24. Tanigawa N, Morimoto H. Colony-forming ability *in vitro* and clonology of colorectal cancer. *Jpn J Cancer Res* 1990, **81**, 687–691.
25. Tanigawa N, Morimoto H. Significance of surgical adjuvant chemotherapy for gastric cancer. *J Surg Oncol* 1991, **46**, 203–207.
26. Preusser P, Wilke HM, Achterrath W, *et al.* Advanced inoperable stomach cancer: a pilot study with the combination etoposide, adriamycin, and cisplatin. *Anticancer Res* 1986, **6**, 1195–1196.
27. Shutt AJ. Chemotherapy of gastrointestinal neoplasms. In: Brodsky I, Benhamkahn S, Conroy JF, eds. *Cancer Chemotherapy* III. Orlando, Grune and Stratton, 1978, 135–142.

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Heterogeneity of Oestrogen Receptor Expression in Normal and Malignant Breast Tissue

K.J. Walker, R.A. McClelland, W. Candlish, R.W. Blamey and R.I. Nicholson

The heterogeneity of oestrogen receptor (ER) expression has been examined in both normal and malignant breast tissue using an immunohistochemical assay. In both instances the ER status and cellular ER negativity were influenced by the patients' menopausal status, with tissues removed from premenopausal women being more often ER-negative, and when ER-positive, containing a high proportion of apparently ER-negative cells. Since the breast is normally regarded as hormone sensitive and since tumour cell ER negativity is apparently under a degree of hormonal regulation, our results suggest that the proportion of breast cancer cells that are ER-negative should be viewed with a degree of caution.
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INTRODUCTION

IMMUNOCYTOCHEMICAL STUDIES on the distribution of oestrogen receptors (ER) in breast cancer specimens have revealed considerable heterogeneity of receptor expression [1, 2]. These tumours may show variation in the quantities of antibody-detected receptor per cell and in the ratio of positive to apparently negative cells. Clinically, these observations are of potential interest since whole tumour receptor negativity has previously been equated with hormone insensitivity and would imply that apparently negative cells within ER-positive breast cancers might also carry with them a poor prognosis for the patient. Indeed, preliminary data from this laboratory have tended to support this notion, with high rates of ER negativity in breast cancer cells being linked to an increased likelihood of a tumour being poorly differentiated, fast growing [1] and hormone-insensitive [3].

Since the therapeutic implications of equating cellular ER negativity in overtly ER-positive tumours with tumour cell autonomy are far reaching and might include combining endocrine and cytotoxic therapeutic regimens, it is essential to eliminate all alternative explanations before clinically acting on this new information. In this light we have undertaken a comparative investigation of the distribution of ER in normal and malignant breast tissues under the physiological influence of the menopause. Our data justify a cautious approach to the interpretation of tumour cell ER negativity and highlight the need for additional markers of hormone sensitivity and independence.

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PATIENTS AND METHODS

218 breast tissue sections from patients of known menopausal status who presented with either primary breast cancer (City Hospital, Nottingham), benign disease (Royal Infirmary, Glasgow) or for reduction mammoplasty (St Lawrence Hospital, Chepstow) were analysed for the presence of ER using the Abbott ER-ICA [1]. Tissue biopsies were trimmed of fat and connective tissue and immediately frozen in liquid nitrogen and stored at −70°C. Specimens were processed according to the Abbott ER-ICA monoclonal kit (Abbott Laboratories, North Chicago, Illinois).

Briefly, tissue sections were cut at 5 μm thickness at −20°C and mounted onto glass slides coated with tissue adhesive. Tissues were fixed by placing them in 3.7% formaldehyde in phosphate-buffered saline (PBS) (v/w) solution for 15 min. The slides were then rinsed in PBS for 5 min and stored in ER-ICA specimen storage medium at −20°C for up to 1 month before assay. At the time of assay the specimens were first washed in PBS prior to the addition of blocking agent (normal goat serum). After incubation for 15 min in a humidified chamber, excess blocking agent was removed. The primary antibody (monoclonal rat anti-human ER H222) was added dropwise to one slide of each specimen and incubated for a further 30 min. Non-specific staining was controlled using parallel sections incubated with an inappropriate normal rat immunoglobulin. Slides were washed twice in PBS for 5 min before incubation with first the bridging antibody (goat anti-rat IgG) for 30 min followed by rat peroxi-

