Study of an Enzyme Immunoassay Kit for Carcinoembryonic Antigen

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We evaluated a commercial enzyme immunoassay kit for carcinoembryonic antigen (CEA) based on a non-competitive "sandwich principle" method (Abbott Laboratories). The serum sample is treated with an acid buffer and the supernate is incubated with an anti-CEA coated polystyrene bead. After washing, the bead is incubated with an anti-CEA/peroxidase conjugate. After a second washing, the activity of the enzyme bound to the solid phase is assayed after addition of a chromogenic substrate. This activity is proportional to the concentration of CEA in the serum sample. The characteristics of the assay are: (a) good sensitivity (around 0.25 µg/L) and (b) satisfactory reproducibility (CV <11% within assay). There is little cross-reactivity between CEA and molecules such as nonspecific cross-reacting antigens that are known for their high potential of cross-reactivity. No nonspecific interference was encountered with anti-globulin factors. We compared results with this enzyme immunoassay kit with those by a radioimmunoassay provided by the Commissariat à l’Énergie Atomique. The correlation (r) was 0.95 (p <0.001). The distribution of CEA values obtained for 1020 normal subjects is given. We conclude that the kit provides a simple and reliable procedure.

Additional Keyphrases: "kit" methods • cancer • reference intervals

Among the known tumor-associated antigens, the oncofetal antigens are particularly important. Normally these antigens are synthesized only during a limited period of fetal life. They apparently disappear completely during adult life but can reappear in a high concentration in association with certain tumors. Carcinoembryonic antigen (CEA),4 a prime example of oncofetal antigen, was first described by Gold and Freedman (1) as a specific marker for adenocarcinomas of the gastrointestinal tract. Thomson et al. (2) then showed by a CEA radioimmunoassay (RIA) that increased circulating concentrations of CEA were found only in patients with colorectal cancer. Further studies by other investigators (3, 4) have shown, however, that increased CEA concentrations are also found in patients with other types of carcinomas such as those of breast, lung, genitourinary tract, and lymphoid tissue, and have been observed in patients with inflammatory nonmalignant diseases, in heavy smokers, and in 10% of apparently normal subjects (4). Although of limited usefulness as a tumor-specific marker, CEA has been especially useful in monitoring and followup of cancer patients. Tumor ablation is usually followed by a decrease in circulating CEA, whereas recurrence of the primary tumor and (or) metastasis is accompanied by increased concentrations of CEA (5).

Many RIAs and, more recently, an enzyme immunoassay (EIA) for CEA have been described. The first fairly sensitive tests for this antigen have been RIAs (2, 3, 6, 8). Three commercial products are currently available in France: two competitive inhibition assays (CIS and Hoffman-La Roche) and a "sandwich"-type assay (Abbott Labs.). In 1975, Hammarstrom et al. (9) described a CEA-EIA. It is a classical competitive-inhibition assay, involving alkaline phosphatase-labeled CEA. In 1976, Frackelton et al. (10) introduced a sandwich EIA involving cellulose discs coated with rabbit anti-CEA immunoglobulin. The four-day procedure limits use of this test for routine hospital work.

Finally, Rose et al. (11) described an EIA made commercially available by Abbott Labs. as the CEA-EIA kit. This technique is the subject of our study.

The Abbott CEA-EIA system is a solid-phase EIA based on the two-site assay principle often referred to as a "sandwich"-type assay. After extraction from the sample plasma or serum, the CEA to be tested is incubated with a polystyrene bead coated with anti-CEA raised in guinea pigs. After incubation, the unbound material is washed away. The solid phase is then incubated with an excess of goat anti-CEA/peroxidase conjugate, and the excess labeled antibody is washed away. The enzyme activity retained on the coated bead is assayed by the addition of a chromogenic substrate, the resulting absorbance being proportional to the CEA concentration of the specimen.

We evaluated the sensitivity, reproducibility and specificity of the EIA assay, and report the absence of nonspecific cross-reactivity with substances known to cross react with CEA. We also illustrate the distribution of CEA concentration in sera from an ostensibly normal population.

Materials and Methods

Instruments

We used a Model 901 photometer (Saitron, Florence, Italy) and a Model 2200 computer (Wang Labs., Lowell, MA 01851).

