Biochemical Analysis of Human Ovarian Cancer-associated Antigens Defined by Murine Monoclonal Antibodies

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ABSTRACT

Two monoclonal antibodies (MOv1 and MOv2) raised against a membrane preparation of a human surgical specimen from a mucinous ovarian cystadenocarcinoma were used to biochemically define their target antigens. The heating of peritumoral mucus-soluble extracts and the sialidase treatment of crude membrane preparations did not affect the binding capacity of MOv1 and MOv2, which, on the contrary, was significantly reduced by periodate oxidation of the same materials. Pronase digestion completely solubilized MOv1-defined antigens, whereas MOv2-defined antigens were only partially solubilized. This, however, did not affect antibody binding with digested products. These data suggest that carbohydrate residues of recognized molecules constitute the antigenic determinants and that sialic acid residues are not involved. Gel filtration on Sephacryl S-200 revealed that most of the antigenic activity eluted in the void-volume fractions with a high carbohydrate content and in the included fractions before the elution volume of the ferritin standard protein. When CsCl gradient equilibrium ultracentrifugation of the solubilized mucus was used, MOv1-recognized antigens sedimented with a density of 1.45 g/ml, while the MOv2-defined epitope was carried by molecules with a density of 1.52 g/ml as well as by molecules with a lower density. Using thin-layer chromatography of organic solvent extracts obtained from mucus and crude membrane preparations, only MOv2-positive molecules could be resolved as a single band of glycolipid. Altogether, these data suggest that the antigens detected by MOv1 are mainly mucins whereas the determinant recognized by MOv2 is carried by both mucins and a glycolipid.

To analyze the diagnostic potential of MOv1- and MOv2-recog-

nized antigens, we tested their presence, as soluble products, in supernatants of tumor cell lines and in peritoneal effusions from cancer patients. Ovarian tumor-associated antigens were studied with the aim of detecting markers which could be clinically useful in establishing initial diagnosis and in monitoring response to therapy. A number of markers associated with ovarian carcinoma have been identified using polyclonal heteroantisera (3, 4, 13, 16) and, more recently, monoclonal antibodies (1, 6). Most of the potential markers are high-molecular-weight glycoproteins rich in carbohydrates expressed on the cell surface and shed or secreted by tumor cells. OCA1 (4, 5), OCA (14, 15), the antigen described by Dawson et al. (10), CA 125 (1, 2), and the antigen defined by the DU-PAN-2 monoclonal antibody (21) may be included in this group. Some of these antigens have been detected in peritoneal effusions and serum.

We have already reported the generation of 2 monoclonal antibodies which, respectively, recognize an antigen present on mucinous ovarian carcinomas (MOv1) and an antigen common to serous and mucinous carcinomas (MOv2) (24). These antigens are not detectable in normal ovaries, whereas they are expressed with different patterns in normal gastrointestinal glands. Detailed histological reactivities and preliminary cytological applications of MOv1 and MOv2 monoclonal antibodies have been described (24). The biochemical characterization and partial purification of the relevant antigens are reported in this work.

MATERIALS AND METHODS

Chemicals. Where not otherwise stated, chemicals were purchased from Merck, Darmstadt, West Germany. Ferritin and Pronase came from Calbiochem, Los Angeles, CA; Sepharose 4B, Sepharose 6B, Protein A-Sepharose CL-4B, CNBr-activated Sepharose 4B, and Blue Dextran 2000 were from Pharmacia, Uppsala, Sweden; cesium chloride, BSA, and phenylmethylsulfonyl fluoride were from Sigma Chemical Co., St. Louis, MO; 2-mercaptoethanol was from Bio-Rad Laboratories, Richmond, CA; Nonidet P-40 was from BDH Chemicals, Ltd., Poole, England; Antigosan was from Behring Spa, L'Aquila, Italy; sialidase (Test-neo-

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Antiserum. The production and the biological characterization of mon-

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BIOTECHNOLOGICAL ANALYSIS OF HUMAN OVARIAN CANCER ANTIGENS

Cell Lines and Tissues. The characteristics of cell lines and tissue culture methods have been reported previously (24). The cell line 1063 of human ovarian mucinous carcinoma origin was kindly provided by Dr. A. Horowitz. CM from surgical specimens were prepared according to the method of Pierotti et al. (22).