dase/anti-peroxidase (PAP) complex. A chromagen substrate solution containing diaminobenzidine tetrahydrochloride and H_2O_2 was added to each specimen for 6 min. The reaction of peroxidase in the PAP complex with hydrogen peroxide converts the DAB to an insoluble reddish brown pigment which may be visualised under the light microscope. Slides were immersed in distilled water before counterstaining with Harris' haematoxylin (1% aqueous) for 6 min. Sections were rinsed in tap water for 5 min, dehydrated in alcohol, cleared in xylene and mounted under cover slips in dibutylpthalate xylene solution.

Specimens were evaluated as previously described [1, 4], with sections examined on an Olympus microscope using a magnification of ×40. Control slides were checked for non-specific binding before assessing the percentage of epithelial cells stained by the primary antibody. Tissues were classified as ER-ICA-positive where greater than 2% of the epithelial cells (tumour or normal) were stained positively. A further subdivision was made at 50% cellular ER negativity to facilitate the analysis [1]. The predominant intensity of ER staining in target cells was assessed semiquantitatively using a scoring system of 0, 1, 2 and 3 corresponding to negative, weak, intermediate and strong staining, respectively. From this an index (I) value was calculated using the equation: I = [(% cells showing an intensity value of 1 × 1) + (% cells showing an intensity value of 2 × 2) + (% cells showing an intensity value of 3 × 3)] ÷ 100.

RESULTS

The ER-ICA procedure was applied to 218 breast specimens, including 144 histologically proven carcinomas. Normal ducts and lobules were removed from patients undergoing breast reduction and from biopsied material from both benign and malignant breasts. In all specimens examined staining, where present, was confined to the nuclei of luminal epithelial cells. No staining was localised in the cell cytoplasm or in myoepithelial and stromal cells.

The immunocytochemical localisation of ER in breast tumours revealed specific binding in the nuclei of 97 of 144 (67%) tumours. Both the proportion of tumour nuclei expressing the antigen and the intensity of stain were highly variable (Fig. 1a). A higher proportion of ER-ICA negative tumours were recorded in premenopausal women (41%) than in postmenopausal (29%) patients (Table 1). This trend was also reflected in the proportion of negative cells detected within ER-ICA positive tumours. Thus, while 16 of the 26 (62%) ER-ICA positive tumours examined in premenopausal women contained more than 50% ER-ICA negative cells, only 21/71 (30%) of ER-positive tumours in postmenopausal patients fell into this category. Interestingly, the increased ER negativity observed in tumours from premenopausal women was also accompanied by an apparent decrease in the intensity of ER expression in positive cells, with the mean index value (sum of the proportion of cells staining at various intensity levels) in premenopausal women being lower than the value recorded in postmenopausal women (0.41 and 0.76, respectively).

Examination of ER-ICA staining in the ductal components of normal breast tissue (Fig. 1b) revealed a similar proportion of wholly ER-ICA negative structures to those seen in breast tumours removed from pre and postmenopausal women (Table 1). Ductal structures, however, contained a higher proportion of specimens with increased numbers of ER negative cells (>50% negative cells). This was again observed in both pre and postmenopausal women (normal, 88% and 57%, vs. tumour, 62% and 30%, respectively). The ER-ICA index values recorded

