Automated Quantitation of Immunocytochemically Localized Estrogen Receptors in Human Breast Cancer


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ABSTRACT

Frozen sections of breast tumor tissue have been stained using an immunoperoxidase [estrogen receptor (ER)-immunocytochemistry] kit incorporating a monoclonal antiserum [H222] to visualize nuclear human ERs. Quantitation of specific staining has been performed by manual procedures using optical microscopy and by a computer-assisted image analysis system (CAS 100).

Initial investigations with a test panel of ER-immunocytochemistry-positive tumors revealed a good qualitative agreement between CAS and manual assessments. Reduced variance was, however, observed between quantified ER-immunocytochemistry results from four experienced investigator using the CAS analysis. An extended study confirmed the relationships between CAS and manual methods of assessment. These findings were evident when studies were scored either by assessment of the percentage of positively stained cells (n = 92; r = 0.913; P < 0.01) or by H-score calculations (n = 92; r = 0.913; P < 0.01). A good correlation was also found between CAS quantification and the results of an ER enzyme immunoassay of 48 primary breast cancer specimens (r = 0.715; P < 0.05).

In 49 cases it was possible to relate CAS-defined ER status and levels to the subsequent response of patients to endocrine therapy. ER was assessed on specimens obtained prior to commencement of treatments for recurrent breast cancer. Presuming the presence of ER to be a prerequisite for successful therapy, very good correlations were found between response and both status and levels of positivity were recorded. None of 16 patients with CAS-ER-negative tumors responded to treatment, while 16 of 33 (48.4%) CAS-ER-positive patients achieved an objective response according to International Union Against Cancer criteria. A relationship between response and the degree of CAS-ER positivity was obtained when the CAS score divisions of 0, 1–100, and >100 (response rates, 0, 41, and 64%, respectively) were used.

These data demonstrate that automated image analysis offers a reliable, reproducible procedure for quantifying ER in immunocytochemically stained sections. It has potential advantages over manual procedures, providing less opportunity for subjective influences in scoring sections. Future advances in software design should further reduce elements of subjectivity and increase both the speed and reliability of results. We anticipate image analysis becoming a valuable tool in investigations concerning, for example, the influence of heterogeneity of steroid receptor distribution on the rate of recurrence of breast cancer after mastectomy and in the clinical course of the disease.

INTRODUCTION

The development of immunocytochemical procedures for ER and progesterone receptor determinations (1–3) in hormone-sensitive tissues has led to both a reevaluation of our concepts of steroid hormone action (reviewed in Ref. 4) and, when applied to breast tumor specimens, has provided a very valuable clinical tool. Simple and reliable to perform, the immunocytochemical assays offer a number of technical advantages over existing biochemical procedures, including their requirement for only small quantities of tissue and their ability to recognize both steroid hormone-occupied and unoccupied forms of the receptors. Moreover, because they are performed on fixed tissue preparations they enable the visualization of receptor proteins in their approximate locality within individual cells. This facilitates an assessment of the distribution of receptors within the tissue in question, an important consideration in breast cancer where malignant cells may invade normal or benign components of the breast, each of which may contain receptor-positive cells (5). Furthermore, the technique differentiates between receptor expression of individual tumor cells, allowing analysis of the intratumoral heterogeneity of such, to be performed. These studies have proved useful in the prediction of early recurrence of breast cancer after mastectomy (6).

A major current problem with immunocytochemistry of steroid receptors, however, lies in the inherent inadequacies in the quantification of the insoluble end products of receptor localization reactions. Estimates of the percentage of tumor cells stained or, more significantly, of staining intensity-based scoring systems have, necessarily, elements of subjectivity within them and thus may be subject to errors in interpretation.

We report here our experiences with a computer-assisted image analysis (CAS 100; Lombard, IL) system (7) for quantification of ER in immunocytochemically stained frozen sections. The machine has specifically designed software to facilitate the analysis of nuclear steroid receptor antigens using immunoperoxidase techniques.

MATERIALS AND METHODS

Patients

Ninety-four samples composed of 50 primary operable or locally recurrent tumor specimens and 44 distant recurrent biopsies were obtained from 65 patients with histologically proven breast cancer presenting to one surgeon (R. W. B.) at the City Hospital, Nottingham during the period of March 1985 to November 1987. Histology was confirmed according to previously described procedures (8). Menopausal status was recorded for 46 patients 36 of which (78.2%) were postmenopausal and 10 (21.7%) premenopausal. No patient had received any form of adjuvant therapy prior to recurrence of their disease.

