Monoclonal Antibody Immunoradiometric Assay for an Antigenic Determinant (CA 125) Associated with Human Epithelial Ovarian Carcinomas


ABSTRACT

CA 125 is an antigenic determinant expressed by greater than 80% of nonmucinous epithelial ovarian carcinomas. An immunoradiometric assay has been developed using a murine monoclonal antibody (OC125) to quantitate CA 125 in human serum. This immunoradiometric assay was optimized for specificity, sensitivity, and performance characteristics. Using a simultaneous immunoradiometric assay, the mean CA 125 concentration in 56 sera from healthy individuals was 11.2 ± 5.4 (S.D.) units/ml, with 9.7 ± 3.2 units/ml for 30 males and 13.1 ± 6.8 units/ml for 26 females. A reference value of 35 units/ml included all 56 normals and excluded 86 of 105 (82%) ovarian carcinoma patients. This reference value also excluded 9 of 142 patients (6%) with benign diseases, but if the upper limit of normal was set at 65 units/ml, only 3 of 142 (2%) patients with benign diseases had elevated serum CA 125 levels, whereas 77 of 105 (73%) ovarian carcinoma patients remained positive. The ability of researchers, with this assay, to discriminate between CA 125 values in sera of patients with ovarian carcinoma and those of healthy individuals and patients with benign disease suggests that the assay deserves continued evaluation for monitoring and early diagnosis of ovarian cancer.

INTRODUCTION

Over the last decade, there have been many attempts to develop antibodies against antigens associated with ovarian epithelial carcinomas (3). Polyclonal heteroantisera have been utilized to isolate several antigens, but radioimmunoassays exist for only 2 ovarian-associated antigens, OCAA (4, 5) and OCA (12-15). Elevated OCAA was reported to be present in serum from 66% of patients with serous and mucinous cystadenocarcinomas of the ovary (4), whereas OCA was elevated in approximately 60% of patients with serous, mucinous, and epidermoid carcinomas of the ovary (13). In studies to date, however, the clinical utility of these assays has not been established.

Recently, a murine monoclonal antibody (OC125) has been developed which reacts with a determinant (CA 125) on 6 epithelial ovarian carcinoma cell lines (1) and with greater than 80% of nonmucinous ovarian epithelial tumors of serous, endo-

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\footnote{2 To whom requests for reprints should be addressed, at Centocor, 244 Great Valley Parkway, Malvern PA 19355.}

\footnote{3 Scholar of the Leukemia Society of America, Inc.}

\footnote{4 The abbreviations used are: CA 125, the antigenic determinant(s) to which the monoclonal antibody OC125 binds; IRMA, immunoradiometric assay; ANOVA, analysis of variance; NCI, National Cancer Institute.}

\footnote{Received August 25, 1983; accepted December 1, 1983.}


\footnote{6 The abbreviations used are: CA 125, the antigenic determinant(s) to which the monoclonal antibody OC125 binds; IRMA, immunoradiometric assay; ANOVA, analysis of variance; NCI, National Cancer Institute.}
reference standard. This antigen preparation, derived from the cell culture supernatant of a human carcinoma cell line (OVCA 433) (1), was partially purified. Therefore, unit value assignments were arbitrary but reflected, on a relative basis, the amount of CA 125 present. Standards of 500, 300, 200, 80, 30, 6.5, and 0 units/ml were calibrated against this reference standard and used in the present studies to generate a standard curve from which quantitative amounts of CA 125 in specimens could be computed. Samples with CA 125 greater than 500 units/ml were diluted 1:10 or 1:100 with a standard serum diluent (11.1 units/ml) and reassayed. Because pooled serum contained small amounts of CA 125, "zero" concentration standards were prepared by adding 5 μg of OC125 antibody/ml of serum. Stability of standards was determined by comparison of CA 125 unit values of standards kept at 0, 20, 37, and 45° with those stored at -20°.