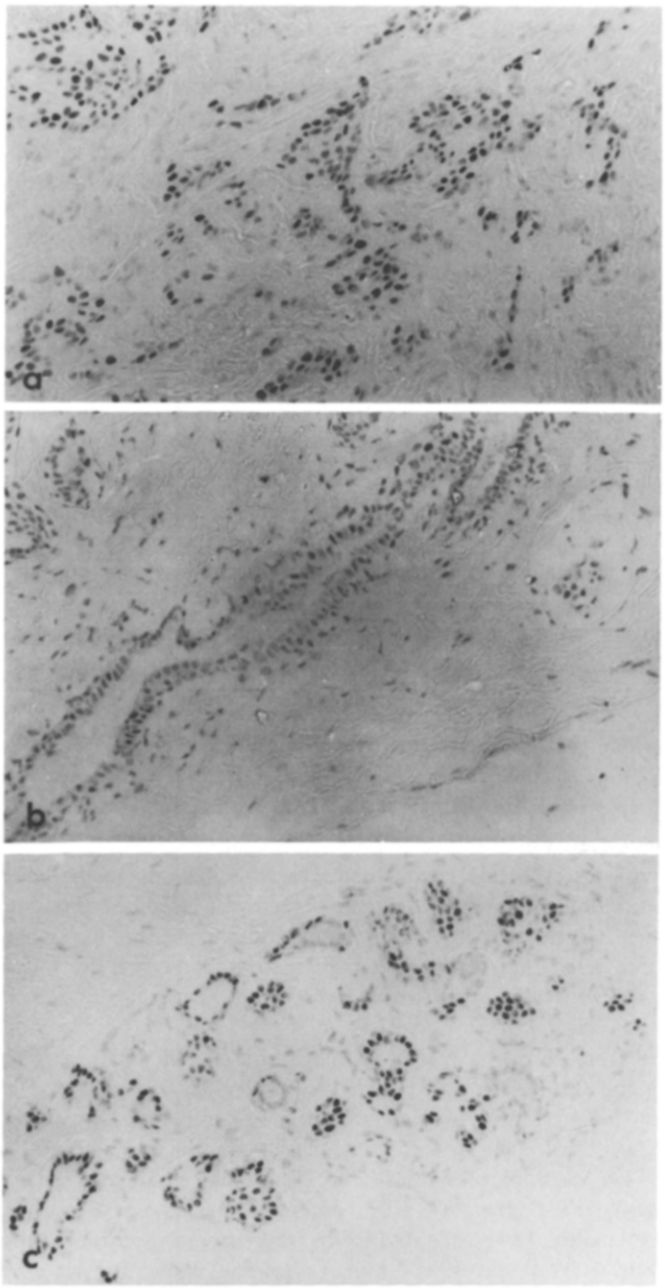

Fig. 1. Oestrogen receptor expression in (a) an infiltrating breast cancer, (b) in a breast duct (c) and in lobular tissue.

for these specimens were lower than those observed in the malignant tissues. Although a lower proportion of the lobules (Fig. 1c) examined were wholly ER-ICA negative in comparison with both normal ductal structures and the breast carcinomas, nevertheless, the ER-positive group appeared to contain an elevated proportion of ER-negative cells relative to the tumour group.

Examination of a small number of benign tissues in premenopausal women revealed a mean % ER-ICA negativity of approximately 25% and low numbers of highly ER-ICA negative structures (>50% negative cells). This was especially evident in fibroadenomas.

DISCUSSION

The data presented in Table 1 confirms our previous observation that many overtly hormone receptor positive breast tumours contain ER-ICA negative cells [1]. This phenomenon,

Table 1. ER-ICA results by menopausal status

Breast tissue	ER-ICA status		Number of ER-ICA positive tissues containing		(S.D.) Mean index value for ER +ve tissues
	Negative	Positive	<50% (−) cells	>50% (−) cells	
Tumours					
Premenopausal	18 (41%)	26	10	16 (62%)	0.41 (0.40)
Postmenopausal	29 (29%)	71	50	21 (30%)	0.76 (0.60)
Ducts					
Premenopausal	11 (41%)	16	2	14 (88%)	0.21 (0.35)
Postmenopausal	7 (33%)	14	6	8 (57%)	0.25 (0.31)
Lobules					
Premenopausal	1 (8%)	10	3	7 (70%)	0.33 (0.40)
Postmenopausal	3 (21%)	11	6	5 (45%)	0.49 (0.50)
Adenosis/blunt duct adenosis/microcysts					
Premenopausal	3 (27%)	8	5	3 (27%)	0.53 (0.05)
Cysts					
Premenopausal	2 (25%)	6	4	2 (33%)	0.23 (0.25)
Fibroadenomas					
Premenopausal	2 (29%)	5	5	0 (0%)	0.25 (0.38)

however, was not restricted to breast cancer specimens and was also observed in normal breast structures. Indeed, both ductal and lobular components of the breast were often either wholly ER-ICA negative or contained large numbers of ER-ICA negative cells. Interestingly, ER expression in both normal and cancerous breast tissue was influenced by the menopausal status of the patient, with increased numbers of ER-ICA negative cells, and possibly lower levels of ER expression in positive cells, being detected in premenopausal women. These data suggest an underlying physiological control of cellular ER negativity which is common to both the normal breast and its cancers.

