nterference by Human Anti-Mouse Antibodies in CA 125 Assay after Immunoscintigraphy: Anti-Idiotypic Antibodies Not Neutralized by Mouse IgG but Removed by Chromatography

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Falsely increased concentrations of the ovarian carcinoma-associated antigen, CA 125, were measured by a monoclonal antibody (MAb)-based double determinant immunoradiometric assay (IRMA) in patients who developed antibodies to mouse immunoglobulins (IgGs) after receiving injections of the same MAb as is used in the CA 125 IRMA. Addition of undiluted mouse serum or purified mouse IgG to the assay mixture failed to eliminate the falsely increased CA 125 concentrations in most of the samples, owing to the presence of anti-idiotype antibody. Because of their anti-idiotype nature, the human anti-mouse antibodies (HAMA) had only little effect on other immunometric assays, and this effect could be completely eliminated by addition of mouse IgG. To eliminate this effect HAMA on the CA 125 assay, we studied the ability of various chromatographic methods to separate the interfering HAMA from CA 125. For measuring HAMA in serum and chromatographic fractions we developed a time-resolved fluorimunoassay. Adequate separation of CA 125 and HAMA was achieved by affinity chromatography of patients’ sera with solid-phase Protein A, Protein G, cation-exchange chromatography on Mono S, and gel filtration on Superose 6. These results demonstrate that the interference can effectively be removed by rather simple chromatographic procedures.

Additional Keyphrases: immunoradiometric assay · time-resolved fluorimunoassay · chromatography, cation-exchange · gel filtration

“Sandwich”-type immunoradiometric assays (IRMAs) based on mouse monoclonal antibodies (MAbs) give falsely increased results when human anti-mouse immunoglobulin antibodies (HAMA) are present in the patients’ sera (1–3). These antibodies simulate the behavior of the antigen by linking the radiolabeled IgG to the solid-phase. Such artificial sandwiches are observed in immunoasays based on both monoclonal and polyclonal antibodies and are indistinguishable from the specific sandwiches (4–6). Development of HAMA is a common complication after intravenous injections of murine MAbs in connection with radiomaging of ovarian tumors. Induction of HAMA response has been found in 62% of the patients (7). Low concentrations of HAMA have also been found in 9% of blood donors (8).

The IRMA of CA 125 has been developed with use of a murine MAb, OC125, (9) and optimized for quantification of the MAb-defined determinant, CA 125 (10). Klug et al. (10) have discussed the possibility that at least some increases in CA 125 concentrations can arise from serum factors other than the defined antigenic determinants and recently identified (6) an interfering human IgM-type antibody in a patient for which the adverse effects were eliminated by using a different iodination technique of the antibody. Addition of murine serum has also been used to diminish false-positive CA 125 results caused by HAMA (10, 11). In other immunoassays, affinity chromatography on Protein A-Sepharose and murine MAb adsorbed onto vinylidene fluoride flocules have proved effective for removing HAMA (8, 12, 13).

We analyzed serum samples from patients who had undergone immunoscintigraphy with the MAb OC125. Falsely increased concentrations of CA 125 in the “sandwich”-type IRMA were found regularly, and the interference correlated directly with the concentration of HAMA in the sera. In most of the samples, addition of mouse serum blocked only part of the interference. We therefore characterized the reactivity of HAMA and evaluated the ability of various chromatographic methods to separate the interfering factor from CA 125 antigen. On the basis of these results we propose methods for separating HAMA from CA 125 antigen.

Materials and Methods

CA 125 analysis, immunoscintigraphy, and blocking experiments. The CA 125 IRMA kits (Abbott Laboratories, North Chicago, IL) were used according to the manufacturer’s instructions. In the CA 125 assay, the same murine MAb to CA 125 is used both as capture and tracer antibody in a simultaneous sandwich format.

Immunoscintigraphy was performed with 131I-labeled Fab(’)
2-fragments of OC125 monoclonal antibody Imacis-2 (ORIS, Gif-sur-Yvette, France).

To attempt to block or reduce the false increase in CA 125 in the patient’s serum, we pre-incubated samples, directly or after dilution, for 1 h at room temperature with 0.02–1.0 mL of mouse serum (Dakopatts A/S, Glostrup, Denmark) per milliliter of serum. Alternatively, mouse IgG, purified by Protein G chromatography (MAbTrap G; Pharmacia LKB, Uppsala, Sweden), was added at final concentrations of 1–50 mg of IgG per milliliter of serum. After this, we performed the CA 125 assay as usual.