IRMA Protocol. All assays were performed in duplicate on serum or citrated plasma, as heparin was found to interfere with the assay. Samples were assayed with a simultaneous sandwich IRMA in which antigen in serum was bound to nonlabeled antibody (OC125) on the bead. Since multiple CA 125 determinants were associated with each antigen molecule, free determinants on the bead-bound antigens were detected by binding of 125I-labeled OC125 antibody. In a reaction tray, 100 μl of serum, standard, or positive control were mixed with 100 μl of tracer buffer (100 mmol of sodium citrate, 50 mmol of EDTA, 150 mmol of sodium chloride, 2 g of bovine serum albumin, and 0.4 g of non-OC125 murine monoclonal IgG/liter, pH 5.90) containing 130,000 dpm 125I-OC125. Bovine serum albumin was obtained as Fraction V powder from Armour Pharmaceuticals, South Plainfield, NJ. One antibody-coated bead was added to each reaction well, and samples were incubated at room temperature (18-22°) for 20 hr. Beads were washed 3 times with deionized water, and the number of counts bound was determined in a gamma counter. Bound radioactivity (cpm) was converted to CA 125 units/ml by reference to a standard curve generated by linear regression, log-log, or 4-parameter transformation (16) of the data with computer programs written for this purpose. For comparison with the simultaneous IRMA, forward 2-step IRMAs were performed by incubating serum and immunoadsorbent for 6 hr at 37°, washing, and incubating with tracer for 20 hr at 18-22°. All reagents were identical to those of the simultaneous IRMA. Additionally, 100 μl of tracer buffer without nonspecific murine monoclonal IgG were added at each step to bring the pH to 6.0 and the final assay volumes to 200 μl.

All statistical analyses were performed by an IBM 370 computer using the Statistical Analysis System (SAS Institute, Inc., Cary, NC) programs. The predictive value of the CA 125 IRMA was evaluated by Bayes’ Decision Theory (10). Briefly, the a posteriori probability of a disease i given a positive CA 125 test result, p(i|R), was calculated from the equation

\[
p(i|R) = \frac{\text{Prevalence} \times \text{sensitivity}}{\text{Prevalence} \times \text{sensitivity} + \sum_{j=1}^{n} \text{prevalence}_j \times \text{sensitivity}_j}
\]

where i to n are specific disease or nondisease states in the population for which one can estimate the probability of obtaining a positive result, i.e., the test sensitivity. Prevalence of any cancer was assumed to be twice the mean cancer incidence rate for females 45 to 74 years of age weighted for a 1970 standard age distribution (17). Sensitivity of the test for any group was approximated by the fraction of the group with CA 125 greater than the reference values.

RESULTS

Optimization of Assay. Factors relevant to the optimization of the present IRMA included assay configuration, incubation times, presence of non-OC125 murine IgG, pH, OC125 concentration on the solid phase, and concentration of 125I-labeled OC125. As multiple CA 125 determinants are expressed on antigen molecules, OC125 could be used both on the immunoadsorbent and as the 125I-labeled probe to detect and quantify bound antigen. Chart 1 illustrates the dose-response curves for optimal simultaneous and forward 2-step IRMAs with identical immunoreagents. At concentrations greater than 2000 units of CA 125/ml, more 125I-OC125 was bound in the 2-step assay than in the simultaneous assay. However, with CA 125 concentrations less than 1000 units CA 125/ml, the simultaneous assay bound more than twice as much 125I-OC125 and was, therefore, the configuration providing highest sensitivity. At CA 125 concentrations greater than 4000 units of CA 125/ml, diminished binding was observed with the simultaneous IRMA but not the 2-step IRMA. This prozone or "hook" effect was not pronounced in that addition of 40,000 units of CA 125/ml was required before an apparent anomalous unit value below 500 units/ml was found. Chart 2 compares typical standard curves for the simultaneous and 2-step IRMAs generated by plotting cpm bound versus CA 125 units/ml for CA 125 standards of 6.5 to 500 units/ml. The slope of the simultaneous assay was more than twice that of the 2-step assay and appeared to increase slightly with increasing dose. Since the simultaneous incubation of all assay reagents proved superior to sequential addition of sample and tracer, further optimization was restricted to this configuration.

The importance of the assay incubation time is demonstrated by data in Chart 3. Serum samples containing CA 125 greater than 35 units/ml were assayed with identical reagents in 4- and 20-hr simultaneous IRMAs. Twenty hr was found to be the shortest time to permit equilibration of the antigen-antibody reaction to include all samples tested. However, the 4-hr IRMA values appeared to be higher in nearly one-half of the samples.

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tested, presumably due to more rapid antigen-antibody binding kinetics in these samples than in reference standards. Therefore, 20 hr was selected as the best time in which to run the assay.

