Characterization of Inhibin Forms and Their Measurement by an Inhibin α-Subunit ELISA in Serum from Postmenopausal Women with Ovarian Cancer


The aim of this study was to characterize the molecular wt forms of inhibins A and B and its free α-subunit present in serum from women with ovarian cancer as a basis for developing improved monoclonal antibody-based inhibin assays for monitoring ovarian cancer. Three new inhibin α-subunit (αC) ELISAs were developed using monoclonal antibodies directed to three nonoverlapping peptide regions of the αC region of the inhibin α-subunit. To characterize serum inhibin molecular wt forms present in women with ovarian cancer, existing inhibin immunoassays (inhibin A, inhibin B, and pro-αC) and the new αC ELISAs were applied to sera from women with granulosa cell tumors and mucinous carcinomas previously fractionated using a combined immunofinity chromatography, preparative SDS-PAGE, and electroelution procedure. The distribution and molecular size of dimeric inhibins and α-subunit detected were consistent with known mol wt forms of inhibins A and B and inhibin α-subunit and their precursor forms present in serum and follicular fluid from healthy women. The αC ELISAs recognized all known forms of inhibin and the free inhibin α-subunit, although differences between αC ELISAs were observed in their ability to detect high mol wt forms. To assess which of the αC ELISAs was preferred in application to ovarian cancer, the αC ELISAs were applied to serum from a range of normal postmenopausal women (n = 61) and postmenopausal women (n = 152) with ovarian (serous, mucinous, endometrioid, clear cell carcinomas, and granulosa cell tumors) and nonovarian (breast and colon) cancers. Despite differences in their ability to detect high mol wt forms of inhibin, the αC ELISAs showed similar sensitivity (i.e. proportion of cancer patients correctly detected) and specificity (proportion of controls correctly detected) indexes in the detection of mucinous carcinomas (84% and 95%) and granulosa cell tumors (100% and 95%) compared with earlier inhibin RIA or polyclonal antibody-based immunofluorometric assays. A combination of the αC ELISAs with the CA125 assay, an ovarian tumor marker that has a high sensitivity and specificity for other ovarian cancers (serous, clear cell, and endometrioid), resulted in an increase in sensitivity/specificity indexes (95% and 95%) for the all ovarian cancer group. These new monoclonal antibody-based inhibin αC ELISAs now provide practical and sensitive assays suitable for evaluation as diagnostic tests for monitoring ovarian cancers. (J Clin Endocrinol Metab 87: 816–824, 2002)

INHIBINS A AND B are glycoprotein heterodimers consisting of α- and either βA- or βB-subunits that are produced by the gonads and act as feedback regulators of the pituitary hormone, FSH (1–3). Inhibins are found in serum as a range of mol wt forms that are the result of differential processing of the larger precursor forms (Fig. 1). In addition to the dimeric (αβ) inhibin forms, the free α-subunit (pro-αC and pro-αN-αC) has been identified in serum. Inhibin forms identified to date are presented in Fig. 1.

Inhibins A and B and their subunits are produced by the developing ovarian follicle and corpus luteum and show cyclical activity during the menstrual cycle, consistent with their role as negative feedback regulators of FSH (4). After menopause with the depletion of ovarian follicles, serum inhibin levels decline to undetectable or near-undetectable levels (2, 3, 5). It was subsequently noted that serum inhibin levels were elevated in postmenopausal women with ovarian cancers (6–11), in particular with granulosa cell tumors (11, 12) and mucinous carcinomas. In contrast, other ovarian cancers (e.g. serous, endometrioid, clear cell) showed limited increases. Immunoassays [inhibin RIA (6, 7, 10, 12) and αC immunofluorometric assay (IFMA) (13, 14)] have been developed and used in the routine detection and monitoring of these ovarian cancers. These assays detect all α-subunit forms containing the αC region and thus detect both dimeric (αβ) and monomeric α-subunit forms, including unprocessed and processed forms (15, 16). Studies undertaken using inhibin ELISAs that detect inhibins A, B, and pro-αC with high specificity were less effective in detecting mucinous cancers compared with the less specific inhibin α-subunit-directed assays (17). This difference was attributed to the large variation in the secretion of inhibin A and B or α-subunit forms between patients. An immunoassay that detected all α-subunit-containing forms was thus the preferred choice.