Mucus Preparation. After removal of tissue fragments, the peritumoral mucus was processed as follows: (a) saline extraction with 200 mm NaCl under intense stirring for 1 hr at room temperature; (b) enzymatic digestion by Pronase (0.2 mg/mucus proteins) in 200 mm ammonium acetate buffer (pH 6.5) for 16 hr at 37°C; (c) reduction of disulfide bridges with 500 mm 2-mercaptoethanol for 2 hr at 37°C. After each treatment, insoluble material was discarded by gauze filtration and centrifugation at 20,000 x g for 30 min; protein and hexose contents of the soluble fractions were determined, respectively, by the method of Lowry et al. (17) and the orcinol method (26).

Gel Filtration of the Solubilized Material. Gel filtration of solubilized mucus preparations was performed on a Sepharose 4B column (1.5 x 100 cm), equilibrated, and eluted in 10 mm Tris-HCl buffer (pH 7.4) containing 200 mm NaCl. Four-ml fractions were collected, and 50 mm of each fraction per well were seeded in triplicate on microtiter plates for solid-phase RIA. Proteins and standards used in the calibration of gel filtration columns included Blue Dextran, purified monoclonal IgM antibodies, ferritin, BSA, and phenol red.

Chemical and Enzyme Treatments. Methanol treatment and periodate oxidation were performed directly on the material seeded on microtiter plates immediately before testing, whereas heat and acetic treatment, Pronase digestion, and sialidase treatment were performed prior to seeding on plates.

Methanol treatment was carried out at 4°C for 30 min; periodate oxidation was carried out at 4°C for 2 hr, by adding to each well 10 mm Tris-HCl (pH 7.4) which contained sodium metaperiodate in concentrations ranging from 0 to 50 mm; heat treatment was carried out at 100°C for different periods of time; acetone treatment was accomplished by slow volume by volume addition of cold acetone (−20°C). After a 10-min incubation at −20°C, the material was centrifuged at 23,000 x g for 20 min, and the pellet was washed twice with cold acetone and once with 10 mm Tris-HCl (pH 8.3).

Pronase digestion was performed at 37°C for different periods of time in 200 mm ammonium acetate buffer (pH 8.5); CM were treated with Pronase (0.2 mg/mg proteins), and the reaction was stopped by adding BSA (5 mg/ml) and phenylmethylsulfonyl fluoride (10 mm). After treatment, CM were either washed with 10 mm Tris-HCl (pH 8.3) and then seeded in microtiter plates or directly seeded without previous washing.

Sialidase treatment was carried out at 37°C for different periods of time in 100 mm sodium acetate buffer (pH 5) containing 100 mm NaCl and 9 mm CaCl2. CM were treated with the enzyme (0.1 IU/mg protein), and the reaction was stopped by adding an excess of 10 mm Tris-HCl (pH 8.3). After 2 washings with the same buffer, the material was seeded in microtiter plates.

Density Gradient Ultracentrifugation. Ultracentrifugation of 5 mg 200 mm NaCl-solubilized mucus was carried out in 5 ml cesium chloride isopyknic density gradients in 200 mm NaCl. The samples were dissolved in a CsCl solution with a starting density of 1.42 g/ml, and the gradients were formed by centrifugation in a Beckman SW50.1 rotor at 150,000 x g for 48 hr at 5°C (23). Fractions of 0.45 ml were collected, and their density was determined by refractive index measurement. Each fraction, diluted 1:50, was assayed for the antigens by solid-phase RIA.

Preparation and Analysis of Lipid Extracts. Lipids were extracted from CM and 200 mm NaCl-solubilized mucus according to the procedure described by Tettamanti et al. (25) already applied to CM (8). The reactivity of lipid extracts with MOv1 and MOv2 antibodies was determined by TLC immunostaining according to the method originally described by Magnani et al. (18).

