Modified Method for Determining Carcinoembryonic Antigen in the Presence of Human Anti-Murine Antibodies

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The increasing use of monoclonal antibodies (MAbs) for disease diagnosis and therapy has created a class of patients at risk for systematic error in clinical testing due to interference by human anti-murine antibodies (HAMA). HAMA interference is often difficult to detect and can cause either an increase or a decrease in apparent concentrations of antigen present. We undertook a clinical study to test a HAMA-resistant enzyme immunoassay (EIA) format for carcinoembryonic antigen (CEA) determination. Using the Food and Drug Administration-approved CEA-EIA Monoclonal One-Step Assay (Abbott) with the addition of an acid/heat extraction of patients' specimens, we found that the resulting CEA values accurately reflected the patients' status. We demonstrated that the acid/heat-extracted specimens yield linear dilution curves and show analytical recoveries of added CEA in the range of 76–123% in HAMA-positive specimens and 86–103% in HAMA-negative specimens. The correlation of CEA values in extracted vs unextracted specimens from 184 patients and control subjects was 0.9963. The CEA detection limit of the assay was 1.6 μg/L for the extracted samples.

Monoclonal antibodies (MAbs) have been increasingly used in cancer patients for both diagnosis and therapy.4 The appearance of human anti-murine antibodies (HAMAs) in sera of such patients after this treatment has caused interference in laboratory methods used to measure carcinoembryonic antigen (CEA) (1–5). This interference is often difficult to detect and has been shown to both increase and decrease measured CEA concentrations (1, 2, 4). CEA assays are used routinely to monitor cancer patients, and an increase in the CEA concentration is regarded as an early indicator of residual or recurrent colorectal cancer (6, 7). Because the presence or absence of HAMA in a given patient's serum is often unknown to the clinician, it is important that the assay used for CEA be resistant to this increasingly common interference.

In this study, we used the Abbott (Abbott Park, IL) CEA-EIA Monoclonal One-Step Assay with an acid/heat extraction step (8). This assay replaced the Abbott CEA-EIA Polyclonal Two-Step Assay, the production of which has been discontinued by the manufacturer. Although more cumbersome, the older assay format, because of its use of guinea pig and goat antisera plus an extraction step to precipitate interfering serum proteins, including immunoglobulins (8), was inherently more resistant to HAMA interference (1, 4, 8; Kinders et al., ms. submitted for publication). However, using the Monoclonal One-Step Assay with heat/acid extraction of the sample, we found that the CEA concentrations measured corresponded with patients' clinical status, whether HAMA was present in the sample or not. We first evaluated the Monoclonal One-Step Assay, with and without the extraction step, for reproducibility, dilution linearity, and analytical recovery experiments. Then we compared the performance of the acid/heat-extracted CEA-EIA Monoclonal One-Step with that of the standard kit format. Finally, normal subjects, patients with nonmalignant disease, and patients with known adenocarcinoma—who were both negative and positive for the presence of HAMA—were tested; some patients were monitored over time.

Materials and Methods

Assay Evaluation

Reproducibility studies. Precision experiments were conducted with panel specimens at six different CEA concentrations (3, 5, 10, 30, 60, and 180 μg/mL) over 2 months of kit evaluation for intra- and interassay variation. The panel specimens were obtained by adding affinity-purified tissue culture CEA from LS174T cells to normal human serum.

Dilution linearity. Nine serum specimens, three each from lung, breast, and colon cancer patients, were tested undiluted and by dilution with zero standard without the extraction step. Then, in parallel, each specimen was acid/heat-extracted and assayed undiluted and after dilution with zero standard. All samples and dilutions were assayed by CEA-EIA Monoclonal One-Step (no. 3520) according to the manufacturer's directions.

