Evaluation of a New Radioimmunoassay Method for Carcinoembryonic Antigen in Plasma, with Use of Zirconyl Phosphate Gel

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A radioimmunoassay of carcinoembryonic antigen in plasma is described and evaluated. The assay can be easily performed and implemented in a clinical laboratory. Assessed by Rodbard's statistical quality-control procedure, the assay is shown to be highly sensitive, precise, and reproducible.

Additional Keyphrases normal values • changes in cancer patients' plasma • diagnostic aid

Carcinoembryonic antigen (CEA), a cancer-specific antigen reported by Gold and Freedman in 1965 (1), is a glycoprotein that is present in malignant entodermal tissue, in fetal colonic mucosa, and in the plasma of patients with gastrointestinal cancer. Recently, CEA has also been detected in the plasma of patients with other malignant diseases (2-4) as well as in the plasma of normal adults (5). The two current methods for radioimmunoasay of CEA in plasma are (a) the original procedure of Thomson et al. (6) and (b) the procedure described by Hansen et al. (7), in which an ion-sensitive antigenic site in the carcinoembryonic molecule is detected. The major difference between the two procedures are: the time and temperature of incubation, and the method of separating free from antibody-bound antigen. In the procedure of Hansen et al., the dialysate need not be lyophilized, the incubation is much shorter, and antibody-bound CEA is removed by zirconyl phosphate gel rather than by a solution of ammonium sulfate (500 g/liter).

Here, we evaluate the specificity and precision of the zirconyl phosphate method, with special emphasis on a quality-control procedure that may be suitably applied to radioimmunoassay of CEA in the clinical laboratory.

Materials and Methods

Principle

CEA, a protein-polysaccharide complex, is extracted from plasma with perchloric acid and dialyzed against distilled and de-ionized water to remove the perchloric acid, ions, and other small molecules. After dialysis, diluted goat anti-CEA antiserum is added to the dialysate and the mixture is incubated to allow the CEA to complex with anti-CEA antibodies. Then radioactive CEA (iodine-125 labeled) is added to the mixture, which is again incubated. Bound CEA is separated from free CEA by adding zirconyl phosphate gel, which precipitates the antibody-bound CEA. The radioactivity in the precipitate is then measured. Bound radioactivity is plotted vs. CEA (in nanograms) in the standards to give a calibration curve from which the concentration of CEA in the plasma extract can be determined.

Samples for Analysis

Ten milliliters of blood is collected in a tube containing sodium ethylenediaminetetraacetic acid ("Evacuated Glass Container," 3240Q; Becton, Dickinson and Co., Rutherford, N.J. 07070). The blood is kept cold and refrigerated within 2 h after collection. Samples are stable for later analysis if the plasma is frozen within 2 h after the blood is drawn. Serum should not be used.

Reagents

Ammonium acetate, 10 mmol/liter. Adjust to pH 6.25 with HCl and NH₄OH.
**Borated buffer, 50 mmol/liter, pH 8.3.**

**Diluted anti-CEA antiserum.** Anti-CEA antiserum, 20 µl, is diluted with 2 ml of normal plasma and 18 ml of borate buffer (1,000-fold dilution). Refrigerate. Stable for at least one month.

**Zirconyl phosphate gel.** Prepare according to the method of Hansen et al. (8). The final slurry is adjusted to pH 6.25 with concentrated ammonium hydroxide.

**Perchloric acid, 1.2 mol/liter.**

**Purified colon CEA and radioactive I251-CEA, 250-300 µC/5 µg** (kindly supplied by Hoffmann-La Roche Inc., Nutley, N.J. 07110) was kept refrigerated.

**Equipment**

*Disposable gamma test tubes,* polystyrene.

*Auto-Gamma Spectrophotometer,* linked to Tri-Carb Scintillation Spectrometer, Model 3380; Packard Instrument Co., Downers Grove, Ill. 60515.

*Samplers and tips,* Oxford or Brinkmann.