Solid-Phase RIA. The test was carried out in microtiter plates on CM or soluble proteins as described previously (20). In short, 50 mm of properly diluted monoclonal antibody were added to each well containing 2.5 mm of glutaraldehyde-fixed proteins and incubated for 45 min at 37°C. MOv1 ascsites was used at 1:2500 dilution, and MOv2 ascsites was used at 1:100 dilution. After washing, 50 mm of 125I-goat anti-mouse 7S immunoglobulins were added and incubated at 37°C for 45 min. The wells were then washed, and 100 mm of 2 NaOH were added in order to harvest the contents, the radioactivity of which was measured by a gamma counter. The binding index was calculated as the average cpm of 3 test replicates divided by the average cpm of 3 control replicates in which targets were treated with medium and 125I-labeled anti-mouse immunoglobulin. Values ≥2 were considered positive.

For each monoclonal, an IRMA was developed to detect antigens in patient's effusions. Purified antibodies were coupled to CNBr-activated Sepharose 4B according to the manufacturer's suggested procedure. Fifty mm of packed gel were mixed with an equal volume of effusion or of phosphate-buffered saline (136 mm NaCl:2.7 mm KCl:8 mm Na2HPO4:1.5 mm KH2PO4:0.9 mm CaCl2:0.5 mm MgCl2, pH 7.4) with 2% BSA for negative control. After 1 hr of incubation at room temperature with gentle shaking, the pellet was washed 3 times with phosphate-buffered saline with 2% BSA and incubated for another hr at room temperature with 100,000 cpm 125I-labeled antibodies. Excess 125I-labeled antibody was then washed with wash buffer (10 mm Tris-HCl:150 mm NaCl:0.4% Nonidet P-40:200 IU. Antigosen:0.02% NaN3, pH 7.4), and the activity associated with the immunoadsorbent was measured by a gamma counter. The binding index was calculated on the average cpm of the 2 test replicates divided by the average cpm of the 2 control replicates. Values ≥2 were considered positive.

RESULTS

Binding Activity on Different Materials from the Immunizing Tumor. The biochemical characterization of the MOv1- and MOv2-defined antigens was carried out on different materials obtained from the immunizing mucinous cystoadenocarcinoma. Previous immunofluorescence tests on cryostatic sections of this tumor indicated that these 2 monoclonals reacted not only with tumor cells but also with the mucinous peritumoral secretion. Therefore, the binding activities of MOv1 and MOv2 were tested on the CM extracted from the tumor which was used as immunogen and on materials solubilized from the mucinous secretion of the same tumor (Table 1).

Three different methods were used to change the macromolecular organization of the mucus in order to facilitate its processing.

To obtain mucus components in their native form, the secretion

<table>
<thead>
<tr>
<th>Antigenic source</th>
<th>MOv1 Binding index</th>
<th>Relative binding activity</th>
<th>MOv2 Binding index</th>
<th>Relative binding activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM</td>
<td>12.4</td>
<td>1</td>
<td>9.2</td>
<td>1</td>
</tr>
<tr>
<td>Mucus solubilized by</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 mm NaCl</td>
<td>13.3</td>
<td>1.07</td>
<td>7.7</td>
<td>0.83</td>
</tr>
<tr>
<td>Pronase</td>
<td>10.5</td>
<td>0.85</td>
<td>9.5</td>
<td>1.03</td>
</tr>
<tr>
<td>500 mm 2-mercapto-ethanol</td>
<td>18.8</td>
<td>1.50</td>
<td>10.7</td>
<td>1.16</td>
</tr>
</tbody>
</table>

The table shows the binding activity of MOv1 and MOv2 with immobilized antigens from CM and solubilized mucus and the relative binding activity as compared to CM. Positive indexes were ≥2.

