Characterization of the CA 125 Antigen Associated with Human Epithelial Ovarian Carcinomas

H. M. Davis, V. R. Zurawski, Jr., R. C. Bast, Jr., and T. L. Klug

Centocor, Malvern, Pennsylvania 19355 [H. M. D., V. R. Z., T. L. K.], and Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710 [R. C. B.]

ABSTRACT

The murine monoclonal antibody OC125 reacts with an antigenic determinant (CA 125) found on a high-molecular-weight glycoprotein complex present in the serum of greater than 80% of women with epithelial ovarian cancer. The antigen expressing CA 125 (CA 125 antigen) isolated from the sera of ovarian carcinoma patients was shown by gel electrophoresis, molecular size exclusion chromatography, and buoyant density ultracentrifugation to have similar immunological and physical characteristics to antigen isolated from an ovarian cancer cell line (OVCA 433) and human milk. A composite sodium dodecyl sulfate:polyacrylamide gel electrophoresis gave rise to a band at approximately 200,000 daltons. Treatment of the CA 125 antigen from OVCA 433 cells with 10 mM periodic acid resulted in no loss of activity. Reduction and alkylation in 6 M guanidine-HCl or treatment at 100°C for 20 min resulted in complete loss of activity. Exoglycosidase treatments did not result in loss of activity, whereas protease digestion eradicated all activity. These data strongly suggest that the CA 125 antigenic determinant is composed of, at least in part, conformationally dependent peptide.

INTRODUCTION

The murine monoclonal antibody OC125 was generated by use of an established human serous cystadenocarcinoma cell line, OVCA 433 (1). Moreover, this antibody has been shown to react with an antigenic determinant (CA 125) expressed on greater than 80% of all nonmucinous ovarian epithelial tumors of serous, endometrioid, clear cell, and undifferentiated histologies (1, 2). Quantitation of this determinant in serum of patients with ovarian cancer has been made possible by the development of immunoradiometric assay with OC125 (3). The CA 125 antigenic determinant has also been reported to be found in human milk (4), normal cervical mucus (5), and the central airway and normal lung tissue (6). In addition, our laboratory has found CA 125 activity in human seminal plasma. The CA 125 antigenic determinant has also been reported to be associated with a mucin-like high-molecular-weight glycoprotein complex (4, 7-10). No studies, however, have been specifically directed towards elucidation of the physical and immunological characteristics of this antigen.

The objectives of this study were: (a) to identify by physical, chemical, and immunological methods the characteristics of this ovarian tumor-associated antigen isolated from various sources; and (b) to determine the biochemical nature of the determinant recognized by the monoclonal antibody OC125.

MATERIALS AND METHODS

Materials. The murine monoclonal antibody OC125, produced by hybridomas grown in pristane-primed BALB/c mice (1), was isolated by Protein A chromatography (11). Serum samples were obtained from women with advanced epithelial ovarian cancer (Stages III and IV). Human milk was obtained from a healthy 7-mo postpartum female. The exoglycosidases and proteases were purchased from Calbiochem, Los Angeles, CA (Pronase) and from Sigma, St. Louis, MO (chymotrypsin, trypsin, chondroitinase ABC, α- and β-galactosidase, α-fucosidase, hexosaminidase, and neuraminidase). Monoclonal antibody 1161NS 19-9 (12) was obtained from Dr. Zenon Steplewski, Wistar Institute, Philadelphia, PA. Polyclonal anti-CEA antibody was obtained from Abbott Laboratories, North Chicago, IL. Sepharose CL-4B and CL-6B and Protein A-Sepharose CL-4B were purchased from Pharmacia, Piscataway, NJ. Electrophoresis reagents were purchased from Bio-Rad, Rockville Centre, NY. SeaKem LE agarose was purchased from FMC Corp., Rockland, ME. Fish gelatin was obtained from Norland Products, Inc., New Brunswick, NJ. All other reagents were of the highest purity commercially available.

Solid-Phase Radioimmunoassays. The simultaneous “sandwich” IRMA was used to measure CA 125 activity (3) and CA 19-9 activity (13). In the CA 125 IRMA, 125I-labeled OC125 (100 μL, 1 x 10^6 cpm) was incubated (20 h, 23°C) with polystyrene-immobilized OC125 and sample (100 μL). The beads were washed (3 times) and counted in a gamma counter. Assay kits were manufactured at Centocor, Malvern, PA.

