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Perspectives and Commentaries

Technical Pitfalls, Methodological Improvements and Quality Control of Steroid Hormone Receptor Assays

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ESTROGEN (ER) and progesterone (PgR) receptor assays are now routinely performed in a large number of laboratories. These assays are mainly run on breast cancer samples in view of their major impact on the management of this disease [1, 2]. Thus, at the time of recurrence, receptor measurements give the most accurate factor for selecting appropriate patients for endocrine therapy. Moreover, receptor status of primary tumors seems a valuable prognostic factor for disease-free survival. Therapeutic impact of receptor assays in other hormone-sensitive neoplasias has not presently been studied to the same extent but it is currently receiving more attention.

Over the last ten years, extensive investigations have been performed to improve the accuracy of receptor assays. It is my purpose to rapidly overview these studies as well as to describe quality control programs developed in parallel to assess the value of routine procedures.

Binding assays

Since the pioneer work of Jensen in the late 1960s [3], several methods have been developed for the measurement of the steroid binding capacity of tumor samples [4]. The most significant step was the introduction in the early 1970s of the dextran-coated charcoal method (DCC method) which made receptor assays accessible to classical clinical laboratories. The homogenization of the tumor samples is the first step of the assay. The homogenate is then centrifuged at 100,000 ***g*** to obtain the cytosol fraction which contains the soluble form of the receptor. The latter, in the next step, is measured by incubating the cytosol fractions with appropriate ^{3}H-labeled steroids (i.e. estradiol for ER, R-5020 or ORG-2058 for PgR); an incubation in the presence of an excess of unlabeled steroid is usually run in parallel to evaluate the amount of non-specific binding. Most unbound and non-specifically bound steroids are then removed with a suspension of dextran-coated charcoal. The remaining radioactivity is finally measured by scintillation counting and the data analyzed by the graphical method of Scatchard. Receptor concentration, which differs from one tumor to another, is usually expressed in fmoles (10^{-15} moles) per mg of cytosol protein (range = 10–1000). This implies a protein measurement most often performed by the Bradford method (also called the BIO-RAD method in view of its commercialization by this company).

During the last decade several efforts were made to improve the accuracy of the DCC method. Dilution of cytosol below a critical level (~ 1–2 mg/ml) frequently, but not always, caused high underestimation of receptor concentration [5–8]. This could be explained by a protein loss through adsorption to charcoal. Addition of a protein expender (i.e. bovine serum albumin or gelatin

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at 1 mg/ml) to the DCC suspension was shown to largely prevent this phenomenon and thereby reduce the loss of binding activity [7, 8]. Another pitfall in receptor assays was found to result from unexpected high or low pH of the cytosols. Although most tumor cytosols prepared with a conventional phosphate buffer (5 mM, pH 7.4) have pH values between 7.1 and 7.5, some of them have weak acidic ($\geq$ pH 6.8) or basic ($\leq$ pH 8.0) pH [9]. These deviations largely reduced the yield of receptors. Moreover, the incubation with the DCC suspension was found to increase the pH of the mixtures by 0.6 units. In certain cytosols, a final pH higher than 8.0 could then be obtained, while acidic cytosols may be adjusted to the optimal pH. Whether or not these variations modify the number of complexes produced during the binding reaction has not been analyzed. Nevertheless, it seems that these potential effects of pH on the binding capacity of the cytosols, which have not been taken into account by most investigators, should be regarded with caution. On the other hand, an increase in the yield of receptors, especially PgR, has been reported when tumor samples are homogenized in buffers containing sodium molybdate [10–12]. This observation has led to the recommendation of using molybdate in routine assays. Raam and Teixeira [13], in a paper published in this Journal, emphasize interference of this oxyanion in the protein measurement giving significantly lower protein values. Therefore, they stress the need for quantifying protein on a standard curve using protein solutions containing molybdate at the same concentration as in the cytosols. This study clearly shows that all potential improvements of assays must be carefully analyzed before being introduced in routine practice. Several meetings have been organized for that purpose [1, 14–16], the EORTC Receptor Group having played a pilot role in this regard [14, 15].