Previously developed inhibin assays [RIA (6, 7) and αC IFMA (13)] were largely research laboratory-based assays and were not suitable for wider use. Thus, monoclonal an-
tibodies were raised to three inhibin epitopes identified by the polyclonal antisera used in the αC IFMA (14). These epitopes were located at the N-terminal region and in two nonoverlapping internal sequences of the αC region (Fig. 1). Antisera and monoclonal antibodies (Mabs) to the N-terminal region have been produced previously, of which the R1 antibody (4, 18, 19) is the most widely known. The other two epitopes are novel. Sensitive sandwich-based ELISAs (termed αC ELISAs) have been developed using combinations of R1 Mab and either Mab directed to one of the other two sites. To date these assays have not been applied to serum samples.

The aim of the present study was to establish the sensitivity and specificity of these αC ELISAs in order to develop an improved immunoassay that can be used as a diagnostic serum test in detecting certain ovarian cancers. Because the various αC ELISAs show different specificities, the various mol wt forms of inhibin in serum from women with ovarian cancer were characterized using previously described procedures (15, 16) with modifications (14, 20) based on a combined immunoaffinity chromatographic, preparative SDS-PAGE, electroelution procedure. The eluted samples were assayed by the various ELISAs. The application of the αC ELISAs to a range of normal postmenopausal women and postmenopausal women with ovarian cancers was then undertaken. The combination of these data with that of CA125, a tumor marker with high sensitivity and specificity for ovarian cancers other than granulosa cell tumors and mucinous cancers, was investigated to assess whether their combination led to an increased detection of all ovarian cancers. The combination of these αC ELISAs with CA125 resulted in the detection of 95% of all ovarian cancers at a 95% level of specificity.

**Subjects and Methods**

**Subjects**

Serum samples were obtained preoperatively from 117 women admitted to major gynecological teaching hospitals in Melbourne with symptoms consistent with ovarian cancer and who subsequently were shown to have ovarian cancers. Classification of the tumors was similar to that used previously (13). The various cancer types investigated are presented in Table 1.

In addition, serum samples were obtained from postmenopausal women with breast and bowel cancers. Serum samples were obtained from 61 healthy postmenopausal women attending a mammography breast cancer-screening clinic as previously described (13). The women studied were asymptomatic and considered postmenopausal based on age (55 yr and older). Serum samples from postmenopausal women with granulosa cell tumors (pool from 2 women, aged 65 and 66 yr), serous

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**TABLE 1.** Geometric means ± 2 sd for serum inhibin as determined by various assays in normal postmenopausal women and postmenopausal women with ovarian and nonovarian cancers

<table>
<thead>
<tr>
<th></th>
<th>No. of subjects</th>
<th>PO#14/R1 ELISA (ng/liter)</th>
<th>PO#23/R1 ELISA (ng/liter)</th>
<th>PO#14+23/R1 ELISA (ng/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>61</td>
<td>15.7</td>
<td>7.09–34.9</td>
<td>8.75</td>
</tr>
<tr>
<td>Ovarian cancers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>42</td>
<td>17.5</td>
<td>9.24</td>
<td>11.2–27.3</td>
</tr>
<tr>
<td>Mucinous</td>
<td>27</td>
<td>185</td>
<td>160</td>
<td>18.2–1880</td>
</tr>
<tr>
<td>Granulosa cell tumor</td>
<td>16</td>
<td>1300</td>
<td>2560</td>
<td>12.6–134000</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>14</td>
<td>38.7</td>
<td>33.1</td>
<td>33.1</td>
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<tr>
<td>Clear cell</td>
<td>9</td>
<td>33.0</td>
<td>21.5</td>
<td>1.51–990</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>9</td>
<td>33.5</td>
<td>24.5</td>
<td>1.36–800</td>
</tr>
<tr>
<td>Nonovarian cancers</td>
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<tr>
<td>Bowel</td>
<td>16</td>
<td>18.0</td>
<td>10.5</td>
<td>18.0</td>
</tr>
<tr>
<td>Breast</td>
<td>19</td>
<td>11.2</td>
<td>9.26</td>
<td>2.45–51.6</td>
</tr>
</tbody>
</table>
carcinomas (pool from 2 women, 55 and 61 yr), and mucinous carcinomas (a single sample from a woman, aged 51 yr, and a pool from 5 women, >52 yr) were used in the fractionation studies. A postmenopausal serum pool (18 ml) was prepared by combining equal aliquots from all 61 normal women used as controls in this study. Human follicular fluid (hFF) and serum from women stimulated by gonadotropins as part of an in vitro fertilization (IVF) program were obtained from Monash IVF (Clayton, Australia). The research and ethics committees of all participating hospitals approved the study protocol.