Significantly, previous studies examining the influence of the menstrual cycle on ER expression in normal breast tissue using cytological samples obtained by fine needle aspiration have shown the presence of ER in breast epithelial cells was only evident during the follicular phase of the menstrual cycle [5]. The absence of ER observed during the luteal phase of the menstrual cycle was attributed to the ability of progesterone to down-regulate the expression of this protein [6]. Thus increasing circulatory levels of progesterone, resulting from the formation of an active corpus luteum, were envisaged as a direct means of suppressing tissue ER levels. Although Markopoulos *et al.* [5] failed to observe a similar cyclical regulation of ER expression in premenopausal breast tumours, lowered circulatory levels of progesterone following the menopause may contribute to the increased ER levels.

It is important to appreciate that within the context of our study a failure of the immunohistochemical assay to detect ER should not be directly equated with ER negativity. Thus it is possible that a proportion of the ER-ICA negative cells are expressing levels of receptors which are below the sensitivity of the assay. Indeed, while the application of the highly sensitive ER-enzyme immunoassay to breast tumours shows that over 85% of specimens contain more than 2 fmol/mg protein, application of the ER-ICA assay to the same specimens produces a 65–70% positivity rate [1]. Since this rate may also be achieved using a cut-off for the ER-EIA of 15 fmol/mg protein, it may be argued that the ER-ICA assay is incapable of detecting receptor concentrations of below 15 fmol/mg protein. These levels, although low, may still be sufficient to mediate some of the cellular responses to oestrogens.

Based on the above, it is therefore an interesting possibility that the large numbers of apparently negative cells observed in normal breast tissue, and possibly also in cancers, are resting or dormant cells which given an appropriate stimulus are capable of entering cell cycle and expressing higher levels of hormone receptors. Certainly, with respect to normal mature breast tissue, rates of cell proliferation are relatively low and do not involve the majority of cells [6, 7]. Our own data on Ki67 immunostaining within the mature breast have revealed that this antibody, which detects cells in cell cycle but not in G_0 [8], is observed in <5% of normal epithelia from premenopausal women, indicating that the majority of cells are relatively quiescent [9]. Indeed, even in breast tumours where we have observed elevated Ki67 staining to be associated with early recurrence of breast cancer after mastectomy, the average percentage of cells exhibiting Ki67 staining is below 20%, leaving more than 80% of tumour cells in a resting stage [10].

It is possible that the further characterisation of the ER-negative cell population in normal tissue may provide useful markers of a similar phenotype in breast tumours. The presence of a dormant or resting ER-negative, yet hormone-responsive cell population in breast tumours may explain why some ER-negative and low ER-positive tumours respond well to endocrine measures [3]. These data, therefore, suggest that until true tumour cell autonomy can be distinguished from cellular dormancy a degree of caution should be applied to the interpretation of ER negativity in breast tumour cells.

1. Walker KJ, Bouzubar N, Robertson J, *et al.* Immunocytochemical localisation of estrogen receptor in human breast tissue. *Cancer Res* 1988, **48**, 6517–6522.