During the early development of this assay, some serum samples from healthy individuals gave elevated CA 125 scores. Normal mouse serum or murine monoclonal IgG was added to the tracer buffer and found to inhibit this discrepant binding in every instance. Optimization of remaining variables, pH, OC125 bead coating, etc. were pursued in a systematic, empirical fashion with the ultimate criteria being their effects upon specificity, sensitivity, reproducibility, and clinical utility.

Table 1 shows the assay parameters for the final, optimized simultaneous IRMA for CA 125 as derived from 7 consecutive assays done with the same reagents. Comparisons of unweighted linear regression, logit-log, and 4-parameter fit transformations of the binding data revealed that simple unweighted linear regression gave the best fit of the standard curve over the range of 6.5 to 500 units/ml with a slope of 24.3 ± 3.45 (S.D.) cpm/unit, correlation coefficient of 0.998 ± 0.001, and one-half maximum binding of about 500 units/ml. Goodness of fit was assessed from the coefficient of variation between data points and the computed line or curve. In some assays, a positive curvature in the dose-response curve was noted. Nonlinear polynomial regression analysis fit the standard curve better than did linear regression in these instances, but similar accuracy of dose interpolation was obtained by simple point-to-point construction of the standard curve. The within-assay variance was uniformly distributed between 30 and 500 units/ml, with a mean coefficient of variation of 7.5%, whereas the coefficient of within-assay variance for the 6.5 units/ml standard was 15.5%. When a single lot of immunoreagents was used, the coefficient of between assay variance was 11.2%. The lowest detectable dose was 1.4 units/ml, when this value was defined as the amount of antigen corresponding to 2 standard deviations in excess of the mean cpm for the “zero” CA 125 standard. Recoveries of CA 125 from patient sera and standards were compared by plotting CA 125 determined from the standard curve versus CA 125 levels calculated from the dilution factor for samples from 13 patients with ovarian carcinoma. If antigen was recovered in parallel, the slope would equal 1.0, and the Y-intercept would equal 0 units (16). The observed slope was 1.002 ± 0.048, and the Y-intercept was 1.82 ± 6.45 units/ml for serum dilutions of 1:2 to 1:16. The CA 125 standards were stable for at least 8 weeks at 0-37°.

Distribution of Serum CA 125 in Control Groups. Distributions of serum CA 125 scores in normal, blood donor, and benign disease groups are presented in Table 2. For the normal group, confidence limits of 95 and 99%, based on moment statistics generated from the actual data set by a standard algorithm (9), were 25 and 28 units/ml, respectively. Ninety-five and 99% confidence limits based on the assumption that the data fit a

### Table 1

<table>
<thead>
<tr>
<th>Assay parameters for IRMA of CA 125</th>
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<tbody>
<tr>
<td>Slope</td>
</tr>
<tr>
<td>Correlation coefficient</td>
</tr>
<tr>
<td>Half-maximal binding</td>
</tr>
<tr>
<td>Least detectable dose</td>
</tr>
<tr>
<td>Coefficient of variation</td>
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<tr>
<td>Within assay</td>
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<tr>
<td>Between assay</td>
</tr>
<tr>
<td>Parallelism (antigen recovery with dilution)</td>
</tr>
<tr>
<td>Slope</td>
</tr>
<tr>
<td>Y-intercept</td>
</tr>
</tbody>
</table>

a Average ± S.D.  
b N, number of determinations.  
c M, number of samples, diluted 1:2 to 1:16.
normal distribution were 20 and 24 units/ml, respectively. An identical analysis of the larger blood donor group yielded 95 and 99% confidence limits of 21 and 37 units/ml, calculated from a normal distribution were 20 and 24 units/ml, respectively. An

distribution of scores in benign disease closely resembled that with a mean of 16.8 and a median of 10.0 units/ml. Ninety five and 99% confidence limits were 42 and 212 units/ml for a with a mean of 16.8 ±27.2 (n = 142), with a mean of 18.7 ±11.7p(age)0.0129c

