Heterophilic Antibodies in Human Sera Causing Falsely Increased Results in the CA 125 Immunofluorometric Assay

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An in-house OC 125 monoclonal antibody based "sandwich" immunofluorometric assay (IFMA) described previously (Clin Chem 1987;33:2191-4) gave higher results for CA 125 in 37 of 123 serum samples than did a commercially available immunoradiometric assay (IRMA). Discordant results between the two assays became concordant when measurements of the samples were repeated with normal mouse serum (100 mL/L) included in the IFMA reagent. The murine immunoglobulins were thought to block the ability of the heterophilic antibodies in the human serum samples to cross-link the labeled antibody with the solid-phase antibody. Using an enzyme immunoassay, we demonstrated human anti-mouse antibodies (HAMA) in most of the discordant samples examined. We tested the ability of nonimmune sera from other animal species to lower the apparent CA 125 concentrations of the spurious samples and observed that rat, goat, and sheep serum was less effective than mouse serum. One serum sample was discovered to give a falsely increased CA 125 result with the IRMA, but this increase could be prevented by adding murine serum to the IRMA reagent. We conclude that falsely increased CA 125 results are best prevented by adding murine serum (or murine antibodies) to the assay buffer.

Additional Keyphrases: variation, source of · immunoradiometric assay compared · species-specific antibodies

The monoclonal antibody-based assay for CA 125 (cancer antigen 125) in serum has become a reliable indicator of tumor status in patients with ovarian cancer.4 Concentrations of CA 125 exceed a cutoff value of 35 arb. units/mL in >80% of patients with epithelial ovarian cancer (1). Rising, stable, or falling values correlate with tumor progression, stability, or regression, respectively, in >90% of cases (2). CA 125 concentrations are predictive of the chance of clinical recurrence (3, 4). At present, ongoing studies are evaluating the use of the CA 125 assay as an adjunct for screening apparently healthy women for ovarian cancer (5-7).

We have recently developed a two-site immunofluorometric assay (IFMA) for CA 125, using europium-labeled OC 125 monoclonal antibody (MAb) as the tracer (8). While using this in-house assay concurrently with a commercially available CA 125 immunoradiometric assay (IRMA), we noted falsely increased concentrations of CA 125 in several samples determined with the IFMA, as compared with the IRMA results. Here we report on the heterophilic antibodies present in human serum samples that caused the spuriously increased concentrations of CA 125.

Materials and Methods

Serum samples. Serum samples from 30 patients with gynecological neoplasms and from 93 healthy female controls were included in the present study. The following nonimmune serum pools were used for supplementation in the CA 125 assays: normal mouse serum 1 (NMS1), normal mouse serum 2 (NMS2), normal rat serum (NRS), mouse serum (NGS), and normal sheep serum (NSS). NMS1 was obtained from Swiss mice kept in the Central Animal Facility of the University of Nijmegen. The other nonanimal sera were obtained from Organon-Teknika-Cappel, Turnhout, Belgium.

CA 125 immunoradiometric assay. The CA 125 IRMA (Centocor Inc., Malvern, PA) was used according to the manufacturer’s instructions. In this assay the MAb OC 125 is used both as capture and tracer antibody in a simultaneous "sandwich" format (9). Serum (100 mL) is added to the OC 125-coated polystyrene bead in a 25-well tray, then the tracer antibody (5 to 10 ng of 125I-labeled OC 125 in 100 mL of assay buffer containing a nongenomic murine MAb) is added. After overnight incubation at room temperature, the beads are washed and their radioactivity is counted in a gamma counter. In our hands, the lower detection limit of the IRMA for CA 125 is 1.4 arb. units/mL.

CA 125 immunofluorometric assay. We performed the IFMA according to the two-step "sandwich" protocol as described previously (8). In brief, the assay buffer contained 100 mL of serum and 100 mL of assay buffer [per liter, 50 mmol of Tris-HCl (pH 7.75), 154 mmol of NaCl, 5.0 g of albumin, 7.5 mmol of Na2HPO4 to OC 125-coated 12-well strips (Eloph Oy, Helsinki, Finland). After overnight incubation at room temperature, we washed the well, added 200 mL of europium-labeled OC 125 (100 mL/L) as tracer, and incubated for 90 min at room temperature. After washing, we added fluorescence enhancement solution (Pharmacia-Wallac Oy, Turku, Finland), and measured the fluorescence intensity in a 1230 Arcus fluorometer with time-resolution (Pharmacia-Wallac Oy). We determined 1.5 arb. units/mL as the lower detection limit of the IFMA in our laboratory.