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was initially solubilized by intense stirring with 200 mM NaCl. In 3 different experiments, an average 2.3 mg of proteins was obtained per g of mucus. In spite of the low protein yield, a good antigenic reactivity was defined. As reported in Table 1, both monoclonals reacted by solid-phase RIA on saline extract with a binding activity relative to the immunogen of 1.07 for MOv1 and of 0.83 for MOv2.

Since proteolytic digestion was described as a very effective method to solubilize the mucoprotein fraction by destroying the free protein components (9), mucus was incubated with Pronase (0.2 mg/mg mucus proteins), and the soluble material was separated by centrifugation. The protein recovery could be only roughly estimated because of the interference of the exogenously added protein, and an average 1.2 mg of proteins were obtained per g of mucus in 3 different experiments. The relative binding activities of MOv1 and MOv2 were, respectively, 0.85 and 1.03 (Table 1). These results suggest that MOv1- and MOv2-defined antigens are insensitive to proteolytic treatments.

Solubilization of the MOv1- and MOv2-defined antigens was also obtained by 500 mM 2-mercaptoethanol treatment. The average protein recovery was 3.5 mg/g of mucus, with an antigenic activity increased for both monoclonals (relative binding activities: MOv1, 1.50; MOv2, 1.16) (Table 1). These results confirm that MOv1- and MOv2-defined antigens were expressed both on CM and on secretion products of the immunizing tumor.

Sensitivity to Physicochemical or Enzymatic Treatments. The behavior of the antigens studied after physicochemical and enzymatic treatment was investigated using CM or solubilized mucus preparations. As shown in Table 2, the MOv1 antigen resisted methanol and acetone treatment; unchanged reactivity was recovered on CM as shown by solid-phase RIA. In contrast, the MOv2-recognized antigen was partially affected by methanol which reduced its binding activity to 70%; the acetone treatment had a borderline effect (86% of the antigenic activity was recovered).

To further investigate the sensitivity of the molecules recognized by the 2 monoclonals with proteolytic enzymes, Pronase digestion for different times was performed on CM. Incubation with Pronase for 1 hr reduced MOv1-relative binding activity to 75% (Table 2, Pronase A), whereas MOv2 activity was unaffected. After 16 hr treatment, the test on washed membranes indicated that MOv1 relative binding activity had almost been abolished, whereas MOv2 activity was reduced to about 50% (Table 2, Pronase B). Nevertheless, when the binding capacity was assayed, after 16 hr treatment, on the total material, i.e., both CM and soluble degradation products without washings, antigenic activity with both monoclonals was almost completely recovered (Table 2, Pronase C). These results indicate that MOv1 and MOv2 antigens are carried by molecules which have different Pronase sensitivity and that the recognized epitopes are not susceptible to proteolytic treatment.

In contrast, the MOv1- and MOv2-recognized antigens were sensitive to periodate oxidation performed on CM with increasing concentrations of the oxidizing agent. As reported in Table 2, with low periodate concentration (12.5 mm) the relative MOv1 binding activity was unaffected whereas MOv2 activity was reduced to 50%; with the highest dose (50 mm sodium metaperiodate) the MOv1 activity was reduced to about 50% and MOv2 activity had been abolished completely. Similar results were obtained when periodate treatment was applied to soluble material from mucus. These data suggest that carbohydrate residues of the molecules confer the antigenic specificity. However, sialidase treatment, which was performed on CM for 1, 6, or 24 hr, did not modify the reactivity with MOv1 and MOv2, as shown in Table 2. This indicates that both antigenic determinants do not contain sialic acid residues.

Heat sensitivity was investigated by heating 200 mM NaCl-solubilized mucus at 100°C for different time intervals. As shown in Table 2, after 10 min of treatment, MOv1 activity was reduced to 86% whereas MOv2 activity was unchanged; after a prolonged treatment (1 hr), MOv1 and MOv2 activities were reduced to 76 and 85%, respectively.

In most experiments, β2-microglobulin was used as control protein antigen. As expected, it was found to be unaffected either by periodate oxidation, even at the highest concentrations, or by methanol treatment, whereas it was inactivated by heating or proteolytic digestion.

