Immunohistology of oestrogen receptor and D5 antigen in breast cancer: correlation with oestrogen receptor content of adjacent cryostat sections assayed by radioligand binding and enzyme immunoassay

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SUMMARY Two monoclonal antibodies recognising epitopes associated with oestrogen receptor protein were evaluated against the assayable soluble oestrogen receptor concentration in a series of 149 breast carcinomas. One antibody (anti-ER) recognises the hormone binding unit of oestrogen receptor and gives nuclear staining; the other antibody (anti-D5) was raised to a component of soluble oestrogen receptor and gives cytoplasmic staining. To minimise variations attributable to tumour heterogeneity and sampling error immunohistology using the two monoclonal antibodies, radioligand binding assays, enzyme immunoassays, and quantitative histology were done on adjacent frozen sections. Thirty nine per cent, 48%, 54%, and 43% of the tumours were found to be oestrogen receptor positive by radioligand binding assay, anti-ER and anti-D5 immunohistology, and enzyme immunoassay, respectively. Strong correlations ($p < 0.0005$) were found between anti-ER immunohistology and the radioligand binding assay. Only weak correlations were found between anti-D5 immunohistology and the results of other assay methods for oestrogen receptor. Nuclear staining of human breast cancers with the anti-ER monoclonal antibody thus seems to be an acceptable alternative to biochemical assays, with the additional advantage of showing intercellular and regional heterogeneity for oestrogen receptor content.

Clinical decisions on endocrine manipulation in the management of breast cancer are often based on the oestrogen receptor content determined by radioligand binding assays of tumour homogenates. Although these assays satisfy theoretical criteria for the biochemical characterisation of oestrogen receptor, various defects have become apparent. In addition to their inability to elucidate the intercellular and regional heterogeneity in oestrogen receptor content within a tumour, these assays preclude simultaneous histological confirmation that viable tumour is present in the assayed sample and an assessment of its morphological features.¹ Radioligand binding assays are also affected by factors such as protein estimation method,² quality of radioactive tracers, and methods of tissue storage and homogenisation. Interlaboratory and intralaboratory variations in the bio-

chemical measurement of oestrogen receptor content on the same tumour sample have been reported and their possible therapeutic implications discussed.³

There is therefore a need for an effective alternative. Histochemical methods based on fluoresceinated ligands or antiligand antibodies have questionable validity.¹ Serious theoretical objections have been raised against these histochemical methods, and the consensus is that while they may have as yet unsubstantiated clinical relevance they do not apparently detect high affinity oestrogen receptor, which, on current evidence, is the main determinant of the endocrine responsiveness of breast cancers.

This study is a comparative evaluation of two monoclonal antibodies against biochemical data. The oestrogen receptor monoclonal antibody recognises the hormone binding unit of oestrogen receptor but reacts with an epitope remote from, and apparently undisturbed by, ligand interactions at the hormone binding site.⁴ The D5 monoclonal antibody was

raised to a component associated with soluble oestrogen receptor and, under certain conditions, forms complexes with it.⁵

To ensure meaningful comparisons between methods and reagents and to assess the influence of tumour cellularity on assay results all discrepant factors including intratumoural oestrogen receptor variations were virtually eliminated by using adjacent frozen sections cut from the same tissue block.

Material and methods

TUMOUR SAMPLES

Tissue blocks from 149 breast carcinomas were trimmed of obvious fat, necrosis, and sclerosis, and snap frozen at -50°C in isopentane. Tissue was stored at -70°C for not more than three months for radioligand binding assay and for up to two years for immunohistology and enzyme immunoassay. All immunohistology and assay procedures were performed on cryostat sections cut adjacently from the same face of these tissue blocks.

CRYOSTAT MICROTOMY

Each tissue block was mounted frozen on a metal chuck using a small volume of ET buffer for microtomy in a cryostat running at -20°C .

Subject to the availability of reagents, the following sections were cut at $4-6\text{ }\mu\text{m}$ from each tumour block and picked up on glass slides: one test and one control section for oestrogen receptor monoclonal antibody immunohistology; one test and one control section for D5 monoclonal antibody immunohistology; one section for quantitative estimation of cellularity.