Sensitivity. Assay sensitivity, that is, the least detectable quantity of CEA, was determined by the formula

\[
\text{Sensitivity} = \frac{2 \times \text{SD}(A_{490}) \times 4.0 \mu g/L}{\text{mean } A_{490} \text{ of } 4.0 \mu g/L \text{ std. } - \text{mean } A_{490} \text{ of zero std.}}
\]

Forty-three determinations of the zero standard and 46 determinations of the 4.0 μg/L standard were performed.
by a single technologist, who used the assay in both the extracted and the unextracted formats separately. The sensitivity of the acid/heat-extracted assay was determined by multiplying the value obtained from the above calculation by 3, to account for the threefold dilution caused by the extraction step.

Analytical recovery. The recovery of CEA in the assay, with and without extraction, was evaluated by adding two known quantities of CEA to matched serum and EDTA-plasma samples from two normal individuals and to the assay zero standard, and then measuring CEA in both assay formats. The mean endogenous and enriched CEA values were calculated, and percent recoveries for both assay formats were determined as follows:

\[
\text{Percent recovery} = \frac{\text{observed [CEA]} - \text{endogenous [CEA]}}{\text{added [CEA]}} \times 100
\]

The recovery of CEA was also evaluated with CEA-supplemented sera from colon cancer patients who had been treated with MAb C110 or B72.3 (both IgG₁κ) and who had subsequently developed measurable concentrations of HAMA of the IgG class in their sera. These HAMA-positive cancer patients’ sera were supplemented with two different concentrations of CEA and then determined in both assay formats, unextracted and extracted.

HAMA Assay

Determination of serum HAMA titers (9) in the presence of antigen was performed in a 96-well plate with a bridging EIA format. For determination of anti-isotype reactivities, MAb B72.3 was used as the capture antibody ("coater") and murine MAb C110 IgG conjugated to horseradish peroxidase (HRPO; Sigma Chemical Co., St. Louis, MO; EC 1.11.1.7) was the probe. For determination of total HAMA, anti-CEA MAb C110 IgG was used as both the capture antibody and the HRPO-conjugated probe.

Immulon-II™ (Dynatek Laboratories, Chantilly, VA) plates were coated with MAb, 100 μL/well, at a concentration of 2 g/L in pH 9.6 carbonate buffer, overnight at 2–8 °C. Wells were then coated for 1 h at 37 °C with 50 mmol/L phosphate-buffered saline, pH 7.4, containing 0.1 g/L bovine serum albumin, to block any nonspecific binding sites. Sera were diluted 1:10 in phosphate-buffered saline containing 50 mL of fetal bovine serum and 10 mg of gentamycin sulfate (Sigma) per liter, then serially diluted 1:2–1:1024 in the wells, with a final assay volume of 100 μL. Samples were incubated for 2 h at 37 °C; the contents of the wells were then aspirated and the wells were washed 3 times. Then 100 μL of conjugate was added to each well. All MAbes were used as HRPO conjugates at a concentration of 2 mg/L in the phosphate-buffered saline/fetal bovine serum buffer. The plates were incubated for 2 h at 37 °C, then washed with distilled water, and developed by adding 100 μL of o-phenylenediamine · 2HCl (Abbott). After 30 min at room temperature, the development was stopped by adding 100 μL of H₂SO₄ (0.5 mol/L) and the absorbance was read at 490 nm on a BioTek™ (Winooski, VT) microtiter plate reader. The reciprocal dilution of serum that gave an absorbance reading equal to 1.0 A in the assay was referred to as the HAMA titer.

Monoclonal antibodies. The MAbes used in this study were produced by standard methods from BALB/C mice with SP2/0 or NS-1 as fusion partners. Purification was on Protein A, essentially as described by Ey et al. (10). Antibodies were conjugated with HRPO according to the method of Nakane and Kawai (11). Antibody fragments were generated essentially as described by Parmham (12) and Mage (13). Monoclonal B72.3, obtained from the National Institutes of Health (Bethesda, MD), is nonreactive with CEA (14, 15).