Gel Filtration of Solubilized Mucus. Gel filtration of the 200 mM NaCl-solubilized mucus was performed on a Sepharose 4B column in order to determine the approximate molecular weight of MOv1- and MOv2-recognized antigens and obtain their partial purification. The protein and hexose content was determined for each fraction, and the antigenic activity was monitored by MOv1 and MOv2 in a solid-phase RIA. As shown in Chart 1, the highest antigenic activity for each monoclonal was recovered in the void volume (V0) fractions; the fractions after V0 and before the elution volume of ferritin were also positive, but at a lower level. The peak of proteins, without antigenic activity, corresponded to the

| Table 2
| Solid-phase RIA reactivities of MOv1 and MOv2 on materials from OvCa 9588/80 after treatment with various chemicals and enzymes
<table>
<thead>
<tr>
<th>Treatment</th>
<th>MOv1</th>
<th>MOv2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM treated with</td>
<td>MOv1</td>
<td>MOv2</td>
</tr>
<tr>
<td>No treatment</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Methanol</td>
<td>1</td>
<td>0.70</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.96</td>
<td>0.86</td>
</tr>
<tr>
<td>Pronaseb</td>
<td>0.75</td>
<td>1</td>
</tr>
<tr>
<td>60 min (A)</td>
<td>0.08</td>
<td>0.46</td>
</tr>
<tr>
<td>16 hr (B)</td>
<td>0.98</td>
<td>0.94</td>
</tr>
<tr>
<td>16 hr (C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sialidasec</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>60 min</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6 hr</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>24 hr</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>12.5 mm sodium metaperiodate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>50 mm sodium metaperiodate</td>
<td>0.52</td>
<td>0.05</td>
</tr>
<tr>
<td>Solubilized mucus treated with</td>
<td>MOv1</td>
<td>MOv2</td>
</tr>
<tr>
<td>No treatment</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Heat 10 min 100°C</td>
<td>0.86</td>
<td>1</td>
</tr>
<tr>
<td>Heat 60 min 100°C</td>
<td>0.76</td>
<td>0.85</td>
</tr>
<tr>
<td>12.5 mm sodium metaperiodate</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>50 mm sodium metaperiodate</td>
<td>0.22</td>
<td>0.30</td>
</tr>
</tbody>
</table>

a Binding activity relative to untreated material.
b After treatment, CM were either washed with 10 mM Tris-HCl (pH 8.3) and then seeded in microplates (A and B) or directly seeded without previous washings (C).c After treatment, the reaction was stopped by adding an excess of 10 mM Tris-HCl buffer (pH 8.3); the CM were washed twice with the same buffer and seeded in microplates.
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Chart 1. Elution profile of 200 mM NaCl-solubilized material from the mucus of an ovarian mucinous tumor (OvCa 9588/80) assessed by different criteria. Absorbance of light at 280 nm representing protein content, hexose content as measured by the orcinol method, and MOv1 and MOv2 antibody reactivities as determined by cpm bound in solid-phase RIA are plotted against fraction number. The sample (6 ml) was applied to a Sepharose 4B column (1.5 x 100 cm) and eluted using 10 mM Tris-HCl (pH 7.4):200 mM NaCl, at a flow rate of 10 ml/hr. Fractions were 4 ml each. Arrows, molecular weight standards. Columns, binding activity by solid-phase RIA of MOv1 (left column) and MOv2 (right column) on the unfractionated material. Top abscissa, H/P, ferritin.

Chart 2. Density gradient ultracentrifugation. The 200 mM NaCl-solubilized material from the mucus of an ovarian mucinous tumor (OvCa 9588/80) was subjected to equilibrium ultracentrifugation in cesium chloride as described in “Materials and Methods.” Fractions obtained from the gradient were monitored for MOv1 and MOv2 reactivity as determined by cpm bound in solid-phase RIA. Density was evaluated by refractive index measurement.

elution volume of BSA, suggesting that most of the mucus proteins solubilized by saline extraction were contaminating serum proteins. The H/P of the V₀ pooled fractions was higher (H/P 0.49) than that of the unfractionated material (H/P 0.07), indicating that an enrichment of highly glycosylated mucoproteins corresponded to an enrichment of antigenic activity. The H/P of included positive fractions was not recorded because of the low protein and hexose content.

