thyroglobulin, which is labeled with as many as 150 BCPDA residues.

The present time-resolved fluorescence detection system is different from the one reported earlier (17) and has a number of advantages described in detail elsewhere (18). There are no Eu^{3+} contamination problems and the final fluorescent complex remains stable for months on the dry solid-phase thus allowing re-measurement at any time.

In conclusion, we are presenting here a new time-resolved immunofluorometric assay for CEA using monoclonal antibodies. The assay is sensitive, specific with good performance characteristics and has a protocol which is easy to perform. It requires only 20 µL of serum per assay and has two incubation steps lasting 210 min. The final fluorescent product is stable for months. Measurement time per well is only 1 s thus allowing rapid sample processing. The assay is suitable for the routine quantification of CEA in clinical chemistry laboratories.

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