The plate assay was performed using 96-well polyvinyl chloride microtiter plates (Dynatech). The OVCA 433/PCA/4B (see Isolation of CA 125 from OVCA 433 Tissue Culture Supernatant) fraction was used to coat the wells (100 μL, 500 units/well). Following the binding of the antigen to the plates (18 h, 4°C), the wells were incubated for 1 h with PBS containing 5% (w/v) bovine serum albumin. After the incubation period, the wells were emptied and washed (2 times) with PBS. 125I-labeled OC125 (20 μL, 2 x 10^6 cpm) was then incubated with the immobilized antigen (4 h, 23°C). The wells were subsequently washed (3 times) with PBS, cut, and counted in a gamma counter.

As the CA 125 IRMA only detects polyvalent antigens, an inhibition assay was developed to quantitate both mono- and multivalent antigens. The inhibition assay was performed similarly to the plate assay described above, the only difference being that 125I-labeled OC125 (20 μL, 2 x 10^6 cpm) was incubated simultaneously (30 μL, 4 h, 23°C) with various antigen preparations which might inhibit binding of radiolabelled OC125 to the plate. The wells were washed (3 times), cut, and counted in a gamma counter. The radiiodinated OC125 used in both the plate and inhibition assays was obtained from Centocor RIA kits.

SDS-Polyacrylamide Gel Electrophoresis. Conventional SDS-PAGE was performed essentially according to the method of Laemmli (14). The sample buffer did not contain sodium dodecyl sulfate reducing agents or SDS and was not heated, as the CA 125 antigen was inactivated by these conditions. Some experiments required a polyacrylamide gel for separation of sample components as the CA 125 antigen did not penetrate a conventional 3% (w/v) polyacrylamide gel. Typically, the composite gels were prepared with 2.5% polyacrylamide and 1.0% agarose. The solutions were heated to 65°C at which time the ammonium persulfate was added. The prewarmed solutions were then

Received 5/27/86; revised 9/4/86; accepted 9/5/86.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

To whom requests for reprints should be addressed.

1 Unpublished results.
immediately poured into the gel apparatus which had been equilibrated at 37°C, and the entire apparatus was then cooled at 4°C until the agarose solidified. After overlying a 2.5% polyacrylamide:1.0% agarose stacking gel at room temperature, the samples (300 units/lane) were applied in 10 µl urea sample buffer which did not contain sulfhydryl-reducing agents or SDS and was not heated. The electrophoresis was performed at 4°C. All buffers used in the preparation and running of the composite gels were also those of Laemmli (14).

Immunoblotting. After electrophoresis the proteins were electrochemically transferred to nitrocellulose [Towbin et al., (15), immunoblotted with radiolabeled OC125, and autoradiographed. Each immuno blot contained at least one negative antigen control lane. The electrophoretic transfer was performed at 100 mA overnight. Immunoblotting was accomplished by overlaying the nitrocellulose with radiodinated OC125 (2 ml, 2 x 10^6 cpm) in fish gelatin buffer [1% fish gelatin:50 mM citrate (pH 6.0:0.05% NP-40) for 6 h. The nitrocellulose sheet was then autoradiographed by exposure to X-ray film with the aid of a Cronex Quanta III fluor screen (Dupont) for 18 h at -80°C.

Fractionation of Human Serum and Human Milk. Whole serum was allowed to clot for 1 h and then centrifuged (3000 x g, 10 min). A portion (2 ml) of the supernatant was fractionated on a 1.2- x 47-cm Sepharose CL-4B column (human serum/4B) equilibrated in PBS. Fractions (1 ml) containing CA 125 activity, as determined by the CA 125 RIA, were pooled and concentrated. Human milk was defatted by centrifugation (3000 x g, 1 h) at 10°C. The supernatant was further purified by column chromatography as described above for serum (human milk/4B).