Apparatus. All chromatographic separations, except Protein G chromatography, were performed with a liquid chromatography system consisting of two 2150 HPLC pumps combined with a high-pressure mixer and a 2152 HPLC controller (all from LKB-Produkter AB, Bromma, Sweden) and a Wisp 710B injector (Waters Associates, Milford, MA). All chromatographic runs were performed at room temperature.

Affinity chromatography. Serum samples, 25 or 100 μL, were applied to a Protein A–Sepharose HR 10/2 (10 × 20 mm) column (Pharmacia LKB) in 0.1 mol/L sodium phosphate buffer, pH 8.5, at a flow rate of 2 mL/min. Fractions
of 2 mL were collected. The bound protein was eluted with 0.1 mol/L citric acid–NaOH buffer, pH 3.0, starting 6 min after sample application.

"Protein G fast flow" (Pharmacia LKB) was packed in an 8 × 35 mm polypropylene column (Bio-Rad, Richmond, CA) to total volume of 1.8 mL and equilibrated with 20 mmol/L sodium phosphate buffer, pH 7.0. Then, 100 µL of serum was applied to the column. The flow rate was 0.5 mL/min with use of hydrostatic pressure. After sample application the column was washed with 10 mL of equilibration buffer, and elution was performed with 0.1 mol/L glycine·HCl solution, pH 2.7. Fractions of 1 mL were collected.

Ion-exchange chromatography. Anion-exchange chromatography was performed with a 5 × 50 mm Mono Q HR 5/5 column (Pharmacia LKB). The column was equilibrated with 20 mmol/L Tris·HCl buffer (pH 8.0), and 100 µL of serum was applied. Elution was achieved with a salt gradient from the starting buffer to buffer B: Tris·HCl 20 mmol/L, NaCl 0.5 mol/L, pH 8.0. Three minutes after sample application, the concentration of buffer B was linearly increased to 100% in 17 min. Fractions of 1 mL were collected.

A 5 × 50 mm Mono S HR 5/5 column (Pharmacia LKB) was used for cation-exchange chromatography. The column was equilibrated with 20 mmol/L sodium acetate buffer, pH 5.0, pH 5.75, pH 6.5, or pH 6.75 (buffer A), and then eluted with a gradient to 20 mmol/L sodium acetate of the same pH containing NaCl, 0.5 mol/L (buffer B). Elution with a linear gradient reaching 100% B in 18 min was started 2 min after sample application. Serum samples of 50 µL were diluted fourfold with buffer A, and 200 µL of the diluted sample was applied to the column. The flow rate was 1 mL/min and fractions of 1 mL were collected. Centricron-10 microconcentrators (Amicon, Danvers, MA) were used according to the manufacturer's instructions for concentration of chromatographic fractions.

**Filtration.** This was performed with a 10 × 300 mm Superose 6, HR 10/30 column (Pharmacia LKB) at a flow rate of 0.4 mL/min. The buffer was 50 mmol/L phosphate, 0.5 mol/L NaCl, pH 7.2. Serum samples of 30–50 µL were diluted with an equal volume of buffer before application. The column was calibrated using low- and high-molecular-mass standard proteins and Blue Dextran 2000 (Pharmacia).

**Hydrophobic interaction chromatography.** This was performed with a 7.5 × 75 mm Ultrasphere TSK Phenyl-SPW column (LKB-Produkter AB) at a flow rate of 1 mL/min. Buffer A was ammonium sulfate 1.7 mol/L, phosphate 10 mmol/L, and buffer B, phosphate 10 mmol/L, pH 7.0. After application of a 25-µL serum sample, the concentration of buffer B was linearly increased from 30% (starting buffer) to 100% in 20 min. Fractions of 1 mL were collected.

**Assay for anti-murine IgG antibodies.** Crude mouse IgGs were isolated from mouse serum by precipitation at 45% ammonium sulfate saturation. The precipitate was dissolved in 0.1 mol/L sodium carbonate buffer, pH 9.0.

Mouse IgG was adsorbed onto polysyryene microtiter wells (Elaston, Helsinki, Finland) by incubation overnight at room temperature. To each well we added 250 µL of a 10 mg/L solution of IgG in 0.1 mol/L sodium carbonate buffer, pH 9.0. After washing the strips with the wash solution, containing 9 g of NaCl, 0.2 mL of Tween 20, and 1 g of Germal II as preservative per liter, we stored the strips in a moist atmosphere at 4 °C.