Statistical methods. The results of the CA 125 determinations from the different immunoassays were compared by orthogonal regression analysis (10). We calculated the
slope and the y-axis intercept of the regression lines, the standard error of estimate (SE_{yx}), the mean values of x and y, and the Spearman rank correlation coefficient (r).

Samples were considered to display high values for CA 125 in the IFMA if:

\[
\text{CA 125 (IFMA)} - \text{CA 125 (IRMA)} > 3 \left( \frac{SE_{\text{IFMA}}}{+ (SE_{\text{IRMA}})^2} \right)^{1/2}
\]

where SE_{IFMA} and SE_{IRMA} represent the standard errors of CA 125 (IFMA) and CA 125 (IRMA), respectively (both assumed to be 15%).

**HAMA determinations.** Serum samples were assayed for the presence of human anti-mouse antibodies (HAMA) by the Enzygnost-HAMA-enzyme immunoassay (Behringwerke AG, Marburg, F.R.G.) according to the manufacturer's protocol. In brief, either nonspecific mouse immunoglobulins, OC 125 MAb (1.0 mg/L), or phosphate-buffered saline was incubated in wells (1 h, 37 °C) that had been precoated with goat anti-mouse antibodies. After washing, 10-fold dilutions of serum samples were incubated for 1 h at 37 °C in the wells, together with the HAMA-positive and HAMA-negative control samples. In this test, two conjugates of goat-anti-human immunoglobulin (specifically directed against either human IgG or IgM) and horseradish peroxidase (EC 1.11.1.7) are used as the tracers. After the 2-h incubation with tracer, we washed the wells and determined the amount of bound tracer by incubation with excess enzyme substrate (3 mmol of H₂O₂ per liter in the presence of 3',3',5',5'-tetramethylbenzidine). To stop the color reaction, we added 100 μL of 0.25 mol/L H₂SO₄, then measured the absorbance at 450 nm with a Titertek Multiscan spectrophotometer (Flow Laboratories, Herts., U.K.).

**Results**

**CA 125 determinations.** We assayed 123 serum samples for CA 125 simultaneously in the CA 125 IRMA and the CA 125 IFMA. The results are plotted in Figure 1A. Orthogonal regression analysis gave the equation CA 125 (IFMA) = 1.35 CA 125 (IRMA) + 3.0 arb. units/mL (S_{yx} = 30.7, r = 0.655, mean x = 30.1 arb. units/mL, mean y = 43.5 arb. units/mL). The slope of 1.35 and the positive y-intercept indicate that the mean IFMA results were higher than those obtained with the IRMA. This demonstrates a discordance between the two assay methods.

Next, we re-assayed the serum samples in the IFMA with the addition of NMS1 (100 mL/L) to the assay buffer. The CA 125 (IFMA^{NMS1}) vs CA 125 (IRMA) plot is shown in Figure 1B. Orthogonal regression analysis revealed the equation CA 125 (IFMA^{NMS1}) = 0.95 CA 125 (IRMA) - 0.15 arb. units/mL (S_{yx} = 10.7, r = 0.947, mean x = 30.1 arb. units/mL, mean y = 28.3 arb. units/mL), indicating a good correlation and concordance between the IFMA^{NMS1} and the IRMA results.

Without the addition of NMS1 to the IFMA reagent, 37 (i.e., 34 from healthy controls and three from patients with malignancies) of 123 serum samples showed falsely increased CA 125 (IFMA) values, on the basis of the statistical criterion stated in Materials and Methods. In contrast, assaying the 123 samples in the presence of NMS1 yielded only four samples with discordant results for IRMA vs IFMA^{NMS1}. Table 1 lists the CA 125 values for these four samples as determined with the IRMA, IFMA, IFMA^{NMS1}, and IFMA^{NMS2}. To exclude methodological errors, we ran these samples twice in each immunoassay. The false increase of two of these four samples (patient 171 and 189) could be prevented completely only by adding normal mouse serum from another mouse serum pool (NMS2). The spuriously increased CA 125 (IFMA) concentration of the third sample (patient 204) was only partly reduced by the addition of normal mouse serum from either pool. The last of these four samples (patient 192) gave high values in both the IRMA and the unsupplemented IFMA (200 and 250 arb. units/mL, respectively), but yielded a significantly lower IFMA value after supplementation with either mouse serum. Thus the IRMA result for this particular sample suggested a false increase due to the presence of heterophilic antibodies, which was not blocked by the monoclonal IgG in the IRMA assay buffer. Supplemen-