Preparation of CA 125 Antigen Concentrate from OVCA 433 Tissue Culture Supernatant. OVCA 433 human ovarian carcinoma cells were grown in Eagle's minimal essential medium supplemented with 2 mM glutamine, 1 mM pyruvate, 1% nonessential amino acids, and 10% heat-inactivated fetal calf serum. T-150 flasks (Costar) were seeded with 1 x 10^6 cells. Growth was permitted to continue until cells reached confluence at which time the medium was removed. Fresh medium was added and collected at 5- to 7-day intervals, for a total of 10 to 12 wk. OVCA 433 cells appeared to produce the maximum amount of CA 125 antigen in G0 growth phase. The concentration of CA 125 antigen produced under these conditions was approximately 1,000 units/ml. Pooled cell supernatants were centrifuged at 10,000 x g, filtered through a Sartorius 0.2-µm-pore-size cascade filter capsule, and concentrated to one-tenth of the original volume with an Amicon 2 cc bottom filter apparatus and filter cartridge (HP 100-200) with a molecular weight cutoff of 100,000. The concentrates were stored frozen at -20°C under conditions which the CA 125 activity was stable for at least 12 mo.

Isolation of CA 125 from OVCA 433 Tissue Culture Supernatant. The spent tissue culture 10x concentrate of the OVCA 433 cell supernatant was first subjected to PCA, (0.6 m final concentration) precipitation (16). The CA 125 activity remained in the PCA-soluble fraction and was completely conserved. The acid-soluble fraction was neutralized with 1 x 10^6 cells. Growth was permitted to continue until cells reached confluence at which time the medium was removed. Fresh medium was added and collected at 5- to 7-day intervals, for a total of 10 to 12 wk. OVCA 433 cells appeared to produce the maximum amount of CA 125 antigen in G0 growth phase. The concentration of CA 125 antigen produced under these conditions was approximately 1,000 units/ml. Pooled cell supernatants were centrifuged at 10,000 x g, filtered through a Sartorius 0.2-µm-pore-size cascade filter capsule, and concentrated to one-tenth of the original volume with an Amicon 2 cc bottom filter apparatus and filter cartridge (HP 100-200) with a molecular weight cutoff of 100,000. The concentrates were stored frozen at -20°C under conditions which the CA 125 activity was stable for at least 12 mo.

Isolation of CA 125 from OVCA 433 Tissue Culture Supernatant. The spent tissue culture 10x concentrate of the OVCA 433 cell supernatant was first subjected to PCA, (0.6 m final concentration) precipitation (16). The CA 125 activity remained in the PCA-soluble fraction and was completely conserved. The acid-soluble fraction was neutralized with potassium hydroxide (1.2 m), dialyzed against distilled water (24 h, 4°C), and concentrated to 20x the original supernatant volume. This sample is referred to as OVCA 433/PCA. The OVCA 433/PCA sample (35 ml) was applied to a Sepharose CL-4B column (3.2 x 70 cm) equilibrated in PBS. The fractions (7 ml) which contained CA 125 activity as determined by the CA 125 RIA were pooled and concentrated. This fraction is referred to as OVCA 433/PCA/4B and is used in all experiments except as indicated.

Further fractionation involved treatment of the OVCA 433/PCA/4B fraction with urea (6 M, 30 min, 45°C) and subsequent chromatography on a Sepharose CL-6B column equilibrated in Tris:urea:SDS [50 mM Tris:6 M urea:0.1% SDS (pH 8.0)]. Final fractionation was accomplished by immunoaffinity chromatography on an OC125:Protein A-Sepharose CL-4B column. The monoclonal antibody OC125 was covalently bound to the Protein A-Sepharose CL-4B column, washed, and coupled essentially according to the method of Schneider et al. (17). Minor modifications included the substitution of citrate buffer (0.05 M, pH 6.0) for Tris-HCl, and taurodeoxycholate for deoxycholate. Repeated passes (3 times) over the affinity column of the CA 125 reactive lower molecular weight fraction from the Sepharose CL-4B column in 0.1% SDS and 6 M urea gave greater than 80% binding of the CA 125 activity. Elution of the CA 125 antigen from the column was accomplished with the use of DEA (50 mM, pH 11.3). This affinity-purified antigen is referred to as OVCA 433/4B/DEA.

