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

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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. The buoyant densities of the CA 125 antigen complexes from human serum, OVCA 433 cells, and human milk were in the range of 1.36 to 1.46 g/ml. Isolation of CA 125 antigen of higher purity from OVCA 433 supernatant was achieved by a series of steps including 245C125 immunoaffinity chromatography. Subsequent resolution of this purified CA 125 antigen complex by 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 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 an 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.2 The CA 125 determinant has 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-CEA2 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 × 10⁶ 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 polystyrene 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 × 10⁶ 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 × 10⁴ cpm) was incubated simultaneously (30 μl, 4 h, 23°C) with various antigen preparations which might inhibit binding of radio labeled OC125 to the plate. The wells were washed (3 times), cut, and counted in a gamma counter. The radiolabeled 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 Laemml (14). The sample buffer did not contain sulfhydryl reducing agents or SDS and was not heated, as the CA 125 antigen was inactivated by these conditions. Some experiments required a polyacrylamide:agarose composite 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

1 To whom requests for reprints should be addressed.
2 Unpublished results.

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The abbreviations used are: CEA, carcinoembryonic antigen; PBS, phosphate-buffered saline (0.01 sodium phosphate:0.15 NaCl:0.005 Na₂HPO₄); PCA, perchloric acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NP-40, Nonidet P-40 detergent; DEA, diethylamine; IRMA, immunoradiometric assay; DT,T, dithiothreitol; RIA, radioimmunoassay.
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 overlaying 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 electrophoretically transferred to nitrocellulose [Towbin et al. (15)], immunoblotted with radiolabeled OC125, and autoradiographed. Each immunoblot 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 radiolabeled 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% FBS. The 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% FBS. The 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% FBS. The 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% FBS. The 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% FBS. The 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% FBS. The 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% FBS. The 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% FBS. The culture supernatant.
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 (O). 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 ( ). Fractions were monitored for CA 125 activity and density as described in "Materials and Methods." G, buoyant density of β-galactosidase.

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. These 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 (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."

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 serum/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). 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.
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 immunoaffinity 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 CA 125 per µg of protein.

Samples of antigen eluting from a Sepharose CL-4B column and from an OC125 immunoaffinity 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 3.6:0.40:3.0:6.6:5.8:2.2 ratio, respectively (data not shown). These data suggest that there are both nitrogen- and oxygen-linked oligosaccharides present. In addition, this immunoaffinity purified CA 125 antigen is found to contain 24% carbohydrate, by mass, in close agreement with the buoyant density 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 of the CA 19-9 carbohydrate determinant, the sialylated lacto-N-fucopentaose II. 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.
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.

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 mucus glycoprotein from human milk. Eur. J. Biochem., 149: 323–330, 1985.


36. Towbin, H., Staehelin, T., and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applic


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