Fifty-four patients were treated with endocrine therapy for locally advanced or systemic breast cancer. Forty-three received tamoxifen (20 mg twice daily) alone and 4 received tamoxifen plus a luteinizing hormone-releasing hormone agonist (Zoladex; ICI Pharmaceuticals, United Kingdom; 3.6-mg depot/28 days). Five patients received the luteinizing hormone-releasing hormone agonist alone and 2 patients received the progesterogen megestrol acetate (Megace; Bristol Myers, United Kingdom). Of the remaining 11 patients 8 received radiotherapy.

Response to therapy was monitored by regular follow-up and assessed by International Union Against Cancer criteria (9) accepting the British Breast Group recommendation that reported response and static disease be of a minimum duration of 6 months (10).
Tissue Preparation

Material for study was obtained routinely at surgery for primary or recurrent breast cancer. Samples were rapidly frozen upon excision, stored at −70°C, and transported on dry ice to the Tenovus Institute, Cardiff, for analysis. A representative portion of the tissue was fragmented from the main specimen and blocked for cryostat sectioning and immunocytochemistry. The remainder was stored at −70°C for subsequent cytosol preparation and enzyme immunoassay for ER.

Receptor Analyses

ER EIA. Details of this assay and its validation are described by Nicholson et al. (11) and by Leclercq et al. (12).

Briefly, the assay is performed using a monoclonal kit from Abbott Laboratories with the use of anti-ER antibody-coated glass beads which are incubated with tumor cytosol. If present, ER binds to the beads while unbound material is removed by washing. A second anti-ER monoclonal antibody conjugated to horseradish peroxidase is incubated with the beads in a “sandwich” procedure. Unbound antibody is removed by washing and color development is achieved through use of hydrogen peroxide and o-phenylenediamine. The resultant colored solution is assessed by spectrophotometry and its optical density is related to receptor concentrations based on standard curves produced on established control specimens.

Protein estimations for cytosolic preparations are made using Bio-Rad protein dye reagents according to the Coomassie blue procedure of Bradford (13). Receptor levels were expressed as fmol/mg cytosol protein and positivity was assumed at levels ≥10 fmol/mg of cytosolic protein.

ER-ICA. All immunological reagents including H222 primary antibody were supplied in the monoclonal ER-ICA kit (Abbott Laboratories). Alternative primary anti-ER antisera, H226 and D547 (1), were supplied by Abbott Laboratories and Dr. G. Greene (Ben May Laboratories, Chicago, IL). H222 staining was carried out according to kit instructions with prediluted antibody. H226 and D547 were substituted into the assay at concentrations of 5 and 20 μg/ml, respectively, in phosphate-buffered saline.

Details of the staining procedures and validation of results have been exhaustively discussed elsewhere (14, 15) and are relatively unmodified here except concerning counterstain and mounting processes. Fixation of 5-μm cryostat sections was in 3.7% formaldehyde in phosphate-buffered saline (0.01 M) for 15 min followed by immersion in cold (−20°C) baths of methanol (5 min) and acetone (2–3 min). Fixed sections were frequently stored at this point for up to 1 week prior to staining, according to the method of Crawford et al. (16).

Nonspecific attachment of antibody was prevented by preincubation of slides for 15 min with a blocking reagent (2% normal goat serum), followed by primary or inappropriate control rat antibody incubations (30 min). Receptor localization was revealed by the indirect peroxidase-antiperoxidase procedure of Sternberger et al. (17).

After color development by DAB and hydrogen peroxide, slides were immersed in distilled water for 5 min and counterstained in 1% methyl green in acetate buffer, added dropwise, for 5 min. Excess counterstain was washed off with distilled water for 2 min and sections were dehydrated through graded ethanol baths to xylene. Slides were mounted in DPX and coverslipped. Comparability between interassay staining intensities was controlled by inclusion of positive control sections in each staining run.

Staining procedures for all anti-ER antisera were as described above and carried out simultaneously on adjacent sections.