Density Gradient Ultracentrifugation. Ultracentrifugation of the CA 125 antigen isolated either from human serum, human milk, or from the OVCA 433 tissue culture supernatant after chromatography on Sepharose CL-4B was performed in a cesium chloride isopycnic density gradient in PBS (2.276 g of CsCl dissolved in 3.541 ml of PBS). The buoyant density of β-galactosidase was determined as a standard. Fractions (0.2 ml) were assayed for β-galactosidase activity following equilibrium by the method of Miller (18). Gradients were formed by ultracentrifugation in a Beckman SW50.1 rotor (33,000 rpm, 68 h, 10°C) under conditions which have been described (19). Fractions (0.2 ml) were collected and assayed for activity using the CA 125 RIA described above. The density of each fraction was determined by weighing a known volume.

Chemical Treatments. Periodate oxidation of the CA 125 antigen was accomplished with 0, 0.1, 1.0, 10.0, and 100 mmoi periodate in acetate buffer (pH 4.5, 50 mM, 4°C) in the dark according to Stahl et al. (20). Reduction and alklylation were performed according to methods described elsewhere (21). Reduction was accomplished with DTT (10 mM, 50 mM Tris, pH 8.1, 4 h, 45°C) either in the presence or absence of guanidine-HCl (6 M). Alkylation was performed with iodoacetic acid (20 mM, 30 min) after the samples had been cooled to room temperature. The samples were immediately dialyzed (4°C, 18 h) against distilled water.

Exoglycosidase Treatments. Exoglycosidase digestions were performed in acetate buffer (0.2 M, pH 4.5, 48 h, 37°C). Unit values of the exoglycosidases were chosen in order to ensure complete digestion of the oligosaccharide residues within an appropriate time frame. All exoglycosidase digestions were performed under conditions whereby the appropriate substrates were shown to be completely hydrolyzed as detected by thin-layer chromatography, CA 125 activity following treatment was measured both by the CA 125 RIA and by the plate assay as previously described.

Exhaustive Protease Digestion. The various protease digestions were performed in Tris-HCl buffer (0.2 M, pH 8.0, 10 mM calcium chloride). The proteases trypsin, chymotrypsin, and Pronase (2%, w/v, 50 µl) were added to wells containing antigen and allowed to incubate (48 h, 37°C). Protease digestions were performed under conditions which caused hydrolysis of albumin as detected by thin-layer chromatography. Samples were assayed for CA 125 activity by both the CA 125 RIA and the plate assay as described above.

Amino Acid Analysis. Samples of OVCA 433/4B/DEA were dissolved in 6 N HCl containing 0.1% phenol, sealed under vacuum, and hydrolyzed for 24 h at 110°C. Amino acids were derivatized with phenylisothiocyanate, separated, and quantitated by high-pressure liquid chromatography using the Waters PICO-TAG column and elution conditions (22).

Exhaustive Protease Digestion. The various protease digestions were performed in Tris-HCl buffer (0.2 M, pH 8.0, 10 mM calcium chloride). The proteases trypsin, chymotrypsin, and Pronase (2%, w/v, 50 µl) were added to wells containing antigen and allowed to incubate (48 h, 37°C). Protease digestions were performed under conditions which caused hydrolysis of albumin as detected by thin-layer chromatography. Samples were assayed for CA 125 activity by both the CA 125 RIA and the plate assay as described above.

Carbohydrate Composition. Samples of the same lot of OVCA 433/4B/DEA that had been subjected to amino acid quantitation were subjected to carbohydrate compositional analysis as described by Yang and Hakomori (23). The samples were subjected to acetylation, followed by hydrolysis and reduction. The resultant alditols were peroxoacetylated with acetic anhydride. Quantitation of sialic acid was accomplished by trimethylsilyl derivatization (24, 25). Both the alditol acetate and the trimethylsilyl-methyl glycosides were separated by a Hewlett Packard 5790 gas chromatograph and identified by a Hewlett Packard 5970 mass selective detector.

