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Development and validation of a sensitive immunohistochemical oestrogen receptor assay for use on archival breast cancer tissue

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Summary. In a preliminary paper (Teasdale et al. 1987) comparing the oestrogen receptor (ER) content of breast cancers by the biochemical dextran coated charcoal (DCC) method and by two histochemical methods, peroxidase immunocytochemistry (ERICA) and immunogold-silver staining (IGSS), it was indicated that ERICA is more sensitive than DCC and that IGSS is as specific as ERICA but less sensitive. This paper describes the comparison of the above three assay methods with two other biochemical methods, iso-electric focusing (IEF) and an enzyme immuno-assay (EIA) on a larger number of cancers. All methods gave statistically comparable results except that IGSS remained less sensitive than the rest. Various modifications to IGSS showed that an immunogold streptavidin enhancement method (IG-SAM) produced sensitivity and specificity equal to that of ERICA. Since IGSS and its modifications are the only methods which can be used on archival paraffin-embedded cancers and IG-SAM gives results highly comparable to ERICA, retrospective studies can be performed on patients whose outcome and response to various treatments are known. Most recent studies have shown that ER positive results can be obtained from 10-year-old paraffin blocks.

Introduction

We have described in a pilot study (Teasdale et al. 1987) the validation of an immunoperoxidase method (ERICA) of assaying oestrogen receptor (ER) against the standard dextran coated charcoal (DCC) method and showed that immunogold-silver staining (IGSS, Holgate et al. 1983a, b) could be applied to ER detection using the ERICA antibody. The specificity of IGSS equalled that of ERICA but the sensitivity was lower. The IGSS method was pursued since it is considerably more sensitive that the corresponding immunoperoxidase methods on lymphocyte markers (Holgate et al. 1983a, b). More important, IGSS, unlike any other ER assay, is effective in detecting antigens in conventionally paraffin-processed tissue. For purposes of studying the ER status of breast cancers with regard to prognosis and response to anti-hormonal treatment, the ability to assess ER in archival material would be invaluable.

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Our current studies reported here were to improve the sensitivity of IGSS and, using larger numbers of breast cancers, to validate by comparison the results of various ER assay methods. These are the above three, which are biochemical (DCC) and immunohistochemical (ERICA and IGSS, using the same monoclonal antibody) together with two other biochemical methods, iso-electric focusing (IEF) and enzyme immuno-assay (EIA), the last using the ERICA antibody.

Materials and methods

To compare the results from the five different methods of ER assay, 75 unselected breast carcinomata (mastectomy or biopsy) which had sufficient tumour were transported from operating theatre to the laboratory on ice up to $\frac{1}{2}$h after operation.

Within this study, in order to detect any possible loss of ER during transport, a piece of tumour from 24 of these specimens was placed in liquid nitrogen immediately after the operation and the remainder transported as above when an adjacent piece was similarly frozen. Assays of these pairs of specimens for comparison were by ERICA and IEF.

In all 75 cases, several pieces of fat- and necrotic-free tumour, $\frac{1}{2}$–1 cm in diameter were frozen in liquid nitrogen and two to three pieces placed in 10% formalin. Of the pieces in liquid nitrogen, one was used for the IEF and ERICA assays and the rest (4–8) were used for preparing the conventional cytosol as used in both the DCC and EIA assays.

The IEF method used, with histological monitoring for presence of tumour and cellularity, was that given by Underwood et al. (1983) and the DCC was by the standard method (Teasdale et al. 1987). EIA and ERICA methods (both Abbott Diagnostics Division) were performed according to the instructions provided with the kits. The IGSS method has been detailed in our previous paper (Teasdale et al. 1987). As described there, its sensitivity was reduced due to increased background staining in both the test and control slides.

To overcome this a separate study was performed on 30 breast cancer specimens treated as for IGSS assays. In order to assess sensitivity improvement, mostly positive cases by ERICA were selected. Firstly, blocking of non-specific binding was attempted by diluting all antibody solutions, including the immunogold, with human AB serum (modification A). Further attempts for improvement included enhancement by streptavidin-biotin (modification B) and a combination of A and B (modification C), as shown in Tables 1 and 2. The results of these modifications were compared with each other, with the standard IGSS method and with ERICA for each specimen. As a result of modification C (immunogold streptavidin modification; IG-SAM) a further 30 specimens were assayed by IG-SAM and ERICA.