CEA Assay and Acid/Heat Extraction Procedure

The CEA concentration of each sample was determined in duplicate with the Abbott CEA-EIA Monoclonal One-Step Assay, with and without acid/heat pretreatment. In the extraction procedure, 1 mL of extraction buffer (0.2 mol/L sodium acetate, pH 5.0, with thimerosal perservative) was added to 0.5 mL of patient's sample or control, thoroughly mixed, incubated in a 70 °C water bath for 15 min, and then centrifuged for 10 min at 1200 × g. The clear supernatant fluid was assayed in duplicate in the One-Step protocol. All samples were read at 490 nm with a Quantum II™ Analyzer (Module B, Mode 1.20; Abbott Laboratories). For samples with absorbance values exceeding that of the highest concentration standard (80 μg/L), portions of the extracted or unextracted sample were diluted with zero standard and reassayed. CEA concentrations obtained on extracted samples were multiplied by 3 to correct for dilution with the extraction buffer.

The acid/heat extraction step was evaluated by analyzing 133 patients' samples chosen without conscious bias. These included 98 patients whose CEA values ranged from 0 to 12 μg/L, 28 patients whose values were from 13 to 80 μg/L, and 12 patients with CEA values ranging from 80 to 3000 μg/L.

Patients/Clinical Specimens

Serum or plasma collected by standard procedures from a group of patients whose diagnosis was known and that had previously been assayed by the Food and Drug Administration (FDA)-approved Abbott CEA-EIA Two-Step Assay were included in this study. These specimens were used to assess the ability of the One-Step Assay with the acid/heat extraction step to accurately reflect CEA concentrations in patients' serum. Specimens in this group were from 20 normal subjects with no known malignancy or benign disease condition; 20 patients with nonmalignant disease, including cervical polyps, ulcerative colitis, pancreatitis, and diverticulitis; 33 patients with malignancies, including those of the colon, lung, breast, stomach, and pancreas; and 37...
patients with various other malignancies. We also assayed samples from 38 cancer patients who had been tested for the presence of HAMA as described above, and were negative. These patients were then enrolled in an immunoscintigraphy trial in which they were injected with a single dose (5 mg intravenously) of anti-CEA MAb C110 labeled with \(^{111}\text{In}\). In (16). This procedure previously was shown to lead to the formation of HAMA in some patients (17; Kinders et al., ms. submitted for publication). The CEA concentrations in five patients in this group who remained HAMA-negative, and in seven patients who later demonstrated positive HAMA titers, were tracked over time and the results compared with these patients’ clinical status.

In addition, the CEA values obtained with the acid/heat-extracted One-Step CEA-EIA were compared with those obtained with the two FDA-approved CEA assays, the One-Step Monoclonal and the Two-Step Polyclonal. Clinical information concerning each patient’s status was obtained from the principal investigators, S. Larson at Memorial Sloan Kettering Cancer Center and T. Griffin at the University of Massachusetts.

Statistics

Linear-regression analyses and correlation of the data were performed according to the method of Sokal and Rohlf (18).

Results

Removal of HAMA by Acid/Heat Extraction

To determine the degree of HAMA removal that could be achieved by the acid/heat extraction method, we took two experimental approaches to the problem.

Experiment 1. Because it seemed appropriate at the onset to demonstrate that the acid/heat extraction method physically removed HAMA from the specimens before assay, we designed a HAMA assay to test this assumption. We acid/heat-extracted two plasma samples of high and low HAMA titers (4, 9; Kinders et al., ms. submitted) from patient A and tested the extracts in a plate ELISA for HAMA, and in the CEA-EIA Monoclonal One-Step Assay for CEA. Unextracted A, normal human plasma, and a HAMA-free plasma specimen (by clinical history) were used as the control and reference samples. Patient A was a cancer patient treated with MAb B72.3 (14), which is specific for a carbohydrate determinant on the mucin antigen TAG (tumor-associated glycoprotein), which is a different macromolecule from CEA (14, 15) [TAG has repetitive epitopes, unlike CEA (19, 20)]. This patient was injected with MAb B72.3 several times as part of both diagnostic and therapeutic regimens, and was suspected of having HAMA on the basis of CEA and TAG assay results that were inconsistent with the physician’s clinical assessment of the patient. We constructed our HAMA ELISA by taking advantage of the specificities of MAbS for the two different antigen molecules. We used the B72.3 MAb as the capture antibody (coated on the microtiter plate) and the anti-CEA MAb C110 as the conjugate (labeled) antibody. Anti-CEA MAb C110 is directed to a peptide epitope in the N-terminal region of CEA and does not react with TAG (19). Both MAbS are IgG1,κ. A positive result in the assay is obtained when an antibody reacts with the capture antibody (B72.3), remains bound after a wash, and then reacts with the C110-HRPO conjugate (9). Such antibodies in human sera would be expected to be IgG1-specific HAMA (21, 22). Thus, the MAb combination B72.3/C110 would be positive in the presence of HAMA (Figure 1A), but negative in the presence of CEA alone (Figure 1B). Titration of the CEA-containing plasma control by use of two CEA-specific MAbS (H8 and C110) (19) is illustrated to indicate a positive result in the assay.