2. De Sombre ER, Thorpe SM, Rose C, *et al.* Prognostic usefulness of estrogen receptor immunocytochemical assays for human breast cancer. *Cancer Res* 1986, **46** (Suppl.), 4256–4264.
3. Nicholson RI, Bouzubar N, Walker KJ, *et al.* Hormone sensitivity in breast cancer: influence of heterogeneity of oestrogen receptor expression and cell proliferation. *Eur J Cancer* 1991, **27**, 908–913.
4. McCelland RA, Finlay P, Walker KJ, *et al.* Automated quantitation of immunocytochemically localised estrogen receptors in human breast cancer. *Cancer Res* 1990, **50**, 3545–3550.
5. Markopoulos C, Berger U, Wilson P, Gazet J-C, Coombes RC. Oestrogen receptor content of normal breast cells and breast carcinomas throughout the menstrual cycle. *Br Med J* 1988, **296**, 1349–1351.
6. Going JJ, Anderson TJ, Battersby S, Macintyre CCA. Proliferation and secretory activity in human breast during natural and artificial menstrual cycles. *Am J Pathol* 1988, **130**, 193–204.
7. Meyer JS. Cell proliferation in normal human breast ducts, fibroadenomas, and other ductal hyperplasias measured by nuclear labelling with tritiated thymidine. *Human Pathol* 1977, **8**, 67–81.
8. Gerdes J, Lemke H, Baisch H, *et al.* Cell cycle analysis of a cell proliferation associated human nuclear antigen defined by the monoclonal antibody Ki_{67}. *J Immunol* 1984, **133**, 1710–1715.
9. Walker KJ, Price-Thomas MJ, Candlish W, Nicholson RI. Influence of the antioestrogen tamoxifen on normal breast tissue. *Br J Cancer* (in press).
10. Bouzubar N, Walker KJ, Griffiths K, *et al.* Ki_{67} immunostaining in primary breast cancer. Pathological and clinical associations. *Br J Cancer* 1989, **59**, 943–947.

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Specific Binding of Anti-N-acetyllactosamine Monoclonal Antibody 1B2 to Acute Myeloid Leukaemia Cells

Lydia Campos, Jacques Portoukalian, Sylvie Bonnier, Zhi Hua Shi, Pascale Calmard-Oriol, Danielle Treille and Denis Guyotat

1B2 is an IgM monoclonal antibody binding to glycoconjugates bearing the terminal N-acetyllactosamine structure. It agglutinates human erythrocytes. Various cell lines, peripheral blood leucocytes, normal marrow and blast cells from 179 acute myeloid leukaemia (AML) and 11 acute lymphoblastic leukaemia (ALL) patients were tested for reactivity with 1B2. Myelomonocytic (CFU-GM), erythroid (BFU-E), mixed (CFU-GEMM) and leukaemic (CFU-L) progenitor cells were tested in clonogenic assays. Granulocytes, monocytes, myeloid cell lines and 152 out of 179 AML were positive. All FAB subtypes were equally recognised. Lymphocytes, T-cell and Burkitt's cell lines, and 10 of 11 ALL samples were negative. 1B2 inhibited partially day 7 CFU-GM, whereas it was not toxic for BFU-E, CFU-GEMM and day 14 CFU-GM. Leukaemic clonogenic cells were killed in 33 out of 36 AML (more than 40% growth inhibition). 1B2 identifies the more mature steps of myeloid differentiation. It may be useful in the diagnosis of AML, and is a candidate for remission marrow purging before autologous transplantation.
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INTRODUCTION

MONOCLONAL ANTIBODIES (Mabs) reactive with myeloid antigens are interesting to identify acute myeloid leukaemias (AML) [1] and a few antibodies directed to antigens expressed on AML cells but not on normal precursor cells have been tested for their ability to purge the graft prior to autologous bone marrow transplantation [2–6]. Many tissue-specific and tumour-specific mouse Mabs recognise carbohydrate determinants such as those of cluster of differentiation (CD) 15 [7] and MY28 [8]. The present report describes the reactivity of a mouse monoclonal antibody, 1B2, directed to glycolipids having the nonreducing terminal N-acetyllactosamine structure [9]. 1B2 identified early myeloid cells, monocytes and granulocytes but not the cells of lymphoid lineage. The reactivity with acute myeloblastic (AML) and acute lymphoblastic (ALL) leukaemias was similar to that of CD15 antibodies. Most normal precursor cells were not recognised.

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MATERIALS AND METHODS

Cells

Normal cells were recovered from peripheral blood and marrow from healthy donors. Leucocytes were separated by centrifugation on double Ficoll density gradient (1.077 and 1.092).