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Estrogen and Progestin Receptor Assay in Fine Needle Aspirates of Breast Cancer: Methodological Aspects

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Abstract—*A methodology, originally built up in our laboratory, for the simultaneous determination of estrogen (ER) and progesterone (PR) receptors on small samples of tumor tissues* [1], *has been adapted to fine needle aspirates (FNA) of breast tumors. The method is based on a simultaneous incubation of aliquots of high-salt* (0.4 M KCl) *cytosols with tritiated estrogen and progestin tags. The mixture of receptor-bound ligands is isolated by dextran-coated charcoal and extracted by ethanol. The extracted ligands are separated quantitatively by HPLC and counted by liquid scintillation.*

FNA provides sufficient cellular material for ER and/or PR assay by single point (5 nM) *dextran-coated charcoal (DCC) assay. FNA samples, being contaminated by blood,* [^{3}H] *R* 2858 *and* [^{3}H] *O* 2058 *are appropriate tags for ER and PR respectively, giving lowest non-specific binding.*

Sixty-five simultaneous estrogen and progestin receptor determinations on FNA at time of diagnosis were compared to the individual assays performed on the surgical sample obtained from the same patients a few weeks after diagnosis. The correlation between the 2 *sets of determinations is excellent in* 60 *cases with sufficient FNA cellularity. This correlation validates the reliability of estrogen and progestin receptor determination on FNA in the majority of clinical cases.*

INTRODUCTION

THERE is a large consensus that the status of estrogen and progesterone receptors should be determined in every case of accessible breast tumor since these two biological parameters have been found essential for predicting response to hormonal therapy [2–5] and as independent prognosis factors in primary as well as metastatic breast cancer [6–10].

Usually, estrogen and progestin receptors are measured on substantial surgical samples. This, of course, supposes surgical treatment of the tumor. There are, however, a number of circumstances where a substantial surgical sample is not available for assay, i.e. when the tumor is not operable, or when a conservative treatment is indicated (radiotherapy or chemotherapy). It is also the case for small tumors, biopsies, as well as material from cell or tissue culture.

Moreover, the optimization of therapeutical protocols associating various modalities of chemo- and hormonal therapy [11, 12] raises many questions related to the modulation of hormone receptors by the therapeutical agents. These questions can only be answered by sequential assays of hormone receptors on small biopsy specimens obtained during the course of treatment with no harm to the patient. In all these cases where surgery is not clinically indicated, one needs a simple and atraumatic means of collecting sufficient cellular material from the tumor for a reliable assay of estrogen and progestin receptors.

In the present work, we propose a methodology which allows the simultaneous determination of estrogen and progestin receptors on the cellular material harvested by fine needle puncture (with or without aspiration) of breast tumors. The method is validated by a comparison, in 65 patients, of the results of estrogen and progestin receptor values assayed, first, simultaneously on a fine needle aspirate at time of diagnosis, then, classically on a surgical sample obtained at time of surgery.

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Abbreviations used—ER, estrogen receptor; PR, progesterone receptor; E_2, estradiol; DCC, dextran-coated charcoal; HPLC, high performance liquid chromatography; FNA, fine needle aspiration.

MATERIALS AND METHODS

Steroids

[^{3}H] R 2858 (87Ci mmol^{-1}) and R 2858 (17-ethynyl-11-methoxy estradiol), [^{3}H] R 5020 (87 Ci m-mol) and R 5020 (17,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione), were purchased from NEN (Boston, Mass.). [^{3}H] O 2058 (40–50 Ci m-mol), and O 2058 (16 ethyl-21-hydroxy-19-nor-pregna-3,20-dione) were purchased from Amersham (Amersham, England).

Buffer salts

Buffer salts were from Merck (Darmstadt, Germany), dithiothreitol and sodium molybdate from Sigma (St Louis, Miss.). Absolute ethanol was from Prolabo (France) and fluorescence grade methanol from Baker (Holland).

Homogenization buffer

Phosphate 10 mM, glycerol 10% (w/v), sodium molybdate 10 mM, KCl 0.4 M, dithiothreitol 0.5 mM; pH 7.6 at 0°C.