Titration of plasma A without acid/heat extraction (Figure 1A) in comparison with the titration with acid/heat treatment (Figure 1C) illustrates a significant decrease (90%) in signal in the HAMA assay after the extraction. HAMA removal was apparently not quantitative: the midpoint titer after extraction was 40 (vs 360 originally) for specimen A1 and 20 (vs 160) for specimen A2. We assumed that the reduction in titer did, however, reflect the HAMA concentration, because the titrations of all specimens were linear at the midpoint (1.0 \(A_{400}\)) of the curve.

Affinity purification of this specimen on Protein G (collecting the bound fraction from the plasma), followed by a second affinity step on a B72.3 column, yielded 1.96 g/L of human IgG (data not shown). These results suggested that acid/heat extraction could remove as much HAMA as 1.76 g/L. Specimen A2, which had a lower HAMA titer than A1, showed a similar reduction in HAMA signal in the plate ELISA.

Experiment 2. The results from the above experiment indicated that acid/heat extraction was effective, but not quantitative in HAMA removal. We therefore tested whether HAMA remaining after extraction was sufficient to interfere in CEA determination. Specimens A1 and A2 were supplemented with tissue culture CEA (42.3 μg/L) and tested with the CEA-EIA Monoclonal One-Step Assay in extracted and unextracted formats; the control was zero standard containing added CEA. As shown in Table 1, the quantity of HAMA remaining after the extraction step did not interfere with measurement of a known quantity of CEA added to these plasma samples.

CEA Assay Validation

Sensitivity. The detection limit of the unextracted kit was 0.53 μg/L for the lot of kits tested. Sensitivity of the acid/heat-extracted assay was taken to be 3 times the calculated value for the unextracted kit (to account for specimen dilution): 1.6 μg/L.

Reproducibility studies. Intra- and interassay CVs for six different CEA concentrations measured by the CEA-EIA Monoclonal One-Step Assay with the acid/heat extraction step were ≤10% except for the lowest-concentration CEA specimen. The intra-assay CVs for six replicates with mean concentrations of 2–57 μg/L ranged from 4% to 8%. These replicates were assayed by a single technologist on a single day. The interassay
Fig. 1. Effect of acid/heat extraction on HAMA content in plasma
(A) Titration of two different plasma specimens—A1 (C) and A2 (Δ)—from patient A injected on multiple occasions with MAb B72.3. The two MAb, B72.3 and C110, are IgG1,κ and specific for two different antigens, TAG and CEA, respectively. The positive signal is due to the presence of HAMA. Affinity purification of HAMA from the starting plasma yielded 1.96 g IgG. (B) Titration of a plasma containing 2415 μg/L CEA, from a patient who had never been exposed to mouse monoclonal antibodies. The HAMA-detecting MAb combination B72.3/C110 (Δ) (R: Kinders et al., ms. submitted) is negative, whereas the combination of anti-CEA MAbs, H8/C110 (C), is strongly positive. (C) Titration of patient A plasma (C, Δ as in panel A) after the acid/heat extraction step used in the CEA assay validation described in this paper. HAMA contents are reduced by >85% for both specimens.

