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NEW MONOCLONAL ANTIBODIES TO OESTROGEN AND PROGESTERONE RECEPTORS EFFECTIVE FOR PARAFFIN SECTION IMMUNOHISTOCHEMISTRY

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SUMMARY

Assessment of oestrogen and progesterone receptors (ER and PgR) in breast cancer is widely used for the prediction of response to endocrine therapy and as a prognostic marker. Cytosolic assays have been replaced in many centres by immunochemical techniques, which have many advantages including applicability to small samples, simplicity, and cost-effectiveness. This study describes the generation and characterisation of two novel murine monoclonal antibodies recognizing ER and PgR, designated NCL-ER-6F11 and NCL-PGR respectively, which are effective in heat-treated formalin-fixed, paraffin-embedded tissue. The antibodies have been characterized by Western blotting and by immunohistochemistry on normal and pathological breast and other tissues. NCL-ER-6F11 has been shown to compare favourably with a currently available ER antibody. These antibodies may prove of value in the assessment of hormone receptor status in human breast cancer. © **1997 John Wiley & Sons, Ltd.**

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KEY WORDS—**oestrogen receptor; progesterone receptor; monoclonal antibodies; paraffin-embedded tissue; breast cancer**

INTRODUCTION

The oestrogen receptor (ER) status of breast cancer is determined in many centres, principally on account of its value as a predictor of endocrine response on relapse,[1] although it has recently been shown that ER may also predict response to chemotherapy.[2] The failure of 50 per cent of women with ER-positive tumours to respond to endocrine therapy on relapse prompted investigations into the value of oestrogen-regulated proteins which might indicate a functional (and thus responsive) ER apparatus. Oestrogen-regulated proteins which have been evaluated include cathepsin D[3–5] and pS2.[6–8] Progesterone receptor (PgR) is also regulated by oestrogen[9] and has been shown to provide additional predictive power for likely response to endocrine therapy.[10]

In addition to its role as a predictor of endocrine response, ER has been shown to be a moderately powerful prognostic factor in breast cancer,[11,12] although some investigators have found that the effect is attributed to longer survival after relapse, perhaps related to superior endocrine response.[13] Other workers found that the predictive power of ER was confined to node-positive patients[14] and furthermore, the same group found that ER was not significant when considered in multivariate analysis.[15] Thus, the usefulness of assay of ER alone as a prognostic marker remains controversial and it has been claimed that ER estimation is not necessary in the routine management of breast cancer.[16] A number of groups have therefore evaluated oestrogen-regulated proteins in order to determine whether they can provide additional prognostic data. PgR does appear to be of predictive value either alone or combined with ER,[17,18] and indeed some investigators have found PgR to have greater power than ER in predicting disease-free interval.[19,20]

Until recently, hormone receptors were conventionally analysed by the dextran-coated charcoal (DCC) competitive binding assay. Immunohistochemical methods have been shown to correlate well with such techniques.[21,22] and indeed to have some advantages, allowing assessment of small biopsies and possibly providing a more accurate classification of receptor status.[23]

Our aim in the present study was to generate monoclonal antibodies to oestrogen and progesterone receptors which are effective in routinely processed tissue. When the present study was planned, monoclonal antibodies giving reliable results on paraffin sections in our laboratory were not available, although some investigators had reported satisfactory results with digestion techniques.[24–26] We initially raised antibodies to peptide antigens and successfully produced an anti-PgR monoclonal antibody, NCL-PGR, effective in paraffin-embedded material. The anti-ER monoclonal antibodies raised against peptides, however, were effective only in frozen tissue. We therefore produced recombinant ER protein and used this to generate monoclonal antibodies

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to the ER. The use of recombinant protein was judged to be likely to give a higher chance of success than the peptide antigen approach, since multiple epitopes are presented at immunization, theoretically resulting in a much greater number of potential hybridomas. Using this approach, we have been successful in producing an antibody effective in paraffin-embedded material, NCL-ER-6F11.

