

Development of new immunoradiometric assay for CA 125 antigen using two monoclonal antibodies produced by immunizing lung cancer cells

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CA 125 is an antigen associated with non-mucinous epithelial ovarian cancer, which is defined by OC 125 antibody developed by immunizing ovarian cancer cells. We have produced two monoclonal antibodies, 130-22 and 145-9, by using the human lung adenocarcinoma cell line PC-9. Both 130-22 and 145-9 antibodies recognized CA 125 antigen. However, the binding sites seemed to be separate from those of OC 125. Testing by 9 immunoradiometric assays (IRMA), using different combinations of the 3 monoclonal antibodies 130-22, 145-9 and OC 125 demonstrated that the best standard curve for detecting CA 125 could be obtained by a "simultaneous sandwich" assay based on a mixture of ^{125}I -labeled OC 125 and 130-22 or 145-9 coated beads. One-step IRMA, using 130-22 as a tracer and 145-9 as an immunoadsorbent, also showed good reproducibility and sensitivity for measuring CA 125. Antigens were detectable in the culture supernatants of PC-9 cells and 5 of 6 ovarian cancer and endometrial adenocarcinoma cells. These results indicate that one-step IRMA using 130-22 and 145-9 is useful for detecting CA 125 antigen.

Key words: Monoclonal antibody, CA 125, Immunoradiometric assay, Ovarian cancer, Lung cancer

INTRODUCTION

RECENTLY NEW CANCER MARKERS, such as CA 19-9, CA 125 and CA 15-3, have been developed by the application of the monoclonal antibody technique.¹⁻³ The immunoradiometric assay (IRMA) of serum concentrations of these cancer-associated antigens has been proved to be useful for monitoring patients with various cancers.⁴ CA 125 is a high-molecular weight glycoprotein defined by OC 125 antibody which was developed by somatic hybridization of

mouse myeloma cells and spleen cells immunized with epithelial ovarian cancer cells.⁵ Bast et al. reported that serum CA 125 concentration was elevated in 82% of the patients with non-mucinous epithelial ovarian carcinoma and that serum CA 125 levels correlated with the clinical course during the treatment.^{2,5-7}

We have produced two monoclonal antibodies, 130-22 and 145-9, by fusing mouse myeloma cells and spleen cells immunized with human lung adenocarcinoma cells. These two monoclonal antibodies were found to react with CA 125 but to bind to separate determinants with OC 125.⁸ Since some monoclonal antibodies have been reported to enhance either the sensitivity or the specificity of various immunoassays,^{9,10} a series of IRMAs using these antibodies was evaluated.

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MATERIALS AND METHODS

Cells

Human lung adenocarcinoma cells PC-9 used for the immunization of mice in this study were established by Dr. Y. Hayata (Tokyo Medical College, Tokyo). Human ovarian cancer cells HTOA, HMOA, HUOA, HUOCA and HTBOA and human endometrial adenocarcinoma cells HOUA were established by Dr. I. Ishiwata (Ishiwata Hospital, Ibaragi).¹¹ All cell lines were grown in RPMI medium (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 5–10% fetal calf serum. Cell culture supernatants of lung cancer cell lines derived from adenocarcinoma A-549 and ABC-1, squamous cell carcinoma SK-MES-1 and RERF-LC-AI, and small cell carcinoma SBC-3, SBC-5, QG-90 and RERF-LC-MA were supplied by Dr. M. Akiyama (Radiation Effect Research Foundation, Hiroshima) through the courtesy of the Japanese Cancer Research Resources Bank (Tokyo).