The enrichment of glycoproteins rich in carbohydrates in the more reactive fractions and the resistance of MOv1- and MOv2-recognized determinants both in CM and in mucus to Pronase treatment influenced us to isolate the mucoproteins by Pronase digestion and subsequent gel filtration. As in the first column, the highest antigenic activity for both monoclonals was found in V₀ fractions and in the fractions preceding the elution volume of ferritin but, since Pronase degradation products with residual antigenic activity were present in all the included volumes of the column according to their molecular size, we could not estimate the molecular weight of MOv1- and MOv2-defined molecules by using this separation.

The material solubilized by 500 mM 2-mercaptoethanol had quite a high H/P (0.33), but we could not prove a further enrichment in glycosylated material after fractionation. In fact, the removal of 2-mercaptoethanol by dialysis caused a strong reaggregation of the solubilized material which could not be separated further by gel filtration.

Analysis of Solubilized Mucus by Density Gradient Ultracentrifugation. The density of MOv1- and MOv2-recognized antigens was investigated by fractionating a 200 mM NaCl-solubilized mucus preparation by equilibrium ultracentrifugation in cesium chloride. As shown in Chart 2, the gradient obtained varied linearly from 1.33 g/ml (top fraction) to 1.57 g/ml (bottom fraction). MOv1-recognized molecules sedimented over a broad range of fractions showing the highest antigenic activity in Fraction 5 (density, 1.45 g/ml); a peak in MOv2-binding activity was found in Fraction 3 (density, 1.52 g/ml), but MOv2-positive material was present in almost all the gradient fractions, particularly in the top fractions where the MOv2 activity reached its highest levels.

Immunoreaction with Lipid Extracts from CM and Solubilized Mucus. In order to verify whether glycolipids were involved in MOv1 and MOv2 reactivities, total lipids were extracted from CM and solubilized mucus and subjected to TLC. The immunoreactions with MOv1 and MOv2 antibodies, revealed by autoradiography, are shown in Fig. 1. MOv2 selectively stained a glycolipid band on lipid extracts from both sources (Fig. 1, Lanes 3 and 4) as well as some material, from the solubilized mucus only, which in our conditions remained at the origin (Fig. 1, Lane 3). This material was also stained by MOv1 (Fig. 1, Lane 1), which, on the contrary, did not recognize any glycolipid band from either sources.

Binding Activity on Cultured Cells and Supernatants. The results reported in Table 1 indicate that the molecules defined by MOv1 and MOv2 were present both on CM and on solubilized tumor cell secretion. To further analyze the localization of these molecules, we evaluated the presence of soluble products in the supernatants of tumor cell lines. The tissue culture medium, harvested when the cells reached the confluence, was clarified by centrifugation (20,000 x g for 30 min) and seeded in microtiter plates. The binding activity of both monoclonals was assayed by solid-phase RIA on culture supernatants and compared to results obtained by IF on live cells grown in the tested medium.

As shown in Table 3, MOv1 monoclonal antibody was positive on the supernatants from the 2 mucinous ovarian carcinoma cell lines SK-OV-4 and 1063 and negative on that from a serous ovarian carcinoma cell line SW626, whereas IF on SK-OV-4 and SW626 cells was negative. In addition, MOv1 was negative by both solid-phase RIA and IF on the mammary carcinoma cell line MCF-7. MOv2 monoclonal antibody gave positive surface mem-

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BIOCHEMICAL ANALYSIS OF HUMAN OVARIAN CANCER ANTIGENS

Fig. 1. TLC of lipid extracts from 200 ml NaCl-solubilized mucus and CM from an ovarian mucinous tumor (OvCa 9588/80) developed with chloroform-methanol-water (50:42:11, by volume), MOv1 (Lanes 1 and 2) and MOv2 (Lanes 3 and 4)-defined antigens were detected by autoradiography. The amount of the extracts chromatographed corresponds to 1:1000 of the material extracted, respectively, from 11 mg of proteins for the solubilized mucus (Lanes 1 and 3) and from 0.2 mg of proteins for the CM (Lanes 2 and 4).