Reagents

CEA kits. The CEA-EIA kits provided by Abbott Labs., North Chicago, IL 60064, included the following reagents: anti-CEA of guinea-pig origin coated on polystyrene beads, anti-CEA (of goat origin)/horseradish peroxidase (EC 1.11.1.7) conjugate in trihydroxymethyl)methylamine buffer (0.1 mol/L, pH 7.2), sodium acetate extraction buffer (0.2 mol/L, pH 5.0), CEA standards (0, 3, 10, and 20 µg/L), CEA-positive serum controls (15 µg/L), o-phenylenediamine, citrate-phosphate buffer diluent (0.1 mol/L, pH 5.5) for o-phenylenediamine (containing hydrogen peroxide, 5.9 mmol/L), hydrochloric acid (1 mol/L), and plastic tubes and trays necessary for the test.

The CEA-EIA kits were obtained from CIS Radiochemicals (Gif-Sur-Yvette, France), and all reagents were...
either used as received from the supplier or prepared as directed in their procedure.

**CEA International Standard.** The International Reference Standard (code 73/601) was provided by the National Institute of Biological Standards and Control, London, U.K. Each vial provided contained 100 international units of lyophilized CEA (1 int. unit is defined as the CEA activity in 0.236 mg of the standard). This material was reconstituted with 0.5 mL of distilled water and further dilutions of this material were made with the 0 μg/L CEA standard solution.

**Samples.** Serum and plasma specimens give identical results with the Abbott CEA-EIA procedure. Samples should be stored in the refrigerator at 2 to 8 °C, or frozen. We assayed 1020 normal specimens obtained from local volunteers at the Medical Prevention Center in Nancy, France, and 165 specimens from hospital patients at the Centre Antoine Lacassagne in Nice, France. Twelve sera containing an EIA developed by Maiolini et al. (12), were also utilized for CEA assays. All specimens were stored at −30 °C before use.

**Other reagents.** Purified CEA, nonspecific cross-reacting antigen (NCA), and NCA-2 preparations were provided by Dr. P. Burtin (National Institute of Cancer Research, Villejuif, France). NCA preparations were purified from a perchloric acid extract of normal lung.

**Test Procedure**

The various incubations are made in a 20-well tray. For the enzymatic step of the assay, the polystyrene beads are transferred to test tubes. Each sample is tested in duplicate.

**Serum or plasma preparation.** Pretreat all of the samples and the control serum by extracting with acid buffer, which precipitates most of the nonspecific proteins and leaves CEA in solution. Dilute 0.5 mL of serum or plasma with 1 mL of extraction buffer in polystyrene tubes, then rapidly vortex-mix. After mixing, incubate the tubes for 15 min in a water bath at 70 °C, then centrifuge at 1200 × g for 10 min. Assay the supernate.

**Incubation with solid-phase antibody.** Add 200 μL of each supernate to an antibody-coated bead. Also add 200 μL of non-pretreated standard CEA solution to single beads placed in each well of the 20-well tray. Cover the trays and incubate for 2 h at 45 °C, then rinse each bead twice with 5 mL of distilled water.

**Incubation with the antibody–enzyme conjugate.** After washing, add 200 μL of antibody conjugate to each well. Cover the plates again and gently agitate to eliminate air bubbles that could be trapped under the beads. Incubate the plates again for 2 h at 45 °C, then remove excess conjugate by washing the beads as before.

**Enzyme color development.** Transfer the washed beads from the well trays to test tubes. To determine the enzyme activity bound to the solid phase, add 0.3 mL of enzyme substrate (H₂O₂ and o-phenylenediamine · HCl) to each tube. After incubations for 30 min at 20 °C, stop the enzymic reaction by adding 2 mL of 1 mol/L HCl. Measure the absorbance at 492 nm on the spectrophotometer calibrated vs distilled water.

**Calculation of results.** The results may be evaluated by two different methods. Either plot CEA concentration as a function of absorbance at 492 nm, or use a double-reciprocal plot of CEA concentration vs absorbance (1/C vs 1/A) to obtain a straight line. Most of the results reported here were calculated by computer according to the second procedure.

**Quality control.** Check the quality control for each run by measuring the CEA value of the CEA-positive control serum (15 μg/L). Follow the distribution of values by use of a Levey–Jennings graph (13).

**Results**

**Sensitivity.** Figure 1 shows typical CEA-EIA standard curves prepared by both methods of calculation (see above). The lower limit of detection of this assay was 0.24 μg/L (sensitivity threshold), defined as the least amount of antigen that could be distinguished from zero within 99% confidence limits; concentrations below this limit are subject to random variations in absorbance. The upper limit of the calibration curve
is 20 μg/L, but because the samples are diluted threefold by the extraction step, the working range of the assay extends from 0.75 to 60 μg/L.