Monoclonal antibodies

Female Balb/c mice were immunized with PC-9. Cell fusion was performed by incubating 1×10^7 NS-1 mouse myeloma cells with 1×10^8 mouse spleen cells in 50% polyethylene glycol #4000. Hybridoma supernatants were tested by an enzyme-linked immunosorbent assay (ELISA) in a screening test.¹² Two monoclonal antibodies, 130–22 and 145–9, were selected because they reacted with PC-9 cells but failed to react with other cancer cells of the stomach, colon, cervix, prostate and lung squamous cell type. Selected clones were injected intraperitoneally to Balb/c mice pre-immunized with pristane (Tokyo Kasei Kogyo Co., Ltd., Tokyo). Monoclonal antibodies were obtained from ascites fluid, and purified by Affi-gel protein A chromatography (Bio-Rad Laboratories, Richmond, CA, USA). Both 130–22 and 145–9 were IgG₁ isotypes. The specificity of staining in tissue sections was examined by indirect immunoperoxidase staining, and these antibodies were found to be reactive not only with lung adenocarcinoma but also with non-mucinous epithelial ovarian cancer (data not shown).

Radioiodination of monoclonal antibodies

Monoclonal antibodies 130–22 and 145–9 were radioiodinated by the chloramine-T method.¹³ Antibodies (40 μ g/180 μ l) in 0.3 M phosphate buffer, pH 7.5, and ¹²⁵I (1 mCi/10 μ l) for protein labeling (New England Nuclear, Boston, MA, USA) were mixed with chloramine-T dissolved just before use. After 5 minutes of vigorous agitation, free ¹²⁵I was separated from the labeled antibody using Sephadex G-50 gel chromatography. Since the specific activity

of the ¹²⁵I-labeled antibody markedly influenced the sensitivity of the assay, labeled antibodies with a rather high specific activity ranging from 10 to 15 mCi/mg antibody were used in this study. ¹²⁵I-labeled antibody was dissolved in 0.05 M phosphate buffered saline, pH 7.5 (PBS) containing 0.5% (BSA) and 1% normal mouse serum, and adjusted to about 50,000 cpm/0.2 ml. ¹²⁵I-labeled OC 125 was obtained from a commercially available ELISA CA 125 kit (CIS, Saclay, France).⁷

Cell binding

Radioiodinated antibody and various concentrations of PC-9 cells were incubated in a total volume of 0.5 ml PBS containing 0.5% BSA, at room temperature for 60 minutes.¹⁴ After centrifugation at $2,500 \times g$, the supernatant was aspirated and the radioactivity of the pellet was measured. The nonspecific binding with ¹²⁵I-labeled irrelevant antibody was subtracted from all tubes. Increasing amounts of unlabeled antibodies were also added to obtain a Scatchard plot analysis from which affinity constant values and number of binding sites per cell were calculated.

IRMA protocol for CA 125

Three monoclonal antibodies were used as capture antibodies on the solid-phase support and as the ¹²⁵I-labeled indicator antibody. Polystyrene beads (outer diameter, 0.64 μ m) were coated with 130–22 or 145–9 by overnight incubation.

In general, samples (0.1 ml) and ¹²⁵I-labeled monoclonal antibodies (50,000 cpm/0.2 ml) were added simultaneously with the antibody-coated beads. After incubation at room temperature for 24 hours, the reaction mixture was aspirated and the beads were then washed twice with saline. The radioactivity bound to the beads was measured by a well-type gamma counter.⁷

The standard antigen was made from serially diluted culture supernatants of PC-9 cells. Assay standards were also obtained from ELSA CA 125 kits and DAIICHI CEA kits (Daiichi Radioisotope Laboratories Ltd., Tokyo).¹⁵ Alpha-fetoprotein (AFP) was purchased from Alpha Therapeutic Corp., Los Angeles, CA, USA.

For comparison with the simultaneous one-step IRMA mentioned above, two-step IRMA was also performed by incubating standard antigen and antibody-coated beads for 5 hours at room temperature, washing beads with saline, and incubating with the tracer for 24 hours. All reagents were identical to those used in the one-step IRMA. The immunological relationships of OC 125, 130–22 and 145–9 were examined by competitive inhibition assays. Cell culture supernatants of PC-9 cells, equivalent to 1,000 U/ml, were incubated with 145–9 coated beads

for 5 hours, and after washing the beads with saline, incubated with the tracer and increasing amounts of unlabeled antibodies for 24 hours. The result was expressed as the percentage of radioactivity bound to the beads in the presence of unlabeled antibodies divided by the radioactivity bound to the beads without unlabeled antibodies.