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**Table 1. CA 125 Determinations with IRMA and IFMA (arb. units/mL) in Four Selected Serum Samples**

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>IRMA</th>
<th>None</th>
<th>NMS1</th>
<th>NMS2</th>
</tr>
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<tbody>
<tr>
<td>171</td>
<td>24</td>
<td>410</td>
<td>91</td>
<td>32</td>
</tr>
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<td>189</td>
<td>89</td>
<td>310</td>
<td>180</td>
<td>100</td>
</tr>
<tr>
<td>204</td>
<td>14</td>
<td>85</td>
<td>56</td>
<td>63</td>
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<tr>
<td>192</td>
<td>200</td>
<td>250</td>
<td>18</td>
<td>15</td>
</tr>
</tbody>
</table>

*Assay buffers were supplemented with normal serum (100 mL/L) from different mouse serum pools. Data represent the mean of two separate duplicate determinations. SD < 13% of the mean for all determinations.*
tion of the IRMA assay buffer with NMS1 (100 mL/L) resulted in a CA 125 (IRMA) concentration (20 arb. units/mL) comparable with the CA 125 (IFMA\textsuperscript{NMS1}) concentration of this sample. CA 125 (IRMA) values of other samples were not significantly affected by this modification.

We investigated whether the interfering antibodies could be suppressed by adding other nonimmune serum pools. The 37 samples giving falsely increased CA 125 (IFMA) results as compared with the CA 125 (IRMA) results were re-assayed with the IFMA, with use of assay buffers supplemented with NMS2, NraS, NGS, or NShS (10 mL/L). The results of the orthogonal regression analysis are shown in Table 2. In these calculations, the CA 125 (IFMA) results \( \gamma \) were expressed as a function of the CA 125 (IRMA) results \( \alpha \). Without any serum supplementation in the IFMA reagent, orthogonal regression analysis revealed high values of the slope \( (\alpha = 1.55) \), y-axis intercept \( (b = 20) \), standard error of estimate \( (S_{\text{error}} = 48.3) \), mean \( \gamma \)-value \( (74.3\)arb. units/mL), and a poor correlation \( (r = 0.565) \). Addition of NMS1 or NMS2 (100 mL/L) to the IFMA assay buffer greatly reduced the discordance between the IRMA and the IFMA results. Furthermore, as Table 2 shows, NraS more effectively suppressed false increases of CA 125 (IFMA) than did the addition of NGS or NShS, but was less effective than NMS1 and NMS2.

**HAMA determinations.** We further analyzed the heterophilic antibodies contained in the serum samples by assaying in the HAMA assay the 17 of the 37 discordant serum samples for which sufficient sample volume remained. Ten of these 17 samples proved to be positive for human anti-murine immunoglobulins, irrespective of whether nonspecific murine antibodies or the MAb OC 125 was used as "catcher" antibodies in the HAMA assay. Application of the heavy-chain-specific (mu or gamma) anti-human antibodies as tracer demonstrated that nine of the sera contained HAMAs belonging to the IgM immunoglobulin class, while one of the samples contained IgG-class HAMAs. Unexpectedly, we obtained identical results if phosphate-buffered saline was added instead of catcher antibodies, suggesting that the HAMAs also react with the goat antibodies pre-coated to the plates by the manufacturer. Finally, the seven remaining serum samples showing discordant CA 125 (IRMA) vs CA 125 (IFMA) results did not respond in the HAMA.

**Discussion**

Interference by human anti-immunoglobulin antibodies in immunoassays has previously been reported in several studies (11–14). Few studies have been undertaken to determine the incidence of heterophilic antibodies in sera from normal human individuals. Measuring creatine kinase isoenzymes in sera from 1008 healthy blood donors, Thompson et al. (15) found interfering heterophilic antibodies in 92 samples (9.1%). Bocato and Stuart (16), using an assay specifically designed to detect the heterophilic antibodies, demonstrated their presence in 40% of 668 normal serum samples. Courtenay-Luck et al. (17) reported anti-murine antibody immune reactivity in sera from all the healthy controls \( (n = 24) \) included in their study. The high variability of the reported incidences of heterophilic antibodies may be a result of the different sensitivities of the assays used.

The present study showed that 37 of 123 serum samples gave falsely increased results for CA 125 in the IFMA. These spurious increases could be suppressed by adding normal mouse serum (or irrelevant murine IgG1 MAb, 0.5 g/L, data not shown) to the probes, suggesting the presence of heterophilic antibodies. These antibodies can mimic the CA 125 antigen in the OC 125-based "sandwich" assay by cross-linking the tracer antibody with the solid-phase antibody.