Reproducibility. Within-assay reproducibility was tested on 12 different samples (standards, sera, or pooled sera, assayed repeatedly in the same run by two technicians). The average curve, derived from 10 separate assays of each calibrator point (± 2 SD), is shown in Figure 1 (top). The coefficients of variation (CV) ranged from 2.78 to 6.34% within the assay range. Within-assay CVs for sera samples (containing CEA between 1.6 and 32.9 μg/L) assayed repeatedly seven to 30 times ranged from 3.85 to 10.63%. To study within-assay reproducibility, we measured separate absorbances obtained for each of 20 samples of the 0 μg/L CEA standard placed in different wells of the same tray. The mean absorbance was 0.041 (SD 0.003) and the CV 8.90%.

We assessed between-assay reproducibility by assaying 17 samples in different runs (Table 1). The CVs were between 5.87 and 13.84% for mean CEA concentrations ranging from 1.34 to 1469 μg/L.

Calibration with the International CEA standard. The assay of six different dilutions (800-, 1600-, 2000-, 4000-8000-, and 10 000-fold) of the International Standard gave a mean concentration of 13 970 μg/L (CV, 11.5%), or 13 970 ng/mL. Because 1 mL of the preparation contains 200 int. units of CEA activity, 1 int. unit is equivalent to 69.8 ng of CEA, in terms of the calibrator of the Abbott CEA-EIA kit.

Specificity. We evaluated the specificity of the assay by three classical procedures. The first, a dilution test (Figure 2), showed parallel curves for dilutions of purified CEA (from Dr. P. Burtin) and for standards provided with the kit. In the second, an analytical-recovery test in which known quantities from two purified CEA solutions were added to a previously adjusted pool of normal sera, the percentage of CEA accounted for in the pooled serum ranged from 92.2 to 103.3% (Table 2); in view of the reproducibility of the assay, these recovery figures may be considered identical. In a third test, to determine possible interference in the assay by NCA and NCA-2 (glycoproteins that possess antigen characteristics similar to those of CEA) different concentrations of NCA and NCA-2 (5 to 10 000 μg/L) were first pre-treated by acid extraction buffer and then assayed with the CEA-EIA kit. Figure 1 shows that only at a concentration of 10 000 μg/L does NCA appear to have significant cross reactivity with CEA (equivalent to a CEA concentration of 6.97 μg/L). Similarly, at concentrations of 5000 and 10 000 μg/L, NCA-2 cross-reacts equivalent to 5.18 and 8.52 μg of CEA per liter.

Nonspecific interference. Because the CEA-EIA is a "sandwich" method of assay, some anti-globulin factors (especially rheumatoid factors) in certain sera may adhere to the insolubilized antibody during the first stage of the assay; later, during the second incubation, these factors may become fixed by their free antibody-binding sites to the CEA-antibody conjugate. This nonspecific interaction could thus give rise to an excessively high CEA estimation. However, it is probable that the pretreatment step precipitates rheumatoid factors along with the rest of the nonspecific proteins and thereby eliminates this interference, which is already minimized by the use of antisera from two different animal species. Of the 12 rheumatoid sera tested, only two showed an apparent CEA concentration > 4.86 μg/L (equivalent to the average CEA value of normal subjects increased by 3 SDs). These two sera had been treated by immunoabsorption with a solid-phase IgG Sepharose 6B adsorbent (activated with cyanogen bromide), to eliminate the rheumatoid factors. Table 3 shows that CEA concentrations after immunoabsorption are essentially equal to those obtained before elimination of rheumatoid factors, if nonspecific adsorption onto Sepharose 6B (generally 15 to 20%) is taken into account.

Table 1. Between-Assay Reproducibility for Various Specimens

<table>
<thead>
<tr>
<th>Specimen</th>
<th>No. cases</th>
<th>Mean (SD) CEA, μg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal serum pool</td>
<td>8</td>
<td>1.34 (0.16)</td>
<td>12.38</td>
</tr>
<tr>
<td>Positive control of kit, lot 1</td>
<td>8</td>
<td>13.82 (0.77)</td>
<td>5.57</td>
</tr>
<tr>
<td>Positive control of kit, lot 2</td>
<td>6</td>
<td>18.22 (1.64)</td>
<td>9.01</td>
</tr>
<tr>
<td>Serum A</td>
<td>6</td>
<td>27.94 (1.71)</td>
<td>6.15</td>
</tr>
<tr>
<td>Serum B</td>
<td>7</td>
<td>63.32 (5.36)</td>
<td>8.47</td>
</tr>
<tr>
<td>Serum C</td>
<td>6</td>
<td>149.71 (20.72)</td>
<td>13.84</td>
</tr>
<tr>
<td>Serum D</td>
<td>3</td>
<td>1469.63 (146.74)</td>
<td>9.98</td>
</tr>
</tbody>
</table>