Table 1. Immunogold streptavidin modification (IG-SAM)

1. Take formalin fixed paraffin sections through xylene and ethyl alcohol to water.
2. Trypsinise in 0.1% Trypsin (Type II: crude from porcine pancreas, Sigma) in 0.1% $CaCl_2$ at 37° C for 20 min. Rinse in tap water.
3. Immerse sections in Lugol's iodine for 5 min.
4. Rinse thoroughly in tap water and decolourise in 2.5% aqueous sodium thiosulphate solution. Rinse in tap water.
5. Block endogenous biotin with avidin, 15 min, followed by biotin, 15 min (Vector Laboratories). Rinse in *Tris* buffered saline (0.05 *M Tris* in isotonic saline, pH 7.6).
6. Block non-specific background staining with normal goat serum for 5 min. Shake off the excess.
7. Apply the primary monoclonal rat antibody against human ER (Abbott ERICA kit), at a dilution of 1 in 2 in *Tris* buffered saline containing 5% human AB serum. Incubate for 1 h.
8. Wash sections in *Tris* buffered saline, pH 7.6 for 20 min (2 × 10 min washes).
9. Apply biotinylated anti-rat Ig (Amersham) at a dilution of 1 in 200 in *Tris* buffered saline containing 5% human AB serum. Incubate for 1 h.
10. Repeat 8.
11. Repeat 6.
12. Apply streptavidin-gold G5 LM (Janssen) at a dilution of 1 in 200 in *Tris* buffered saline containing 5% human AB serum. Incubate for 2 h.
13. Rinse sections in *Tris* buffered saline, pH 7.6 for 10 min followed by distilled water for 10 min.
14. Immerse sections in silver enhancing solution (Table 2). Develop for up to 15 min, under light-microscopic control, in a dark room using Ilford safe light S902 or F904. When silver intensification is sufficient wash slides thoroughly in distilled water.
15. Immerse sections in 2.5% aqueous sodium thiosulphate solution for 3 min to fix the preparations.
16. Wash sections in tap water.
17. Counterstain with haematoxylin and eosin.
18. Dehydrate with ethyl alcohol, clear in xylene and mount in a synthetic mounting medium.

Results: ER positive cells show black nuclear stippling

Table 2. Silver development

Solution	Vol. required for 100 ml developer
1. Gum acacia (500 g/l)	7.5 ml diluted to 60 ml in distilled water
2. Citrate buffer, pH 3.5	10 ml
3. Hydroquinone (0.85 g/15 ml)	15 ml
4. Silver lactate (0.11 g/15 ml)	15 ml

All solutions are made in distilled water and mixed in the above order. All solutions containing silver lactate should be protected from light. For preparation of solution 1, stir overnight and filter through gauze. For preparation of solution 2, add 23.5 g trisodium citrate $2H_2O$ and 25.5 g citric acid 1 H_2O to 100 ml distilled water. Solutions 3 and 4 should be prepared immediately prior to use

In the case of each biochemical estimation (DCC, IEF or EIA) a figure ≥ 10 fmol/mg was considered to represent ER positivity. Duplication of results is automatically obtained by the methods when two pieces of tissue are assayed by IEF and two samples of the cytosol by EIA. Biochemical results were corrected for the cellularity of the tumour by point counting cells in whole paraffin sections of representative pieces of the tissue used for DCC and EIA and in the adjacent frozen section from the tissue used for IEF as described in that method.

Table 3. Comparison of IGSS and ERICA

		IGSS		Total
		+	−	
ERICA	+	28	14	42
	−	0	33	33
	Total	28	47	75

McNemar's test $p < 0.001$. Significantly different

Controls for the histochemical methods were, for ERICA, the slides supplied in the kit and for IGSS and its modifications, duplicate slides treated identically but for the replacement of the primary antibody by the ERICA control antibody. Histochemical assays (ERICA and IGSS and its modifications) were all scored by three independent observers who, while scoring each slide with its control, were unaware of the results of ER content by any of the other histochemical or biochemical methods. Scoring of slides, as described in Teasdale et al. (1987), was by grading them −, ±, +, ++ or +++ depending on whether staining was absent, minimal or positive in varying degrees. This was based essentially on numbers of cells staining but intensity of staining was also considered. The cellularity of the tumour was thus automatically taken into account. Grades − and ± were taken to be ER negative and the others positive. Disagreements between observers were few and were resolved by consensus, again without knowing results from any other assays.