The following sections were cut at $40\text{ }\mu\text{m}$, picked up on small glass coverslips, and transferred to plastic test tubes containing buffer: sections to give a total section area of about 1 cm^2 into $200\text{ }\mu\text{l}$ ET buffer for oestrogen receptor by assay radioligand binding; a similar number of sections into $400\text{ }\mu\text{l}$ Tris buffer for enzyme immunoassay using the oestrogen receptor monoclonal antibody.

ER AND D5 IMMUNOHISTOLOGY

The oestrogen receptor monoclonal antibody (Abbott Laboratories Ltd) and the D5 monoclonal antibody (Amersham International plc) were both used in accordance with the manufacturers' protocols. In summary, cryostat sections were treated sequentially at room temperature, unless stated otherwise, as follows:

Oestrogen receptor monoclonal immunohistology Sections were fixed in 3.7% neutral phosphate buffered saline-buffered formalin for 10–15 minutes; phosphate buffered saline wash; methanol at -20°C for three to five minutes; acetone at -20°C for one to

three minutes; phosphate buffered saline washes; normal goat serum for 15 minutes; oestrogen receptor monoclonal antibody (rat) for 30 minutes; phosphate buffered saline washes; goat antirat immunoglobulin antibody for 30 minutes; phosphate buffered saline washes; rat antiperoxidase-peroxidase complex for 30 minutes; phosphate buffered saline washes; diaminobenzidine (DAB) in hydrogen peroxide for six to eight minutes; water wash; haematoxylin counterstain, dehydrate, cleared, and mounted.

D5 monoclonal immunohistology Sections were briefly air dried; 0.3% hydrogen peroxide in methanol for seven to 10 minutes; phosphate buffered saline washes; normal goat serum for 20 minutes; D5 monoclonal antibody (mouse) for 30 minutes; phosphate buffered saline washes; goat antimouse immunoglobulin antibody for 20 minutes; phosphate buffered saline washes; mouse antiperoxidase-peroxidase complex for 20 minutes; phosphate buffered saline washes; DAB in hydrogen peroxide for six to eight minutes; phosphate buffered saline washes; haematoxylin counterstain, dehydrate, cleared and mounted.

Control sections were treated with normal rat or mouse serum substituted for the oestrogen receptor and D5 monoclonal antibodies, respectively. Positive control sections from known oestrogen receptor and D5 positive tissues were included in each batch; in addition, known oestrogen receptor positive controls provided by Abbott Laboratories for use with their reagent were included in each batch.

Staining intensity was scored and recorded on a 4 point scale by one observer throughout (DDG) with independent verification (JCEU): 0 = no staining; + = weak staining; ++ = intermediate; +++ = strong staining. An estimate of the proportion of tumour cells positive for oestrogen receptor and D5 was also recorded in each case. For comparison with the results of the radioligand binding assay and the enzyme immunoassay, tumour sections showing ++ or +++ staining intensity in more than 25% of the tumour epithelial population were regarded as positive; the rest of the sections were regarded as negative.

A staining index was calculated and used to examine linear correlations with quantitative assays:

$$\text{staining index} = \frac{\text{staining intensity} \times \% \text{ of cells positive}}{\% \text{ cellularity}}$$

QUANTITATIVE HISTOLOGY

Tumour epithelial cellularity was estimated by point counting the haematoxylin and eosin stained cryostat sections.

RADIOLIGAND BINDING ASSAY

The radioligand binding assay of the oestrogen receptor content of cryostat sections has been described

elsewhere.⁶ Briefly, 2×10^{-9} M ^3H -oestradiol in 200 μl of ET buffer (1.5 mmol disodium edetic acid 10 mmol Tris-hydrochloric acid, 0.5% bovine serum albumin, 0.02% sodium azide; pH 7.4) was added to the tubes containing cryostat sections immersed in 200 μl ET buffer. After one hour at room temperature the tubes were centrifuged and the clear supernatant, enriched with diffusible oestrogen receptor now occupied by ^3H -oestradiol, aspirated into clean tubes. Fifty μl was withdrawn for protein assay by Bradford's method⁷ and calculated volumes (about 10 μl) of trypsin (1 mg/ml) and calcium chloride (to give final concentration of 2 mmol) added to encourage limited proteolysis over a further 30 minutes' incubation at 10°C. Two hundred μl was then withdrawn and subjected to isoelectric focusing in polyacrylamide gel. Radioactivity in gel slices was measured by liquid scintillation counting and the receptor content calculated by measuring the area of the peak of radioactivity attributable to specific binding above the diagrammatic baseline. Results were expressed as fmol/mg protein and values >10 were regarded as positive.