**αC ELISAs**

The Mabs used in this study consist of Mab R1 (directed to amino acids 235–259 of the α-subunit) (18), PO#14 (amino acids 306–329) (14), and PO#23 (amino acids 342–356; Fig. 1) (14). These Mabs were tested in various combinations in an ELISA system based on the procedure employed by Groome and colleagues (4, 18, 19, 21) previously with inhibins A, B, and pro-αC. Serum samples were initially boiled in the presence of 6% SDS before assay as undertaken by Groome and colleagues (4, 19). Serum sample (100 μl) or inhibit standard (100 μl in inhibin-free serum; see Ref. 13 for procedure) and Tris buffer (100 μl, 0.13 mol/liter Tris-HCl, pH 7.5, containing 0.154 mol/liter NaCl, 2 mmol/liter EDTA, 1% Triton-X-100 (vol/vol), 1% BSA, and 0.05% NaN₃) were added to Mab-coated 96-well microtiter plates that were incubated overnight with shaking at room temperature. Alkaline phosphatase-labeled second antibody [50 μl R1 Mab in 0.13 mol/liter Tris-HCl, pH 7.5, containing 0.154 mol/liter NaCl, 5% Triton-X-100 (vol/vol), 1% BSA, and 0.05% NaN₃] was added, and the plate was incubated for an additional 3 h. The enzymatic response was amplified with an AMPAK kit (DAKO Corp., Carpinteria, CA). The data were analyzed using the Multicalc line-fitting program (Wallac, Turku, Finland).

The procedure was modified in the assay of fractionated samples. Sample or standard diluted in Tris buffer (100 μl) and Tris buffer (100 μl) were incubated directly with the Mab-coated 96-well plate without sample pretreatment. The remainder of the procedure remained unchanged. Three ELISAs were devised using Mab PO#14 or PO#23 or a combination of both Mabs (PO#14+23) as coating antibodies, alkaline phosphatase-linked R1 as label, and recombinant human (rh) 30K inhibin A reference preparation (WHO 91/624) as standard. The sensitivity, working range, and between-assay variation of the PO#14/R1 ELISA were 15–1000 ng/liter and 19% (n = 4); those of the PO#23/R1 ELISA were 8–1000 ng/liter and 6.5% (n = 4); and those of the PO#14+23/R1 ELISA were 6–1000 ng/liter and 7% (n = 4). The specificity of these assays has been presented previously (14) and showed cross-reactivity of less than 0.2% with activin A, 40–90% with pro-αC, and 320%, 253%, and 138% for human 30K inhibin B for the three ELISAs, respectively.

**Pro-αC ELISA**

The pro-αC ELISA (21) was employed using reagents (INPRO Mab as capture and R1 Mab as label) and standard preparation provided by Oxford Bio-Innovation Ltd. (Upper Heyford, UK). Samples obtained from the fractionation of serum (100 μl) or standard (100 μl) and Tris buffer (100 μl) were incubated directly with the Mab-coated 96-well plate without sample pretreatment. The remainder of the procedure remained unchanged. The sensitivity of the assay was 2 ng/liter. The within-assay variation and the between-assay variation were 7.5% (11 assays) and 12% (11 assays), respectively. In addition, a second pro-αC ELISA using INPRO Mab as capture antibody, PO#14 as label, and pro-αC standard as used above, was employed using the same assay design as that used for αC ELISAs. The sensitivity of the assay was 3 ng/liter, and the working range was 3–2000 ng/liter.

**Inhibin A ELISA**

The inhibin A ELISA of Groome and O’Brien (18) was used with modifications (22), using kit reagents provided by Oxford Bio-Innovation. Inhibin A (91/624) was used as standard. The assay procedure consisted of addition of sample or standard (100 μl), Tris buffer (100 μl), and 30% H₂O₂ (20 μl) to the Mab-coated microtiter plate (20); the remainder of the procedure remained unchanged. The sensitivity of the assay was 6 ng/liter.