In addition, factors relevant to the optimization of the IRMA for detecting CA 125 included pH and incubation time. The effect of pH on the IRMA was examined by diluting the tracer and standard antigen with 8 different pH buffers: 0.05 M glycine/HCl buffer pH 3.0, acetate buffer pH 4.0 and 5.0, phosphate buffer pH 6.0, 7.0, 7.5 and 8.0, and borate buffer pH 9.0. All buffers contained 0.14 M saline and 0.5% bovine serum albumin. The effect of the incubation time was studied by simultaneously incubating the tracer, standard antigen and antibody-coated beads for 0, 1, 3, 5, 24 and 48 hours at room temperature.

RESULTS

Binding of ^{125}I -labeled 130-22 and 145-9 to PC-9 cells
 Radioiodinated 130-22 and 145-9 produced against PC-9 cells showed a specific binding to PC-9 cells (Fig. 1). The percent binding of the added radioactivity nonlinearly increased as the number of incubated cells increased, reaching to more than 50% and 30% of the input cpm, respectively: whereas the percent binding of ^{125}I -labeled control antibodies was less than 5%. Furthermore, the binding of ^{125}I -labeled 130-22 and 145-9 to PC-9 cells was inhibited completely by the addition of unlabeled antibodies (data not shown), and the affinity constants to PC-9

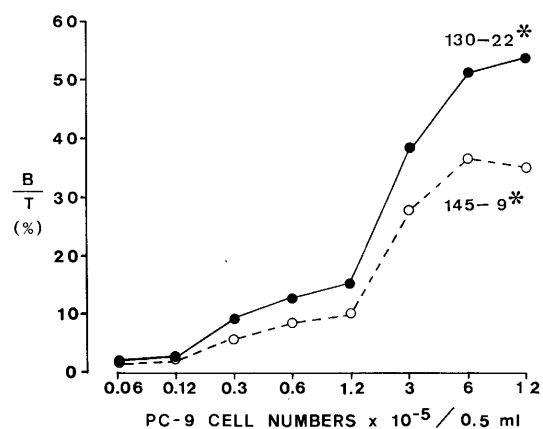


Fig. 1 Cell binding of ^{125}I -labeled 130-22 and 145-9 to PC-9 cells at various concentrations.

cells were calculated to be $1.2 \times 10^9 \text{ M}^{-1}$ for 130-22 and $0.5 \times 10^9 \text{ M}^{-1}$ for 145-9 by the Scatchard-plot analysis.¹⁶ The number of binding sites on a PC-9 cell was estimated as 5.4×10^5 per cell for 130-22 and 4.3×10^5 per cell for 145-9, respectively.

Immunological relationship of 130-22, 145-9 and OC 125

Immunostaining of tumor tissues with 130-22 and 145-9 revealed the antigenic expression not only in lung adenocarcinoma but also in ovarian cancer cells, and there were no differences in staining patterns with that of OC 125 (data not shown). To examine the immunological relationship of 3 monoclonal antibodies (130-22, 145-9 and OC 125) we developed 9 IRMAs by using different combinations of antibodies as radiolabeled indicator and solid-phase support. Specific binding of ^{125}I -labeled anti-