RESULTS

Physical and Immunological Characteristics of the CA 125 Antigen. The antigen isolated from OVCA 433 and from human ovarian cancer patient serum by perchloric acid precipitation elutes primarily in the void volume of a Sepharose CL-4B column (Fig. 1). In addition, a smaller peak of CA 125 activity elutes from the column later, indicating a much lower molecular weight cutoff.
CA 125 ANTIGEN CHARACTERIZATION

Fig. 1. Sepharose CL-4B column chromatography of the CA 125 antigen isolated from the OVCA 433 tissue culture supernatant (O) and from human serum (C). The chromatography was performed as described in "Materials and Methods." Fractions were monitored for CA 125 activity by a solid-phase RIA.

Fig. 2. Density gradient ultracentrifugation following Sepharose CL-4B column chromatography of the CA 125 antigen isolated from human serum (O), OVCA 433 tissue culture supernatant (•), and from human milk (D). Fractions were monitored for CA 125 activity and density as described in "Materials and Methods." G, buoyant density of β-galactosidase.

Fig. 3. Sodium dodecyl sulfate:2.5% polyacrylamide:1.0% agarose composite gel electrophoresis of the CA 125 antigen isolated from human milk/4B (200 units/lane) (Lane 1), OVCA 433 passage 52/4B (200 units/lane) (Lane 2), OVCA 433 passage 69/4B (300 units/lane) (Lane 3), human ovarian cancer patient sera/4B (100 to 200 units/lane) (Lanes 4 to 7), and from a negative control serum/4B (23 units/lane) (Lane 8). The molecular weight standards used were: M, 200,000, myosin heavy chain; M, 440,000, undenatured myosin; and M, 900,000, IgM. Refer to "Materials and Methods" for conditions concerning electrophoresis and immunoblotting techniques.

Fig. 4. Conventional SDS:PAGE (3 to 12% gradient) of the CA 125 antigen isolated from OVCA 433. The Western blots were overlaid with radioiodinated monoclonal antibody OC125 (Lane A), radioiodinated monoclonal antibody 19-9 (Lane B), and with radioiodinated anti-CEA (Lane C). Electrophoresis and immunoblotting conditions are described in "Materials and Methods."

weight. These peaks of CA 125 activity correspond to molecular masses of greater than 1 million and about 200,000 to 400,000 daltons. The CA 125 antigen elution pattern of human milk is similar to that shown for OVCA 433 and human cancer patient serum (data not shown).

In an effort to compare the physical characteristics of the antigen isolated from OVCA 433 cell supernatants, ovarian cancer patient serum, and human milk, a buoyant density was determined for each (Fig. 2). The average buoyant density of the antigen isolated from OVCA 433 after passage over a Sepharose CL-4B column (OVCA 433/4B) is approximately 1.42 g/ml, whereas the buoyant densities of the patient serum/4B and the milk/4B are 1.46 and 1.39 g/ml, respectively. As a standard, the buoyant density of β-galactosidase was determined and found to be 1.32 g/ml. This agrees well with the published value of 1.316 g/ml (26). Fig. 3 compares the electrophoretic mobility of immunoreactive species from OVCA 433/4B, human milk/4B, and ovarian cancer serum/4B on a composite 2.5% polyacrylamide/1.0% agarose gel. The samples were applied in 10 M urea sample buffer which did not contain DTT or SDS and was not heated. The immunoblotted OC125 reactive antigen from each of the sources is present as high-molecular-mass complexes of between 200,000 and 1 million daltons with similar electrophoretic profiles. This data, which suggest multiple aggregated states of the CA 125 antigenic complex, correlate well with the Sepharose CL-4B elution profile shown in Fig. 1. Both experiments indicate that antigen exists as a high-molecular-mass species of greater than 1 million daltons and lower-molecular-mass species of approximately 200,000 to 600,000 daltons.

When the OVCA 433/PCA/4B fraction is subjected to SDS:PAGE electrophoresis using a 3 to 12% polyacrylamide gradient gel followed by immunoblotting (Fig. 4), the lane which is reactive with radioiodinated monoclonal antibody OC125 gives rise to a band of greater than 1-million dalton molecular mass and a lower-molecular-mass band of approximately 400,000. The sample buffer used contained only 10% glycerol, 0.08 M Tris (pH 6.8), and bromophenol blue. After overlaying the adjacent lane with radioiodinated monoclonal antibody 19-9, which recognizes the sialylated lacto-N-fucopentaose II carbohydrate determinant, only the higher-molecular-mass band is observed. The lane which is overlaid with radioiodinated anti-CEA does not show any immunoreactivity. Furthermore, Western blots using monoclonal antibody 19-9 as an overlay with the OC125 affinity-purified CA 125 antigen (OVCA 433/4B/DEA) fraction do not give rise to any bands. Also, there is no CA 19-9 activity present when measured by the CA 19-9 RIA (data not shown). This result clearly demonstrates that the antigenic determinants CA 125 and CA 19-9 are located on the same high-molecular-mass glycoprotein complex, but the CA 125 and CA 19-9 determinants are not present on the same glycoprotein molecule.