MATERIALS AND METHODS

PgR antigen: peptide preparation

Two peptides, representing sites of predicted high antigenicity on the human PgR molecule, were determined by the Protean I molecular modelling system (Proteus International plc.). The peptides were synthesized on a semi-automatic peptide synthesizer (Cambridge Research Biochemicals Pepsynthesiser II) using Fmoc chemistry.[27] The peptides were covalently bound to a carrier molecule, keyhole limpet haemocyanin (KLH), to increase their antigenicity. The peptides were also conjugated to bovine serum albumin (BSA) for immunoassay.[28,29] Conjugates were produced in small amounts and stored in 500 μl aliquots at -20°C.

ER antigen: recombinant protein preparation

Total RNA was extracted from MCF-7 cells with guanidinium thiocyanate and purified by ultracentrifugation in caesium chloride.[30] cDNA was prepared using the AMV Reverse Transcription System (Promega) and the ER gene was amplified by polymerase chain reaction (PCR). The DNA sequence of the PCR product was determined to confirm the integrity of the amplification reaction. The ER gene was ligated into plasmid pET15b, allowing expression of a $(His)_6$-ER fusion protein in *Escherichia coli* strain BL21 (DE3) (Novagen). The His-tagged ER was purified on His-bind resin (Novagen), according to the manufacturer's instructions. The ER gene was also ligated into plasmid pMalC2 (New England Biolabs) and expressed as a maltose-binding protein–ER (MBP–ER) fusion protein for immunoassays. The MBP–ER fusion was purified by maltose affinity. Purified protein was stored at -70°C.

Immunization and hybridoma fusion

Balb/c mice were immunized with PgR peptides or $(His)_6$-ER recombinant antigen. Spleen cells were then fused, using polyethylene glycol, with P3-Ns1/1-Ag-1 cells and plated out into HAT selective growth medium. Cultures were incubated for 10–14 days, or until colony growth was apparent.

Screening

Supernatant from each well was removed and assayed by enzyme-linked immunosorbent assay (ELISA) for reactivity against the appropriate antigen. Individual colonies from positive wells were transferred to separate wells in 24-well growth plates. After further incubation, supernatant from each well was assayed by ELISA. ELISA-positive supernatants were tested immunohistochemically using an indirect immunoperoxidase technique on formalin-fixed, paraffin-embedded sections of breast carcinoma of known receptor status. Colonies demonstrating positive immunohistochemical staining were cloned by limiting dilution.

Immunohistochemistry

Routinely processed formalin-fixed, paraffin-embedded tissue from the files of the Department of Pathology, Royal Victoria Infirmary was employed for assessment of immunostaining. The staining pattern in breast cancer of known hormone receptor status was assessed and labelling of non-neoplastic breast epithelial elements was also evaluated. A range of normal tissues was also assessed: endometrium, tonsil, skin, and skeletal muscle. For comparative studies, a series of 55 sequential breast carcinomas were stained using both NCL-ER-6F11 and monoclonal antibody ER1D5 (Dako Ltd.). Staining in these cases was assessed using the 'quickscore' method.[41]

For paraffin section immunohistochemistry, 5 μm sections were mounted on slides coated with Vectabond (Vector Laboratories, CA, U.S.A.). Sections were dewaxed, rehydrated, and treated with H_2O_2, then washed in running tap water. Prior to addition of the primary antibody supernatant, sections were heat-treated in citrate buffer, using a microwave oven[31,32] on high power for 2×5 min followed by a 20 min standing time. Sections were blocked with normal goat serum, diluted 1:5 in TBS (dNGS), before incubation with the primary antibody, diluted in dNGS. NCL-ER-6F11 was diluted 1:20 and NCL-PGR was diluted 1:40. Frozen section immunohistochemistry was employed during the initial screening of supernatants. Five micrometre sections were thaw-mounted and supernatants were applied neat. Sections were washed in TBS and then incubated with goat anti-mouse IgG (NCL-GAMP, Novocastra Laboratories Ltd., U.K.) diluted 1:40 in dNGS. After further washing in TBS, bound antibody was revealed using a DAB/H_2O_2 substrate.