Cell Analysis System 100

Equipment and Principles of Analyzer. Immunochemically stained cell images were analyzed using a CAS 100 image analyzer. The hardware for this has been described fully by Bacus and Grace (7) and comprises an enhanced IBM AT personal computer with allied printer, two video monitors, and a calibrated imaging system. The imaging system is formed from a digital camera affixed to a standard optical light microscope, image acquisition, and storage modules and optical computers, to produce high speed digital images for cell measurement.

Various software packages for image analysis applications are available, including specific programs for ER-ICA analysis.

In ER assessment a nuclear masking technique is employed which allows the interpretation of specifically stained and counterstained two color images. This process is well described by Bacus et al. (18) but briefly, analysis is enabled by utilizing the differing light adsorption spectra of the chromogen DAB and the counterstain methyl green when exposed to light of differing wavelengths. Both absorb similarly at 650 nm (as achieved by use of a red filter), while methyl green will transmit light only at 500 nm (green filtered light). This phenomenon allows a “mask” to be made and stored electronically of the nuclear area of all methyl green- or DAB-stained nuclei in an analyzed image using red filtered light. Masked areas can then be analyzed under green filtered light where only DAB-stained (i.e., ER-ICA-positive) cells are visible and thus assessable.

Analysis Procedure. Slides stained by ER-ICA procedures and counterstained as described were first examined under a standard light microscope to assess their suitability for CAS analysis. Heavily folded or otherwise damaged sections or those with nonspecific background staining were either resectioned and stained or excluded from the study. Low power examination enabled the localization and subsequent avoidance of normal or benign breast areas within the section. This initial examination also allowed the degree of ER heterogeneity within the tissue to be assessed, and thus ensured adequate sampling of all areas was subsequently performed. CAS analysis was accomplished by first analyzing the methyl green-counterstained, control slide, using the ×40 objective magnification. Light levels using the green filter on a blank area of the slide were set to the required specified range for reproducible analysis. Areas of good tissue structure and representative of the whole section were then studied and a suitable field was selected for masking.

A nuclear mask was produced using the red filter by electronically adjusting levels of pixelling displayed on the video monitor until all nuclei were masked gray and distinct from background unmasked (i.e., blue) stromal areas. This level was then stored. Antibody threshold levels are then set on this control slide using the green filter. Adjustments were made to pixelling levels until the nuclei (which are only counterstained and hence invisible) just began to appear upon the blue screen. This level was then also stored. Repeat sampling of different areas of the nonstained control section allowed minor adjustments to nuclear and antibody thresholds to be made. When possible, 10 different fields or 10,000 μm² of nuclear area were sampled, with less than 1% of this area being recorded to the positive side of the threshold levels.

The primary antibody-stained section was then assessed utilizing threshold levels set for the control slide. Here only DAB-stained nuclei, expressing immunolocalized ER, which are visible under the green filter, will be scored positive. Again at least 10 fields (or >10,000 μm²) were assessed. In this study <15% of sections were analyzed on less than this area, due to small sample size. All, however, were assessed on at least 8 fields.

Fig. la illustrates a histogram obtained from a CAS ER-ICA positive breast specimen. The threshold levels set are depicted by the dotted area and ER-positive nuclear areas are represented by the shaded area to the right of this threshold. To the left of the threshold line are effectively only counterstained cells, as is also found in Fig. lb which shows a clear majority of cells in this position and is hence an ER-negative tumor.

Quantification of ER-ICA Results

Manual Assessments. Manual assessment was carried out by recording estimates of the overall percentage of tumor cells expressing receptor and also of levels of staining at a number of intensities (i.e., no staining, apparent = 0, weak staining but above background levels = 1, moderate = 2, strong/intense = 3). From these semiquantitative estimates of immunostaining, a score was derived according to a modifi-
This formula produces an H-score in the range of 0-300 where 300

culation of previously reported methods (19, 20), to give an histological

ER-ICA H-score = (% of cells stained at intensity category 1 x 1) + (% of cells stained at intensity category 2 x 2) + (% of cells stained at intensity category 3 x 3)

This formula produces an H-score in the range of 0-300 where 300 equals 100% of tumor cells stained strongly (i.e., 3+).