We added 25 µL of fourfold-diluted serum samples, or undiluted chromatographic fractions, and 200 µL of assay buffer (50 mmol/L Tris buffer containing 9 g of NaCl, 1 g bovine IgG, 0.1 mL of Tween 20, and 0.5 g NaN₃ per liter) to the coated microtiter wells and incubated for 2 h at room temperature. The wells were washed four times with wash solution, and to each well 20 ng of Eu-labeled polyclonal monoclonal anti-human IgG (Pharmacia Wallac, Turku, Finland) in 200 µL of assay buffer was added and incubated at room temperature for 1.5 h. Again, six washings were performed and 200 µL of enhancement solution (Pharmacia Wallac) was added (44). After gentle shaking for 5 min, we measured the fluorescence with a 1230 Arcus fluorometer (Pharmacia Wallac), using a measuring time of 1 s. Positive and negative control serum was included in every determination. The antibody concentration was expressed in arbitrary units, one arb. unit corresponding to 10 000 counts/s. The mean fluorescence ± SD in sera from 4 apparently healthy subjects was 8040 ± 2460 counts/s corresponding to 32 ± 10 kilo-arb. units/L with the polyclonal Eu-label.

**Quantitative assays.** Human α-fetoprotein (AFP), carcinoembryonic antigen (CEA), and chorionic gonadotropin (hCG) were measured by time-resolved fluorimmunoassay (TR-IFMA) with commercially available kits (Pharmacia Wallac).

**Binding studies.** Binding of HAMA to different mouse and rat antibodies was determined by TR-IFMA. Polystyrene beads coated with mouse Mab to CA 125, CEA, or with rMAb to estrogen receptor (Abbott Laboratories) were used. We added 25 µL of fourfold-diluted sera followed by 200 µL of assay buffer and performed the assay as for the antinmurine IgG antibodies. After counting we added 500 µL of enhancement solution and transferred 200 µL to microtiter wells for counting. Results were expressed in counts/second. To study the specificity of the antibodies, we preincubated the polystyrene balls coated with OC125 with 500 arb. units of CA 125 (per ball) that had been obtained from ovarian cyst fluid after removing cells and debris by centrifugation. To these, 25 µL of fourfold-diluted serum sample was added.

**Results.**

Apparent concentrations of CA 125 and HAMA in patients injected with Mab. We observed dramatic increases in apparent CA 125 concentrations in six of nine patients after immunostaining. Figure 1 shows serum CA 125 and HAMA concentrations in two patients after injection of the Mab OC125. About one month after the injection we detected an increase in the apparent CA 125 concentrations, which rapidly increased by 20–450-fold over the next two months. This increase in apparent antigen concentrations paralleled a similar rise in HAMA content. Generally, both the CA 125 and antibody concentrations decreased in a similar fashion three to six months after the immunostaining. The similar pattern of these two suggests that bridging of the two Mabs by HAMA in the CA 125 immunoassay was responsible for the increase in CA 125 concentrations.

We first attempted to suppress the false increase of CA 125 in patients’ sera by adding increasing amounts of normal mouse serum. Concentrations as high as 1.0 mL per milliliter of sample serum only slightly suppressed the apparent CA 125 concentrations (Figure 2). To evaluate the proportion of the antibodies that could be inhibited by addition of mouse IgG, we diluted the patient’s serum to
CA 125 concentration in the measuring range of the assay and added increasing amounts of purified IgG. The maximum reduction was only 28%, attained with 10 mg of mouse IgG per milliliter of serum. Larger amounts of mouse IgG did not further reduce the apparent CA 125 concentrations.

Interference in other assays. To study the specificity of the OC125-induced HAMA, we measured the concentrations of CEA, AFP, and hCG by MAb-based immunometric assays. HAMA did not interfere in the TR-IFMA of CEA. Minor interferences were observed in the AFP and hCG assays. In the AFP assay HAMA decreased the true AFP values, and in the hCG assay it increased them. The interference in the hCG assay was totally eliminated by adding 0.3 mL of mouse serum to 1 mL of patient serum.