Effect of Age and Sex on CA 125. Table 3 lists the mean serum CA 125 found for healthy individuals and patients with benign disease. ANOVA for the effects of age and sex was performed for each of these groups to determine the relative contribution of these variables to the distribution of CA 125 values (Table 2). A large blood donor group (2) was included in this statistical analysis to increase the sampling accuracy and corroborate the results found in the normal (NCI) panel group. Mean CA 125 concentration in normals (NCI panel) was 11.2 ±8.9 (n = 56), with a mean of 9.7 ±3.2 (n = 30) for males and 13.1 ± 6.8 units/ml for females. Mean CA 125 concentration in the blood donors was 8.7 ± 8.9 (n = 888), with a mean of 8.0 ± 9.4 for males (n = 537), and 9.9 ± 8.0 units/ml for females (n = 351). Among patients with benign disease, the mean CA 125 was 16.8 ± 27.2 (n = 142), with a mean of 18.7 ± 33.6 for nongastrointestinal disease (n = 86) and 14.0 ± 11.7 units/ml for gastrointestinal disease (n = 56). ANOVA revealed a significant (p < 0.05) dependence of CA 125 score on sex in both NCI normals and blood donor group, due to greater CA 125 levels in females at all ages. There may also have been a consistent dependence of serum CA 125 levels upon age, since ANOVA found probabilities of 0.0145 and 0.0628 for correlation of age and CA 125 score in the NCI normal and blood donor groups, respectively. While the latter appeared to result from decreasing CA 125 levels with increasing age in both sexes (data not shown), this ANOVA was not done with age-matched groups of males and females. CA 125 levels in benign disease did not appear to depend upon either age or sex except in benign gastrointestinal disease, in which a significant (p < 0.001) dependence on age and sex was found.

Selection of Reference Values. The previous considerations suggest that a reference value of 35 units/ml is an upper limit on serum CA 125 levels, with at least 99% confidence in disease-free individuals. A reference value of 65 units/ml should include normal individuals and patients with benign disease with 98% confidence.

The influence of the choice of CA 125 reference values upon the sensitivity and specificity of the IRMA for ovarian cancer is illustrated in Table 4. With a reference value of 35 units/ml, 82 (86 of 105), 6 (9 of 142), and 1% (9 of 888) of the sera of subjects with ovarian carcinoma, benign disease, and sera collected from blood donors, respectively, were positive. In contrast, 0% (0 of 56) of healthy individuals in the NCI serum panel had CA 125 values greater than 35 units/ml. Using 65 units/ml as the reference value, 73% of the ovarian carcinoma group were still positive, whereas the fraction of benign disease and blood donor samples greater than 65 units/ml decreased significantly to 2 and 0.2%, respectively. With this group of specimens, the complete elimination of benign disease as a potential source of positivity would require a reference value of 220 units/ml, but

Table 2
Distribution of serum CA 125 scores (units/ml) in normal healthy individuals and patients with benign disease and CA 125 unit values for 95 and 99% confidence limits that any member of a group would have a CA 125 score less than that shown

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Units/ml</th>
<th>Median</th>
<th>Range</th>
<th>Units at the following confidence limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (NCI)</td>
<td>56</td>
<td>11.2 ± 5.4</td>
<td>10.0</td>
<td>3.1–28.0</td>
<td>25c 28c</td>
</tr>
<tr>
<td>Blood donors</td>
<td>888</td>
<td>8.7 ± 8.9</td>
<td>7.1</td>
<td>0–161.0</td>
<td>20c 24c</td>
</tr>
<tr>
<td>Benign disease(NCI)</td>
<td>142</td>
<td>16.8 ± 27.2</td>
<td>10.1</td>
<td>2.5–212.0</td>
<td>42c 212c</td>
</tr>
<tr>
<td>Benign disease(CA 125 &lt; 65 units/ml)</td>
<td>138</td>
<td>12.9 ± 9.4</td>
<td>10.1</td>
<td>2.5–60.5</td>
<td>62c 80c</td>
</tr>
</tbody>
</table>

a NCI, National Cancer Institute Serum Panel.
b Mean ± S.D.c CA 125 units at given confidence limits calculated from a distribution fitted to data (9).
d CA 125 units at given confidence limits calculated, assuming that CA 125 levels are normally distributed within that group.