A fully quantitative score (CAS score) which incorporates staining intensity was made for each slide according to the formula of Bacus et al. (18) where

\[
\text{CAS score} = \frac{\% \text{ of cells positive} \times \text{positive stain intensity}}{10}
\]

Here the percentage of cells positive is defined as nuclei with staining levels above those of the control antibody threshold (with the above

A maximum potential CAS score was determined to be 600 based on assessment of small homogeneously strong positive fields. This was presumed to be equivalent to the highest levels of ER staining possible and comparable to an H-score of 300.

Analysis of Results

Data from ER-EIA and ER-ICA analyses were generated in a manner which ensured that results from each could not be evaluated until completion of the study.

EIA were routinely performed for clinical management purposes and results were recorded. ER-ICA analyses were performed on sections of tissue obtained from adjacent areas of the tumor to those used in EIA.

Nonautomated evaluations were carried out in an initial interassessor variation study by four independent observers, experienced in breast pathology, on an Olympus microscope equipped with both x10 and x40 objectives. Later studies were carried out by two investigators using the double-headed facility of this microscope.

Familiarization with the functioning of the CAS 100 was performed by the four investigators with a test batch of slides of established receptor content (data not shown). A further panel of previously unassessed but EIA-positive tumors were then examined by each of the assessors, using CAS and manual techniques to establish effects of interassessor variability on interpretation of staining.

Subsequent studies with CAS were carried out by one suitably experienced technician (R. M.) without prior knowledge of manually determined results. In all studies assessments were performed in a double-blind manner without prior knowledge of either the results obtained by the other investigators in the study or access to the corresponding result of either the manual or the CAS assessment. Indeed, 2 weeks were allowed to elapse between the forms of assessment, making previous recollection of the recorded values unlikely.

The various ER-ICA assessments were carried out, EIA were determined whenever possible, and the relationships between them were analyzed to assess the validity of the CAS procedure as a means of quantifying immunolocalized ER.

A subgroup of samples were assessed as described and their results compared with the subsequent responses of patients with recurrent breast cancer to endocrine therapy. Such relative predictive abilities of the techniques described were also recorded.

RESULTS

Initial Assessment of the Variability of ER-ICA Analysis Procedures. A test panel of 13 slides were chosen which covered the range of ER-ICA (and ER-EIA)-positive results routinely obtained in the Breast Cancer Unit of the Tenovus Institute for Cancer Research, Cardiff. These slides were examined by four suitably experienced personnel, used to assessing ER-ICA-stained breast tumors, and who had previously undergone a period of initial familiarization with the CAS instrumentation in order to determine the extent of interassessor variations for both CAS and manual procedures. The assessments revealed a good qualitative agreement between the procedures and between the assessors (Fig. 2). The manual quantification of slides, however, led to a greater degree of discordance between operators (larger standard deviations from mean values; i.e., for manual assessment of percentage positivity, SD n – 1 ranged from 3.7 to 28.1% and for CAS from 1.8 to 13.8% only. For comparable scores, SD n – 1 ranged from 14.1 to 58.7 for manual assessment and from 2.7 to 43.7 for CAS score), and a higher proportion of stained cell estimates, than when the CAS instrumentation was used. This was evident for both the percentage of positively stained cells (manual) or nuclear area
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Fig. 2. Comparison of interassessor variations using automated (CAS) and manual assessment procedures for evaluating ER-ICA-stained breast tumor specimens. Variations between (a) assessments of percentage of positive cells and (b) scoring systems are presented. □, mean CAS-determined results from four assessors; □, manually derived results. Bars, SD from mean values. CAS and manual scores are presented together in b with scale adjustments of 2:1 for direct comparison.

(CAS) (Fig. 2a) and manual and CAS scores (Fig. 2b). Indeed, in the latter group, after compensatory adjustments of scale were made for the two ranges of the scoring systems, reduced deviations for CAS-assessed slides were recorded in 12 of 13 cases.

Fig. 3 illustrates further the relationship between CAS and manual procedures on 92 primary or recurrent breast samples. Significant associations were obtained between the procedures when comparisons of the percentage of positively stained cells were made on all tumors [Fig. 3a] \( r = 0.919; P < 0.01 \) and for ER-positive tumors only \( r = 0.821; P < 0.01 \) [not illustrated]. Regression analysis showed a bias toward higher manually assessed percentages. Similarly, comparison of CAS and H-score methods indicated an excellent correlation between procedures \( r = 0.913; P < 0.01 \) for all tumors (Fig. 3b) and \( r = 0.861; P < 0.01 \) for positive tumors only [not illustrated]. Again a bias toward higher manually assessed scores was, however, observed.