**Procedure**

The procedure, with minor modifications, is that of Hansen et al. (7), of which a step-by-step outline follows:

**CEA titration curve.** It is necessary to obtain a titration curve for each preparation of radioactive CEA, to determine the optimal amount of diluted anti-CEA antiserum to be used. The titration curve is constructed as follows: (a) Deliver 10 ml of distilled, and de-ionized water into a disposable polystyrene test tube. (b) Add 0.1 ml of pooled normal plasma to each tube. Mix by use of a vortex-type mixer, and add 0, 10, 20, 50, 100, or 200 µl of diluted antiserum to properly labeled test tube. Mix by vortex. (c) To each tube, add 100 µl of I251-CEA solution (about 400,000 cpm). Mix by vortex, and incubate all tubes at 45°C for 30 min in a water bath. (d) Add 5 ml of zirconyl phosphate gel to each tube, invert three times, then centrifuge at 500 × g for 10 min. (e) Discard the supernatant fluid, and wash the sediment with 15 ml of ammonium acetate solution. (f) Centrifuge again, discard the supernatant fluid, and measure the radioactivity of the sediment in a solid crystal gamma-ray spectrometer. (g) I251-CEA, 100 µl, serves as the total radioactivity in the final calculation. (h) Plot percent bound I251-CEA vs. amount of antiserum to determine the optimal volume to be used in step g of the following procedure and subsequently for the radioimmunoassay.

**Radioimmunoassay of CEA.** (a) To a mixture of 1 ml of plasma and 3 ml of saline (9 g/liter) add 4 ml of perchloric acid solution (1.2 mol/liter). Immediately mix by vortex for 30 s. (b) For the CEA standard curve, use pooled plasma from six to 10 normal healthy subjects. (c) Let stand at room temperature for 10 min, mix again for 30 s, and centrifuge at high speed (1,400 × g) for 20 min. (d) Decant the supernatant fluid into a dialysis tube. Dialyze against distilled, de-ionized water for 48 to 72 h. Change the water at least once every 2 h. (e) During the last 12-18 h of dialysis, ammonium acetate buffer solution (10 mmol/liter, pH 6.25) is used instead of distilled, de-ionized water. (f) The dialyzed perchloric acid extract is transferred to a plastic tube. (g) To the standard tube, add purified CEA: 0, 10, 20, 50, or 100 ng. Mix by vortex, and add 50 µl of diluted antiserum (or the volume previously determined to be optimal) to each tube. Mix by vortex and incubate all tubes for 30 min at 45°C. (h) To each tube, add 100 µl of I251-CEA and mix well, and re-incubate all tubes again (45°C, 30 min). (i) Proceed as in the method for the titration curves, steps d to g. Always include a blank, i.e., 100 µl of I251-CEA in 10 ml of distilled water with 5 ml of zirconyl phosphate gel. (j) For the standard curve, plot nanograms of CEA vs. percent bound I251-CEA (or counts per minute). Samples are calculated by interpolating the unknown percent bound in the vertical axis and reading the unknown CEA from the horizontal axis.

**Results**

**Titration Curve for Anti-CEA Antiserum**

A typical titration curve is shown in Figure 1A, which also illustrates that nonspecific binding of zirconyl phosphate gel to the radioactive CEA molecule amounts to about 12% of the total. Our experience for over one year has shown that this nonspecific binding, or blank, varies between 10 and 15%. Maximal binding, as seen in the Figure, is about 65 to 70%. A series of calibration curves, derived from the titration curve in Figure 1A, are shown in Figure 1B. According to Berson and Yalow (9), the sensitivity of any radioimmunoassay procedure is maximal when the bound (B) radioactivity is about 33% of the total and the free (F) radioactivity is about 67% (B/F = 0.5). In the curve shown in Figure 1A, this point corresponds to 20 µl of the 1,000-fold dilution of antiserum. In practice, we have chosen to use 50 µl rather than 20 µl of the same antibody dilutions. The resulting initial B/F is closer to 1.0 than to 0.5. The initial or zero point in the standard curve equals a binding of about 50% of the total radioactivity, instead of 33%. By doing so, we can use 38% (50% - 12%) of working ranges for standard curves rather than 21% (33% - 12%); for example, a specimen containing 20 ng of CEA can be easily measured without further dilution.
Fig. 1. A, titration curve obtained by addition of various amounts of a 1,000-fold diluted goat anti-CEA antiserum and a constant amount of $^{131}I$-CEA. B, Standard curves of radioimmunoassay for CEA by using 50 µl of 1,000-fold diluted antiserum, $^{131}I$-CEA (10$^{-13}$ mol/liter, 400,000 cpm) and known amounts of standard CEA. These curves were obtained during five weeks. C, Logarithmic transformation of the standard curves for CEA radioimmunoassay. $B/T^*$ vs. log CEA concentration. D, Sigmoidal transformation of the standard curves for CEA radioimmunoassay. Logit $B/T^*$ vs. log CEA concentration.

