Recognition of Ovarian Cancer Antigen CA125 by Murine Monoclonal Antibody Produced by Immunization of Lung Cancer Cells

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ABSTRACT

In studies aimed at developing monoclonal antibodies against lung adenocarcinomas, we produced a murine monoclonal antibody designated 130-22 by immunizing mice with lung cancer cells. Since in immunoperoxidase staining experiments this antibody was reactive not only with lung adenocarcinomas but also with ovarian carcinomas, we examined its relationship to the ovarian cancer marker CA125, an antigen recognized by monoclonal antibody OC125 produced by immunization of mice with ovarian carcinoma cells. Although CA125 antigen was adsorbed by 130-22 antibody, 125I-labeled 130-22 did not compete with OC125, indicating that although these two antibodies recognized CA125 antigen, they reacted with separate antigenic determinants. The antigen defined by both antibodies was thought to be heat-stable glycoprotein with a molecular weight of over 1,000,000. A series of immunoradiometric assays was developed using combinations of two monoclonal antibodies in a simultaneous sandwich mode. Mixed monoclonal antibodies may provide a more sensitive assay for the detection of CA125 than the homologous assay, in which OC125 was used both as a tracer and as a catcher. These results indicate that CA125 is an antigen with two separate epitopes present in both ovarian and lung adenocarcinomas and that combination use of monoclonal antibodies reactive with different antigenic determinants will give certain advantages to the immunoradiometric assay of cancer markers.

INTRODUCTION

The hybridoma technique has been widely applied to the production of new cancer marker antibodies that may serve as aids in diagnosis of various cancers. Koprowski et al. (1) developed a monoclonal antibody 19-9 by the fusion of myeloma cells and spleen cells from mice immunized with colon carcinoma cells. By use of ovarian carcinoma cells, Bast et al. (2) described a monoclonal antibody, OC125, which reacted with derivatives of the coelomic epithelium. The antigen defined by the former antibody was designated CA19-9 (3) and that recognized by the latter CA125 (4). Both have been found to be clinically very useful as cancer markers, especially in the management of patients with carcinomas of the pancreas and ovary, respectively (5-8).

In developing monoclonal antibodies reactive with lung adenocarcinomas, but not with squamous cell carcinomas, we selected monoclonal antibody 130-22. In this report, we describe in detail its production and characterization and show that it defined CA125 antigen but reacted with an epitope separate from that recognized by antibody OC125. We conclude that CA125 is an antigen shared by lung and ovarian carcinomas and that monoclonal antibody 130-22 may be useful in the immunodiagnostic evaluation of patients with lung and ovarian cancers.

MATERIALS AND METHODS

Cells. The 11 cell lines used in the present study are listed in Table 2. PC-9 cells, injected into mice for the production of monoclonal antibody 130-22, were derived from a patient with lung adenocarcinoma and established by Dr. Y. Hayata (Tokyo Medical College, Tokyo, Japan). All cell lines were grown in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, NY) and 0.03% l-glutamine (Nakarai Chemicals, Kyoto, Japan).

Preparation of Monoclonal Antibody. BALB/c mice were immunized by i.p. injection of 2-4x10^6 PC-9 human lung adenocarcinoma cells every 2 weeks. Four weeks after the fifth injection, 1x10^6 PC-9 cells were injected i.v., and 3 days later extracted spleen cells (1x10^7) were fused with NS-1 mouse myeloma cells (1x10^5), using 50% polyethylene glycol 4000 (Merck Co., Rahway, NJ). After gradual dilution over a period of 4 min at room temperature, the fused cells were washed with PBS, resuspended in RPMI 1640 containing 10% fetal calf serum, and seeded into 384 wells of 96-well microculture plates (Corning Glass Works, Corning, NY). Hybridomas were selectively grown in hypoxanthine-aminopterin-thymidine medium for 14 days following fusion. The culture supernatants were screened by the ELISA test. Since the aim was to produce monoclonal antibodies against lung adenocarcinomas, positive clones were selected which reacted with PC-9 cells but failed to react with both non-lung tumor cells and lung squamous cell carcinomas. The antibody-secreting hybridomas were cloned by the limiting dilution method. Monoclonal antibodies were purified by application of ascitic fluid to a protein A affinity column (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. Monoclonal antibodies OC125 and 19-9 were obtained commercially from ORIS/CEA (Saclay, France). Monoclonal antibody to CEA was a gift from Dr. S. Nishi (Hokkaido University, Sapporo, Japan).