**Inhibin B ELISA**

The inhibin B ELISA method (4) was employed using kit reagents and inhibin B standard provided by Oxford Bio-Innovation. Modifications to the assay as outlined in the inhibin A ELISA were included. The sensitivity of the assay was 6 ng/liter. The within-assay and between assay variations were 8.6% (11 assays) and 7.9% (11 assays), respectively.

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**Fig. 2.** Mol wt profiles of inhibins and pro-αC in serum from a woman with a granulosa cell tumor as determined by αC ELISA and inhibin B and pro-αC ELISAs. Horizontal dashed lines refer to the sensitivity of the respective assays.
Fractionation of serum and hFF inhibin

Serum samples (2–18 ml, depending on inhibin content) were fractionated according to their mol wt by a combined immunoaffinity, preparative PAGE, and electroelution procedure based on modifications (20) to previously described procedures (14–16). These studies (14–16) included details of the reproducibility of the fractionation procedure.

General statistical analyses

For statistical purposes the patient sera were divided into seven main groups: controls; serous, mucinous, endometrioid, clear cell, and undifferentiated carcinomas; and granulosa cell tumors. The cancers of nonovarian origin (breast and bowel) were considered separately. The data were normalized with log transformation as established previously (13).

Receiver operating characteristic (ROC) curves

The specificity and sensitivity of the various assays as diagnostic tests were assessed using ROC curve analysis (23). Sensitivity is defined as the proportion of cancer patients identified correctly by the cancer test, whereas specificity is the proportion of control patients detected correctly. The area under the ROC curves was used to compare the performance between any two or a combination of assays. Logistic regression was used to form linear combinations of the assay values that, after retransformation, optimally estimate the probability that a subject has cancer (24, 25).

Results

Inhibin forms in serum from women with ovarian cancer

Serum from normal postmenopausal women and postmenopausal women with granulosa cell tumors and mucinous and serous carcinomas were fractionated by a combined immunoaffinity, Prep-PAGE procedure (Figs. 2–4). The serous carcinoma sample was chosen for its detectable inhibin levels (Fig. 4).

The various mol wt forms of inhibins A, B, and pro-αC detected in the granulosa cell tumor samples were similar to those previously identified in serum and hFF from women undergoing gonadotropin treatment (Table 2) (14) and in normal women during early pregnancy (20). Mature (28–36K) forms of inhibin B and, to a lesser extent, inhibin A and pro-αC (25–35K) were identified, each consisting of two peak regions attributed to different glycosylated forms. In addition, high mol wt forms of inhibins A and B attributed to precursor dimeric forms were identified. The αC ELISAs (PO#14/R1 and PO#23/R1) showed some differences in specificity, with the PO#23/R1 ELISA more readily detecting the high mol wt forms as seen previously with this assay with hFF and serum from women stimulated with gonadotropins as part of an IVF program (IVF serum) (14). The free α-subunit, believed to be precursor and processed forms, was detected at low levels in postmenopausal serum, similar to that reported previously (16).

Fractionation of the mucinous and serous cancer serum samples showed predominantly the presence of the free α-subunit (Figs. 3 and 4 and Table 2). A comparison of the αC ELISAs showed that the PO#23/R1 ELISA more readily detected the high mol wt forms (Fig. 3). Little inhibin A and B was identified in any of these chromatograms.

As the two αC ELISAs appeared to show differing specificities, a further αC ELISA was devised (termed PO#14+23/R1 ELISA) whereby PO#14 and PO#23 antibodies were coated in equal amounts onto microtiter plates to form the capture antibody with #R1 Mab used as label. The resulting patterns of immunoactivity with the PO#14+23/R1 ELISA showed some averaging of the values obtained with the other two αC ELISAs (Figs. 2 and 3).