The results of Sepharose CL-4B column chromatography and of SDS:PAGE analysis suggests that the lower-molecular-weight material was probably derived from the higher-molecular-weight species. Attempts to disaggregate the high-molecu-
lar-weight material with both ionic (SDS) and nonionic (NP-40) detergents proved futile. However, treatment of the pooled and concentrated void volume fraction of the Sepharose CL-4B column of OVCA 433/PCA with 6 M urea for 30 min at 45°C, followed by column chromatography on Sepharose CL-6B in 0.1% SDS and 6 M urea, yields two peaks, as shown in Fig. 5. Following this step, the majority (80%) of the CA 125 activity is found associated with a much lower molecular mass peak of approximately 200,000 daltons. This is verified by electrophoresis and immunoblotting of fractions from the Sepharose CL-6B column chromatography (Fig. 5). Some of the antigen still remains in the high-molecular-mass aggregated form.

Immunofinity Purification of the CA 125 Antigen from OVCA 433 Cells. Sepharose CL-4B column chromatography followed by treatment with 6 M urea and heat with subsequent column chromatography on Sepharose CL-6B in the presence of 6 M urea and 0.1% SDS (Fig. 5) results in a 1400-fold purification of CA 125 antigen from OVCA 433 supernatants (data not shown). This preparation has a specific activity of 117 units of CA 125 per µg of protein. The specific activity is determined by measuring the CA 125 activity using the Centocor CA 125 RIA kit and determining the amount of protein by amino acid analysis on this same lot of purified CA 125 antigen. Final fractionation of the antigen is accomplished by immunofinity on an immobilized OC125:Protein A:Sepharose CL-4B column. The antigen which eluted from the column with DEA has a specific activity of 317 units of CA 125 per µg of protein.

Samples of antigen eluting from a Sepharose CL-4B column and from an OC125 immunofinity column were subjected to density gradient ultracentrifugation. This procedure reveals different average buoyant densities for the two antigen preparations (Fig. 6). The more highly purified DEA eluate has a buoyant density of approximately 1.36 g/ml, whereas the buoyant density of the OVCA 433/4B is approximately 1.42 g/ml. This suggests that the less pure antigen is associated with more highly glycosylated proteins which would result in the polydisperse nature of the buoyant density profile as well as the higher average buoyant density observed.

Carbohydrate Composition of the CA 125 Antigen Isolated by Affinity Chromatography. Preliminary carbohydrate composition of OVCA 433/4B/DEA reveals that sialic acid, fucose, mannose, galactose, N-acetyl glucosamine, and N-acetyl galactosamine are present in the 3.6:0.4:0.3:0.6:6.5:8.2 ratio, respectively (data not shown). These data suggest that there are both nitrogen- and oxygen-linked oligosaccharides present. In addition, this immunopurified CA 125 antigen is found to contain 24% carbohydrate, by mass, in close agreement with that calculated from its buoyant density of 1.36 g/ml. Therefore, the CA 125 antigen is not a typical mucin and does not have a significant amount, if any, of lipid associated with it.

Nature of the CA 125 Determinant. The nature of the CA 125 determinant was investigated using a number of chemical and physical treatments, as well as exhaustive exoglycosidase and protease digestions of the antigen. Periodate oxidation (Table 1) of the CA 125 immunoreactive antigen isolated from OVCA 433/4B and from human milk/4B has no effect on activity at periodate concentrations and reaction times that totally destroyed activity at 0.1 mM. In fact, at the lowest periodate concentrations which destroyed CA 19-9 activity (0.1 mM) there actually appears to be an increase in CA 125 activity.