Results

Assaying the 24 specimens whether immediately frozen in liquid nitrogen at operation or after transporting to the laboratory on ice showed no loss of receptor during transport.

Comparison of ER assay methods

The IGSS method showed 37% of the tumours to be ER positive and the other four methods gave from 57.3% to 62.7% positivity. Since no single method of ER assay could be assumed to be the most accurate, the results of the five methods were compared in pairs by McNemar's test. If the IGSS method were excluded, comparisons involving the other four methods showed no significant differences. The IGSS results were significantly different from all of the other 4 ($p < 0.001$). Of the 28 positive cases by IGSS, all were positive by ERICA (Table 3) and 27, 26 and 24 were positive by DCC, IEF and EIA respectively. The results for differences between pairs were found, as for IGSS and ERICA, by contingency tables as exemplified in Table 3 and are summarised in Table 4. Thus the sensitivity and specificity of ER assays were found to be the same for all except that the sensitivity of IGSS was poorer.

Improvement of sensitivity of IGSS

Of the 30 specimens used to modify IGSS, 28 were positive and 2 were negative by ERICA. Fifteen were positive by IGSS but the numbers of positive results increased to 24, 25 and 27 when IGSS modifications A, B and C respectively were employed. As well as producing more ER positive

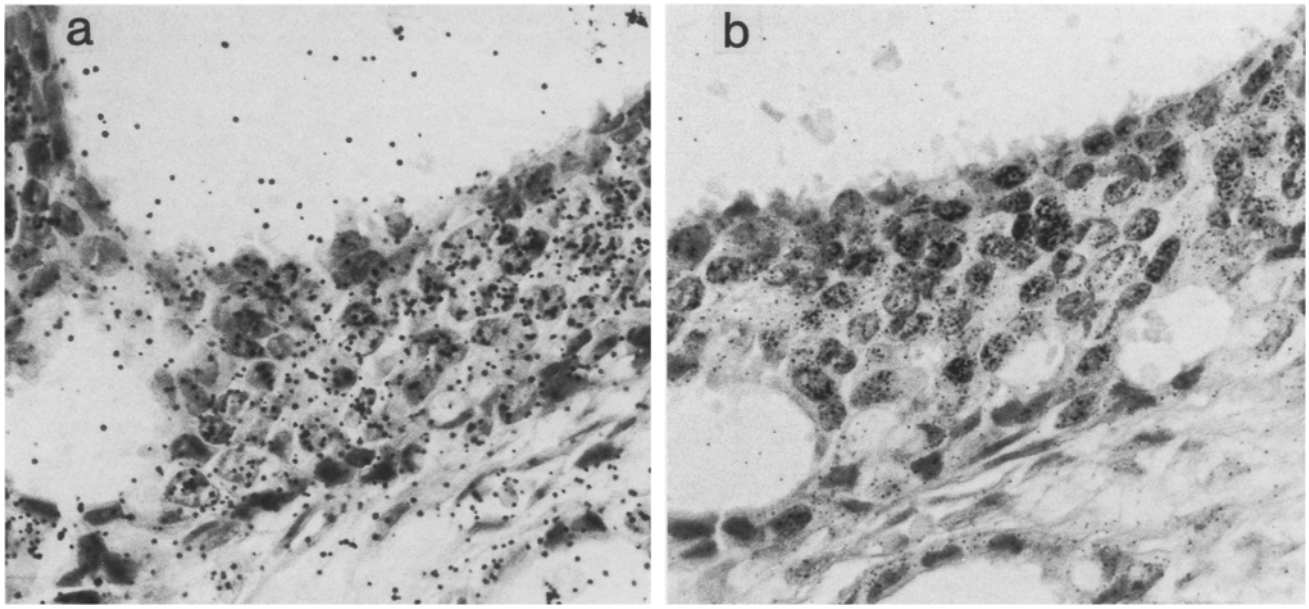

Fig. 1a and b. Serial sections of breast cancer stained (**a**) IGSS, scored negative and **b** IG-SAM scored + +. In (**a**) no significant nuclear staining is seen and background is unacceptable. Specific nuclear staining is seen in (**b**) with reduced background. Counterstain H&E, ×700

Table 4. Significance of differences between pairs of assays Results of contingency tables (McNemar's test)