The R1 Mab was used as the common labeled antibody in the above αC ELISAs and inhibin A, B, and pro-αC ELISAs. To assess the specificity of an alternative α-subunit Mab, a pro-αC ELISA employing INPRO Mab as capture antibody and PO#14 Mab as label was formulated. When applied to the fractionated granulosa cell tumor serum sample (Fig. 2)

![Table 2: Apparent molecular weights (k) of inhibin forms identified in serum from normal postmenopausal women and postmenopausal women with ovarian cancers (Figs. 2–4)](image)

<table>
<thead>
<tr>
<th>Inhibin form</th>
<th>Assay</th>
<th>Human follicular fluid (pool)</th>
<th>IVF serum (pool)</th>
<th>Postmenopausal serum (n = 12)</th>
<th>Granulosa cell tumor 1 (n = 1)</th>
<th>Granulosa cell tumor 2 (n = 1)</th>
<th>Mucinous carcinoma (n = 5)</th>
<th>Serous carcinoma (n = 2)</th>
<th>Presumed inhibin forms</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>53–82</td>
<td>49–65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibin B</td>
<td>ELISA</td>
<td>27–37</td>
<td>28–42</td>
<td>ND</td>
<td>35–43</td>
<td>26–36</td>
<td>52–76</td>
<td>ND</td>
<td>αβB, αβB, high mol wt forms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68–74</td>
<td>49–65</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro-αC</td>
<td>ELISA</td>
<td>25–34</td>
<td>76–90</td>
<td>ND</td>
<td>70–82</td>
<td>24–33</td>
<td>28–37</td>
<td>22–30</td>
<td>Pro-αC, Pro-αC, high mol wt forms</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26–37</td>
<td>20–35</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Subunit</td>
<td>forms</td>
<td>14+23/R1 ELISA</td>
<td>26–40</td>
<td>60–75</td>
<td>26–39</td>
<td>26–36</td>
<td>28–37</td>
<td>22–30</td>
<td>high mol wt forms</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>23–33</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

Inhibin forms in human follicular fluid and serum (IVF serum) from women undergoing gonadotropin treatment as part of an IVF program are included as a reference (15). n. Number of individual serum samples in the sample for fractionation; ND, nondetectable levels.

* and **, Diglycosylated form.
and mucinous carcinoma serum pool (Fig. 3), a pattern similar to pro-αC ELISA with R1 Mab as label was observed, except for a high proportion detected of some higher mol wt forms. Similar findings were observed with the second granulosa cell tumor sample with this ELISA (data not shown).

Validation of αC ELISAs for measuring serum inhibin

Serial dilutions of IVF serum and hFF gave parallel responses with the inhibin A standard in all three αC ELISA systems. Examples of these dose-response curves for the PO#23/R1 ELISA are presented in Fig. 5. Additivity studies were undertaken whereby 5–10 pg inhibin A standard was added to normal postmenopausal serum samples with undetectable inhibin levels (23/R1 ELISA and 14+23/R1 ELISA), and the percent inhibin recovered was determined. Recoveries (mean ± sd) of 84.1 ± 11.3% (n = 27; 23/R1 ELISA) and 90.2 ± 11.8% (n = 10; 14+23/R1 ELISA) were obtained.

Application of αC ELISAs to ovarian cancer

The PO#14/R1, PO#23/R1, and PO#14+23/R1 ELISAs were applied to sera from normal postmenopausal women and postmenopausal women with ovarian cancers (Table 1), and the results were expressed as the geometric mean ± 2 sd. The data were grouped into a healthy control group and a series of ovarian cancer groups and breast and bowel cancer groups.

ROC curves for each ovarian cancer were established for
each αC ELISA, including the ovarian cancer marker, CA125 (for example, see Fig. 6), and the area under the respective curves was calculated (Table 3). A comparison among the three αC ELISAs for the all ovarian cancer group showed that the PO#23/R1 and PO#14+23/R1 αC ELISAs were more sensitive than the PO#14/R1 ELISA (P < 0.001, as assessed by ROC curve analysis), whereas no significant difference was observed between PO#23/R1 and PO#14+23/R1 αC ELISAs for the all ovarian cancer group.

The percentage of cancers detected at 95% specificity for each assay (including CA125) as derived from the ROC curves is presented in Table 4. Granulosa cell tumors were readily detected (100%) by all αC ELISAs, whereas 72–84% of mucinous cancers were detected by the αC ELISAs. The percentage of the all ovarian cancer group detected at 95% specificity by CA125 alone was 82% and that detected by αC ELISAs was 41–50%, with the highest percentage detected by the PO#14/R1 ELISA (50%). For the nonovarian cancers, the percentages of breast and bowel cancers detected at 95% specificity were 0% and 7%, respectively.