Dextran-coated charcoal suspension

1.25% Norit A (Fluka, Switzerland) + 0.625% Dextran T 70 (Fluka) adjusted to desired volume with homogenization buffer containing 1% gelatine (Cooper, Penn.).

Fine needle aspiration

Fine needle aspiration was performed either by aspiration with a 0.6 or 0.7 mm needle (22–23 gauge) fixed to a 10 ml disposable syringe mounted on a Cameco Pistol, or simply by puncture with the bare needle, without aspiration. The cellular material was rinsed out of the needle in 0.5 ml phosphate buffer and immediately frozen in liquid nitrogen.

Preparation of cytosols

The surgical samples, weighing 200 mg or more, were homogenized with a Polytron PT7 homogenizer (Brinkman, Switzerland) in 6–10 vol. of homogenizing buffer in order to obtain a protein concentration between 2 and 4 mg/ml. Homogenates were centrifuged 1 hr at 105,000 ***g***, at 0° C. The supernatant (cytosol) was pipetted and processed for separate ER and PR assays. The pellet was processed for DNA assay by fluorimetry [13]. Standard single concentration DCC assays were used for surgical samples, as described elsewhere [14]. Briefly, 100 µl aliquots of cytosol were incubated 16 hr at 0° C, with 5 nM [^{3}H] R 2858 or 5 nM [^{3}H] R 5020 in the presence of 500 nM cortisol, in the absence or in the presence of 100-fold excess of the corresponding unlabeled tag. Hormone receptor complexes were isolated after treatment of the incubates with 120 µl of dextran-coated charcoal suspension (as below). One hundred and fifty microliters of charcoal-treated supernatant were counted for radioactivity after addition of 3.5 ml of Econofluor (NEN, Boston, Mass.) and overnight extraction.

The fine needle aspirates in buffer were thawed to 0°C and homogenized without transfer by 4–8 sec sonication with a 3 mm probe, at minimal setting of a Branson B15 Sonifier (Branson, U.S.A.). The homogenate was carefully transferred to a thick-walled 4.5 ml polycarbonate tube (Beckman, U.S.A.), weighed to accurately determine its volume, and centrifuged 1 hr at 105,000 ***g***, at 0°C. The supernatant was processed for the simultaneous assay of ER and PR, and the pellet was used for DNA assay by fluorimetry [13].

Simultaneous assay of ER and PR

Four 100 µl aliquots of the 105,000 ***g*** supernatant were incubated 20 hr at 0°C, with a *mixture* of 5 nM [^{3}H] R 2858 and 5 nM [^{3}H] O 2058 in the presence of 500 nM cortisol.Two of these aliquots were incubated in the presence of a 100-fold excess of the unlabeled tags, to measure non-specific binding.

Unbound ligands were separated by the addition of 120 µl dextran-coated charcoal suspension, agitation for 10 min and centrifugation at 800 ***g*** for 10 min.

An aliquot of 70 µl of each supernatant was measured by scintillation in order to determine those samples which contain at least 1 receptor, to avoid processing of negative samples and to calculate later for positive samples the recovery yield of HPLC separation. One hundred µl of 1% trichloracetic acid in ethanol were added to 130 µl aliquots of DCC supernatant to extract bound ligands and precipitate proteins. One hundred µl aliquots of the ethanolic extract were injected in a high-pressure liquid chromatograph (HPLC) system (Laboratory Data Control, U.S.A.) and the radioactive ligands were collected separately for scintillation counting.

High performance liquid chromatography (HPLC)

One hundred µl of the ethanolic extract were injected by means of an automatic HPLC injector Wisp (Waters, U.S.A.). The separating column (4 mm × 250 mm) was filled with 5 µm reverse phase C 18 beads (Spherisorb, U.S.A.). Elution was isocratic (methanol:water, 85:15, v:v) at 1 ml/min. Retention times were: E_2: 5 min, R 2858: 3.8 min, progesterone: 8.2 min, R 5020: 8.2 min, O 2058: 6 min, R 27987: 5.5 min.

Eluates were collected by a microprocessor-controlled fraction collector (Gilson, France) synchronized with the injector and the retention times of unlabeled R 2858 and O 2058 aliquots (3 nmol) injected every 12 samples.