Binding of HAMA to various antibodies. To further study the specificity of the OC125-induced HAMA, we measured its binding to solid-phase mouse MAb against CA 125 and CEA, to a rat MAb raised against the estrogen receptor, and to polyclonal mouse IgG isolated by affinity chromatography. HAMA bound strongly to purified polyclonal mouse IgG and to OC125 (Figure 3). In two samples HAMA bound significantly to the CEA MAb. HAMA did not react with the rat MAb to the human estrogen receptor. The binding to mouse IgG is not directly comparable with that of the other assays because different solid phases were used, microtitration strips for mouse IgG and polystyrene balls for the other assays. The background in the assays with coated beads was much higher than in our standard assay with microtiter wells.

The idiotypic nature of HAMA was further studied by adding 5000 arb. units of partially purified CA 125 to 25 µL of fourfold-diluted sample in the HAMA assay with OC125 as solid-phase. CA 125 reduced the binding of HAMA to OC125 by 40%.

Separation of CA 125 and HAMA

Cation-exchange chromatography. On the cation-exchanger Mono S at pH 6.5, CA 125 did not bind to the column, whereas all the interfering immunoreactivity (HAMA) was retained (Figure 4a). At pH 6.75 part of HAMA (40%) eluted together with CA 125 as a broad peak with buffer B at 7–11 mL. When the pH was lowered to 5.75 and 5.0, part of CA 125 (30% and 75%, respectively) was retained together with HAMA. At pH 6.5, CA 125 was recovered in the first 0.5–2 mL (Figure 4a) and this caused
Fig. 4. (a) Cation-exchange chromatography on Mono S at pH 6.5 and (b) anion-exchange chromatography on Mono Q at pH 8.0 of a patient's serum with HAMA (CA 125, total) for comparison the elution pattern of CA 125 of a control serum is shown (CA 125, true)

Fig. 5. Affinity chromatography on Protein A (a) or on Protein G (b) of a patient's serum with HAMA (CA 125, total). The elution pattern of CA 125 in a control serum is shown for comparison (CA 125, true). Bound protein is eluted (at arrow) with 0.1 mol/L citric acid-NaOH buffer, pH 3.0 (a), or with 0.1 mol/L glycine-HCl, pH 2.7 (b)

a 30-fold dilution of the original CA 125 concentration. These results indicate a lower isoelectric point of CA 125 compared with HAMA, thus complete separation at pH 6.5 is possible.

To study the reproducibility of cation-exchange chromatography for removal of HAMA, we studied serum samples from patients who had undergone immunoscintigraphy 4–30 weeks earlier. In all samples the CA 125 concentration in the nonretained fraction was decreased and approached prescintigraphic values (Figure 1). The HAMA concentration in this fraction was not measurable. After chromatography at pH 6.5 of serum from the control patient, 90% of CA 125 was recovered in the nonretained fraction, demonstrating that the effects on CA 125 concentrations noted for the immunized patients were related to specific removal of HAMA.

Anion-exchange chromatography. The results obtained by anion-exchange chromatography on Mono Q at pH 8.0 of HAMA and CA 125 are demonstrated in Figure 4b. The main native CA 125 peak eluted after IgG as a broad peak at about 0.5 mol/L NaCl. HAMA was mainly recovered in the nonretained fraction but there was some overlapping with native CA 125.

Affinity chromatography on Protein A and Protein G. By affinity chromatography on Protein A (Figure 5a) or Protein G (Figure 5b) HAMA was bound and could be eluted at low pH, whereas CA 125 was not retained. This treatment caused a marked decrease in apparent CA 125 concentration of patients' sera, CA 125 concentrations approaching pre-treatment values. In the patient's samples (Figure 5) most of the apparent CA 125 reactivity and the antibody concentrations were in the retained fraction, whereas no HAMA was detected in the nonretained fraction. After affinity chromatography 80–90% of the CA 125 was recovered. Protein A chromatography caused a 20-fold dilution of original serum CA 125 when a 100-μL sample was used that with Protein G chromatography was 30-fold. After chromatography of serum from control patients, the recovery of CA 125 was complete, demonstrating that the effects noted for the patients were related to specific depletion of interfering immunoglobulins.

Gel filtration. In gel filtration on Superose 6, native CA 125 antigen eluted at a volume of 7–9 mL corresponding to an $M_r$ of more than $10^6$ and HAMA at 12–15 mL corresponding to an $M_r$ of about 150 000 (Figure 6a). The recovery of CA 125 was 78%. This shows that HAMA can be separated from native CA 125 by gel filtration on the basis of the difference in molecular mass.