Western blot analysis

MCF-7 cell proteins were separated by SDS polyacrylamide gel electrophoresis on a 10 per cent gel and transferred to nitrocellulose membrane using a Biorad SD semi-dry electrophoretic transfer cell. The blots were blocked in phosphate-buffered saline/0·1 per cent Tween 20 (PBST) containing 1 per cent BSA and then incubated with the primary antibody, diluted 1:100 in PBST/10 per cent fetal calf serum (FCS). After washing in PBST, the blots were incubated with rabbit anti-mouse IgG alkaline phosphatase conjugate (Dako), diluted 1:1000 in PBST/10 per cent FCS. The blots were again washed in PBST, prior to detection of bound antibody using bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT) as the chromogen.

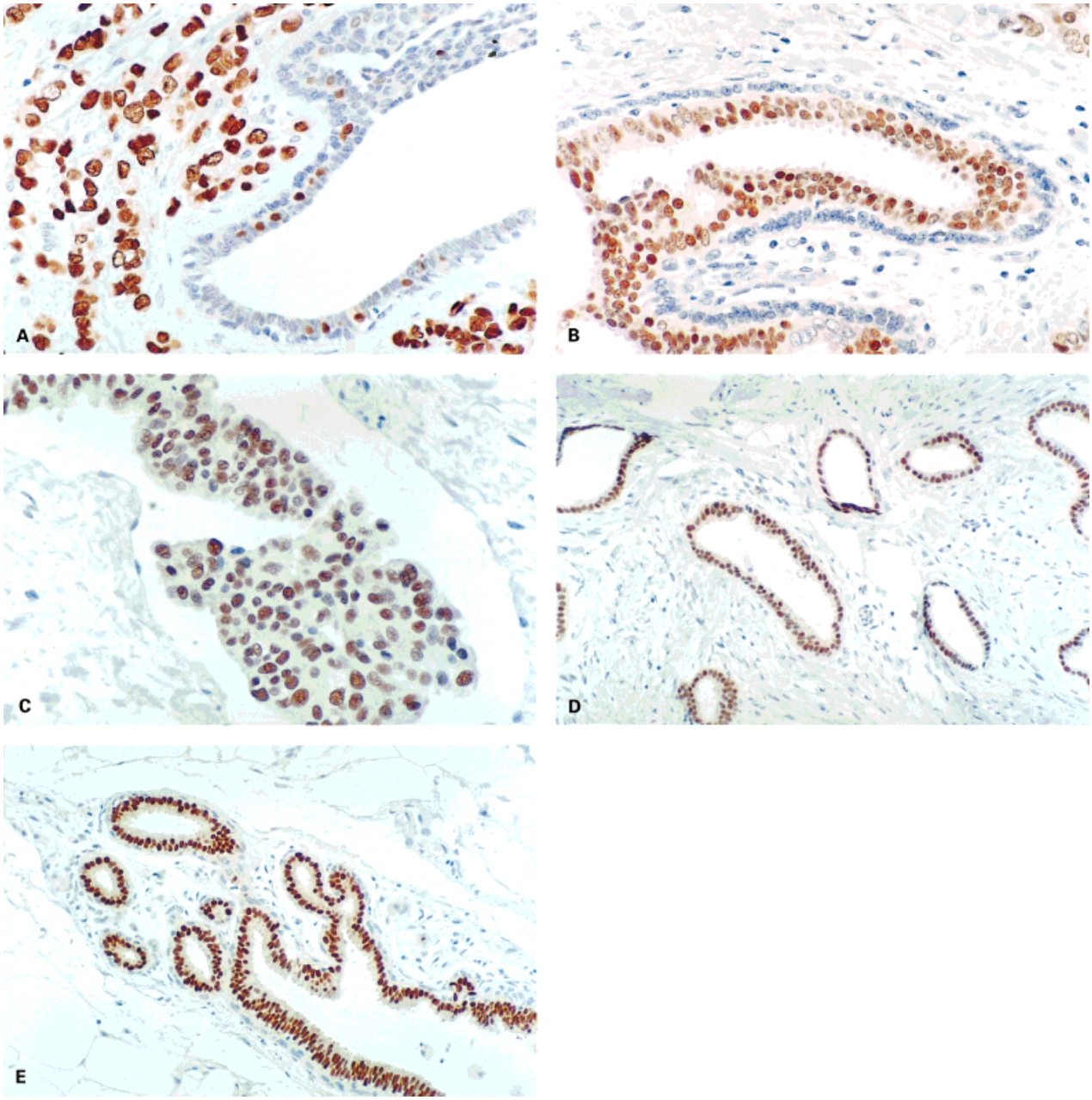

Fig. 1—(A) Grade 2 invasive ductal carcinoma of the breast. Immunohistochemical staining for PgR using NCL-PGR. Note the intense staining of tumour cell nuclei. (B) Same case as A. Immunohistochemical staining for PgR using NCL-PGR. This field shows a normal duct. Note the staining of epithelial cell nuclei, with absence of labelling of the myoepithelial layer. Compare with the non-neoplastic duct in A where only a few epithelial cells show staining. (C) Grade 3 invasive ductal carcinoma of the breast. Immunohistochemical staining for ER using NCL-ER-6F11. Note the intense nuclear staining of cells within this intravascular focus of tumour. (D) Tubular carcinoma of the breast. Immunohistochemical staining for ER using NCL-ER-6F11. Note the intense staining of