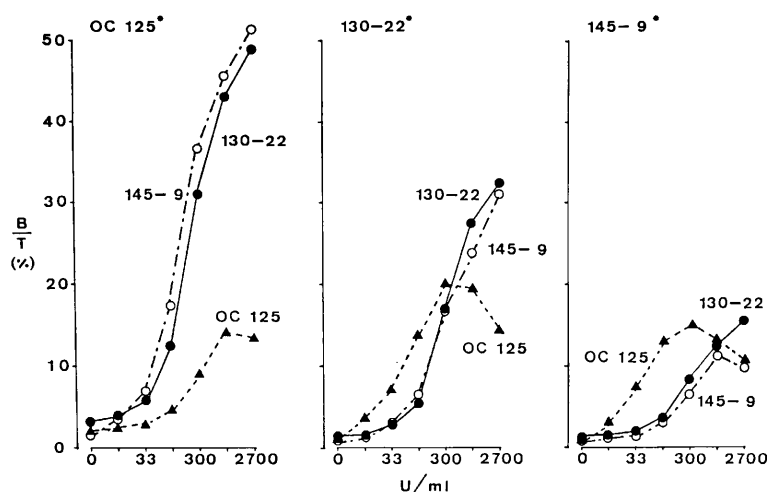


Fig. 2 Comparison of standard curves of 9 one-step IRMAs using combinations of 130-22, 145-9 and OC 125 antibodies. Polystyrene beads were coated with 130-22 (●—●), 145-9 (○---○) or OC 125 (▲---▲), and simultaneously incubated with PC-9 cell culture supernatants and ^{125}I -labeled OC 125 (Left), 130-22 (Middle), or 145-9 (Right) antibodies.

bodies to the antibody-coated beads was observed in cell culture supernatants derived from PC-9 cells. In all 9 one-step IRMAs, as shown in Fig. 2, it was possible to detect CA 125 ranging from 10 to 2,700 U/m. However, CA 125 standard curves were very different. The optimal assay configuration with respect to the sensitivity was found in a heterologous system where the antibody bound to the beads and ^{125}I -labeled antibody were not the same. The highest binding to the beads was obtained in the IRMA that used OC 125 as the ^{125}I -labeled probe and 130-22 or 145-9 as the immunoabsorbent, demonstrating a much higher sensitivity for detecting CA 125 than the homologous IRMA in which OC 125 was used both as a tracer and as an immunoabsorbent.⁷ Furthermore, at CA 125 concentrations exceeding 1,000 U/m, decreased binding to OC 125-coated beads or the high-dose "hook-effect" was observed. Both unlabeled 130-22 and 145-9 inhibited the binding of ^{125}I -labeled 130-22 to 145-9 coated beads (Fig. 3). On the contrary, there was no inhibitory effect on the binding of ^{125}I -labeled OC 125 to CA 125 previously bound to the beads even by increasing the amount of unlabeled 130-22 and 145-9 added. The inhibitory effect was not observed

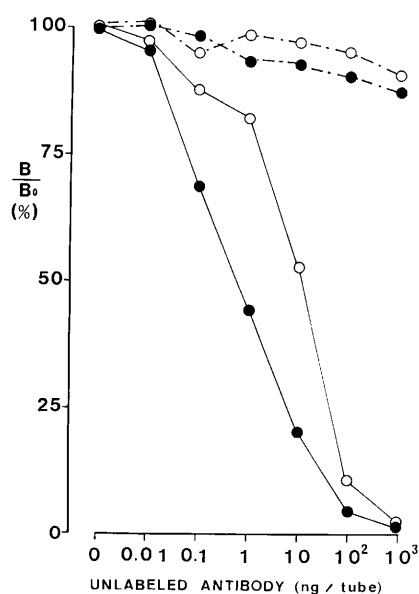


Fig. 3 Effect of unlabeled monoclonal antibodies on IRMA for CA 125. Cell culture supernatants of PC-9 cells were preincubated for 5 hours with 145-9 coated beads, after beads were washed, incubated again with ^{125}I -labeled OC 125 or ^{125}I -labeled 130-22 with increasing concentrations of unlabeled 130-22 or 145-9. ^{125}I -labeled OC 125 and unlabeled 130-22 (●---●), ^{125}I -labeled OC 125 and unlabeled 145-9 (○---○), ^{125}I -labeled 130-22 and unlabeled 130-22 (●—●), ^{125}I -labeled 130-22 and unlabeled 145-9 (○—○). Cell culture supernatants of PC-9 equivalent to 1,000 U/ml and 145-9 coated beads were used in all assays.

either, when these antibodies were added to the commercially available assay for CA 125 determination (data not shown). These results indicated that epitopes of both 130-22 and 145-9 were different from those of OC 125 and that 130-22 and 145-9 bound to sites similar to those of CA 125 antigen molecules.