After initial surgery, serum inhibin levels in women with mucinous carcinomas fell markedly to reach the sensitivity of the αC ELISA (23-R1 ELISA; Table 5). A dramatic decline was also observed with granulosa cell tumors; however, as surgery was undertaken after recurrence of the disease, not all cases resulted in undetectable levels, suggesting the presence of residual disease. Serum FSH levels were elevated after surgery.

The percentage of mucinous cancers detected at 95% specificity for the αC ELISA (23-R1) was assessed by FIGO Stage (26) (Table 6). Although the limited numbers preclude detailed analysis, this cancer was detected by this ELISA in the early stages of the disease.

A combination of CA125 and each of the αC ELISAs as determined by ROC curve analysis in conjunction with a logistic regression technique resulted in a significant increase (P < 0.001) in the detection of mucinous cancers from 82% to 94%. Increases were also observed for the other ovarian cancer group (>89%) including the all ovarian cancer group. This relationship between CA125 and PO#23/R1 ELISA and their combination in differentiating between controls and ovarian cancers is presented as a scatterplot in Fig. 7. The diagonal line was derived by logistic regression and represents the sensitivity values at 95% specificity.

**Discussion**

The inhibin forms identified after the mol wt fractionation of serum from women with granulosa cell tumors and mucinous carcinomas were similar to those previously observed in serum from men and women and hFF (15, 16, 20). These forms include the two presumed glycosylated forms of the αC-subunit as seen with pro-αC and 30K inhibin forms as well as high mol wt forms consisting of partially processed or full-length precursor forms. Inhibin A and B forms could not be characterized in mucinous tumors in this study because of dilution during fractionation procedures and limitations in assay sensitivities.

Other studies have isolated inhibin forms with the N-terminal region (16 amino acids) of the αC region deleted (27, 28). Furthermore, a recent study examining inhibin forms in late pregnancy serum (20) showed evidence of inhibin and pro-αC forms of lower mol wt than those recognized for pro-αC and 30K inhibin, which was postulated to be due to sequence deletions in the αC region. It would appear from
TABLE 3. Area under receiver operating characteristic curves (± SE) for the major ovarian cancer groups between assays and combination of assays determined by logistic regression

<table>
<thead>
<tr>
<th>Cancer group</th>
<th>CA125</th>
<th>PO#14/R1 ELISA 29.0 units/liter</th>
<th>PO#23/R1 ELISA 42.7 ng/liter</th>
<th>PO#14 + 23/R1 ELISA 14.3 ng/liter</th>
<th>PO#14 + 23/R1 ELISA 36.7 ng/liter</th>
<th>CA125 + PO#14/R1 ELISA</th>
<th>CA125 + PO#23/R1 ELISA</th>
<th>CA125 + PO#14 + 23/R1 ELISA</th>
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</thead>
<tbody>
<tr>
<td>Serous</td>
<td>94</td>
<td>26.3 (4.2–165)</td>
<td>50.6 (5.06–505)</td>
<td>9.8 (2.0–47)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucinous</td>
<td>71</td>
<td>27 (9.3–2640)</td>
<td>9.8 (2.0–47)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulosa cell tumor</td>
<td>30</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Endometrioid</td>
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<td>54</td>
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<td>Clear cell</td>
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<td>33</td>
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<td></td>
</tr>
<tr>
<td>All ovarian cancers</td>
<td>82</td>
<td>41</td>
<td>50</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

*The corresponding analyte concentrations at 95% specificity are included.