Table 1 Effect of periodate oxidation on CA 125 activity at various concentrations and reaction times

<table>
<thead>
<tr>
<th>Sample</th>
<th>Periodate concentration (mM)</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVCA 433/PCA/4B</td>
<td>0</td>
<td>100</td>
<td>99</td>
<td>102</td>
<td>102</td>
</tr>
<tr>
<td>0.1</td>
<td>100</td>
<td>110</td>
<td>115</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>100</td>
<td>134</td>
<td>144</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>100</td>
<td>151</td>
<td>135</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>100.0</td>
<td>100</td>
<td>72</td>
<td>48</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Human milk/4B</td>
<td>0</td>
<td>100</td>
<td>101</td>
<td>103</td>
<td>98</td>
</tr>
<tr>
<td>0.1</td>
<td>100</td>
<td>103</td>
<td>93</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>100</td>
<td>107</td>
<td>110</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>100</td>
<td>111</td>
<td>69</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>100.0</td>
<td>100</td>
<td>7</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Positive control 19-9 GPM</th>
<th>0</th>
<th>100</th>
<th>100</th>
<th>97</th>
<th>99</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>100</td>
<td>16</td>
<td>11</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>100</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

* cGPM, cellular glycoprotein (expressing 19-9 determinant).
Only at very high concentrations of periodate (100 mM) or at very long reaction times (24 h) is there a significant decrease in CA 125 activity, which is likely due to nonspecific oxidation of the antigen protein backbone.

Chemical and physical treatments (Table 2) which denature most proteins, that is, reduction and alkylation in 6 M guanidine-HCl:8 M urea, and boiling all reduce the CA 125 immunoreactivity substantially. Reduction alone, however, does not seem to affect CA 125 immunoreactivity. Thus, the decrease in activity observed with either reduction or alkylation in the presence of guanidine-HCl is mainly the result of guanidine-HCl acting on the antigen. There is almost complete loss of activity with reduction and alkylation in the presence of guanidine-HCl. In addition, neither the anionic detergent SDS nor the nonionic detergent NP-40 affects the CA 125 immunoreactivity. Thus, the decrease in activity with periodate oxidation is mainly the result of guanidine-HCl acting on the antigen. There is almost complete loss of activity with reduction and alkylation in the presence of guanidine-HCl. In addition, neither the anionic detergent SDS nor the nonionic detergent NP-40 affects the CA 125 immunoreactivity.

Various combinations of exoglycosidase treatments were performed on the CA 125 antigen (Table 3). The solid-phase IRMA indicates only slight losses of CA 125 immunoreactivity with either α-galactosidase and/or β-galactosidase treatments. On the other hand, no loss of immunoreactivity is demonstrated using the plate assay. In fact, there is an increase in the ability of the immobilized antigen to bind radiolabeled OC125 antibody following most of the exoglycosidase treatments. This result corroborates that obtained with periodate oxidation; that is, removal of terminal carbohydrate moieties may actually increase access of OC125 to the CA 125 determinant.

Finally, exhaustive protease digestion with Pronase, trypsin, or chymotrypsin causes complete loss of antigenic activity as measured with either the IRMA or the plate assays (Table 3).

**DISCUSSION**

The murine monoclonal antibody OC125 recognizes a human ovarian carcinoma-associated antigenic determinant (CA 125). We have isolated glycoprotein complexes from the ovarian cancer cell line OVCA 433, human serum, and human milk all of which express CA 125-determinant activity. In addition, we have evidence of CA 125 activity in seminal plasma which is in contrast to the observations of de Bruijn et al. (5). Chemical treatment and chromatography of the high-molecular-weight complex isolated from OVCA 433 cell supernatants gave rise to a M, 200,000 immunoreactive species. It is possible, however, that the actual protein which expresses the antigenic determinant may be of still lower molecular weight. Further attempts to isolate a lower-molecular-weight immunoreactive species have thus far proven ineffective. Moreover, the isolation scheme described here does not give rise to a completely homogeneous and pure species.

