A time-resolved immunofluorometric assay (IFMA) is described for quantifying the ovarian carcinoma-associated antigenic determinant CA 125 in human serum. Monoclonal antibody to CA 125 is immobilized onto a microtiter well, and the same antibody labeled with a europium chelate is used as a tracer. After the immunoreaction the bound portion of the labeled antibody is quantified by dissociating the Eu³⁺ in a fluorescence-enhancement solution and measuring its fluorescence with a time-resolved fluorometer. The detection limit of the IFMA is 1.5 arb. units/mL, being about the same as that of a commercially available immunoradiometric assay (IRMA) for CA 125 (1.4 arb. units/mL). The analytical range of the IFMA extends to 2000 arb. units/mL, whereas the range of the IRMA is 500 arb. units/mL. For 29 serum samples from ovarian-cancer patients measured simultaneously in the IFMA and IRMA, orthogonal regression analysis gave the equation CA 125 (IFMA) = 0.9937 CA 125 (IRMA) – 1.211 arb. units/mL ($S_x = 6.8681, r = 0.9932$). Apparently, the IFMA for CA 125 is a convenient alternative to the IRMA for CA 125 because of short counting times, the use of nonradioactive, stable reagents, and the much-extended measuring range. Additionally, the microtiter format should lend itself to more fully automated procedures in laboratories doing many such analyses.

Additional Keyphrases: cancer - immunoradiometric assay compared

Monoclonal antibodies generated against antigens associated with a broad range of malignancies (1) have been used to develop serodiagnostic immunoradiometric assays (IRMAs) for gastrointestinal carcinoma (carbohydrate antigen CA 19-9) (2), for breast carcinoma (cancer antigen CA 15-3) (3), for cervical squamous-cell carcinoma (4), and for ovarian carcinoma (cancer antigen CA 125) (5, 6).

The specificity and the sensitivity of the CA 125 IRMA have sufficed to support its evaluation in several clinical applications for ovarian-cancer patients, including detection of disease prior to second-look surgery (7, 8), monitoring patients' response to therapy (9), differential diagnosis (10), and even early detection of ovarian carcinoma (11). The consensus has been that increased concentrations of CA 125 in serum are a reliable indicator of the presence of disease.

Such concentrations have been measured in most cases with the one-step IRMA (5) in which ¹²⁵I-labeled antibody to CA 125 is used as a tracer. An enzyme immunoenzyme for CA 125 determinations has also been developed by Abbott Laboratories, Chicago, IL, in which anti-CA 125–horseradish peroxidase (EC 1.11.1.7) conjugate is a tracer.

Here we take advantage of recent developments indicating the widespread applicability in immunoenzyme assays of a europium chelate; the time-resolved fluorescence signal can be measured with high accuracy and low background (12, 13). We describe a two-step immunofluorometric assay (IFMA) for the quantitative determination of CA 125 concentrations in serum. The use of dissociation-enhanced lanthamide fluorescence provides an assay with sensitivity equivalent to the already available IRMA, with no need for radioactive reagents.

Materials and Methods

Labeling the monoclonal antibody. The anti-CA 125 monoclonal antibody (OC 125) was purified by affinity chromatography on Protein A-Sepharose (Pharmacia, Uppsala, Sweden) (14). OC 125 was labeled with Eu³⁺-N-(p-isothiocyanatobenzyl)diethylenetriamine-N,N,N',N'-tetracacetate, resulting in the incorporation of 77 Eu atoms per antibody molecule, as described by Hemmilä et al. (12).

Sera and standards. Serum samples from 30 ovarian-cancer patients were included in the present study. As a reference we used the standards from a commercially available IRMA kit (Centocor, Malvern, PA). These standards, in a matrix of calcium-supplemented human plasma, contain respectively 7.7, 30, 80, 200, and 500 arb. units of CA 125 per milliliter. (The units in which the CA 125 concentrations are expressed are not internationally standardized.) As a diluent for patients' sera and standards, we used fetal calf serum (batch no. 116139; Flow Laboratories, Herts, U.K.).

Preparation of ovarian carcinoma extract. We prepared an extract of a human ovarian carcinoma tissue by homogenizing 1.0 g of a serous cystadenocarcinoma in 20 mL of 1.0 mmol/L phosphate-buffered (pH 7.4) saline containing 137 mmol of NaCl per liter in a glass homogenizer with a Teflon pestle. After centrifuging the homogenate (9000 x g, 20 min, 4 °C), we stored the supernate in small aliquots at –70 °C.