Optimization of assay

Among 4 IRMAs using combinations of the 130-22 and 145-9 we have generated, the optimum assay configuration for CA 125 was the one that used a mixture of ^{125}I -labeled 130-22 and 130-22 or 145-9 coated beads. At low CA 125 concentrations, the assay employing ^{125}I -labeled 130-22 and 145-9 coated beads was a little more sensitive than the assay employing 130-22 both as a tracer and as an immunoabsorbent. The coating of beads with 145-9 was technically easier and needed less antibody concentrations than that with 130-22 (data not shown). Therefore, the following study was performed by employing 130-22 as a tracer and 145-9 as an immunoabsorbent. The effect of pH on the assay was examined by diluting ^{125}I -labeled 130-22 and standard antigens with 8 different buffers with pHs between 3.0 and 9.0 (Fig. 4). A neutral pH of between 6.0 and 8.0 was optimum for the measurement of CA 125. The assay was also dependent on the incubation time, and more than 24 hours was found

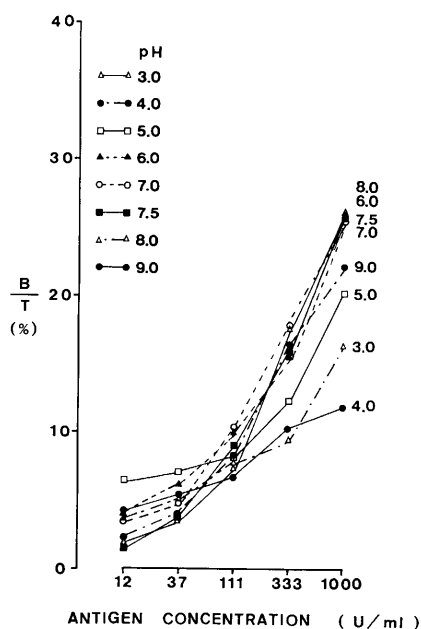


Fig. 4 Effect of pH on IRMA. Various concentrations of standard antigens and ^{125}I -labeled 130-22 were dissolved in 0.05 M glycine buffer (pH 3.0), acetate buffer (pH 4.0, pH 5.0), phosphate buffer (pH 6.0, pH 7.0, pH 7.5, pH 8.0) or borate buffer (pH 9.0), and incubated with 145-9 coated beads for 24 hours.

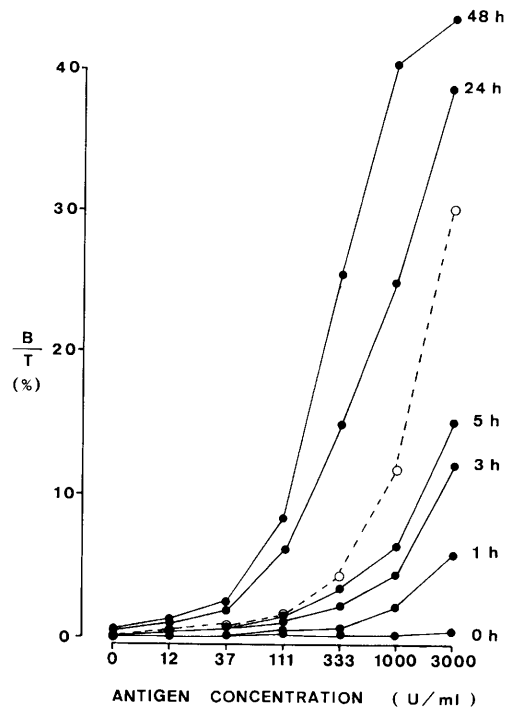


Fig. 5 Effect of incubation time. IRMA was performed with one-step assay (●—●) and two-step assay (○---○). In one-step assay, ^{125}I -labeled 130-22, standard antigen and 145-9 coated beads were incubated simultaneously for 0-48 hours. In two-step assay, standard antigen was preincubated with 145-9 coated beads for 5 hours, beads were washed, and incubated again with ^{125}I -labeled 130-22 for 24 hours.