<table>
<thead>
<tr>
<th>Specimen HAMA titer (μg/L)</th>
<th>Unextracted assay recovery (%)</th>
<th>Extracted assay recovery (%)</th>
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</thead>
<tbody>
<tr>
<td>A1</td>
<td>62.9</td>
<td>1.14</td>
</tr>
<tr>
<td>A1 + CEA, 42.3 μg/L</td>
<td>64.8</td>
<td>4.5</td>
</tr>
<tr>
<td>A2</td>
<td>7.97</td>
<td>0.65</td>
</tr>
<tr>
<td>A2 + CEA, 42.3 μg/L</td>
<td>49.16</td>
<td>97.4</td>
</tr>
</tbody>
</table>

* * Specimen titration curves shown in Fig. 1A
  * Calculated as described in text.

CVs were calculated from the results of eight runs (a total of 48 observations) done by two technologists over a 4-day period. The CVs ranged from 6% to 8% for mean CEA concentrations of 2–57 μg/L. The intra- and inter-assay CVs for the lowest CEA concentration (1.1 μg/L) were 12% and 14%, respectively.

**Dilution linearity and recovery.** Linear dilutions of the extracted and unextracted data were first analyzed by linear regression. Specimens from patients with diagnosed colon, breast, or lung cancer (three different patients for each disease, n = 9) all yielded correlation coefficients >0.99 for both assay formats.

We then tested the linearity of dilution of the extracted format of the assay by fitting the raw assay concentration data from each series to a second-order polynomial equation with the reciprocal of the dilution as the dependent variable. Plots of the resulting data indicated that all nine specimens exhibited linear dilutions. The probabilities that a second-order coefficient was not needed to explain any deviation of the data from expected results ranged from 0.0001 to 0.0729 for four specimens and from 0.1276 to 0.1977 for three additional specimens. One specimen had too few dilutions to be analyzed by this method; the remaining specimen yielded a seemingly high value of 0.4508, although the maximum deviation between the polynomial and straight-line fit of the data for this specimen was 1.3 μg/L CEA over a tested concentration range of 4.0–67.1 μg/L.

Analytical recovery of added CEA, with and without extraction, in the presence of normal human serum or EDTA–plasma, was close to 100% in all instances. Recovery of two different known quantities of CEA added to matched serum and EDTA–plasma samples from two apparently healthy individuals averaged 100% (CV 5.9%) for the unextracted CEA-EIA Monoclonal One-Step procedure and 96% (CV 6.1%) for the extraction version of the assay. No correlation was found between the quantity of CEA added and the percentage of CEA recovery from serum. For normal sera, recovery of CEA ranged from 97.4% to 108.3% (average 102.3%, CV 4.0%) for the unextracted assay; and from 97.6% to 102.5% (average 99.2%, CV 1.9%) for the extracted version. For normal plasma, recovery of CEA ranged from 87.6% to 100.5% (average 97.1%, CV 5.6%) for the unextracted assay; and from 86.2% to 102.5% (average 93.5%, CV 6.7%) for the extracted version.
Table 2 shows the results of analytical recovery studies of CEA in sera from HAMA-positive cancer patients with and without extraction. Recovery of CEA without extraction ranged from 0% to 143.9% (mean recovery of 66%); in these same supplemented HAMA-positive cancer-patient sera treated with the acid/heat extraction step, recovery of CEA ranged from 76.1% to 122.8% (mean 101%). The increased range of values in this format was principally associated with the lower quantity of CEA added (5.52 μg/L), reflecting the loss of precision in the assay at low CEA concentrations associated with the threefold dilution that accompanies the extraction step. Table 2 also shows that the presence of HAMA decreased apparent CEA values (without extraction) in four specimens (217-1, 217-2, SK12, and UM10), whereas in five specimens (217-3, 221, 214, CH-03, and 200) the apparent CEA values were increased by the presence of HAMA. This artificial increase or decrease in apparent CEA value, obtained with the unextracted CEA-EIA procedure, was unrelated to actual HAMA titer.