Table 1. *Cell type content of* 20 *FNA samples*

	Malignant cells (%)	Polynuclear (%)	Lymphocytes (%)	Stromal cells (%)
Mean ± S.D.	93 ± 7	3.6 ± 4.7	2.9 ± 3.5	0
Range	74–99	0–18	0–13.5	0–3

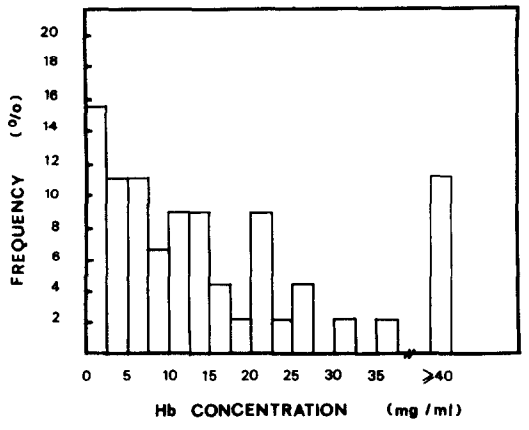

Fig. 1. *Frequency distribution of blood contamination in* 53 *FNA samples.*

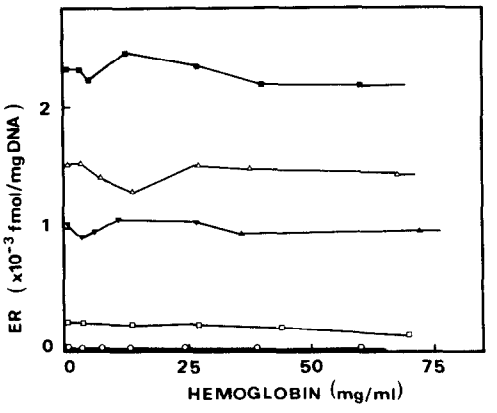

Fig. 2. *Influence of blood contamination on ER assay with* [^{3}H] *R* 2858. *The different curves relate to cytosols containing various levels of ER, to which increasing amounts of blood (hemoglobin,* mg/ml) *were added.*

Collected fractions were evaporated to dryness at 40°C under vacuum. Radioactivity was counted by liquid scintillation after addition of 3.5 ml of Econofluor.

RESULTS

Cellular content of DNA

FNA samples from 20 primary breast cancer were cytologically analyzed, considering the relative proportion of stromal cells. As indicated in Table 1, FNA are rich in tumor cells, moderately contaminated by peripheral blood cells and mostly devoid of stromal cells.

Blood contamination

Figure 1 shows the frequency of blood contamination in 53 FNA expressed by the concentration of hemoglobin in the cytosol. Thirty-six per cent of these samples had a concentration of hemoglobin equal to or above 15 mg/ml (about 10% blood contamination).

Estrogen receptor assay and blood contamination

Known amounts of blood were added to non-hemorrhagic surgical samples containing various levels of ER. The dextran-coated charcoal assay of estrogen receptors, with [^{3}H] R 2858 as the ER tag, was not significantly altered by blood contamination up to 50% contamination (70 mg Hb/ml) as shown in Fig. 2.

Progestin receptor assay and blood contamination

[^{3}H] R 5020 and [^{3}H] O 2058 were compared

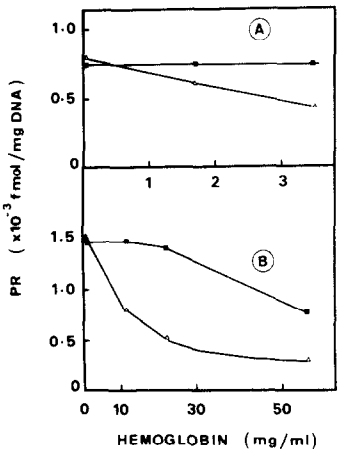

Fig. 3. *Influence of blood contamination on PR assay with* [^{3}H] *R* 5020 (△) *or* [^{3}H] *O* 2058 (■). *A: low concentrations of whole blood were added to a cytosol containing* ca. 750 fmol *PR/*mg *DNA. B: high concentrations of whole blood were added to a cytosol containing* ca. 1500 fmol *PR/*mg *DNA.*