ENZYME IMMUNOASSAY

The coverslips bearing the 40 μm sections immersed in 400 μl Tris buffer were gently crushed to facilitate

pelleting of the section debris by centrifugation at 1000 g for 10 minutes at 0°C. Two 150 μl aliquots of the supernatant, now enriched with diffusible oestrogen receptor, were placed in separate tubes and a further 50 μl used for protein assay by Bradford's method.⁷ The oestrogen receptor content of the supernatants was assayed by enzyme immunoassay using the oestrogen receptor monoclonal antibody and other reagents supplied in kit form for this purpose (Abbott Laboratories Ltd). Diluent (100 μl) and 100 μl supernatant were added to each well and followed by a bead coated with the oestrogen receptor rat monoclonal antibody. After incubation for 18 hours at 0°C the bead was washed twice in distilled water and then incubated with 200 μl of oestrogen receptor conjugate solution for one hour at 37°C. After two further washes in distilled water the bead was transferred to a test tube and 300 μl OPD (o-phenylenediamine, 2 HCl) substrate added and incubated for 30 minutes. The reaction was stopped by the addition of 1 ml 1N H_2SO_4 to each tube. The reaction product was read colorimetrically within two hours at 492 nm.

The χ^2 test and Spearman's rank correlation test were used for all the comparative analyses.

Results

OESTROGEN RECEPTOR MONOCLONAL ANTIBODY
Immunohistology with the oestrogen receptor monoclonal antibody produced exclusively nuclear staining (fig 1): 71 (47.6%) of the tumours were assessed as positive (graded as ++ to +++ staining); 78 (52.4%) were assessed as negative (graded as 0 to + staining). Cytoplasmic staining was noted in three tumours only; it was ascribed to endogenous peroxidase activity, which was not blocked by the staining procedure followed routinely, because it was also present in negative control sections. No staining of stroma or necrotic areas was observed. Intratumoural heterogeneity with respect to staining was noted as follows, but not quantified: cell to cell, different staining intensities in adjacent cells within the same region (fig 2); regional, different staining intensities in different cell groups (fig 3); benign v malignant, different staining intensities for benign and malignant epithelial cells.

Strong correlation was obtained between staining grades and radioligand binding oestrogen receptor assay results ($p < 0.0005$, χ^2 test) (table 1). Similarly, a good linear correlation was obtained between staining indices and radioligand binding assay results ($r = 0.801$, Spearman's rank correlation test) (fig 4). A threshold staining index of 10 units segregated most oestrogen receptor positive and negative cases with little overlap. Strong correlations were also found

Fig 1 Invasive ductal adenocarcinoma of breast stained with anti-oestrogen receptor monoclonal antibody. Tumour cell nuclei are uniformly stained. Stromal cell nuclei are unstained. (Immunoperoxidase with weak haematoxylin nuclear counterstain.)

Fig 2 Heterogeneity of nuclear oestrogen receptor expression shown by anti-oestrogen receptor monoclonal antibody. Stained and unstained nuclei are evident in this group of invasive carcinoma cells. (Immunoperoxidase with weak haematoxylin nuclear counterstain.)

Fig 3 Regional heterogeneity of oestrogen receptor expression shown by anti-oestrogen receptor monoclonal antibody. Nuclei of cribriform intraduct component of this carcinoma are uniformly stained; surrounding invasive component is unstained. (Immunoperoxidase with weak haematoxylin nuclear counterstain.)

between the oestrogen receptor monoclonal antibody staining grades (table 1; $p < 0.005$) and index ($r = 0.803$), and the results of the enzyme immunoassay using the same oestrogen receptor monoclonal antibody. No significant correlation was observed between the immunohistochemistry with oestrogen receptor and D5 monoclonal antibodies (table 2; $p > 0.1$).