IRMA for quantitative measurement of CA125 antigen was performed using ELISA CA125 kits according to the manufacturer's instructions.

ELISA Test. Human leukocytes and cells of 11 human tumor cell lines were attached to separate wells of 96-well plastic microculture plates by the method of Cobbold and Waldmann (9) and were incubated for 30 min with 50 μl of culture supernatants, followed by 50 μl of 1:1000 diluted peroxidase-labeled rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark) for 30 min. Finally, 0.02% o-phenylenediamine solution containing 0.015% hydrogen peroxide was added to the wells, which were then washed extensively with PBS. All reactions were performed at room temperature.

Indirect Immunoperoxidase Staining of Tissue Sections. The reactivities of monoclonal antibodies 130-22 and OC125 with cryostat sections of malignant and nonmalignant tissues were analyzed by the avidin-biotin-peroxidase complex technique (10). In brief, 2-μm sections of malignant and normal tissues fixed with 0.1% NaOCl and 0.075 M lysine in 0.05 M phosphate buffer, pH 7.4-2% paraformaldehyde were incubated with 130-22 (10 μg/ml) or OC125 (ORIS/CEA) for 30 min. After two washes in PBS and deactivation of endogenous peroxidase activity, sections were incubated with biotinylated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA) for 30 min. Sections were then washed three times in PBS and incubated with avidin-biotinylated peroxidase complex (Vector Laboratories) for 45 min. After three additional washes in PBS, color was developed by addition of a solution

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: PBS, phosphate-buffered saline; IRMA, immunoradiometric assay; ELISA, enzyme-linked immunosorbent assay; CEA, carcinoembryonic antigen; BSA, bovine serum albumin.
of 3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan) and 0.005% hydrogen peroxide in 0.5 M Tris buffer, pH 7.6. Sections were then counterstained with hematoxylin. All reactions were performed at room temperature.

Radioiodination of Antibody and Cell-binding Studies. Purified antibody was labeled with 125I (New England Nuclear, Boston, MA) by the chloramine-T method, as described previously (11–13). Iodinated antibodies with specific activities ranging from 10 to 15 μCi/μg were used for the following studies.

Studies into the binding of radioiodinated antibodies to tumor cells were performed with PC-9 cells for antibodies 130-22 and OC125 and KATO-III cells for antibody 19-9. Radioiodinated antibody and about 1 x 107 target cells were incubated with increasing amounts of unlabeled 130-22 antibody in a total volume of 300 μl 50 mM PBS containing 0.5% BSA at room temperature for 60 min. After centrifugation, the supernatant was aspirated and the radioactivity of the pellet was measured. The binding data were used to make a Scatchard plot from which affinity constant values were obtained (14).

To examine the physical properties of antigens defined by these antibodies, the target cells were heated at 56°C for 30 min and treated with 0.1% trypsin (Difco Laboratories, Detroit, MI) at 37°C for 30 min, 0.1% Pronase (Sigma Chemical Co., St. Louis, MO) at 37°C for 30 min, 0.1 units neuraminidase (Nakarai Chemicals) at 37°C for 60 min, and 10 mM sodium metaperiodate (Kanto Chemical Co., Tokyo, Japan) at 23°C for 60 min. Finally, cell-binding studies were performed using PBS treatment as a control. The result was expressed as percentage of radioactivity bound to the treated cells divided by radioactivity bound to the PBS-treated cells.

CA125 and CA19-9 antigens were partially purified from culture supernatants of PC-9 and KATO-III cells, respectively. Periodate oxidation of these partially purified antigens was also accomplished with 0, 0.1, 1.0, 10, or 100 mM periodate in 50 mM sodium acetate buffer (pH 4.5, 4°C) in the dark. Antigenic activity was determined by using commercially available kits for CA125 and CA19-9 from ORIS/CEA and for 130-22 antigen by simultaneous IRMA with 130-22.

Determination of Antigen Concentrations by IRMA. To determine antigen concentrations, a simultaneous sandwich IRMA was developed, in which antibody 130-22 was used both as a catcher attached to a solid-phase immunosorbert and as an iodinated tracer. Polystyrene beads were coated with purified antibody in 1 mM phosphate buffer, pH 6.5, containing 0.01% sodium dodecyl sulfate for 20 h, were washed extensively to remove loosely bound antibodies and stored in PBS containing 0.5% BSA. In a reaction tube, 100 μl of standards or samples were mixed with 200 μl of 125I-labeled 130-22 (50,000 cpm) in 50 mM PBS, pH 7.5, containing 0.5% BSA and 1.2% normal murine serum. One antibody-coated bead was added to each reaction tube and samples were incubated at room temperature for 60 min. After the beads were washed twice with physiological saline, bound radioactivity was measured.