Evaluation of Acid/Heat Extraction Procedure

The performance of the acid/heat extraction step (y) was tested against the unextracted assay format (x) for samples from 184 individuals that were HAMA-free (by patient histories). The linear correlation coefficient for all specimens tested was 0.9963, with root-mean-squared (RMS) error of 6.83 μg/L, y-intercept of −1.08 μg/L (SE 0.54 μg/L), and slope 0.975 (SE 0.006).

Separate analysis of samples from 20 normal subjects and 20 patients with benign disease (CEA content 0–13 μg/L) gave a linear correlation coefficient of 0.9403 (RMS error 0.81 μg/L), y-intercept 0.94 μg/L (SE 0.15 μg/L), and slope 0.711 (SE 0.042). Moreover, the extracted specimens in this group had consistently lower results than did the unextracted samples for CEA concentrations of <3 μg/L, perhaps reflecting a loss of assay sensitivity attributable to the specimen dilution in the extraction procedure.

Analysis of the specimens collected before MAb injection from the 38 cancer patients enrolled in a tumor detection program yielded a linear correlation coefficient of 0.9794 (RMS error 6.30 μg/L) over a CEA concentration range of 1.4–178 μg/L. The y-intercept (extracted) was 0.75 μg/L (SE 1.33 μg/L) and the slope was 0.868 (SE 0.030). Comparable results were obtained from separate analysis of specimens from 33 other cancer patients (chosen without conscious bias) from the Michael Reese Hospital specimen collection.

From the group of 38 patients, 5 who remained HAMA-negative throughout the program and 7 others who subsequently became HAMA-positive were monitored for 2–10 months for CEA concentrations. The CEA concentrations from the CEA-EIA Polyclonal Two-Step Assay and the CEA-EIA Monoclonal One-Step Assay, with and without acid/heat extraction, were then checked against patients' status as reported by their physician. The results for two of the monitored HAMA-negative patients and three of the tracked HAMA-positive patients whose clinical history was complete and readily available are included here, and are representative of the results for the other patients who were monitored.

The HAMA-negative patients' results (Figure 2) demonstrate that, in the absence of HAMA, CEA assay results are similar whether the assay includes the acid/heat pretreatment or not. However, Figure 3 shows that, without the acid/heat extraction, the CEA value would be significantly misleading in some patients with HAMA titer. Figure 3A depicts a tracking series for a patient (SK07, peak HAMA titer of 1:30) from presurgery through a 7-month follow-up. In this case, cancer recurrence was masked at the 210th day postsurgery in the CEA-EIA Monoclonal One-Step Assay performed without acid/heat extraction. This false-negative result was not a problem for either assay when the extraction procedure was included. Confirmation of disease recurrence was obtained by physical examination and computerized tomography scan. For the patient tracked in Figure 3B (SK11), the increase in CEA concentration reflects recurring cancer, as diagnosed by computerized tomography. In the first 90 days after injection of the antitumor MAb, the patient did not exhibit a HAMA response. After reinjection of the MAb, the patient's HAMA content rose to a titer <1:100. The much higher CEA measurement by the unmodified assay at 300 days corresponded to the appearance at the same time of a positive HAMA titer in that patient. In sharp contrast to patient SK07, the results here were falsely positive, even though both patients were imaged with the same MAb, C110. The patient received chemotherapy. Patient UM10 (Figure 3C) remained ambulatory and was diagnosed in a stable disease state, not in remission. The effects of HAMA in this patient (with a peak titer of 1:471) were a false-negative CEA value when assayed by the CEA-EIA Monoclonal One-Step Assay without the heat/extraction method.