tumour cell nuclei. (E) Non-neoplastic breast ducts. Immunohistochemical staining for ER using NCL-ER-6F11. Note the staining of the epithelial cell nuclei. This feature can usually be identified in breast tissue and serves as a useful in-built positive control (see also B above)

RESULTS

Monoclonal antibody production and characterization

Anti-ER monoclonal antibody NCL-ER-6F11 and anti-PGR monoclonal antibody NCL-PGR were produced as described. Both antibodies gave strong nuclear staining of receptor-positive breast cancers in heat-treated, formalin-fixed paraffin-embedded tissue sections (Fig. 1). Labelling of a proportion of non-neoplastic lobular or ductal cells was observed when these elements were present on the tissue section. Strong staining was also observed in tissues known to express high levels of ER and PgR; for example, glandular epithelial cells of endometrium. No staining was observed in any tissues which do not express ER or PgR; for example, tonsil, skin, and skeletal muscle. Both antibodies were also effective for immunohistochemistry using frozen sections of normal breast and breast carcinomas.

Western blotting studies showed in each case reactivity with molecules of the appropriate molecular

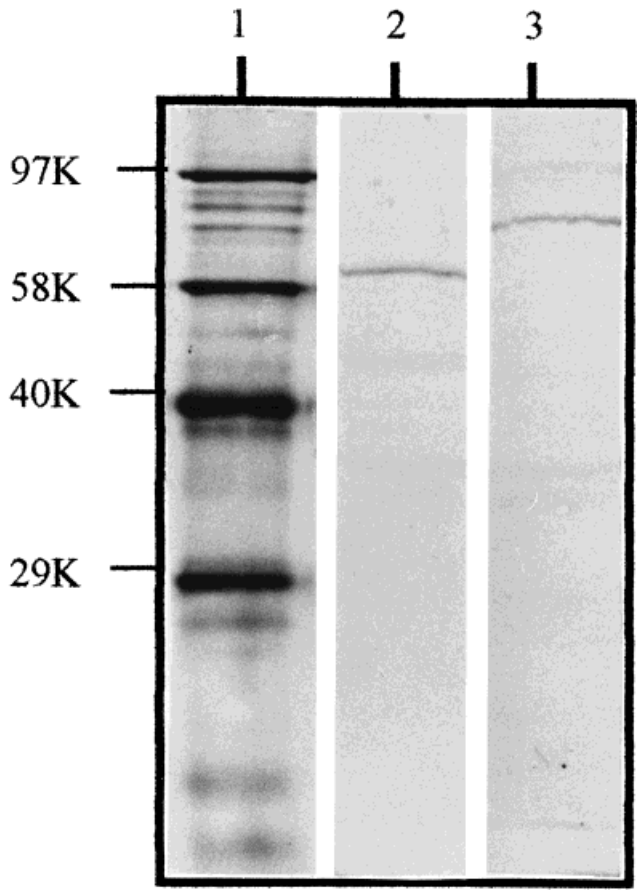

Fig. 2—Western blot of MCF-7 cell lysate. Lane 1: molecular weight markers; lane 2: MCF-7 cell lysate probed with NCL-ER-6F11; lane 3: MCF-7 cell lysate probed with NCL-PGR

weight (Fig. 2). NCL-PGR reacted with bands at 87 and 110 kD and NCL-ER-6F11 reacted with a band at 66 kD.