Table 3

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>% of positive cells by IF with MOv1</th>
<th>MOv1</th>
<th>MOv2</th>
<th>MOv1</th>
<th>MOv2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-OV-4</td>
<td>Mucinous ovarian carcinoma</td>
<td>0</td>
<td>60</td>
<td>3.4</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>1063</td>
<td>Mucinous ovarian carcinoma</td>
<td>NT*</td>
<td>NT</td>
<td>5.6</td>
<td>25.6</td>
<td></td>
</tr>
<tr>
<td>SW626</td>
<td>Serous ovarian carcinoma</td>
<td>0</td>
<td>80</td>
<td>1.5</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td>Mammary carcinoma</td>
<td>0</td>
<td>0</td>
<td>1.4</td>
<td>1.8</td>
<td></td>
</tr>
</tbody>
</table>

* NT, not tested.

When solubilized mucus, previously demonstrated to contain MOv1 and MOv2 molecules, was used as source of antigen, a highly specific and titrable binding activity was obtained in both MOv1-IRMA and MOv2-IRMA. In further experiments, this material was used for positive control. Only MOv2-IRMA, however, was able to detect antigenic molecules in the supernatant from the SW626 line, giving the same results obtained by solid-phase RIA (data not shown).

A limited series of effusions from patients with ovarian carcinoma, selected among cases previously tested by conventional cytology and IF (24), were also tested by MOv1- and MOv2-IRMA.

When using MOv1-IRMA, all the effusions gave negative results; therefore, only the data obtained by MOv2-IRMA are reported in Table 4. Among 10 effusions from patients with well-differentiated ovarian tumors, either mucinous or serous, 8 (80%) were positive; whereas in effusions from patients with poorly differentiated ovarian tumors, only 5 of 11 effusions (45%) were positive. Furthermore, only 1 of 7 mammary carcinoma effusions and none of the 10 control effusions from other diseases were positive.

The average binding index of positive cases was high (21.2) for well-differentiated tumors and intermediate (7.9) for poorly differentiated tumors, which suggests that the secretion of MOv2-recognized molecules corresponds to the degree of differentiation of the ovarian tumors. In addition, in the effusions tested, a complete correlation between the presence of neoplastic cells labeled by MOv2 IF and that of secretion products recognized by MOv2-IRMA was found (results not reported).

DISCUSSION

Two monoclonal antibodies, MOv1 and MOv2, produced and characterized in our laboratory (24) and directed against epithelial ovarian tumors, were used to biochemically characterize their target antigens.

The data presented here suggest that both monoclonals are directed to oligosaccharide determinants. This conclusion is based on the following observations: (a) MOv1- and MOv2-binding activities were unaffected by heating but significantly reduced by periodate oxidation of the target molecules; (b) Pronase digestion solubilized the MOv1- and MOv2-defined antigens from CM and peritumoral mucus but did not affect the antibody binding on digested products; (c) antigenic activities related to MOv1 and MOv2 were distributed in fractions with carbohydrate-rich molecules, as demonstrated by gel filtration

Table 4

<table>
<thead>
<tr>
<th>Effusions from patients with</th>
<th>No. of positive cases</th>
<th>%</th>
<th>Mean binding index of positive cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian carcinoma</td>
<td>8/10</td>
<td>80</td>
<td>21.2 (4.2–49.0)*</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>5/11</td>
<td>45</td>
<td>7.9 (2.7–17.5)</td>
</tr>
<tr>
<td>Total</td>
<td>13/21</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Mammary carcinoma</td>
<td>1/7</td>
<td>14</td>
<td>3.1</td>
</tr>
<tr>
<td>Other diseases</td>
<td>0/10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses.