Table 2. Analytical Recovery of CEA in HAMA-Positive Patients with Abbott CEA-EIA One-Step Assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Unextracted</th>
<th>Extracted</th>
<th>Unextracted</th>
<th>Extracted</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>217-1</td>
<td>3.33</td>
<td>5.34</td>
<td>78.5</td>
<td>91.9</td>
<td></td>
</tr>
<tr>
<td>217-2</td>
<td>7.43</td>
<td>21.39</td>
<td>35.0</td>
<td>78.8</td>
<td></td>
</tr>
<tr>
<td>217-3</td>
<td>63.72</td>
<td>41.87</td>
<td>75.8</td>
<td>106.9</td>
<td></td>
</tr>
<tr>
<td>221</td>
<td>56.38</td>
<td>3.24</td>
<td>74.3</td>
<td>94.9</td>
<td></td>
</tr>
<tr>
<td>214</td>
<td>7.65</td>
<td>4.47</td>
<td>61.2</td>
<td>105.7</td>
<td></td>
</tr>
<tr>
<td>SK-12</td>
<td>2.83</td>
<td>4.47</td>
<td>94.6</td>
<td>110.9</td>
<td></td>
</tr>
<tr>
<td>CH-03</td>
<td>18.51</td>
<td>1.50</td>
<td>143.9</td>
<td>120.1</td>
<td></td>
</tr>
<tr>
<td>UM-10</td>
<td>2.73</td>
<td>6.66</td>
<td>30.6</td>
<td>122.8</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>74.76</td>
<td>3.24</td>
<td>0</td>
<td>78.1</td>
<td></td>
</tr>
</tbody>
</table>

* Range of HAMA titers in the specimens was 1:10 to 1:1000.
* Quantity of CEA added to specimen: 20.15 μg/L in unextracted, and 17.0 μg/L in extracted sera.
* Quantity of CEA added to both unextracted and extracted sera was 5.52 μg/L.

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Fig. 2. CEA values in two cancer patients in the tumor-detection program who did not demonstrate a HAMA titer in the observed period after injection with MAb

Φ, CEA-EIA Polyclonal Two-Step Assay; Δ, CEA-EIA Monoclonal One-Step Assay; O, CEA-EIA Monoclonal One-Step Assay with acid/heat extraction

Discussion

The Abbott CEA-EIA Monoclonal One-Step Assay performed acceptably at all CEA concentrations in HAMA-free human sera or plasma, whether or not the acid/heat extraction step was used. This is the first report of recovery and dilution data for a HAMA-resistant CEA assay performed with an extraction step. Data in this paper also demonstrate the reliability of the assay at all concentrations examined ($r = 0.9963, n = 184$) and indicate that the addition of the extraction step does not affect the CEA assay accuracy or precision adversely, although sensitivity at low CEA concentrations (to $\sim 1.6 \mu g/L$) is decreased. This reduction in

Fig. 3. CEA values in three cancer patients with positive HAMA titers in the observed period after injection with MAb

Assays identified as in Fig. 2
sensitivity will not be important in determining CEA concentrations for patients with active disease, the group most likely to be treated with MAb. However, patients with CEA <5 µg/L will require more conservative interpretation of data obtained with the extraction format assay. The intra- and interassay CVs are >10% only at low CEA concentrations for the extraction assay format, presumably because of the threefold dilution performed. The analytical recovery experiments showed that, although samples from patients known to be HAMA-negative gave acceptable recoveries of CEA with or without the extraction step, this was not true for HAMA-positive sera. In patients known to have circulating HAMA, recovery of CEA in the acid/heat-extracted specimens was 76–123% (mean 101%, CV 16%). Without the extraction step, the HAMA-containing specimens had CEA recoveries of 0–144% (mean 66%, CV 63%). That such an erratic recovery is the result of the HAMA interference is shown by the recoveries for the identical CEA-supplemented samples after the HAMA was destroyed by the acid/heat extraction step (Table 1) or after HAMA removal with Protein G chromatography (data presented elsewhere: 4).

Hansen et al. (1) refer to two cases in which falsely increased values in CEA assays resulted in costly diagnostic procedures and great emotional stress to the patients. Neither patient in their study had an abnormally high value for CEA when their samples were first heat-extracted and reassayed. Price et al. (2) also mention six cancer patients with measurable HAMA titers who had increased CEA concentrations but no other indication of disease.