Table 2. Analytical Recovery for Two Preparations of Purified CEA

<table>
<thead>
<tr>
<th>CEA (Dr. Burtin)</th>
<th>CEA (Abbott Labs.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Added, μg/L</td>
<td>Recovered, μg/L</td>
</tr>
<tr>
<td>34.05</td>
<td>32.65</td>
</tr>
<tr>
<td>17.02</td>
<td>16.19</td>
</tr>
<tr>
<td>8.51</td>
<td>8.29</td>
</tr>
<tr>
<td>4.25</td>
<td>3.92</td>
</tr>
<tr>
<td>2.12</td>
<td>2.19</td>
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Table 3. Serum CEA Concentration after Adsorption with IgG Sepharose Immunoadsorbent

<table>
<thead>
<tr>
<th>Rheumatoid factor concn. in serum, int. units/L</th>
<th>CEA concn., μg/L</th>
<th>CEA removed by nonspecific adsorption, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before adsorption</td>
<td>After adsorption</td>
<td></td>
</tr>
<tr>
<td>32 300</td>
<td>5.09</td>
<td>4.28</td>
</tr>
<tr>
<td>138 000</td>
<td>7.94</td>
<td>6.54</td>
</tr>
</tbody>
</table>
Retention of CEA activity in serum or plasma extracts. A study of the preservation of the supernatant of an extract showed that after storage at 4 °C, the concentration of an extract containing 2.4 μg of CEA per liter decreased by 0.4 μg/L after five days and by 0.8 μg/L after 10 days (i.e., 3.3% each day). Moreover, the apparent concentration in an extract containing 0.85 μg of CEA per liter had decreased by 0.2 μg/L after storage at 4 °C for 10 days (i.e., 2.3% each day). We observed no decrease of CEA after storage of the extract at −20 °C for 15 days, nor was there any effect on CEA concentrations of nonextracted sera after storage at 4 °C for the same period.

Correlation study. We assessed the correlation between the Abbott CEA-EIA kit (x) and the CIS-RIA kit (y), using 165 sera selected to contain different CEA concentrations, with the following results: correlation coefficient, 0.95; equation of linear regression, \( y = 2.33x + 5.63 \); and standard error of estimate, 196.55. Thus, correlation between results with the kits was good, especially in the higher concentrations. However, differences were observed in the lower range, especially when the CEA concentrations measured by the Abbott CEA-EIA were <20 μg/L. A paired t-test and a coefficient of correlation calculated for those 144 samples judged to have low concentrations showed a lack of correlation between the two assays (\( r = 0.69, t\)-value = 13.86, \( p = 0 \). Particularly, CEA concentrations of three sera were found normal by the Abbott CEA-EIA (<4 μg/L) but abnormal by the CIS assay (between 30 and 48 μg/L).

Distribution of CEA concentrations in a normal population. Figure 3 shows that the distribution of CEA concentrations in a normal population was close to a normal distribution curve. The median is 1.26 μg/L, and 87% of the subjects had a CEA of less than 2.5 μg/L.

Discussion

Our data show that the CEA-EIA has good sensitivity, reproducibility, and specificity. The mean sensitivity of this assay is 0.25 μg/L, which is equivalent to that described by other authors. The CEA-RIA based on the “sandwich” principle described by Hirai (8) and distributed commercially by Dainabot Laboratories, Tokyo, Japan, has a detection threshold of 0.1 μg/L. Chu and Reynoso (6) have described an RIA involving zirconyl phosphate (sold by Hoffman-La Roche, Nutley, NJ 07110), by which CEA is detected at 0.5 μg/L. Frackelton et al. (10) have reported a sensitivity of 0.2 μg/L for their EIA method, which is quite comparable with that found in our study.

In our laboratories the within-assay variation CVs of the Abbott EIA kit are lower (2.78 to 10.63%) than those described elsewhere. For example, Martin et al. (14) and Onizawa et al. (15), who used the zirconyl phosphate RIA, had CVs of 8.95 to 19.35% and 12.00 to 25%, respectively. Similarly, the between-assay CVs of 5.2 to 13.94% that we obtained with the Abbott kit are better than those described by Martin et al. (14) and Hirai (8), who reported CVs of 9.91 to 13.05% and 9.29 to 17.90%, respectively. These CVs also seem to be independent of the CEA concentrations in the sera tested.