To study the relationship of antigens defined by 130-22 and OC125, a various combination of antibodies was used in a simultaneous sandwich IRMA, such as a mixture use of 125I-labeled 130-22 antibody and OC125-coated beads or 125I-labeled OC125 antibody and 130-22-coated beads. Furthermore, two-step IRMA was also performed by incubating assay standards and immunosorbents for 6 h at room temperature, washing, and then reincubating with a tracer and various concentrations of unlabeled 130-22 for 20 h at room temperature. Values for assay standards obtained from the conditioned culture supernatants of PC-9 cells were almost comparable to standards used in the ELSA CA125 IRMA kits.

Fractionation of PC-9 Cell Culture Supernatants. A portion (1 ml) of the 10-fold concentrated culture supernatants from PC-9 cells was fractionated on a 1.6- x 100-cm Sephacryl S-300 column equilibrated with elution buffer (0.01 M Tris-HCl, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA, pH 7.4). Antigen concentrations were determined by using a homologous sandwich IRMA, in which OC125 or 130-22 antibody was used both as a tracer and as a catcher.

RESULTS

Female BALB/c mice were immunized with PC-9 single-cell suspensions, which had been removed from cell cultures with trypsin-free EDTA-PBS, to develop antibodies against native cell surface-associated antigens. Monoclonal antibody 130-22 was selected from the library of anti-PC-9 cell monoclonal antibodies by the procedure described in "Materials and Methods." It was found to be an IgG1 murine immunoglobulin reactive with plasma membranes of PC-9 and weakly reactive with Ruwellar lung adenocarcinoma cells, but not with nine other kinds of human tumor cells examined or with normal human leukocytes. The specificity of its staining in tissue sections was further explored by indirect immunoperoxidase staining. No specific staining was observed in such tumor tissues as squamous cell carcinomas of the lung or carcinomas of the stomach, colon, or breast (Table 1). However, it was reactive with sections not only of lung adenocarcinomas but also of ovarian serous cystadenocarcinomas (Figs. 1 and 2). Normal tissues, such as lung, stomach, colon, liver, kidney, pancreas, spleen, and ovary, failed to react with 130-22, although bronchial epithelium, fallopian tube, endocervix, and endometrium were positive with both 130-22 and OC125 antibodies.

Since the antibody showed a clear immunohistological cross-reaction with ovarian carcinomas, its specificity was further explored by ELISA using a panel of cells as shown in Table 2, and the results were compared with those obtained in similar conditions.
Fig. 2. Cryostat section of ovarian carcinoma stained with 130-22 antibody. The luminal surface of the papillary projections was intensely stained with 130-22. × 150.

Table 2 Reactivity of four monoclonal antibodies with human cells as tested by ELISA

<table>
<thead>
<tr>
<th>Cells</th>
<th>Source</th>
<th>Binding of antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-9</td>
<td>Lung adenocarcinoma</td>
<td>+++</td>
</tr>
<tr>
<td>PC-5</td>
<td>Lung adenocarcinoma</td>
<td>+</td>
</tr>
<tr>
<td>Ruwellar</td>
<td>Lung adenocarcinoma</td>
<td>-</td>
</tr>
<tr>
<td>A01</td>
<td>Lung squamous cell</td>
<td>++</td>
</tr>
<tr>
<td>KATO-III</td>
<td>Gastric carcinoma</td>
<td>-</td>
</tr>
<tr>
<td>LoVo</td>
<td>Colon carcinoma</td>
<td>-</td>
</tr>
<tr>
<td>PC-3 (ATCC)</td>
<td>Prostatic carcinoma</td>
<td>-</td>
</tr>
<tr>
<td>T-24</td>
<td>Bladder carcinoma</td>
<td>-</td>
</tr>
<tr>
<td>RCC-K1</td>
<td>Renal cell carcinoma</td>
<td>+</td>
</tr>
<tr>
<td>HeLa</td>
<td>Cervical carcinoma</td>
<td>-</td>
</tr>
<tr>
<td>M1</td>
<td>Malignant melanoma</td>
<td>-</td>
</tr>
<tr>
<td>Peripheral blood leukocytes</td>
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- Kindly supplied by Dr. S. Nishi.
- Kindly supplied by Dr. M. Akiyama.
- Kindly supplied by Dr. T. Kasuga.