The antigen expressing the CA 125 determinant isolated from several sources exists as a high-molecular-weight glycoprotein complex with an average buoyant density of between 1.36 and 1.46 g/ml. Moreover, these average densities indicated that each of the antigens isolated from three sources may have had a slightly different protein and carbohydrate composition. If a mucin is defined as a high-molecular-weight glycoprotein composed of 50% or more carbohydrate with a majority of oxygen-linked oligosaccharides containing little or no nitrogen-linked chains, then the CA 125 antigen is not a typical mucin. This conclusion is based on the CA 125 carbohydrate composition of 24%, the high amount of mannose present, the majority of nitrogen-linked oligosaccharides, and the CA 125 antigen buoyant density. The average buoyant density of unglycosylated protein is between 1.25 and 1.35 g/ml (26), while the average buoyant density of mucins (19) is approximately 1.50 g/ml. This finding is in contrast to that reported for other epithelial tumor-associated antigens recognized by monoclonal antibodies such as 19-9 (19, 27), B72.3 (28), DU-PAN-2 (29), and F36/22 (30), all of which have been classified as high-molecular-weight mucin-like glycoproteins based on their higher buoyant densities.

The higher-molecular-weight antigen complex isolated from the supernatant of OVCA 433 was reactive with the monoclonal antibody 19-9 (12, 27), suggesting that the CA 19-9 determinant is present on this complex. However, we have clearly shown by electrophoresis and immunoblotting that the CA 19-9 and the CA 125 determinants were not located on the same glycoprotein, since the OC125 immunoaffinity-purified CA 125 antigen showed no reactivity with the monoclonal antibody 19-9. This observation is contrary to that suggested by Hanisch et al. (4) who had isolated both CA 19-9 and CA 125 activity from human milk.

Chemical and physical treatments of CA 125 antigen were undertaken to better understand the nature of the antigenic determinant recognized by the monoclonal antibody OC125. Periodate oxidation of CA 125 reduced the immunoreactivity only at high concentrations of periodate or with prolonged reaction times. In fact, the activity of the antigen actually increased at concentrations and reaction times which caused total loss of immunoreactivity of the CA 19-9 determinant. Nonspecific oxidation of the protein backbone probably caused
the loss of CA 125 activity at higher concentrations of periodate. During CA 125 antigen purification, there was a loss of 82% of the original activity following urea and heat treatment. This apparent loss in activity was most likely due to breakdown of antigen complex to a less aggregated form or to partial denaturation of the antigen. A lower aggregated state may lead to a lower unit value as the CA 125 RIA is sensitive to CA 125 antigen valency, i.e. the number of OC125 binding sites per antigen molecule.

The observations of Hanisch et al. (4), which suggested that the CA 125 determinant is carbohydrate in nature, were based on two criteria: its sensitivity to periodate oxidation (at a concentration of 100 mM and a reaction time of 18 h); and its loss of activity under conditions which would selectively cleave N-acetylneuraminic acid (pH 3.3, 100°C). Their results also indicated that neuraminidase treatment alone caused only slight reduction of immunoreactivity even though approximately 97% of the mucin-linked sialic acid was cleaved. Our results clearly show that concentrations of periodate sufficient to oxidize carbohydrates do not affect CA 125 activity, and that heating the antigen at 100°C totally destroys activity. It is not surprising, therefore, that pH 3.3 at 100°C destroyed CA 125 antigenic activity. In addition, greater than 95% of the activity was lost upon reduction and alkylation treatment in the presence of guanidine-HCl. Lastly, exoglycosidase treatments actually caused an increase in CA 125 activity, while antigen activity was completely eradicated with exhaustive protease digestion. These data strongly suggest that the CA 125 determinant is proteinaceous in nature, or at the very least, is protein associated with carbohydrate in a conformationally dependent epitope. This may explain the similarity of the antigen isolated from the various sources such as human serum, OVCA 433, and human milk. A peptide determinant would be expected to be more highly conserved than a carbohydrate determinant; that is, a protein sequence is more likely to be associated with a single unique protein, whereas a carbohydrate structure may exist on several different proteins. These results may not be completely unique as the nature of the tumor-associated glycoprotein epitope (TAG-72) recognized by the monoclonal antibody F36/22. Cancer Res., 43: 4980–4988, 1983.


Characterization of the CA 125 Antigen Associated with Human Epithelial Ovarian Carcinomas

H. M. Davis, V. R. Zurawski, Jr., R. C. Bast, Jr., et al.