TABLE 4. Percentage of cancers detected at 95% specificity for the various assays and combination of assays for linear combinations formed from logistic regression

<table>
<thead>
<tr>
<th>Cancer group</th>
<th>CA125</th>
<th>PO#14/R1 ELISA 29.0 units/liter</th>
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<th>PO#14 + 23/R1 ELISA 14.3 ng/liter</th>
<th>PO#14 + 23/R1 ELISA 36.7 ng/liter</th>
<th>CA125 + PO#14/R1 ELISA</th>
<th>CA125 + PO#23/R1 ELISA</th>
<th>CA125 + PO#14 + 23/R1 ELISA</th>
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<tr>
<td>Serous</td>
<td>94</td>
<td>26.3 (4.2–165)</td>
<td>50.6 (5.06–505)</td>
<td>9.8 (2.0–47)</td>
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<tr>
<td>Mucinous</td>
<td>71</td>
<td>27 (9.3–2640)</td>
<td>9.8 (2.0–47)</td>
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<td></td>
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<tr>
<td>Granulosa cell tumor</td>
<td>30</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Endometrioid</td>
<td>91</td>
<td>31</td>
<td>54</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clear cell</td>
<td>75</td>
<td>22</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>100</td>
<td>50</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All ovarian cancers</td>
<td>82</td>
<td>41</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a vs. b P = 0.01.

TABLE 5. Serum FSH and inhibin levels in women with mucinous carcinomas and granulosa cell tumors (GCT) before and after surgery

<table>
<thead>
<tr>
<th>Cancer group</th>
<th>n</th>
<th>Presurgery (IU/liter)</th>
<th>Postsurgery (ng/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucinous carcinoma</td>
<td>13</td>
<td>26.3 (4.2–165)</td>
<td>50.6 (5.06–505)</td>
</tr>
<tr>
<td>Inhibin (PO#23/R1 ELISA)</td>
<td>14</td>
<td>157 (9.3–2640)</td>
<td>9.8 (2.0–47)</td>
</tr>
<tr>
<td>GCT</td>
<td>4</td>
<td>0.40 (0.25–318)</td>
<td>48 (1.2–1890)</td>
</tr>
</tbody>
</table>

For mucinous tumors, presurgery refers to initial surgery. For GCT, presurgery refers to surgery following recurrence of the disease. Geometric mean ± 2 SD are presented.

a 13 of 14 and b 2 of 5 cases were below the sensitivity of the αC ELISA (8 ng/liter).

the present studies that there is little evidence of these small mol wt forms in ovarian cancer serum, as similar ELISAs were used in both the pregnancy and the present studies. Evidence for the presence of N-terminally truncated αC forms in ovarian cancer sera is less clear. The R1 Mab is the common Mab for all of these ELISAs (including inhibin A, B, and pro-αC ELISAs), and this Mab detects the N-terminal segment of the αC region, which presumably would be deleted in these truncated forms. Preliminary data using either the PO#14 (Figs. 3 and 4) or #23 Mabs (data not shown) instead of R1 as one of the Mabs in an ELISA format (e.g. pro-αC ELISA) did not provide evidence for the presence of smaller inhibin forms not detected by the R1-based ELISAs. Of interest, higher mol wt pro-αC forms (pro-αN-αC) were observed that were not readily detected by the R1 Mab-based pro-αC ELISA. Similar observations were previously observed with hFF and IVF serum (14).

Overall, these studies show that the inhibin forms identified in serum from women with ovarian cancers are similar to those seen in healthy subjects.

A comparison of mol wt profiles among the various αC ELISAs showed that PO#23/R1 ELISA more readily detected high mol wt forms of inhibin compared with the PO#14/R1 ELISA. In addition, quantitatively, inhibin levels showed large differences between assays, suggesting that the two ELISAs are differentially detecting the 30K inhibin A standard compared with the samples under assay. Until these high mol wt forms are isolated in a pure form, and thus a reliable mass determination can be made, quantitation of the various mol wt forms in these assays is open to doubt, as it is unclear whether the high mol wt forms are overestimated in the PO#23/R1 ELISA or underestimated in the PO#14/R1 ELISA.

To offset these differences in specificity between ELISA formats, an ELISA was devised in which the PO#14 and #23 Mabs were combined as coating antibody, and R1 Mab was used as labeled antibody (termed PO#14+23/R1 ELISA). The resulting ELISA was more sensitive (6 ng/liter) than the other αC ELISAs (8–15 ng/liter). When applied to the fractionated ovarian cancer samples, an averaging (to some degree) of the results of the PO#14/R1 and PO#23/R1 ELISAs was observed. This ELISA (PO#14+23/R1) was thus thought to have the broadest specificity in the detection of ovarian cancers and thus the preferred assay.