Reproducibility of Standard Curve

Five CEA standard calibration curves obtained during five consecutive weeks are illustrated in Figure 1B, and show that the curves originate between 38 and 51%, and that these standard curves are quite nonlinear. Figures 1C and 1D show these same standard curves after logarithmic1 and sigmoidal1 transformation. Other data, such as the slope of the curve, 50% intercept (when $B/T$ is equal to half of $B/T_0$), and 90% intercept, as well as $(B/T_0)$, are presented in Table 1.

1 $B$, bound $^{131}I$-CEA; $F$, free $^{131}I$-CEA; $T$, total counts, i.e., $B + F$; $B/F$, ratio of bound to free; $B/T$, ratio of bound to total; $(B/T_0)$, the percentage bound in the absence of unlabeled CEA; $B/T^*$ = $(B/T)/(B/T_0)$; assume $(B/T) = 100%$; Logit $B/T^*$ = $\log_e \frac{B/T^*}{100(B/T^*)} = 2.303 \log_e \frac{B/T^*}{100(B/T^*)}$

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Precision of CEA Radioimmunoassay

The reproducibility of the CEA radioimmunoassay was ascertained from 20 consecutive analyses of a plasma pool prepared from plasma with known CEA values, and by studying the difference between replicate analysis in 50 different samples of CEA ranging from 0 to 50 ng/ml. The mean of the 20 determinations was 26.4 ng/ml; in fact, 25.0 ng/ml was the expected value; the range was 22.0 to 31.0 ng/ml. The coefficient of variation was 9.8%. The combined coefficient of variation for the difference between duplicate determinations for 50 samples (range 0–50 ng/ml) was 13.5%.

Sensitivity

The sensitivity of CEA radioimmunoassay was evaluated from the least amount of CEA we could measure. By using a plasma pool and using different dilutions, it is possible to show that there is a significant difference ($P < 0.01$) between the mean percentage (four replicates) of radioactivity bound at zero concentration of CEA ($B/T = 61.3 \pm 0.3$ SE) and the mean percentage of radioactivity bound at a concentration of 0.5 ng ($B/T = 54.2 \pm 0.2$, 1 SE). Hence, the assay procedure can reliably discriminate 0.5 ng clearly from zero.

Specificity of CEA

Results for normal individuals. Normal healthy individuals have plasma CEA concentrations of less than 2.5 ng/ml (5). The value of 2.5 ng/ml that we chose as the upper normal limit is the same as that reported by Thomson et al. (6).

Figure 2 shows the distribution by sex and age of the 138 normal controls. These are the blood donors in our plasmapheresis program. There were 27 women and 111 men in this group. Complete physical examinations and biochemical profiling were available in each case. The youngest individual is 18 years old and the oldest is 58. Of the 138 donors, 100 were 31 years old or older and may be considered as part of the "at risk" cancer population. All but one man had CEA values of less than 2.4 ng/ml. The one positive case is a 28-year-old man plasmapheresis donor in ap-
parent good health. Extensive physical and radiological examinations, sigmoidoscopy, and routine blood-test profiles provided no positive information.

Results in Cancer Patients

The results of CEA assay in cancer patients, its clinical usefulness and implication, are published in detail elsewhere (4). Of patients with malignant tumors of the gastrointestinal tract, 82% are positive. Other positive results include: male genitourinary tract cancer, 30%; female genitourinary, 39%; breast, localized disease, 10%, and disseminated, 60%; osteogenic sarcoma, 50%; neuroblastoma with active disease, 100%. Abnormally high concentrations of plasma CEA also were found in patients with hepatoma, leukemia, Hodgkin's disease, rhabdomyosarcoma, Ewing's sarcoma, carcinoma of the thyroid, teratoma, myeloma, and macroglobulinemia of the Waldenström type.