** Other diseases included: 3 melanomas; 4 lymphomas; 1 cordoma; 1 hepatitis; 1 cirrhosis.
experiments.

The results of fractionation on Sepharose 4B column of the peritumoral mucus, solubilized either by 200 mw NaCl or Pronase, indicate that the antigenic activities for both monoclonals were associated with high-molecular-weight glycoproteins but did not resolve the approximate molecular weight of the studied molecules. Most of the antigenic activity was associated with the $V_o$ ($M_o > 2 \times 10^5$); nevertheless, a significant part of the antigenicity was present in a large tract of the included volume, particularly after Pronase digestion.

With regard to the antigens recognized by MOv1 and MOv2, besides the different tissue distribution reported previously (24), peculiar biochemical differences have been found. In fact, Pronase digestion of CM completely solubilized MOv1-recognized antigenicity but reduced to only 50% the MOv2-binding reactivity. In contrast, MOv2 reactivity on CM was abolished by periodate oxidation, whereas MOv1 binding was only reduced to 50%. MOv2 reactivity was only partially affected by methanol.

Analysis of the density of the molecules recognized by MOv1 and MOv2 as determined by equilibrium ultracentrifugation in cesium chloride indicated that MOv1-recognized epitope was carried by molecules with a high density (1.45 g/ml) characteristic of mucins (12), whereas MOv2-recognized determinant was expressed both on molecules with a high density (1.52 g/ml), i.e., mucins (23), and on molecules with a lower density, i.e., glycoproteins and/or glycolipids. The results obtained by lipid extraction from CM and solubilized mucus and immunoreactions on TLC with both monoclonals were in keeping with these findings.

Glycoproteins and glycolipids can share the same carbohydrate antigens, as seen for blood group antigens and also for antigens recognized by monoclonal antibodies, like the 19.9-defined epitope, which has been reported to be present in glycolipids as well as in mucins (18, 19). In addition, preliminary results, obtained by immunoblotting with MOv2 on different materials from the immunizing tumor and on supernatants from tumor cell lines, indicate that this antibody may recognize antigenic determinants present on various glycoproteins of different sizes. These data agree with those reported by Burchell et al. (7), who described monoclonals reacting with several components of different molecular weight, each carrying the same oligosaccharide determinants. Further purification of molecules recognized by MOv2 from different sources is warranted to determine if there is a peculiar antigenic pattern depending on the cellular or extracellular localization of the antigen and/or the tumor histotype.

Various ovarian carcinoma-associated antigens belonging to the family of carbohydrate-rich glycoproteins (5, 14, 15) were studied for their diagnostic value, and encouraging results were obtained with the monoclonal antibody CA 125 (2). In fact, more than 80% of patients with nonmucinous ovarian tumors showed elevated CA 125 antigen levels in the serum, and a significant correlation was found between antigen levels and the regression, stability, or progression of the disease (2). We developed an IRMA with our monoclonals, which was used in initial experiments to detect antigens in effusions from cancer patients. The results obtained on tumor cell lines and their supernatants indicate that MOv2-defined molecules are present not only on the surface membrane of tumor cells and mucus components but also as soluble forms, whereas MOv1-defined molecules are preferentially expressed on secretions but poorly represented in cell membranes of live cells and as soluble forms. Accordingly, MOv1-IRMA always resulted negative, whereas MOv2-IRMA was useful for detecting circulating antigens in peritoneal fluids from 80% of patients with well-differentiated ovarian tumors and from 45% of patients with undifferentiated lesions. If these preliminary results are accepted and technically improved on wider series of cancer and normal sera, the MOv2 antigen could become a useful marker in patients with ovarian cancer. Also, since the carbohydrate composition of mucins varies in different patients, monoclonal antibodies directed against other carbohydrate sequences might be used together with monoclonal antibody MOv2 in obtaining more sensitive serum tests for epithelial ovarian cancer.

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