To test this possibility, the αC ELISAs were applied to a range of ovarian cancer serum samples and compared with data published previously using the inhibin RIA and the αC IFMA (7, 13). Similar responses were seen among the various
Inhibin assays based on the similarity in area under the ROC curves for the number of cancer groups. Determination of the area under the ROC curves for the three \( \alpha \)C ELISAs showed that the PO\#23/R1 and PO\#14+23/R1 ELISAs were significantly different from the PO\#14/R1 ELISA for the mucinous cancer group, although no differences were seen among the other \( \alpha \)C ELISAs. These results suggest that as there were no significant differences between PO\#23/R1 and PO\#14+23/R1 ELISAs, PO\#23/R1 was the preferred ELISA based on its simplicity for use in the detection and monitoring of ovarian cancer.

When the numbers of ovarian cancers detected at 95% specificity between cancer groups were compared, it was clear that inhibin as a marker shows a high sensitivity for granulosa cell tumors and mucinous cancers, whereas CA125 had a high sensitivity for the other cancers. Thus, the combination of the two markers should result in an enhanced detection for a combination of all ovarian cancers. It is noted that at 95% specificity, CA125 assay and \( \alpha \)C ELISAs individually detected 84% and 50%, respectively, for the all ovarian cancer group, whereas the combination of CA125 and \( \alpha \)C ELISAs resulted in 95% of all cancers detected. These data are comparable with previous comparisons between CA125 and \( \alpha \)C IFMAs (13). Nonovarian cancers (breast and bowel) were poorly discriminated.

In the previous study (13), the combination of CA125 and \( \alpha \)C IFMA data were assessed either using a MultiROC analysis (29) or a canonical variant analysis (CVA) (30). The researchers found the methods to be similar, but noted a number of advantages of CVA over MultiROC. Logistic regression also possesses those advantages compared with MultiROC. Many papers in the statistical literature have compared discriminant analysis to logistic regression (30). Discriminant analysis is a method for classifying subjects into one of two groups. In that sense discriminant analysis is similar to CVA, but discriminant analysis makes additional assumptions, such as the data must be normally distributed, and the variance structure must be homogeneous. Discriminant analysis and logistic regression produce similar results in terms of separating groups of subjects if the data are normally distributed and variances homogeneous. However, logistic regression is superior if the data are nonnormal, and variances are heterogeneous. Thus, logistic regression has, over the last 20 yr, become the standard method of analysis for binary data, including the classification of subjects into one of two groups. Logistic regression possesses inferential advantages compared with CVA. The serum inhibin data as determined by the inhibin \( \alpha \)C ELISA (13) were nonnormal given the preponderance of tied data for controls. Further the areas under the curve for the logistic regression assay combinations were slightly better than the areas under the curve for the CVA combinations. Therefore, the results of the logistic regression have been reported in this study.

Serum inhibin levels after surgery of mucinous tumors show a marked decline to very low or undetectable levels. After surgery in women with recurrent granulosa cell tumors, inhibin levels also decreased markedly in most cases; however, the decline in some cases was less marked apparently suggestive of incomplete removal of the cancer. Previous studies with an inhibin RIA (6, 12) have shown a rapid decline in inhibin levels after initial and recurrent surgery.

One of the objectives of this assay was to provide a test that would assist in the early diagnosis of ovarian cancer. The inhibin ELISA can detect early stages of the disease for mucinous tumors, but it is unclear whether the proportion of cancers detected is different from that at latter stages. A larger size analysis would need to be undertaken to clarify this question.

It is of interest to note that Mab PO\#23 detected the same region of the \( \alpha \)C region as previously observed (14) with the inhibin RIA using #1989 As (31), although conflicting results have been reported (32). Thus, it is not too surprising that there is a similarity in specificity when comparing the ovarian cancer data obtained with the inhibin RIA (8) with those obtained with the PO\#23/R1 ELISA.

In summary, these studies showed that serum inhibins in women with ovarian cancer are structurally similar in terms of mol wt to known inhibin forms in serum from healthy women. An ELISA using Mabs directed to two regions of the \( \alpha \)C fragment provides a suitable inhibin assay for detecting granulosa cell tumors and mucinous carcinomas, and in con-
junction with CA125 resulted in increased sensitivity/spec-
ificity indexes of clinical relevance.

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