Fig. 3. Lung adenocarcinoma, as in Fig. 1, but stained with OC125. Cancer nests were markedly stained as in Fig. 1. × 150.

Fig. 4. Ovarian carcinoma, as in Fig. 3, but stained with OC125. The reactivity was most intense on the luminal surface, as in Fig. 3. × 150.

Fig. 5. Competitive inhibition studies using PC-9 cells. Radiiodinated OC125 (■) and 130-22 (□) antibodies were incubated with increasing amounts of unlabeled 130-22 antibody. The results were expressed as the percentage of radioactivity bound (B) in the absence of unlabeled 130-22 antibody.

cyanoxygen bromide-activated Sepharose 4B (data not shown).

These results indicated that 130-22 antibody reacted with CA125 antigen but bound to antigenic sites separate from those recognized by OC125.
Binding of both $^{125}$I-labeled 130-22 and OC125 antibodies to PC-9 cells was destroyed by the heating and by treatments with trypsin, Pronase, and periodate but was resistant to neuraminidase (Fig. 6). CA19-9 antigen, which contains a sialyl derivative of lacto-N-fucopentaose II (3), was resistant to heating and to treatments with trypsin and Pronase but was easily destroyed by neuraminidase and periodate. Since the effect of periodate oxidation on the binding of $^{125}$I-labeled antibody to cultured cancer cells was not clearly different among three antibodies, CA125 and CA19-9 antigens were partially purified from culture supernatants of PC-9 and KATO-III cells, respectively (18). Treatment of partially purified antigens with 0.1 and 1 mM periodate completely eradicated the immunoreactivity of CA19-9. On the other hand, antigenic determinants defined by both 130-22 and OC125 antibodies were conserved by the treatment of 0.1, 1 and 10 mM periodate and were destroyed by increasing the periodate concentration to 100 mM (data not shown).

After identification and partial characterization of the antigens, studies were performed to detect soluble antigens in the culture supernatants from PC-9 cells and in serum of patients with various cancers. We therefore developed a series of IRMAs in which various combinations of 130-22 and OC125 antibodies were used in a simultaneous forward sandwich mode (Fig. 7). Studies to optimize the reaction conditions were pursued, including conditions for 130-22 antibody bead coating, as well as the incubation time, pH, and the specific activity of $^{125}$I-labeled 130-22. The latter greatly influenced the sensitivity of antigen detection, and a specific activity ranging from 10 to 13 μCi/μg was selected as a tracer (data not shown). By using IRMAs with all the possible antibody combinations, high antigen binding activities were found in the culture supernatants from PC-9 cells, in standard samples of CA125 IRMA kits, as well as in the serum of patients with ovarian carcinomas. With respect to the sensitivity of the assay, a heterologous system, in which different antibodies were used as a radiiodinated tracer and immunosorbent, was found to give optimal results. A maximum binding of about 50% of input cpm was observed when OC125 was used as a tracer and 130-22 as a catcher, the sensitivity for the detection of CA125 antigen being much higher than when OC125 was used both as tracer and immunosorbent. Furthermore, at antigen concentrations greater than 1000 units/ml, diminished binding was observed by using OC125-bound immunosorbent.

Additional experiments were done to confirm the immunological similarities of the antigens defined by 130-22 and OC125 (Fig. 8). Unlabeled 130-22 completely inhibited the binding of...
125I-labeled 130-22 to antigen which had been bound previously to both 130-22- and OC125-coated beads, whereas it did not compete with 125I-labeled OC125, indicating that 130-22 and OC125 reacted with the same antigen but with the same determinant.

In homologous assays for the two antibodies, the antigen defined by both was found to be eluted in the void volume of Sephacryl S-300 gel chromatography indicating that this antigen had a high molecular weight of over 1,000,000 (Fig. 9).

**DISCUSSION**

Immunization of mice with the lung adenocarcinoma cell line PC-9 resulted in the production of monoclonal antibody 130-22. Several lines of evidence suggest that monoclonal antibodies 130-22 and OC125 recognize distinct antigenic determinants on the same antigen, CA125, which is associated with human ovarian carcinomas (15). In addition to the similar expression and similar chemical nature of antigen defined by both 130-22 and OC125 antibodies, CA125 antigen could be adsorbed by incubation with 130-22. Combinations of two antibodies can be used in a sandwich IRMA (Fig. 7). However, two antibodies competed neither for binding sites on PC-9 cell (Fig. 5) nor in the IRMAs of CA125 (Fig. 8). We, therefore, concluded that 130-22 antibody recognized CA125 antigen but reacted with antigenic determinants different from those recognized by OC125.