for their capacity of accurately measuring specific progestin binding sites in the presence of blood contamination. As shown in Fig. 3, [^{3}H] R 5020 yields highly irreproducible and underestimated progestin receptor values, even at low hemoglobin concentration (10 mg/ml). In contrast [^{3}H] O 2058 yielded almost constant progestin receptor values from 0 to 30 mg Hb/ml contamination. The inaccuracy of [^{3}H] R 5020 was the consequence of an unacceptably high non-specific binding, due to an unidentified component of red blood cell lysates (data not shown). As a consequence, [^{3}H] O 2058, in the presence of cortisol (500 nM), was chosen as the progestin receptor tag in FNA.

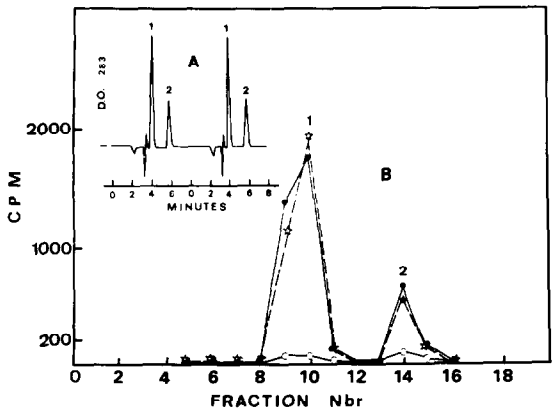

Fig. 4. HPLC elution profiles of R 2858 (1) and O 2058 (2). A (insert): 2 successive injections of the mixture of 3 nmol of unlabeled R 2858 (1) and 3 nmol of unlabeled O 2058 (2). B: elution profile of [^{3}H] R 2858 (1) and [^{3}H] O 2058 from an FNA sample processed for simultaneous DCC–HPLC assay of ER and PR; • and ⋆: total binding; ○: non-specific binding.

Assay of estrogen or progestin receptor in FNA

Dextran-coated charcoal assays of either ER or PR were therefore undertaken in a large series of FNA samples from patients not treated by surgery. Results from these series have already been published [15, 16]. As additional information, we could show that (a) the distribution of ER and PR levels in different subgroups of FNAs according to the level of blood contamination (5 subgroups from 0 to 25 mg hemoglobin/ml, 178 FNAs for each receptor) were identical (data not shown); (b) there was no correlation between the level of either ER (129 samples) or PR (330 samples) and the cellularity of FNA expressed as DNA/per ml cytosol. The correlation coefficients were 0.046 for ER and 0.009 for PR, respectively.

Simultaneous assay of estrogen and progestin receptor on FNA

The simultaneous assay of estrogen and progestin receptor was practised on FNA samples as described in Materials and Methods. The synchronization of the automatic injector and the programmed fraction collector was based on the reproducibility of the elution profile of unlabeled R 2858 and O 2058 (3 nmol of each steroid) as detected by u.v. absorbance at 283 nm (Fig. 4, insert). This standard mixture was injected every 12 FNA samples to control any shift in retention times. For each processed FNA sample separated by HPLC, each radioactive peak was collected in 3 fractions of 0.5 ml. The recovery of the radioactive tags injected in the chromatography column was quantitative (100 + 10% due to statistical variations in scintillation counting). A typical elution profile is shown in Fig. 4.

Simultaneous Scatchard plots on FNA sample

Scatchard plots could be derived from 4 FNA samples with only 3 incubation concentrations (1.2 and 5 nM of each labeled steroid), evidencing a unique dissociation constant, K_d, of 5×10^{-10} M for the estrogen receptors and 2 sets of K_d values for the corresponding progestin receptors: 2 samples with K_d around 2×10^{-9} M and 2 samples with K_d around 5×10^{-10} M (Fig. 5.)

In one case Scatchard plots were derived from an FNA sample as above and from the corresponding sample obtained at surgery. Both samples showed no [^{3}H] O 2058 saturation, and Scatchard plots with similar affinity constants for estrogen receptors (Fig. 6).