Hydrophobic interaction chromatography. The hydrophobic properties of CA 125 antigen and HAMA were quite similar as the main immunoreactive peak of both components eluted at 17 mL, corresponding to 0.17 mol/L (NH₄)₂SO₄. The broad elution profile of CA 125 antigen indicated a fairly high degree of heterogeneity in hydrophobicity, overlapping that of HAMA (Figure 6b).

Discussion

This study demonstrates that the antibodies that develop after injection of MAO OC125 can be only partially neutralized by absorption with mouse serum and mouse IgG because a major part of HAMA consists of anti-idiotype antibodies. Several studies claim that addition of mouse serum eliminates the false increase of CA 125 caused by HAMA (10, 11, 15). In sera with only moderately increased concentrations of HAMA, this treatment may reduce the falsely increased CA 125 concentrations, but it is important
Fig. 6. (a) Gel filtration on Superose 6 and (b) hydrophobic interaction chromatography on Ultropac TSK Phenyl-5PW of a patient’s serum with HAMA (CA 125, total), with the elution pattern of “true” CA 125 in a control serum shown for comparison. Same symbols are used in (a) and (b).

rapid and simple to perform. The complete binding of the nonspecific immunoreactivity to these columns (Figure 5) and especially to Protein G suggests that the interfering factor in the patient serum was IgG. Gel filtration could be used because the molecular mass of CA 125 is greater than that of IgG; however, this procedure is slow and requires a larger dilution, about 40-fold, than the other methods.

Cation-exchange chromatography at pH 6.5 was a suitable method for routine removal of HAMA. Because CA 125 was not retained by Mono S at this pH, the chromatography was simple to perform and CA 125 recovery was good. The dilution of serum CA 125 in cation-exchange, Protein A, and Protein G chromatography was similar, about 30-fold. Before quantifying CA 125 after chromatography, we concentrated the nonretained fractions about fivefold, but the total dilution of the original serum reduced the sensitivity to 30–50 kilo-arb. units/L. The recovery of the procedure, determined with sera containing native CA 125 antigen, was about 90%. However, a larger sample volume and a larger column can be used. An economical approach would be to use cation-exchange chromatography with open-column technique. Preliminary studies suggest that 200 μL of serum can be processed on an 8 × 55 mm column of S-Sepharose with the same recovery, but with a smaller dilution, as a Mono S column. This allows concentration of the sample to its initial volume.

The background of the HAMA assay was relatively high and varied from patient to patient, probably due to the high sensitivity of our assay technique in combination with the presence of natural antibodies reacting with mouse IgG (8). Because of this, each patient should serve as his own control when the assay is used to monitor HAMA after immunoscintigraphy.

The best specificity and sensitivity of the HAMA assay could apparently be achieved by using the same MAb on the solid-phase as that given to the patient. We tested this by using the solid-phase bead from assays for CA 125, CEA, and estrogen receptor, and europium-labeled anti-human IgG. This comparison showed that the HAMA of our patients reacted much more strongly with OC125 than with the other MAb’s. However, HAMA of different patients showed large differences in reactivity with various MAb’s. A rat MAb showed the weakest reaction.

As the use of diagnostic immunoscintigraphy with MAb’s increases, the presence of HAMA in sera should be determined, as should methods devised to eliminate the falsely increased results. Although cation-exchange chromatography is effective for CA 125, removal of HAMA with Protein A or Protein G, as reported here, may be more universally applicable. However, problems may be expected with sera containing antibodies of IgA or IgM type, which apparently was the case with some of our samples (not shown). Protein A and Protein G affinity matrices are also relatively expensive, which will be important in large-scale use. Cation-exchange chromatography is more economical, especially when applied as a simple open-column method, but requires that the antigen being measured have a clearly lower isoelectric point than HAMA. Consequently, the use of this method will be limited to relatively acidic antigens. Although most proteins are acidic, the applicability of the method must be tested for each antigen.

The dilution of the sample caused by the chromatographic methods used in the present study poses a problem; e.g., normal concentrations of CA 125 could not be measured without concentration of the fractions obtained by

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chromatography. This problem may be solved by concentrating the samples.

Although the adverse effects of HAMA can be reduced, the additional work required is considerable. Therefore, the benefits of using immunoscintigraphy have to be carefully weighed against its potential adverse effects.

References