Our study expands these data substantially and provides an independent quantitative assessment of assay performance. For the patients in the present study who remained HAMA-negative and were monitored over time, the results of the CEA-EIA Monoclonal One-Step Assay, whether the extraction step was used or not, essentially agreed with both the previously used assay, the CEA-EIA Polyclonal Two-Step, and with the actual clinical status of the patient according to the physician’s report. However, for six of the seven patients in the tracking study who developed positive titers for HAMA, the assays did not agree. The Monoclonal One-Step Assay results for these six patients differed not only from the results by the same assay done with the extraction step and by the previously used Polyclonal Two-Step Assay but also with the observed clinical condition of the patient (see Figure 3).

In addition, in this and other (20) patient populations, it has been observed that these HAMA titers can be very long lasting (>10 months), even after a single injection of MAb. Other investigators (23) have reported that HAMA titers appear to cycle up and down over time. These observations are consistent with the history of patients SK11 and UM10 (Figure 3 and other data not shown), patients from two different clinical sites. These data further compound the difficulty of dealing with potential HAMA interference.

HAMA's are predominantly IgGs that arise in response to immunizing doses of MAb. Because HAMA's are polyclonal and of complex specificity, they can result in many different types of interference, both positive and, in some circumstances, negative (21, 22, 24, 25). The positive interference by HAMA is probably due to binding of conjugate and solid-phase antibodies, similar to the effects we used to measure HAMA (Figure 1).

The negative interference by HAMA is more difficult to pin down. Two possible causes are blocking of the solid-phase antibody by essentially “overcoating” with HAMA or, equally likely, binding of conjugate by HAMA. In either case, we presume that the HAMA has a substantially greater affinity for one MAb or the other. It may be that the HAMA is specific for MAb variable-region sequences that are present in one MAb but not in the other. Either explanation is consistent with the literature data showing that HAMA are polyclonal and of complex specificity (21, 24). Because of this potential of HAMA to interfere in an unpredictable way in laboratory tests used to monitor patients’ CEA concentrations, any patient known to have been exposed to immunizing doses of animal antibodies should be analyzed for CEA by an assay resistant to HAMA interference. However, the clinician is often unaware of the presence of an interfering antibody such as HAMA. A resulting false increase in the reported CEA value could lead to extensive unnecessary testing or even unnecessary surgical procedures. Because of the possibility of HAMA interference, any patient known or suspected of animal antibody exposure for diagnosis or treatment should be assayed with a HAMA-resistant assay for CEA. If a patient is known to be negative for HAMA, either the unmodified CEA-EIA Monoclonal One-Step Assay or the same assay with the acid/heat extraction step gives reliable results.

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Determination of Vitamin D Status by Radioimmunoassay with an 125I-Labeled Tracer

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We report here the first radioimmunoassay for a vitamin D metabolite utilizing a radiiodinated tracer. Antibodies were generated in a goat immunized with the vitamin D analog 23,24,25,26,27-pentanor-C(22)-carboxylic acid of vitamin D, coupled directly with bovine serum albumin. The 125I-labeled tracer was prepared by reacting a 3-amino-propyl derivative of vitamin D-C(22)-amide with Bolton–Hunter reagent. The primary antisera, used at a 15 000-fold dilution, cross-reacted equally with all cholecalciferol and ergocalciferol metabolites tested except 1,25-dihydroxychocolcaliferol metabolites and the parent calciferol; the antisera did not cross-react with dihydro-cholecholsterol. Calibrators were prepared in vitamin D-stripped human serum. 25-Hydroxycholecalciferol was quantitatively extracted from serum or plasma (50 μL) with acetonitrile. The assay consists of a 90-min incubation at room temperature with primary antisera, followed by a 20-min incubation with a second antisera and separation of bound from free fractions by centrifugation. The detection limit of the assay was 2.8 μg/L for 25-hydroxycholecalciferol. Results with the present assay compared well with those from a liquid-chromatographic procedure involving specific ultraviolet detection of 25-hydroxycholecalciferol in plasma.

Indexing Terms: 25-hydroxyvitamin D · vitamin D metabolites

It is well-established that vitamin D nutritional status is a function of circulating 25-hydroxycholecalciferols...