Table 3
ANOVA for the effects of age and sex on serum CA 125 in normal, healthy individuals and patients with benign disease

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>CA 125 (units/ml)</th>
<th>p (age)</th>
<th>p (sex)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normals (NCI)</td>
<td>56</td>
<td>11.2 ± 5.4</td>
<td>0.0145c</td>
<td>0.0129c</td>
</tr>
<tr>
<td>Males</td>
<td>30</td>
<td>9.7 ± 3.2</td>
<td>0.0628</td>
<td>0.0014c</td>
</tr>
<tr>
<td>Females</td>
<td>26</td>
<td>13.1 ± 6.8</td>
<td>0.0628</td>
<td>0.0014c</td>
</tr>
<tr>
<td>Blood donors</td>
<td>888</td>
<td>8.7 ± 8.9</td>
<td>0.0628</td>
<td>0.0014c</td>
</tr>
<tr>
<td>Males</td>
<td>537</td>
<td>8.0 ± 9.4</td>
<td>0.0628</td>
<td>0.0014c</td>
</tr>
<tr>
<td>Females</td>
<td>351</td>
<td>9.9 ± 8.0</td>
<td>0.0628</td>
<td>0.0014c</td>
</tr>
<tr>
<td>Benign disease(NCI)</td>
<td>142</td>
<td>16.8 ± 27.2</td>
<td>0.0628</td>
<td>0.9872</td>
</tr>
<tr>
<td>Nongastrointestinal</td>
<td>86</td>
<td>18.7 ± 33.6</td>
<td>0.8192</td>
<td>0.4957</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>56</td>
<td>14.0 ± 11.7</td>
<td>0.0014c</td>
<td>0.0006c</td>
</tr>
</tbody>
</table>

a NCI, National Cancer Institute Serum Panel.
b Mean ± S.D.c p < 0.05.d Ref. 2.
DISCUSSION

This report describes the development of a sensitive, reliable, and reproducible simultaneous IRMA for an ovarian tumor-associated antigen in serum. While the simultaneous configuration was adopted because of its greater sensitivity, very high levels of CA 125 could, in principle, result in anomalously low values due to a prozone effect. In practice, however, no serum sample has yet been found which contained sufficiently elevated CA 125 to produce such an effect. In evaluating the clinical utility of the assay, one must consider the possibility that at least some elevated CA 125 scores could arise from the presence of serum factors other than the defined antigenic determinant(s). A principal cause for such a discrepant or "false-positive" result might be a human serum factor linking iodinated IgG tracer to immunoabsorbent IgG. The IRMA buffer contained sufficient murine IgG to inhibit nonspecific binding in all such samples tested.

In previous radioimmunoassays for ovarian tumor-associated antigens, a large fraction of control sera contained elevated antigen activity. Moreover, antigen levels among ovarian cancer patients were only 2 to 3 times greater than in normal individuals (4, 5, 12-15). Using the IRMA described in the present report, consistently low levels of CA 125 were found in sera from healthy individuals, and CA 125 levels 10 to 100 times normal levels were detected in a majority of ovarian carcinoma patients. Statistical analysis of CA 125 levels in sera supported setting the upper limit of normal values at 65 units/ml maximized the difference between patients with benign disease and ovarian carcinoma without substantial loss of sensitivity. Slight dependency of mean serum CA 125 levels on age and sex in healthy individuals did not interfere with the establishment of these reference values. Among females, slight elevation among younger individuals may stem from frequently elevated serum CA 125 levels, observed principally during the first trimester of pregnancy.

CA 125 is also expressed on antigens shed into the sera of individuals with nongynecological cancers, including carcinomas of the pancreas, colon, and lung (2). Since nearly all of the sera tested in this and previous studies were collected from patients with advanced malignancies, the excellent specificity of the IRMA for ovarian cancer relative to nongynecological tumors actually may be significantly underestimated in this study.

Making some reasonable assumptions, we have estimated the potential use of the CA 125 IRMA as a diagnostic adjunct. Using available data, at serum levels in excess of 220 units/ml, the estimated probabilities of ovarian cancer (52%) or any cancer...
(100%) being present are significant. The probability of a positive result due to a benign disease was not considered in these calculations, as there was insufficient information on both disease prevalence and test specificity. However, it is not ruled out that certain benign diseases may give rise to CA 125 serum levels greater than 220 units/ml or that the assumptions made regarding the prevalence of cancer in a population are too inaccurate to provide precise evaluation. These caveats notwithstanding, it is possible that application of the present IRMA to a population at high risk for ovarian cancer might yield useful information as to the preclinical course and duration of the disease. The utility of the test for screening, however, will depend upon how often early-stage disease is associated with CA 125 levels between 220 and 35 units/ml. Sequential monitoring will undoubtedly provide additional key information in this regard.

Admitting certain limitations, the excellent specificity and sensitivity of the CA 125 IRMA for ovarian carcinoma and its demonstrated clinical utility in monitoring ovarian carcinoma patients (2) suggest that further exploration of the diagnostic applications of this test is warranted.

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