Comparison between simultaneous estrogen and progestin receptor assays in FNA and in the corresponding surgical samples

As described in Materials and Methods, estrogen and progestin receptors were determined in 65 FNA samples obtained at the time of diagnosis by the simultaneous DCC–HPLC method and in the 65 corresponding tumor samples obtained at surgery, 2–4 weeks after FNA, by the classical individual assays (single saturation concentration, 5 nM).

As shown in Fig. 7, where receptor values are expressed in fmol/mg DNA, the overall correlations were satisfactory for either estrogen receptor or progestin receptor. When the 5 samples with lowest DNA content (i.e. with less than 10 μg DNA/ml cytosol) were excluded from the regression analysis to avoid any bias due to paucicellularity, the correlation coefficients for estrogen and progestin receptor (log values) were 0.864 and 0.868 respectively. These coefficients were even higher when estrogen and progestin receptor values from the surgical sample were expressed in fmol/g of fresh tissue, being 0.871 and 0.888, respectively.

DISCUSSION

There is a general consensus that estrogen and progestin receptors should be systematically assayed in every breast tumor [17]. Surgery usually provides a sample of tumoral tissue which is adequate for both assays by well-standardized methods. Such methods use specific radioligands for each receptor, at several concentrations (Scatchard plots), which require a substantial amount of tissue (at least 200 mg per receptor).

However, there are a number of situations where such assays are inadequate: small tumors shared with the pathologist, unoperated tumors, or metastatic localization. It is also the case for cell cultures where tumoral material is scarce.

It has been shown [18] that the reduction in the number of radioligand concentrations used in the classical assay is acceptable, even though it leads to

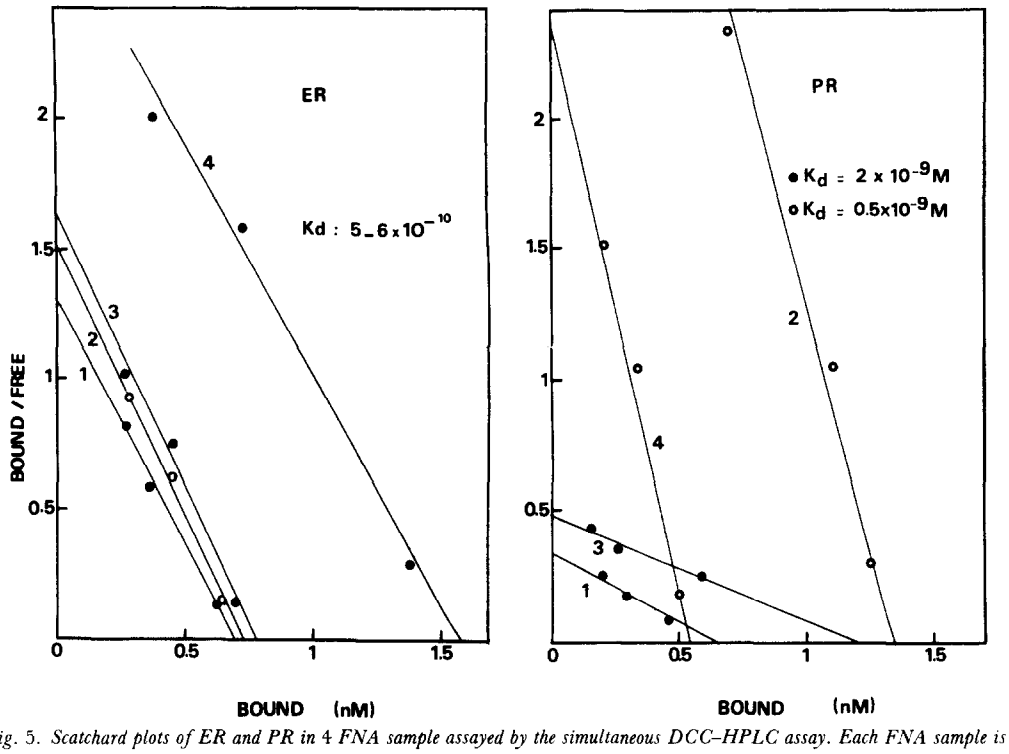

Fig. 5. Scatchard plots of ER and PR in 4 FNA sample assayed by the simultaneous DCC–HPLC assay. Each FNA sample is indicated by a number (1 to 4).