An important issue of the meetings on receptor assays has been a consensus on a standard procedure by all investigators participating to national or international co-operative clinical trials. Quality control programs [2, 17–23] have also been designed in order to test the possibility of introducing receptor data in these clinical studies (i.e. European national programs [2]; ECOG [17]; EORTC [2, 20–23]). Pilot investigations indicated that pulverized tissues and lyophilized cytosols could be used for that purpose since they could be dispatched by mail from a central institution to each laboratory without major loss of binding capacity (Fig. 1; left). Lyophilized material was shown to be the material of choice since it could be stored for several months in the refrigerator of each investigator allowing additional inter-laboratory checks [23]. However, controls of tissue handling to obtain cytosols should also be taken into account since differences in extraction procedures may lead to important inter-laboratory variations [22, 24, 25]. In general, regular controls revealed a good agreement among investigators regarding the presence or absence of receptors in the samples provided by the central institution (i.e. the number of false-positive or false-negative samples was low). On the contrary, with regard to the quantitative assessment of the binding capacity, large variations were usually noticed. However, the rank of values was often the same in most laboratories so that quite a good correlation could be obtained when the samples were arbitrarily grouped into main categories of "low", "medium" and "high" binding. This observation led to the suggestion of an adjustment factor for each laboratory to reduce the inter-laboratory variability [17]. Reasons for these differences between laboratories are numerous and obviously difficult to completely abolish since after several years of co-operative efforts they do still occur. The experimental procedures, as well as the calculation of the binding data (i.e. analysis of non-linear Scatchard plots found at low receptor concentration), were recognized as a possible source of errors (Table 1). Whatever the real origins of the differences, these quality control checks allowed the introduction of receptor data into clinical trials. Logically, institutions providing unsatisfactory values on regular checks should be excluded from the analysis of final results (values lying largely out of the inter-laboratory variations).

Among other requirements, tumor fragments must be stored at very low temperature (freezer at $-80°$ C, liquid nitrogen) as soon as possible after sampling in order to prevent a loss of binding capacity. Therefore, processing and delivery conditions of the samples must be regularly controlled especially in the case of tumors provided by district hospitals (Fig. 1: right). Unfortunately, to our knowledge, only marginal attention has been devoted to this important point. This type of control was performed recently under the auspices of our Institute on a large number of samples sent to our laboratory from 10 district hospitals (over a one-year period) [26]. Analysis of the data revealed that the distribution of ER concentrations varied among hospitals (PgR was not assessed). Samples sent in liquid nitrogen were shown to contain, on average, a higher receptor concentration than those sent in ice-cold saline. Upon review of the clinical records, no satisfactory explanation for the differences between hospitals could be found, strongly suggesting that transportation conditions were at their origin. Storage and subsequent transportation in liquid nitrogen was therefore recommended. After one year of such experience [27], the two hospitals previously characterized by the highest

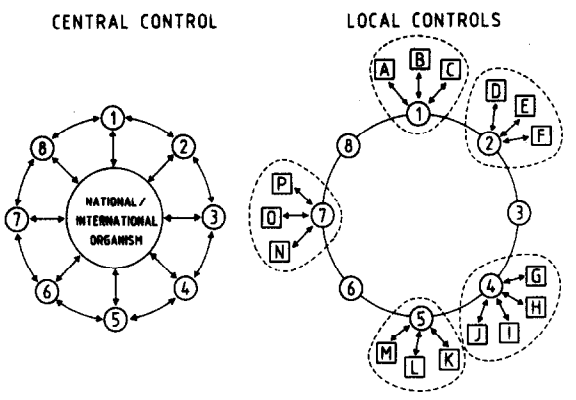

Fig. 1. Schematic representation of quality control programs for steroid receptor assays. The left panel describes a national/international program in which an institution organizes regular control checks by providing standard frozen or lyophilized samples to each laboratory (control of receptor assays). The right panel describes local programs in which regular control checks are organized by laboratories receiving tumor samples from district hospitals (control of handling and transportation of tissue samples). In both panels the arrows define the relationships between institutions.

(liquid nitrogen) and lowest (ice-cold saline) mean ER values provided the same mean high value; receptor concentrations were also similarly distributed. This study clearly showed that systemic storage and transportation of the samples in liquid nitrogen (or dry ice, if not available) could largely reduce differences between hospitals and suppress incorrect results. It also confirmed the need for organizing regular controls of tissue handling and transportation of tumors in co-operative clinical trials, especially those based on stratification according to receptor values.