Bast et al. (5, 16, 17) reported that elevated serum CA125 antigen levels could be detected in serum of more than 80% of patients with epithelial ovarian carcinomas and that levels correlated with progression or regression of the disease. CA125 antigen has been found to be expressed not only in epithelial ovarian neoplasms but also in the derivatives of the coelomic epithelium, i.e., the Müllerian epithelium and the lining cells of the peritoneum, pleura, and pericardium (4). The CA125 antigenic determinant has also been reported to be found in human milk and normal cervical mucus (18). Therefore it may not be surprising that 130-22 antibody produced against lung cancer cells was reactive with the ovarian cancer-associated antigen.

It is noteworthy that mixed use of 130-22 and OC125 antibodies as a tracer and a catcher provides a more sensitive assay for the detection of CA125 antigen than with OC125 alone (Fig. 7). It has been reported that IRMA becomes more sensitive and specific if combinations of monoclonal antibodies recognizing different epitopes are used (19). In the present studies, a heterologous assay using 130-22 and OC125 as a tracer and as an immunosorbent, respectively, was found to give optimal sensitivity. Clinical evaluation of this sensitive IRMA is now in progress in our laboratory.

It is probable that monoclonal antibodies will be clinically useful in transporting large quantities of radionuclides, toxins, and anticancer drugs to target tissues, and their radionabeled forms have already been clinically applied to the diagnosis and therapy of cancers (20). Radiolabeled OC125 antibody has itself been used in the immunoscintigraphy of ovarian cancers (21). The combined use of labeled monoclonal antibodies directed against separate epitopes may increase both the number of antigenic sites on tumor cells and the uptake of injected labeled antibodies into the tumor, as was observed in the IRMA for CA125 antigen.

In previous studies, the gynecological aspects of OC125 were mainly reported. In the present avidin-biotin immunoperoxidase study, 130-22 was reactive with tissues of 4 of 6 lung adenocarcinomas but failed to react with 7 lung squamous cell carcinomas. Antibody 130-22 was selected in an attempt to produce monoclonal antibodies to lung adenocarcinomas, and these may have some clinical importance in the diagnosis of lung cancers as well as of ovarian cancers.

Recent studies have provided some data concerning the antigenic nature of CA125. Masuho et al. (22) reported that OC125 bound to a heat-labile glycoprotein with a molecular weight in excess of 200,000. Davis et al. (18) also obtained data that CA125 antigenic determinant is composed of, at least in part, conformationally dependent peptide, since exoglycosidase treatment did not result in loss of activity, whereas Pronase digestion eradicated all activity. On the contrary, Uhlenbruck et al. (23, 24) recently presented data showing that the carbohydrate structure of CA125 antigen was closely related to that of CA19-9 by performing enzymatic digestion of mucin-bound oligosaccharide chains. Our preliminary studies indicated that the antigen defined by both 130-22 and OC125 antibodies was a heat-labile glycoprotein with a molecular weight measured by gel chromatography of over 1,000,000 and that its response to chemical treatment was quite different from that of CA19-9 (Fig. 6). We do not have enough data to explain this difference, and the exact nature of the CA125 antigen remains to be elucidated. As far as we are aware, this is the first report describing a monoclonal antibody that recognizes CA125 antigen but binds to determinants different from those recognized by OC125 antibody. These studies indicate that CA125 is a antigen shared by ovarian and lung adenocarcinomas and that antibody 130-22 may be useful not only for the study of natures of CA125 antigen but also for the immunodiagnostic evaluation of patients with ovarian and lung cancers. In the radioimmunooassay of hormones, the antigenic structures of which are well characterized, combinations of monoclonal antibodies reactive with different epitopes have been used to achieve greater specificity and sensitivity (25). Although it is hard to produce a variety of monoclonal antibodies against cancer-associated antigens due to their low antigenic expression on tumor cells, the combined use of monoclonal antibodies reactive with separate epitopes will give certain advantages to in vitro IRMAs for cancer markers, and also for the in vivo use of labeled monoclonal antibodies.

**REFERENCES**


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TWO SEPARATE EPITOPES ON CA125 ANTIGEN

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