In comparative studies, 69 per cent of the 55 cases scored positive (quickscores>0) using NCL-ER-6F11 and 60 per cent scored positive with ER1D5. There was complete concordance of staining for 50 cases. The five discrepancies scored positive for NCL-ER-6F11 but negative for ER1D5. The mean quickscore was 8·4 for NCL-ER-6F11 and 5·5 for ER1D5.

DISCUSSION

The monoclonal antibodies generated in this study, NCL-PGR and NCL-ER-6F11, recognizing progesterone receptor and oestrogen receptor respectively, have been characterized. Both show appropriate tissue reactivity, giving nuclear staining in the epithelial cells of tissues known to express PgR and ER and each exhibits reactivity with a predicted band on Western blotting using cell lines known to express PgR and ER. Both NCL-PGR and NCL-ER-6F11 are also effective for frozen section immunohistochemistry. The anti-ER monoclonal antibody H222, available as the ER-1CA kit (Abbott), was the first to be shown to work in paraffin-embedded tissue, but monoclonal antibody ER1D5 (produced by Dako, using recombinant ER) has proved more robust.[33,34] In a comparative study between ER1D5 and NCL-ER-6F11, a high degree of correlation was obtained. The mean quickscore for NCL-ER-6F11 was higher than that for ER1D5, and in a small number of cases, NCL-ER-6F11 gave positive results where no staining was observed with ER1D5. Whilst this result suggests that NCL-ER-6F11 may be a superior reagent, it must be conceded that it is likely that the staining conditions used would favour an antibody generated in our own laboratory and further comparative studies in other laboratories will be required. Antibodies recognizing PgR effective on paraffin sections have also been previously described. The most widely used PgR antibody is clone KB68,[35] produced using partially purified receptor. Comparative studies will be required to establish the efficacy of NCL-PGR compared with this reagent and with cytosolic assays.

The present paper does not address the clinical usefulness of these antibodies. However, in a separate study of 294 cases of breast carcinoma stained for ER and PgR using the antibodies described here and scored by the category score system,[36] we found that 67 and 51 per cent were positive for Er and PgR respectively, which is in accord with previous studies.[37] Staining for ER and PgR correlated strongly, with 49 per cent of cases positive for both antibodies.

Although the value of routine hormone receptor assessment in breast cancer has been questioned,[16] other groups feel that their determination is useful in certain circumstances.[36,38] In addition, the finding in a large meta-analysis that adjuvant tamoxifen is of doubtful value in ER-negative premenopausal women suggests that the analysis could be of clinical value in this subgroup.[39] Using paraffin section immunohistochemistry, it is possible to generate information on the Er and PgR status of tumours very simply and quickly. Furthermore, paraffin section immunohistochemistry for ER has recently been shown to be superior to frozen section immunohistochemistry or ligand binding assays for prediction of response to endocrine therapy.[40] The value of immunohistochemical estimation of both receptors as opposed to ER alone has been demonstrated by Barnes and Millis,[36] who showed significant enhancement of predictive power for response to tamoxifen on relapse, when compared with ER alone. We conclude that assessment of hormone receptors in breast cancer may be of value in patient management and that the monoclonal antibodies described here may prove to be effective reagents in the determination of oestrogen and progesterone receptor status.