Of the 16 cases with grades $++/+++$ staining grades that were negative by radioligand binding oestrogen receptor assay, nine were tumours from premenopausal women and three had unusually low cellularity ($< 10\%$ compared with average cellularity of about 20%). Two of the four cases exhibiting only weak staining but positive on radioligand binding oestrogen receptor assay had unusually high cellularity (38% and 48%).

Cryostat sections from 93 carcinomas were assayed using the oestrogen receptor monoclonal antibody in the enzyme immunoassay and the results compared with those obtained from the radioligand binding assay. A high degree of correlation was found ($p < 0.0005$, $r = 0.895$). Of the 10 tumours that were positive on the enzyme immunoassay but negative on the radioligand binding assay, seven were from premenopausal patients.

D5 MONOCLONAL ANTIBODY

Of the 128 tumours examined with the D5 monoclonal antibody, 68 (53.9%) and 56 (46.1%) exhibited $++/+++$ and $0/+$ staining grades, respectively. Staining was always cytoplasmic (fig 5). Unexpected staining attributable to the D5 monoclonal antibody was seen occasionally in stromal fibroblasts, inflammatory cells, and vascular endothelium (fig 6). While cell to cell heterogeneity of staining intensity was less than that observed with the oestrogen receptor monoclonal antibody, regional differences and differences between benign and malignant epithelium within the same tissue sample were noted.

There were no significant correlations ($p > 0.05$) between staining grades or indices and oestrogen receptor concentrations determined by either radioligand binding assay or enzyme immunoassay (table 3).

Discussion

The highly significant correlation between the results of the radioligand binding assay and immunohistochemistry with the oestrogen receptor monoclonal antibody verifies the suitability of this reagent for the routine determination of the oestrogen receptor content of breast carcinomas. Although the result is not quantitative to the same extent as that obtained from a radioligand binding assay, immunohistochemistry permits confirmation of the presence of viable tumour

Table 1 Correlation between immunohistology staining grades obtained with oestrogen receptor monoclonal antibody and oestrogen receptor content of adjacent cryostat sections assayed by radioligand binding and enzyme immunoassay

Oestrogen receptor monoclonal immunohistology grades	No of tumours			
	Oestrogen receptor content by: Radioligand assay		Enzyme immunoassay	
	Negative	Positive	Negative	Positive
0/+	70	4	49	6
++/+++	16	47	4	33
	p < 0.0005		p < 0.0005

Fig 4 Correlation between oestrogen receptor assay by radioligand binding and anti-oestrogen receptor monoclonal antibody staining index on adjacent cryostat sections ($r = +0.801$). Sixty nine tumours had both oestrogen receptor concentrations and staining indices less than 10 fmol/mg protein and units, respectively. Of 14 tumours with oestrogen receptor concentrations of < 10 fmol/mg protein but with staining indices > 10, most were either of low cellularity or from premenopausal women.

tissue in the assessed sample and an estimate of the degree of cellular heterogeneity with respect to oestrogen receptor content.

The use of adjacent cryostat sections for simultaneous immunohistology and radioligand binding assay enabled us to determine the extent to which tissue sampling and variations in tumour cellularity contributed to apparent discrepancies between the two methods. Thus we were able to show that some of those tumours, which were positive on immunohistology but negative (< 10 fmol/mg) by radioligand binding assay, gave these discrepant results because they were of unusually low cellularity, a factor which does not influence the immunohistological staining grade. Other tumours that were oestrogen receptor negative on assay but positive by immunohistology were from premenopausal women; endogenous oestrogens occupy the radioligand binding site in these tumours, but this does not render unavailable or disturb the conformation of the epitope recognised by the oestrogen receptor monoclonal antibody.