to be necessary in the simultaneous sandwich assay (Fig. 5). In the two-step assay, where the standard antigen was previously incubated with 145-9 coated beads for 5 hours, the beads were washed and incubated again with ^{125}I -labeled 130-22 for 24 hours, the radioactivity bound to the beads was much lower than that obtained by the one-step assay, particularly at low CA 125 antigen concentrations. The simultaneous one-step assay was considered superior to the two-step assay for detecting the CA 125 antigen.

To determine if antigen concentrations measured by this IRMA system were comparable to those obtained by commercially available CA 125 kits, CA 125 standard antigens and culture supernatants of PC-9 cells were incubated with ^{125}I -labeled 130-22 and 145-9 coated beads. Obtained values were quite comparable up to CA 125 concentrations of 500 U/ml as depicted in Fig. 6. There was no cross-reactivity with AFP or carcinoembryonic antigen (CEA). After optimizing IRMA, CA 125 concentrations expressed as the bound radioactivity were linear between 4 and 333 U/ml and the coefficients of variation of each standard antigen were 5.1-12.1% (N=6) (Fig. 7). Intra- and inter-assay variations were determined using pooled normal human serum to which known

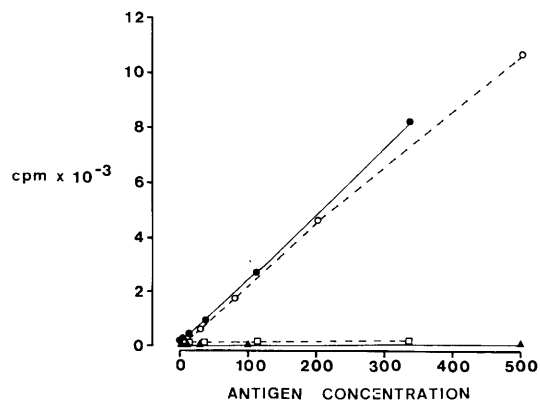


Fig. 6 Comparison of standard curves using CA 125 antigen (○---○), culture supernatants of PC-9 cells (●—●), AFP (□---□) and CEA (▲—▲). Maximum AFP and CEA concentration added were 1,000 ng/ml and 500 ng/ml, respectively. CA 125 antigen was obtained from ELSA CA 125 kit.

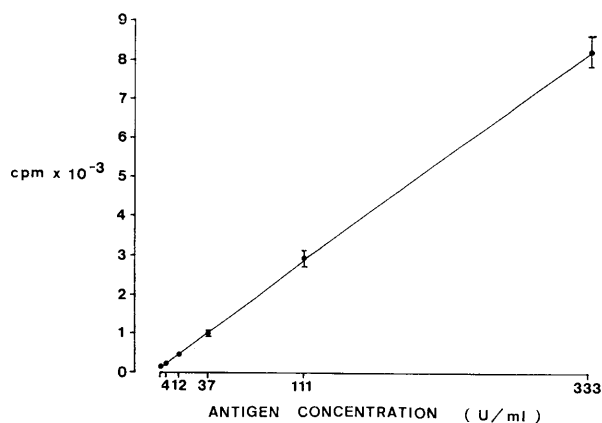


Fig. 7 Reproducibility of standard curve. Each point represents mean \pm SD (N=6).