Assays	Probability	Difference
DCC v EIA	$p>0.50$	Not significant
DCC v IEF	$p>0.50$	Not significant
DCC v ERICA	$p>0.50$	Not significant
DCC v IGSS	$p<0.001$	Significant
IEF v EIA	$p>0.50$	Not significant
IEF v ERICA	$p>0.05$	Not significant
IEF v IGSS	$p<0.001$	Significant
EIA v ERICA	$p>0.50$	Not significant
EIA v IGSS	$p<0.001$	Significant
ERICA v IGSS	$p<0.001$	Significant

At one degree of freedon $\chi^2>1.96$ ($p<0.05$) is significantly different

Table 5. Comparison of results of 60 cases using ERICA and IG-SAM

		IG-SAM		
		+	−	
ERICA	+	42	3	45
	−	8	7	15
		50	10	60

McNemar's test $p>0.10$. No significant difference

cases, these modifications, in most cases, also showed a trend to an increase in score from − to + + +. The improvement in results by modification C (IG-SAM) was due to a reduction in background staining. Figure 1 illustrates the effect of improvement of the IGSS by IG-SAM in serial sections. IG-SAM was then taken to be the most satisfactory and using another 30 breast specimens (total of 60) a McNemar's test showed a high degree of significance between results for ERICA and IG-SAM together with a very comparable specificity and sensitivity (Table 5). There is even a suggestion in that table that IG-SAM is the more sensitive since it gave more positive results.

Discussion

The oestrogen receptor content of breast cancers has consistently shown a significant relationship to prognosis and to response to anti-oestrogen treatment (McGuire and Clark 1983; Wittliff 1984; Williams et al. 1987), a relationship which is of more value in respect of a population of patients than in an individual case. The latter may be due to the sensitivity and specificity of the DCC method of assay, in which case a search for more accurate methods is justified. We began by comparing the results of five methods (DCC, IEF, EIA, ERICA and IGSS) in order to validate them, particularly the last which was developed in this laboratory and found to have considerable potential since it is the only one of the five which can make use of conventional paraffin-embedded material. In addition, like ERICA, it is a histochemical method which is much easier and economical to apply than DCC, IEF and EIA which are biochemical.

We found that DCC, IEF, EIA and ERICA gave remarkably consistent results but that IGSS revealed fewer of the ER positive tumours. However, it produced no more positives than the ERICA, that is, IGSS was as specific as ERICA but less sensitive. In view of this, together with the fact that ERICA is a peroxidase method and that IGSS was found to be considerably more sensitive than conventional peroxidase methods for immunoglobulins (Holgate

et al. 1983a, b), improvement of the IGSS method was attempted. It was hoped that the use of cryostat sections would overcome any loss of receptor which might be destroyed by paraffin processing but no staining was obtained at all. This is in keeping with observations on IGSS used for other antigens, the explanation being that the iodine treatment of the section which is necessary in the IGSS method destroys certain antigens (Pollard et al. 1987). In any case, the advantages of paraffin over frozen sections are apparent in that the former give better morphology and are easier to prepare though taking longer. Most important, it was intended that paraffin embedded breast cancers in departmental files could be assayed for ER, thus making retrospective studies possible. References to difficulties encountered by other workers using methods other than IGSS in detecting oestrogen receptor in paraffin sections of breast cancer have been discussed in our previous paper (Teasdale et al. 1987).

Difficulties with our original IGSS method arose from a high level of background staining which interfered with scoring. To overcome this, diluting all antibody solutions with human AB serum to block non-specific immunologic reactions was found to give more positive results. Amplification of the immunologic reaction was then attempted by using the streptavidin-biotin method. Biotinylated anti-rat serum was applied to the ERICA (rat) anti-ER monoclonal antibody and streptavidin gold reacted with it. In the standard IGSS method, anti-rat antibody adsorbed on gold is applied directly to the ERICA antibody. In both cases, the colloidal gold particles are visualised microscopically by developing with silver. The streptavidin-biotin modification again gave more positive results and, as with the AB serum, converted lower scored cases to higher. When AB serum is used in conjunction with the streptavidin-biotin modification to give IG-SAM, the results on paraffin sections are at least as good as those using ERICA which are highly comparable with those from biochemical assays of ER thus validating the IG-SAM. We have currently applied IG-SAM to more than 50 ten-year-old paraffin blocks in our files and find an expected proportion of the tumours to be ER positive. Clinical details are being obtained for these.

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