Our specificity tests on the Abbott CEA-EIA showed the virtual absence of cross reactivity with NCA and NCA-2 antigens, which had been previously found by Mach and Pusztazeri (16) and Burtin et al. (17), respectively, in perchloric acid extracts of normal lung. NCA is present in the sera of normal subjects in concentrations of 100 to 150 μg/L and can increase to as much as 1000 μg/L in disease [Burtin (18)]. At these concentrations we observed no cross reaction with NCA. Only at 10-fold greater NCA (10 000 μg/L) was detectable cross reactivity observed, equivalent to 7 μg/L of CEA. NCA-2 likewise showed no significant interference with the Abbott EIA kit unless the concentrations were much higher than those found in disease. It is very likely that the absence of cross reaction with NCA and NCA-2 may be explained by the low affinity of CEA antibody for these two antigens, in comparison with the high affinity of these antibodies for CEA. Among substances capable of causing nonspecific interference, rheumatoid factor was ruled out by negative results obtained with 10 of 12 rheumatoid factor-positive sera. Increased CEA concentrations in the remaining two rheumatoid sera were attributed to non-rheumatoid factors, because the activity could not be removed by solid-phase IgG immunoadsorbent.

The ability of CEA to undergo the heat-treatment extraction step of the EIA assay was demonstrated in the CEA supplementation and recovery experiments.

Our experiments on the storage life of the acid buffer supernate showed that the CEA extract lost only 2 to 3% of its activity during 10 days.

The international reference preparation no. 73/801 is delivered without any official indications of its CEA content. However, it has been assayed by different RIA's at the Chester Beatty Research Institute, London, U.K., and found to contain between 98 and 124 ng of CEA per unit. Our own estimation, based on the Abbott standard, is in the lower range of these estimations.

Previous results of population studies on presumably normal populations (8, 19, 20) have shown that 99 to 97% of the normal population have CEA concentrations of <2.5 μg/L. These percentages are slightly higher than we found (87%), but are perhaps explained by our use of a less homogeneous sampling (1020 males and females, between 4 and 70 years old and including smokers and those with variable drinking habits).

The satisfactory correlation between the Abbott CEA-EIA assay and the CIS-RIA kit (which is the most frequently used and studied CEA test in Europe) shows that the CEA-EIA can be used for the follow-up of patients. However, a few differences were found in CEA values obtained by the two techniques. There is apparently a systematic difference in the ratio of values by the two tests; values are roughly threefold greater in the CIS assay than in the Abbott kit. Variations of 0.9 to 7.5 (mean = 3.1) in ratios of CEA concentrations by RIA and by EIA were observed. This may be explained by the facts that the two kits are based on radically different principles (direct assay by competition in serum vs sandwich assay after a precipitation step) and involve different calibrators. In addition, the dilution medium of the CIS-RIA kit consists of normal
human serum, which is likely to introduce an error by excess in the lower CEA concentrations, as demonstrated by the low coefficient of correlation \((r = 0.69)\) and by the \(y\)-intercept of the linear regression equation.

It is unlikely that the differences are due to the presence of some relatively well-defined antigens (NCA, NCA-2, biliary \(\beta\)-glycoprotein, etc.) that cross react with CEA. The antisera used in the assays did not show any substantial cross reactivity with these molecules. Furthermore, all these antigens are glycoproteins which do not precipitate in an acid medium and thus would remain in the same phase as CEA. The precipitation step, which is a feature of several methods, including two commercial assays, is likely to eliminate other ill-defined molecular species that may interfere with the assay, as has been shown by Frackelton et al. (10). This type of as-yet ill-defined cross reactivity may explain some of the discordant results in the lower range of concentrations which are always in the same direction, i.e., higher values for the CIS-RIA assay, even if the systematic difference is taken into account.

In addition to all the features common to all EIA's, the Abbott CEA-EIA kit has the following advantages: (a) Ease and flexibility in use. Each assay needs only the minimum of handling; in particular, semi-automated washing steps allow 100–200 tests to be performed in a day. However, one must pay close attention to avoid errors in identifying the sera when transferring the polystyrene beads. It is also very important to seal the plates to avoid evaporation, especially of the conjugate, which would initiate a nonspecific conjugation on the bead surfaces and subsequently yield increased readings. (b) Rapidity. The whole of the test can be performed within 6 h.

In conclusion, this CEA kit should be of equal interest to the biochemist and the clinician, in that it allows rapid and reliable estimation of an antigen that is useful in diagnosis and follow-up of patients with different forms of malignant disease.

We are very grateful to Dr. P. Burtin and Mrs G. Chavanel, who kindly supplied us with samples of NCA, NCA-2, and purified CEA.

References