Table 1 Results of intra-assay reproducibility

No. of experiments	Mean (U/ml)	S.D. (U/ml)	C.V. (%)
6	78.8	2.2	2.8
6	263.5	9.0	3.4

Table 2 Results of inter-assay reproducibility

No. of experiments	Mean (U/ml)	S.D. (U/ml)	C.V. (%)
6	84.8	8.6	10.1
6	103.5	19.8	6.5

concentrations of CA 125 were added. The coefficients of variations were 2.8-10.1% (N=6) (Tables 1 and 2).

IRMA of cell culture supernatants

Cell culture supernatants derived from human gynecological

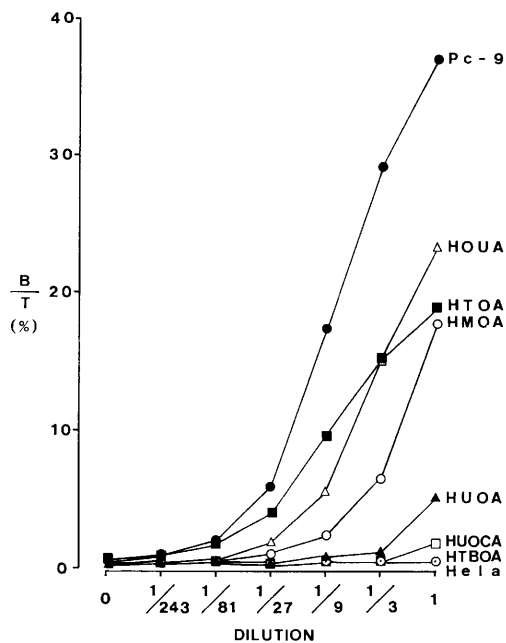


Fig. 8 Antigenic activities in supernatants of various cancer cell lines determined by using ^{125}I -labeled 130-22 and 145-9 coated beads.

colological and lung cancers were added in this IRMA system and the antigen activity was measured (Fig. 8). In good accordance with CA 125 values determined by OC 125 antibody (data not shown), high binding activity was demonstrated in the cell culture supernatants of ovarian cancer cell lines HTOA and HMOA, endometrial cancer cell line HOUA, lung adenocarcinoma cell line ABC-1 as well as in PC-9 cells. A low level of binding activity was also observed with other ovarian cancer cell lines HUOA and HUOCA. However, specific binding was not detected in the cell culture supernatants of the ovarian cancer cell line HTBOA, cervical cancer cell line HeLa, lung adenocarcinoma cell line A-549, lung squamous cell carcinoma cell lines SK-MES-1 and RERF-LC-AI, or lung small cell carcinoma cell lines SBC-3, SBC-5, QG-90 and RERF-LC-MA (data on lung cancer cells not shown).

DISCUSSION

We described the development of a new sensitive one-step IRMA for CA 125 using 2 monoclonal antibodies designated 130-22 and 145-9. CA 125 antigen is expressed on more than 80% of ovarian cancers,¹⁷ and exists as a high-molecular weight glycoprotein complex. However, immunopathological studies revealed that OC 125 is reactive not only with ovarian cancers but also with cancers of the endometrium, endocervix, peritoneum, pericardium and pleura, which are thought to be derived from the coelomic antigen during embryonic development.¹⁷

Furthermore, CA 125 antigenic determinants have been found in human milk, normal cervical mucus, normal lung tissues and seminal plasma.⁵ We tried to produce monoclonal antibodies reactive with lung cancer but lacking reactivity with other types of lung cancers.⁸ Two monoclonal antibodies 130-22 and 145-9 were selected. Reactivity of these antibodies with both lung and ovarian cancers and similar antigenic expression as OC 125 led us to the development of new IRMA.