Discussion

The principle of the radioimmunoassay for CEA can be summarized as follows:

\[
\text{CEA}^* (\text{free, labeled CEA; } F) + \text{Antibody} \rightarrow \text{CEA}^* - \text{Antibody} (\text{specific anti CEA}) (\text{bound, labeled complex; } B)
\]

+ 

(standards or unlabeled CEA in plasma)

\[
\text{CEA} - \text{Antibody} \rightarrow \text{Precipitated by zirconyl phosphate gel counted}
\]

CEA is extracted from the plasma specimen (recovery, 80-90%) and is allowed to compete with 

\[^{125}\text{T}\]-radioactive labeled CEA for the goat monoclonal anti-CEA antibody. The bound labeled CEA is then separated from free radioactive CEA by precipitation with zirconyl phosphate gel and its radioactivity is counted, the partition of the labeled CEA between the free and bound fractions is calculated, and because this is a function (as shown in the above scheme) of the mass of the CEA present in unknown plasma, the concentration of CEA is determined from a standard calibration curve.

There are alternative ways to plot the standard curve. It can be expressed in terms of a free to bound, bound to free, or bound to total radioactivity. With data on these variables, the kinetics of the reaction system can be easily monitored. For routine purposes, we calculate the concentration of CEA in the unknowns from a simple plot of concentration vs. counts per minute. Because all tests are done in plastic disposable tubes, the efficiency of counting remains a constant from test to test.

For quality-control purposes, we plot the standard curve in terms of the ratio of bound to total radioactivity (Figure 1B). As shown in the Figure, these standard curves are nonlinear. Therefore, the classical statistical methods cannot be applied for the validation of CEA radioimmunoassay. Rodbard et al. (10) have reported a unique approach for quality control of radioimmunoassay, which we use to evaluate the present procedure.

By using logarithmic and sigmoidal transformation, we are able to linearize the nonlinear standard curves. Figure 1C shows the standard curves partially linearized by use of a logarithmic transformation of the CEA (ng) vs. B/T. These standard curves are much more nearly linear, but still slightly sigmoid. However, when a sigmoidal transformation is next applied, i.e., logit B/T vs. log CEA, more nearly linear curves are obtained (Figure 1D).

As shown in Figure 1D, a straight line was obtained in portions of the curve but not in the whole curve. The curves diverge beyond log CEA = 1.3 ng; i.e., logit transformation fails to linearize the standard curve when the amount of standard CEA is greater than 20 ng/ml (log 20 = 1.3). Therefore, in those samples with CEA values greater than 20 ng, we may repeat the assay with a diluted sample. The deviation from a straight line that occurs after 1.3 of log CEA may be due to the fact that the B/T ratio for standard CEA of more than 20 ng has reached the range almost equal to that of the blank (B/T = 10-15%), i.e., the nonspecific binding of radioactive CEA preparation by zirconyl phosphate gel in the absence of antibody. In other
words, the available binding sites on antibody have been much saturated by "cold" standard CEA, and the (indicative) radioactive CEA competes insignificantly for the binding sites.

The specific radioactivity of $^{125}$I-CEA that we used was 50-60 $\mu$Ci/$\mu$g, about 400 times greater than the value (0.14 $\mu$Ci/$\mu$g) reported by Thomson et al. (8). The sensitivity of the CEA radioimmunoassay is therefore greatly increased. According to the theory of Berson and Yalow (9) and from the titration curve shown in Figure 1A, it appears that maximum sensitivity is obtained with about 20 $\mu$l of antiserum, i.e., $B/F = 0.5$ or $B/T = 33\%$; we use 50 $\mu$l in our assay, so the sensitivity will not be maximum, although it is sufficient to discriminate 0.5 ng of CEA from zero. These data, along with those presented in Table 1, reveal a very good sensitivity and precision for this CEA radioimmunoassay procedure.

We have used the described procedure routinely for over one year. One technician can assay 50 samples in duplicate at one time without difficulty. Because there is no transfer of reagent or sample after the specimen is dialyzed, technical variation is greatly decreased. In examining the plasma of more than 652 patients, we have found the test to be reliable and useful in the immunologic diagnosis of cancer.

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References