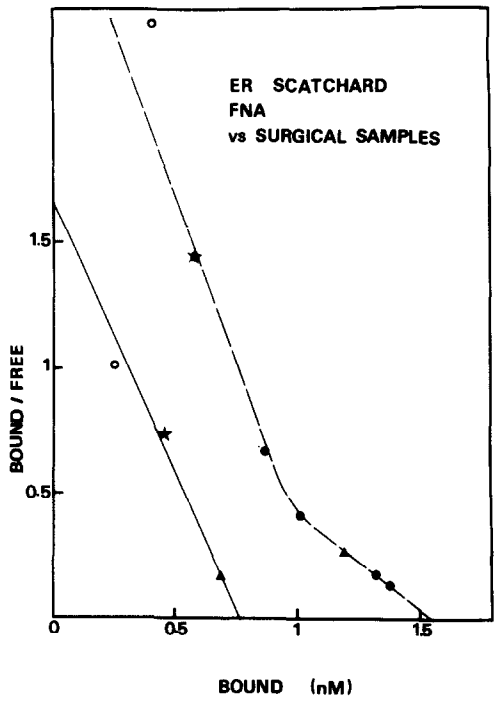

Fig. 6. Scatchard plot for ER in an FNA sample (straight line) and in the corresponding surgical sample (dashed line). Identical incubation concentrations are indicated by identical signs (○, ★, ▲).

to a loss of information concerning the dissociation constant of a given receptor. In particular, the 'single saturation concentration' assay, as used in the present study, provides a correct estimation of the level of receptors in breast pathology [14, 19].

The use of a high salt (0.4 M KCl) buffer in the assay is justified by a more efficient extraction of chromatine-bound steroid receptors, as may occur in premenopausal patients. No noxious effect of the high salt concentration has been observed in any phase of the DCC assay as proved by the excellent agreement we constantly observed between our DCC assay and the standard low salt procedure in EORTC Quality Controls (personal values available). In addition, DCC–HPLC-derived ER levels are highly correlated with those obtained by ER enzymo-immuno-assay in the same FNA [20].

In the case of unoperated tumors, FNA is a convenient means of sampling tumor tissue. It has been used for over 30 years for diagnosis. It is atraumatic, safe [21] and quite reliable in expert hands. In our hands, it provides a homogenous material, essentially composed of tumor cells, with little, if any, contamination by stromal cells (see Table 1).

Estrogen receptor assay on FNA of breast tumors was first described by Silfversward *et al.* in 1980 [22], with the use of electrofocusing. We showed [15] that ER assay in FNA was also reliable with a single concentration DCC assay. However, to our knowledge, no conclusive results were reported for progestin receptor assay on FNA, until ours [23].

Steroid receptor assays required a slight modification of the initial FNA technique used for diagnosis, i.e. a longer time of aspiration. This resulted in a non-negligible contamination by blood, which was the main obstacle to the rapid development

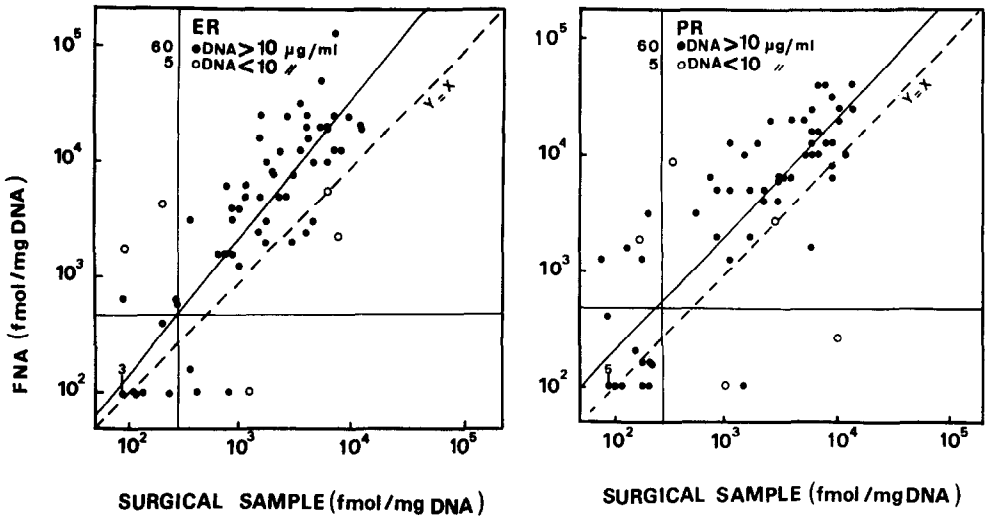

Fig. 7. Correlation between ER (left) and PR (right) in 65 FNA samples (simultaneous assay) and in the corresponding surgical samples (single assays).