Coating of the microtitration strips. Monoclonal antibody OC 125 was adsorbed to the walls of polyethylene microtitration wells (Eflab Oy, Helsinki, Finland) by incubating overnight at room temperature 200 µL of purified antibody (0.1–5.0 mg/L) in sodium bicarbonate buffer (50 mmol/L, pH 9.6) per well. After the incubation we washed the strips, saturated them overnight with 250 µL of bovine serum albumin (Sigma Chemical Co., St. Louis, MO) solution—5.0 g of albumin, 50 mmol of Tris (pH 7.75), 154 mmol of NaCl, and 7.5 mmol of Na₂CO₃ per liter—and stored them in a humidified atmosphere at 4 °C.

Immunofluorometric assay. Pipet 100 µL of CA 125 standard or serum sample and 100 µL of assay buffer (the
albumin solution above, plus 0.2 mL of Tween 20 surfactant per liter) into the coated microtiter wells. After incubation overnight at room temperature, aspirate the reaction mixture and wash the wells four times with wash solution (0.5 mL of Tween 20 surfactant and 7.5 mmol of NaNO₃ per liter of isotonic saline), using a 12-channel washer (LKB-Wallac Oy, Turku, Finland). In the second step, add to each well 210 μL of Eu-labeled OC 125 antibody (diluted to 100 μg/L in assay buffer). After incubating the strips for 1.5 h at room temperature, aspirate the contents of the wells and wash the plates seven times with wash solution. Finally, dispense into each well 200 μL of enhancement solution (containing 1.0 g of Triton X-100 surfactant, 6.8 mmol of potassium hydrogen phthalate, 100 mmol of acetic acid, 50 μmol of tri-n-octyl-phosphineoxide, and 15 μmol of 2-naphthoyltrifluoroacetone per liter). Shake the strips for 5 min on a microshaker (Dynatech, Zug, Switzerland), and after another 10 min measure the fluorescence in a 1230 Arcus time-resolved fluorometer (LKB-Wallac Oy) using a 1-s counting time and a 400-μs time delay (13).

**Immunoradiometric assay.** The simultaneous (one-step) immunoradiometric assay was performed as described by Klug et al. (5). Reagents, buffers, and standards from the Centocor CA 125 IRMA kit were used. The kit contained 125I-labeled anti-CA 125 (specific activity approximately 10 Ci/mg), standards, and anti-CA 125 coated polystyrene beads. 125I-labeled OC 125 (100 μL, 1 x 10⁶ cpm) was incubated (20 h, room temperature) with polystyrene-immobilized OC 125 and sample (100 μL). The beads were washed three times and their radioactivity was counted in a gamma scintillation counter.

**Results**

**Standardization.** We tested the suitability of various CA 125-containing preparations as standards by using the standards from the CA 125 Centocor IRMA kit as a reference. We determined analytical recoveries from a human serum sample with a very high CA 125 concentration as well as from an extract of human serous ovarian carcinoma tissue in saline, using fetal calf serum as a diluent. The curves for the serum and the tissue-extract dilution series paralleled the Centocor standard curve (Figure 1). In subsequent determinations we therefore used the ovarian carcinoma tissue extract diluted in fetal calf serum as a working CA 125 standard.

**Assay optimization.** To determine the effect of the amount of immobilized OC 125 antibody (solid phase) on the assay characteristics, we coated strips with 50 mmol/L sodium bicarbonate buffer (pH 9.6) containing amounts of OC 125 ranging between 0.1 and 5.0 mg/L per liter. Each strip was incubated overnight at room temperature with a series of tissue-extract standards. The assay was then completed according to the standard protocol described above. Each coating concentration gave a different standard curve (Figure 2). The optimal standard curve was reached at an antibody concentration of 0.5 mg/L in the sodium bicarbonate buffer. Use of lower coating concentrations decreased the measuring range of the assay, whereas use of antibody concentrations >0.5 mg/L decreased the sensitivity of the assay.

In contrast, variation of the amount of Eu-labeled antibody negligibly affected the standard curve (data not shown). Therefore, we used an arbitrary concentration of 20 ng of Eu-labeled OC 125 per well in further testing.

**Assay sensitivity.** The lower detection limit of the IFMA was 1.5 arb. units/mL, as determined by calculating the mean plus 3 SD for 10 replicates of the zero-dose standard prepared from assay buffer or fetal calf serum.

**Dynamic range.** The apparent working range of the IFMA was determined by assaying a series of CA 125 tissue-extract standards covering the concentration range from 0 to 7200 arb. units/mL. Fluorescence intensity was plotted vs the concentration of standard CA 125 (Figure 3). Above
2000 arb. units/mL we saw no further proportional increase of fluorescence signal. Thus the working range of the IFMA ranges from 1.5 to 2000 arb. units/mL.