REFERENCES

1. Hawkins RA, Roberts MM, Forrest APM. Oestrogen receptors and breast cancer: current status. *Br J Surg* 1980; **67:** 153–169.
2. Scottish Cancer Trials Breast Group and ICRF Breast Unit GH London. Adjuvant ovarian ablation versus CMF chemotherapy in premenopausal women with pathological stage II breast carcinoma: the Scottish trial. *Lancet* 1993; **341:** 1293–1298.
3. Maudelonde T, Khalaf S, Garcia M, *et al*. Immunoenzymatic assay of Mr 52,000 cathepsin D in 182 breast cancer cytosols: low correlation with other prognostic parameters. *Cancer Res* 1988; **48:** 462–466.

4. Henry JA, McCarthy AL, Angus B, *et al*. Prognostic significance of the estrogen-regulated protein, cathepsin D, in breast cancer. An immunohistochemical study. *Cancer* 1990; **65:** 265–271.
5. Tetu B, Brisson J, Cote C, Brisson S, Potvin D, Roberge N. Prognostic significance of cathepsin-D expression in node-positive breast carcinoma: an immunohistochemical study. *Int J Cancer* 1993; **55:** 429–435.
6. Luqmani YA, Ricketts D, Ryall G, Turnbull L, Law M, Coombes RC. Prediction of response to endocrine therapy in breast cancer using immunocytochemical assays for pS2, oestrogen receptor and progesterone receptor. *Int J Cancer* 1993; **54:** 619–622.
7. Henry JA, Piggott NH, Mallick UK, *et al*. pNR-2/pS2 immunohistochemical staining in breast cancer: correlation with prognostic factors and endocrine response. *Br J Cancer* 1991; **63:** 615–622.
8. Hurlimann J, Gebhard S, Gomez F. Oestrogen receptor, progesterone receptor, pS2, ERD5, HSP27 and cathepsin D in invasive ductal breast carcinomas. *Histopathology* 1993; **23:** 239–248.
9. Horwitz KB, McGuire WL. Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor *J Biol Chem* 1978; **252:** 2223–2228.
10. Clark GM, McGuire WL. The clinical usefulness of oestrogen-receptor and other markers of hormone dependence. *Proc R Soc Edinburgh* 1989; **95B:** 145–150.
11. Henry JA, Angus B, Horne CHW. Oestrogen receptor and oestrogen regulated proteins in human breast cancer: a review. *Keio J Med* 1989; **38:** 241.
12. Rayter Z. Steroid receptors in breast cancer. *Br J Surg* 1991; **78:** 528–535.
13. Howell A, Barnes DM, Harland RNL, *et al*. Steroid-hormone receptors and survival after first relapse in breast cancer. *Lancet* 1984; **1:** 588–591.
14. Williams MR, Todd JH, Ellis IO, *et al*. Estrogen receptors in primary and advanced breast cancer: an eight year review of 704 cases. *Br J Cancer* 1987; **55:** 67–73.
15. Galea MH, Blamey RW, Elston CE, Ellis IO. The Nottingham Prognostic Index in primary breast cancer. *Breast Cancer Res Treatment* 1992; **22:** 207–219.
16. Barnes DM, Millis RR, Fentiman IS, Rubens RD. Who needs steroid receptor assays? *Lancet* 1989; **1:** 1126–1127.
17. Mason BH, Holdaway IM, Mullins PR, Yee LH, Kay RG. Progesterone and estrogen receptors as prognostic variables in breast cancer. *Cancer Res* 1983; **43:** 2985–2990.
18. Chevallier B, Heintzmann F, Mosseri V, *et al*. Prognostic value of estrogen and progesterone receptors in operable breast cancer. Results of a univariate and multivariate analysis. *Cancer* 1988; **62:** 2517–2524.
19. Clark GM, McGuire Wl, Hubay CA, Pearson OH, Marshall JS. Progesterone receptors as a prognostic factor in stage II breast cancer. *N Engl J Med* 1983; **309:** 1343–1347.
20. Thorpe SM, Rose C, Rasmussen BB, *et al*. Prognostic value of steroid hormone receptors: multivariate analysis of systemically untreated patients with node negative primary breast cancer. *Cancer Res* 1987; **47:** 6126–6133.
21. Hawkins RA, Sangster K, Krajewski A. Histochemical detection of oestrogen receptors in breast carcinoma: a successful technique. *Br J Cancer* 1986; **53:** 407–410.