of progestin receptor assay on FNA. Recently, a method for sampling without aspiration was introduced, which gives comparable amounts of cellular material with less blood contamination. Both sampling techniques were used in this study.

The problem of blood contamination was solved by selecting a tritiated progestin tag which gave a minimum non-specific binding, independent of the level of blood contamination. [^{3}H] O 2058 fulfilled this requirement whereas [^{3}H] R 5020 did not (see Fig. 4). [^{3}H] R 27987, the 21 hydroxy derivative of R 5020, was much more appropriate than [^{3}H] R 5020 but slightly less so than [^{3}H] O 2058 (data not shown), which was therefore chosen as the progestin tag for FNA. ER assay has been described on FNA with [^{3}H] E_2 as the tag [22] but the influence of blood contamination was not discussed. We propose [^{3}H] R 2858 as an alternative to [^{3}H] estradiol since it does not bind to contaminating plasma proteins [24] and gives a quantitative measurement of ER over a wide range of blood contamination (see Fig. 3).

We further envisaged the simultaneous assay of ER and PR in a single FNA. Initially [1], the mixture of hormone receptor complexes (ER and PR) was absorbed on hydroxylapatite before the ethanolic extraction of the titriated ligands and their separation by HPLC. However, blood contamination in FNA led to a non-quantitative recovery of bound ligands by hydroxylapatite. The assay was therefore modified according to Milano *et al.* [25]. The hormone receptor complexes were isolated by DCC and the bound hormones were extracted with 10% TCA–ethanol and separated by HPLC.

The procedure allowed us to perform simultaneous Scatchard plots in a few cases where the cellularity of the sample was high, i.e. above 50 µg DNA/ml cytosol (2.5–3 × 10^6 cells), and K_d values in the nanomolar range were characteristic of high-affinity receptors (see Fig. 5). When compared to the corresponding surgical sample, the saturation curve for 1 FNA sample gave consistent results as regards the absence (PR) or presence (ER) of receptors and similar Scatchard plots for ER (see Fig. 6).

Finally, we tested the reliability of the simultaneous determination of ER and PR in FNA in clinical situations, by the comparison between this assay on FNA samples at time of diagnosis and the classical assays on the surgical sample at time of surgery. ER and PR levels in FNA were found highly correlated with the levels in the surgical sample (see Fig. 7). Unexpectedly, some FNA samples (4 out of 65) were found to be ER- or PR-positive whereas the surgical sample was found to be negative. This may depend on the choice of the thresholds for positivity, a problem which has not yet been fully discussed. But this may also emphasize the importance of sample processing—FNA samples are frozen within seconds after sampling, which is not the case for surgical samples (20–30 minutes). Moreover, FNA is multidirectional within the tumor (which is not the case for drill or 'thru-cut' biopsy), giving a representative sample of the whole tumor. Recently, Azavedo *et al.* [26] demonstrated with immunocytochemical assays of ER (ER-ICA) that the distributions of estrogen-positive cells were identical in the FNA and in the corresponding surgical sample. In contrast, a surgical sample can be dispatched between different laboratories and the specimen for steroid receptor assay may not always be representative of the whole tumor if a rigorous sampling procedure is not applied.

One should be cautious with FNA samples with

low cellularity (less than 20 μg DNA/ml cytosol) and negative results in such cases should not be interpreted.

In conclusion, the simultaneous assay of ER and PR on FNA samples, by the DCC–HPLC method described above, appears reliable. It is useful in clinical situations where surgical samples are not available and in sequential studies of steroid receptor modulation by medical treatments, since FNA can be repeated with minimum harm to the patient.

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