**Precision.** Intra-assay precision was determined by assaying fivefold replicates of five different human sera with various amounts of CA 125 in the same run (Table 1 and Figure 3). Inter-assay precision was estimated by determining, in duplicate, CA 125 in three serum samples on five different occasions. Table 1 summarizes the results of these determinations.

**Correlation with immunoradiometric assay.** We assayed, in duplicate, the CA 125 in serum samples from 30 patients, by both the present method (IFMA) and the IRMA. Orthogonal regression analysis of these data gave the linear equation CA 125 (IFMA) = 0.9937 CA 125 (IRMA) - 1.1211 arb. units/mL, with a standard error of estimate ($S_y$) of 6.8681 arb. units/mL and a Spearman rank correlation coefficient ($r$) of 0.9932.

**Discussion**

Radioimmunoassays are valuable tools in the clinical laboratory, but they have their own set of limitations. The limited maximum radioactivity possible for labeled haptons or proteins may have an important effect on the specifications of a particular assay in terms of its sensitivity, specificity, breadth of the analytical range, etc. Furthermore, the shelf-life of a $^{125}$I-labeled protein, which depends on radiation damage, will be shortened as increasing numbers of $^{125}$I atoms are incorporated into the protein, and it is generally limited to six to 10 weeks. Together with radiation hazards and the special requirements for laboratories, these limitations have stimulated the investigation of alternative labels in immunoassay methodology. In the case of the CA 125 assay, Abbott Laboratories have recently made available an enzyme immunoassay based on the "sandwich" principle. In our laboratory a method-comparison analysis has shown that it is less sensitive than the Centocor IRMA (7.5 arb.units/mL vs 1.4 arb.units/mL, respectively). As an alternative, the present report deals with the development of an immunoassay for CA 125 based on the DELFIA™ principle with the same sensitivity as the IRMA (5).

Our results indicate that high sensitivity can be achieved by optimizing the amount of immobilized OC 125 antibody on the solid phase. If coating concentrations exceed 0.5 mg of OC 125 per liter, the sensitivity of the assays is remarkably decreased. Perhaps at higher coating concentrations all the available epitopes present on the CA 125 antigen are captured or masked by the solid-phase antibody, which would limit the numbers of these CA 125 determinants available to bind the Eu-labeled antibody.

The wider measuring range of the IFMA offers obvious important practical advantages. This advantage is probably principally ascribable to the assay design: the IRMA is performed as an one-step assay (5), whereas the IFMA follows a two-step procedure. This leads to a broader measuring range and avoidance of the high-dose "hook" effect (15) characteristic of one-step assays. The sensitivity of the IFMA (1.5 arb. units/mL) does not differ significantly from that of the one-step IRMA (1.4 arb. units/mL) as reported by Klug et al. (5). With use of Eu-labeled antibodies, increased sensitivity has been reported for the immunoassays for epidermal growth factor (16), insulin (17), and choriogonadotropin (18). Especially the IFMA for choriogonadotropin has been performed under exactly similar conditions as the IRMA, and the authors showed the IFMA to be more sensitive than the IRMA because of a higher specific activity of the tracer molecule. Regarding the similar detection limits of the CA 125 IFMA and IRMA, the gain in sensitivity by applying Eu-labeled antibodies may be counteracted by the introduction of a two-step instead of a one-step assay design, leading to a decreased assay sensitivity. This is in line with Klug et al. (5), who showed that the IRMA was less sensitive in its two-step configuration.

The parallelism between the curves of the IRMA standards and the dilutions of the ovarian carcinoma extract or the human serum sample shown in Figure 1 demonstrated that the CA 125 antigen in serum and the CA 125 antigen extracted from tissue behave in an immunochromically similar fashion in the IFMA. This suggests that the epitopes of the CA 125 "tissue antigen" remain well preserved upon release into the circulation. Recently, David et al. (19) reported that CA 125 antigen isolated from the culture supernate of an ovarian carcinoma cell line was biochemically indistinguishable from CA 125 antigen in human serum, also supporting the notion that the number and immunochemical nature of the CA 125 epitopes remain relatively intact in antigen derived from different sources.

In conclusion, the use of Eu-labeled instead of $^{125}$I-labeled OC 125 allows construction of an immunoassay with an extended shelf life and reduced analysis time with no loss of sensitivity and an extended analytical range. It will be of
interest to test this assay on a more extensive set of clinical specimens to see if the IRMA matches the IRMA in its ability to monitor regression and progression of disease (6) and identify patients with residual disease after first-line therapy (7, 8).

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References

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