22. Giri DD, Dangerfield VJM, Lonsdale R, Rogers K, Underwood JCE. Immunohistology of oestrogen receptor and D5 antigen: correlations with radioligand binding assays and enzyme immunoassays. *J Clin Pathol* 1987; **40:** 734–740.
23. Thorpe SM. Monoclonal antibody technique for detection of oestrogen receptors in human breast cancer: greater sensitivity and more accurate classification of receptor status than the dextran-coated charcoal method. *Cancer Res* 1987; **47:** 6572–6575.
24. Soomro S, Shousha S. Demonstration of progesterone receptors in paraffin wax sections of breast carcinoma. *J Clin Pathol* 1990; **43:** 671–674.
25. Snead DRJ, Bell JA, Dixon AR, *et al*. Methodology of immunohistological detection of oestrogen receptor in human breast carcinoma in formalin-fixed, paraffin-embedded tissue: a comparison with frozen section methodology. *Histopathology* 1993; **23:** 233–238.
26. Raymound WA, Leong AS-Y. Oestrogen receptor staining of paraffin-embedded breast carcinomas following short fixation in formalin: a comparison with cytosolic and frozen section receptor analyses. *J Pathol* 1990; **160:** 295–303.
27. Chang C-D, Meienhofer J. Solid-phase peptide synthesis using mild base cleavage of *N*-fluorenylmethyloxycarbonyl amino acids, exemplified by a synthesis of dihydrosomatostatin. *In J Peptide Protein Res* 1978; **11:** 246–249.
28. Liu F-T, Zinnecker M, Hamaoka T, Katz DH. New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. *Biochemistry* 1979; **18:** 690–697.
29. Green N, Alexander H, Olsen A, *et al*. Immunogenic structure of the influenza virus hemagglutinin. *Cell* 1982; **28:** 477–487.
30. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning; A Laboratory Manual. 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989: 7.19–7.22.
31. Cuevas EC, Bateman AC, Wilkins BS, *et al*. Microwave antigen retrieval in immunocytochemistry: a study of 80 antibodies. *J Clin Pathol* 1994; **47:** 448–452.
32. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin fixed paraffin embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J Histochem Cytochem* 1991; **39:** 741–748.
33. AlSaati T, Clamens S, Cohen-Knafo E, *et al*. Production of monoclonal antibodies to human estrogen-receptor protein (ER) using recombinant ER (RER). *Int J Cancer* 1993; **55:** 651–654.
34. Saccani Jotti G, Johnston SRD, Salter J, *et al*. Comparison of new immunohistochemical assay for oestrogen receptor in paraffin wax embedded breast carcinoma tissue with quantitative enzyme immunoassay. *J Clin Pathol* 1994; **47:** 900–905.
35. Press MF, Udove JA, Greene GL. Progesterone receptor distribution in the human endometrium. *Am J Pathol* 1988; **131:** 112–124.
36. Barnes DM, Millis RR. Progress in Pathology 2. Edinburgh: Churchill Livingstone, 1995; 89–114.
37. Al-Tamimi S, Cowan WK, Horne CHW, Angus B. Are interval cancers detected in breast screening programmes biologically distinct from those presenting clinically: an immunohistochemical study. Manuscript in preparation.
38. Forrest APM, Hawkins RA, Anderson EDC, Chetty U, Stewart HJ. Who needs steroid receptor assays? *Lancet* 1989; **2:** 40.
39. Early Breast Cancer Trialists' Collaborative Group. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. *Lancet* 1992; **339:** 1–15, 71–85.
40. Pertshuk LP, Feldman JG, Kim YD, *et al*. Estrogen receptor immunocytochemistry in paraffin embedded tissues with ER1D5 predicts breast cancer endocrine response more accurately than H222Spγ in frozen sections or cytosol based ligand binding assays. *Cancer* 1996; **77:** 2514–2518.
41. Detre S, Saccani Jotti G, Dowsett M. A 'quickscore' method for immunohistochemical semiquantitation: validation for oestrogen receptor in breast carcinomas. *J Clin Pathol* 1995; **48:** 876–878.