Immunohistology with the D5 monoclonal antibody, which reacts with an epitope on a component of soluble oestrogen receptor not associated with the hormone binding site, correlated poorly with the oestrogen receptor content of adjacent sections assayed either by enzyme immunoassay with the oestrogen receptor monoclonal antibody or by radioligand binding assay. Although the protocol we followed for D5 immunohistology (Amersham

Table 2 Correlation between immunohistology staining grades obtained with oestrogen receptor and D5 monoclonal antibodies on adjacent cryostat sections

Oestrogen receptor monoclonal immunohistology grades	No of tumours		
	D5 monoclonal immunohistology grades 0/+	++/+++	Total
0/+	35	32	67
++/+++	21	36	57
Total	56	68	124
	p > 0.1

Fig 5 Invasive ductal adenocarcinoma of breast stained with anti-D5 monoclonal antibody. There is strong uniform cytoplasmic staining of tumour cells. Stromal cells are unstained. (Immunoperoxidase.)

Fig 6 Stromal staining by anti-D5 monoclonal antibody. Tumour cells are unstained. (Immunoperoxidase.)

International plc) gave investigators the option of using either frozen sections or paraffin wax sections, it is reported that false positive staining may be obtained with the former (King RJB, personal communication 1986). This is unexpected; artefactual results in immunohistology are more likely with sections of processed fixed tissue than with frozen sections, though paraffin sections of tissue fixed in methacarn are now advocated for D5 immunohistology.⁸ Loss of D5 antigen by diffusion from the frozen sections is unlikely, and the range of positive and negative staining within our series of tumours seems more likely to be attributable to inherent differences between tumours rather than to methodological problems.

Stromal staining with the D5 antibody has not been reported previously; we observed it in a small number of tumours even when the carcinoma cells were negative. Although this may denote oestrogen responsiveness of the stromal cells in these lesions, nuclear staining of stromal cells with the oestrogen receptor antibody was never seen.

Most of the unoccupied oestrogen receptor within oestrogen responsive cells is now thought to reside within the nucleus,^{9,10} and the oestrogen receptor monoclonal antibody gave consistent nuclear staining. Consistent cytoplasmic staining by the D5 antibody contributes to the suspicion that the D5 antigen is not associated with the hormone binding activity of oestrogen receptor, though there is evidence that the presence of D5 antigen, a 29000 dalton phosphoprotein, may be a useful indicator of oestrogen responsiveness.⁸ Unlike oestrogen receptor, D5 immunostaining shows poor correlation with histological differentiation and tumour ploidy (Walker R, personal communication 1986).

Our results show a strong positive correlation between immunohistology with the oestrogen receptor monoclonal antibody and the radioligand binding assay data; this is consistent with other reports.¹¹⁻¹⁷ Our methodology, using cryostat sections for all the immunohistology and assays, permitted us to examine some of the reasons for discrepant results, particularly in tumours where the cellularity is unusually low or high. Tumours consisting of sparsely distributed oestrogen receptor positive cells on immunohistology may give misleadingly low or negative radioligand binding assay results because of dilution by receptor negative stromal elements.¹⁸

We conclude that the oestrogen receptor monoclonal antibody used in this study is the only reagent currently available that permits the histological determination of the oestrogen receptor content of breast carcinomas. Although the information obtained from its use is not quantitative, it has the advantages of giving simultaneous confirmation that viable tumour

Table 3 Correlation between immunohistology staining grades obtained with D5 monoclonal antibody and oestrogen receptor content of adjacent cryostat sections assayed by radioligand binding and enzyme immunoassay

D5 monoclonal immunohistology grades	No of tumours			
	Oestrogen receptor content by: Radioligand assay		Enzyme immunoassay	
	Negative	Positive	Negative	Positive
0/+	40	16	21	12
++/++	36	32	24	21
	0.05 < p < 0.1		p > 0.2	

is present in the assessed sample; of enabling an assessment to be made of tumour cell heterogeneity with respect to receptor content; and of avoiding the problem of false negative results when using homogenates due to dilution by stromal elements. Immunohistology and enzyme immunoassay with the oestrogen receptor monoclonal antibody also permit the detection and quantification of oestrogen receptor in tumours from premenopausal women. Endogenous oestrogens may saturate all the available oestrogen receptor in these lesions, thus often resulting in misleadingly low or negative radioligand binding assay results.

The D5 monoclonal antibody showed poor correlation with oestrogen receptor content, but this does not exclude the possibility that positive immunostaining for D5 antigen may have clinical and therapeutic importance.⁸

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