CA 125 antigen was detectable by using all 9 IRMAs we developed, employing different combinations of 3 monoclonal antibodies (130-22, 145-9 and OC 125) as immunoadsorbent and ^{125}I -labeled probe. Because of the extraordinary specificity of the IRMA, these 3 monoclonal antibodies seemed to recognize CA 125 antigen, but 130-22 and 145-9 bound to different epitopes of CA 125 antigen molecules than OC 125, as proposed in Fig. 9. PC-9 cells express CA 125 on their surface cell membranes and release high concentrations of CA 125 into the cell culture supernatants. Therefore, it is not surprising that monoclonal antibodies generated against PC-9 cells are reactive with CA 125.

The most sensitive assay for CA 125 was a one-step IRMA that used OC 125 as the tracer and 130-22 or 145-9 as the immunoadsorbent. It is generally accepted that the sensitivity and specificity of an assay can be improved by combining monoclonal antibodies that recognize separate antigenic determinants.^{9,10} In the present study the radioactivity bound to the beads was more than 3 times higher than that of commercially available homologous assays where OC 125 was used both as the tracer and immunoadsorbent. These results were compatible with our proposal that 130-22 and 145-9 bound to sites different from those of OC 125.

Of the 4 IRMAs using combinations of 130-22 and 145-9, the optimal assay was achieved by in-

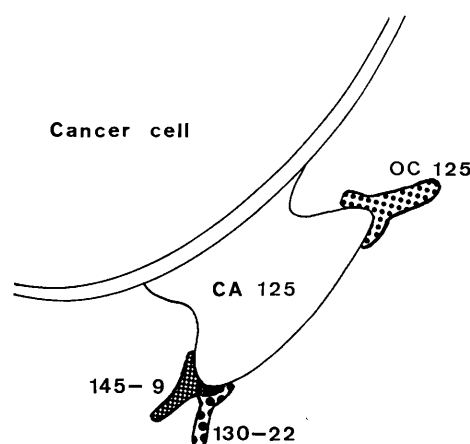


Fig. 9 Proposed model of CA 125 antigen and epitopes defined by OC 125, 130-22 and 145-9 antibodies.

cubating ^{125}I -labeled 130-22, standard antigens and 145-9 coated beads simultaneously for 24 hours at room temperature. Bast et al. used sodium citrate buffer pH 5.9 in the IRMA of CA 125 with OC 125.⁶ In the present study, optimal pH was from 6.0 to 8.0, and the high-dose "hook-effect" was not observed even at CA 125 concentrations greater than 1,000 U/ml by employing newly produced antibodies.⁷ The assay configurations that use 130-22 as a tracer and 145-9 as a catcher are most likely due to the binding affinity of both antibodies for CA 125¹⁸ (K_a to PC-9 cells: $1.2 \times 10^9 \text{ M}^{-1}$ and $0.5 \times 10^9 \text{ M}^{-1}$, respectively). The sensitivity and reproducibility of the assay was satisfactory, enough to detect 4 U/ml. Cell culture supernatants derived from ovarian cancer, endometrial adenocarcinoma and lung adenocarcinoma cell lines contained detectable levels of antigenic activity. Clinical studies are in progress to evaluate this IRMA for detecting serum CA 125 concentrations in patients with ovarian and lung cancers.

It is clear that CA 125 is different from CEA and AFP, as judged by the lack of binding of ^{125}I -labeled 130-22 to 145-9 coated beads in the presence of these antigens. However, CA 125 antigen is not purified and the chemical nature of CA 125 is under investigation.⁵ These two monoclonal antibodies that are reactive with different epitopes than OC 125 will be useful for studying the chemical and immunological nature of CA 125 antigen. In addition to immunoassays, radiolabeled monoclonal antibodies have also been widely applied for tumor targeting in patients with various cancers. Recently F(ab')₂ fragments of ^{131}I - or ^{111}In -labeled OC 125 have been administered to patients with ovarian cancer for the localization of recurrence.¹⁹ In a preliminary study, specific uptake of radioiodinated 130-22 was demonstrated in xenografted tumors transplanted in nude mice. Further studies are needed to determine the eventual *in vivo* use of radiolabeled 130-22 and 145-9.

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