In 10 of 17 discordant samples, human anti-murine antibodies could be demonstrated by an enzyme immunoassay primarily designed to detect HAMAs in sera from patients who receive murine MAb for diagnostic or therapeutic purposes. Only one of the HAMA-positive patients displayed an IgG HAMA response, whereas the other nine serum samples contained heterophilic antibodies belonging to the IgM subclass, in line with the observations of other investigators (11, 17–19). However, IgG antibodies against murine immunoglobulins also have been demonstrated in human serum samples (20).

The effect of heterophilic antibodies was not noticed in our previous study (8) because for the IRMA–IFMA method comparison we used serum samples from 30 patients with ovarian cancer, for whom the mean CA 125 value was 93 arb. units/mL. The present study demonstrates that the spurious increases are most striking in samples with relatively low CA 125 concentrations, the differences between CA 125 (IFMA) and CA 125 (IRMA) results being generally <25 arb. units/mL.

The effect of the heterophilic antibodies could be suppressed only in part with normal sera from other species (i.e., rat, sheep, and goat), which suggests that the interfering heterophilic antibodies present in human sera are also directed against murine-specific epitopes. Moreover, the spurious CA 125 (IFMA) increase in one particular sample could be suppressed completely only by adding normal murine serum from one particular mouse strain, which suggests a strain-specific anti-immunoglobulin immune reactivity. The contradictory CA 125 results in the IRMA and IFMA\textsuperscript{NMS1} for three samples (patients 171, 189, and 204) may be ascribed to the presence of heterophilic antibodies directed against epitopes not present on the

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**Table 2. CA 125 Determinations (arb. units/mL) with IRMA (\( \alpha \)) and IFMA (\( \gamma \)) in 37 Discordant Serum Samples**

<table>
<thead>
<tr>
<th>IFMA supplementation( ^{a} )</th>
<th>Slope</th>
<th>( y )-intercept</th>
<th>( S_{\text{error}} )</th>
<th>( r )</th>
<th>( \bar{\gamma} )</th>
<th>( \gamma )</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.55</td>
<td>20</td>
<td>48.3</td>
<td>0.565</td>
<td>35</td>
<td>74</td>
</tr>
<tr>
<td>+ NMS1</td>
<td>1.00</td>
<td>2.5</td>
<td>14.1</td>
<td>0.955</td>
<td>35</td>
<td>38</td>
</tr>
<tr>
<td>+ NMS2</td>
<td>0.98</td>
<td>3.0</td>
<td>13.6</td>
<td>0.957</td>
<td>35</td>
<td>37</td>
</tr>
<tr>
<td>+ NraS</td>
<td>1.10</td>
<td>0.47</td>
<td>23.3</td>
<td>0.886</td>
<td>35</td>
<td>39</td>
</tr>
<tr>
<td>+ NGS</td>
<td>1.13</td>
<td>14</td>
<td>28.7</td>
<td>0.829</td>
<td>35</td>
<td>54</td>
</tr>
<tr>
<td>+ NShS</td>
<td>1.27</td>
<td>9.3</td>
<td>26.4</td>
<td>0.863</td>
<td>35</td>
<td>52</td>
</tr>
</tbody>
</table>

\( ^{a} \) Assay buffer supplementations (100 mL/L) to suppress heterophilic antibody reactivity.
murine immunoglobulins contained in the NMS1. We therefore suggest that interfering heterophilic antibodies are best prevented by adding pools of immunoglobulins from various strains from the same species as the applied catcher and tracer antibody. Thus, MAbs obtained from the same fusion experiment may also prove to be suitable additives. Apart from the source, the amount of immunoglobulins added is important, as has been discussed in detail in other reports (16, 21).

During a study involving 496 blood donors (22), one patient's serum was discovered to contain highly specific human anti-idiotypic antibodies to the MAb OC 125 (23). Because anti-idiotypic immune reactivity cannot be blocked with nonspecific immunoglobulins, the addition of murine antibodies does not prevent the appearance of false-positive CA 125 results in all cases.

The present study shows that in immunoassays such as the CA 125 assay, nonspecific antibodies should be included in the assay reagent to block interference by heterophilic antibodies. In addition the source of the nonimmune immunoglobulins seems critical. We think it advisable to add immunoglobulins from at least the same species and strain as the reagent antibody.

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References