Immunoassays

The DCC method discussed here (as well as all other binding methods) [4] cannot reliably measure receptors in the presence of high concentrations of endogenous high-affinity ligands (estrogens and antiestrogens for ER, progestins for PgR). In the case of ER, this difficulty has been recently overcome by the introduction of a new assay independent of the estradiol binding site. This assay which derives from the production of monoclonal antibodies to human ER [28] uses the direct antigenic recognition of the receptor. It has been developed by Abbott Laboratories as a solid phase enzyme immunoassay (ER-EIA) based on the "sandwich" principle. In a first step, beads coated with one anti-ER antibody are incubated with the tumor cytosol or appropriate standards containing known ER concentrations. During the incubation, ER binds to the beads; unbound material is then removed by aspiration and washing. In a second step, a second anti-ER antibody conjugated with horseradish peroxidase is incubated with the beads to measure the amount of bound ER. After aspiration of the excess of conjugates, the beads are incubated with hydrogen peroxide and *ortho*-phenylenediamine. The intensity of the color developed under these conditions, which is proportional to the amount of ER in the sample, is read with a spectrophotometer. A straight line is obtained by plotting the absorbance of the standards vs. their ER concentrations; receptor concentrations of the tumor cytosols can easily be determined from this line.

Quality control checks conducted under the auspices of Abbott Laboratories on lyophilized cytosols indicated that this new method provided a somewhat better reproducibility than is usually found with routine DCC assays [29, 30]. More-

*Table 1. Sources of errors in biochemical hormone receptor assays**

Binding assays
Interference of endogenous high-affinity ligands.
Incorrect estimation of ^{3}H-ligand concentrations.
Impurity of ^{3}H-ligands.
Inappropriate concentration of unlabeled ligands (incorrect evaluation of non-specific binding).
Inappropriate DCC treatment (preparation of suspension and/or time of incubation).
Errors in counting efficiency.
Insufficient protein concentration (< 1 mg/ml).
Incorrect interpretation of Scatchard plots.
Immunoassays
Difference in binding reactions among estrogen-sensitive tissues (reactivity of human mammary tumor anti-ER not established towards receptors from all human estrogen-sensitive tissues).
Use of reagents not provided by the manufacturer (requirement to stick to the standard procedure).

*Histochemical assessment of steroid receptors is presently insufficient to define any source of potential errors.

over, on a large series of mammary tumor cytosol samples, an excellent correlation was found between both methods suggesting that the passage from DCC to ER-EIA should not cause serious problems for the clinical interpretation of the receptor data. Another advantage of this new method is its requirement for only a minimal amount of cytosol which makes it especially appropriate for micro-tumor samples (ER-EIA = 2 × 100 µl cytosol at 1–2 mg/ml; DCC = 10 × 100 µl at ⩾ 1 mg/ml). In view of the simplicity of this new procedure, its total standardization (available in kit form), and its low requirement for tumor material, it seems that its introduction in routine practice might present a new step forward in the characterization of hormone sensitivity. However, extensive practice and regular quality control checks are still needed to definitely accept its general use (Table 1).

Important variations in ER levels from samples taken at different regions in the tumor mass were reported by several authors [31, 32]. This observation stresses the important role played by the pathologist who must select tissue pieces representative of the tumor. In this context, it seems that the introduction of histological tests using anti-ER monoclonal antibodies might be extremely powerful (ER-ICA kits produced by Abbott). This approach should provide valuable information on the hormone receptivity at the cellular level. The very low requirement of tumor material for such tests (i.e. fine needle aspirations) [33] makes receptor determinations possible for almost all tumors. Preliminary correlation studies [33–38] revealed a good parallel between the histological and biochemical assessments of the receptor contents of the tumors. However, these results still need confirmation. As a matter of fact, in the past, several other histological methods using fluorescent ligands were also claimed to specifically detect steroid receptors [2]. Careful subsequent analyses did not justify early enthusiasm [39–41].

Conclusion

The data reported here show that important investigations were made to detect possible pitfalls in routine receptor assays and that improved methodologies were developed. In this regard, the most significant progress is the introduction of commercial kits of monoclonal antibodies for the biochemical and histochemical measurement of ER. Similar kits for the detection of PgR will most probably be available in the near future. The diagnostic value of other probes for hormone sensitivity is presently under study (i.e. estrogen-induced proteins [42–43], a protein related to ER [44]). One may legitimately hope that the introduction of this new diagnostic material will lead to significant clinical progress.

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