Examination of the relationship between CAS-defined and EIA measurements of ER from 48 primary tumor specimens is illustrated in Figure 4. Qualitative and quantitative associations were observed \( r = 0.715; P < 0.05 \) for all tumors.

CAS: Assessment and Hormone Sensitivity. Fig. 5a illustrates the relationship between CAS-defined ER status and subsequent response to endocrine therapies based on a study of 49 previously untreated breast cancer specimens. The majority of tumors (14 of 22, 63.6%), which clinically progressed despite endocrine manipulations and which may therefore be characterized as hormone unresponsive, were found to be CAS-ER-ICA negative. Conversely only 2 of 11 (18.2%) and 0 of 16 (0%) of patients with static disease and hormone-responsive disease, respectively, were classified as having ER-negative tumors. Thus all clinically responding patients had demonstrable ER-positive cells by CAS analysis.

When the percentage of positively stained cells present was analyzed (Fig. 5b) a relationship between increased ER positivity and improved response rates was observed. Forty-four % of low positive samples (i.e., between 1 and 40% of cells stained) were found to be responsive to therapy. In the highly ER-positive group the proportion of responders was greatest at 53% with a further 40% (6 of 15) showing stabilization of their disease. Similarly an increased proportion of responding tumors was observed among those tumors with higher CAS scores (Fig. 5c). Sixty-four % of those cases with CAS scores above 100 were found to be responsive, compared with 41% of low positive
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Fig. 5. Influence of CAS-defined ER-ICA status and of CAS quantifications of ER on the response of recurrent breast carcinoma to endocrine therapies. ER-ICA was performed on pretreatment lesions in all cases and response to therapy assessed according to International Union Against Cancer criteria. a, relationship between ER-ICA status and response; b, complete or partial remission of disease; c, progressive disease. The percentage of patients in each group may be obtained from the ordinate and numbers of patients recorded alongside the relevant categories. d, influences of the percentage of ER-ICA-positive cells; e, that of CAS score.

CAS scored tumors (CAS score, 1–100) and 0% in ER-ICA negative tumors.

A number of samples were stained using anti-ER antibodies H226 and D547. Analysis of staining data from these revealed, in the case of H226, results very similar to those obtained by H222 at a similar antibody concentration. Staining results with D547 required a much greater concentration of antibody and often presented interpretation problems for CAS since nonspecific background staining of primary antibody on tissue stroma was often observed.

DISCUSSION

Several reports have recently shown the validity of immunocytochemical assay procedures for determining steroid hormone receptors and have demonstrated good qualitative correlations with radioligand binding assays and enzyme immunoassays (14, 15, 22, 23). Attempts have also been made to give semiquantitative assessments of ER-ICA-stained sections and to portray expressions of linearity between them by regression analysis (15). Criticism has, however, been expressed in the past on the degree of subjectivity which may be involved in such attempts at quantification and on the need for forms of automated means of assessment (24). Our analyses of interassessor variation express justification for such reservations concerning scoring procedures. Data presented here show that while qualitative manual assessments of ER-ICA-stained slides between a panel of experienced observers are very good, quantitation of these by estimates of percentage of stained tumor cells or particularly attempts to incorporate staining intensity factors into scores, are subject to wide variations.

Many reports have indicated relationships between levels of steroid receptors and prognosis (25, 26), duration of endocrine therapy-induced disease remission, and survival (27). The importance of accuracy and consistency within histochemical assessment methods is therefore obvious. This is becoming increasingly important since current trends are away from the more complex, time- and tissue-consuming biochemical methods of receptor analysis and toward the simpler, rapid, monoclonal antibody-based procedures. Such immunocytochemical methods of analysis have further advantages in that they have demonstrable applications in assessing the receptor content of fine needle aspirate specimens (28, 29), samples of normal breast (5) and may allow treatment effects to be monitored by repeat sampling of lesions currently exposed to antiestrogen therapy.

With regard to these factors we report the value of a computer-assisted image analysis system in quantification of receptor levels in breast cancer sections. Subjective semiquantitative microscopic determinations of percentage of cells stained and H-score are compared with CAS equivalents and found to correlate well. CAS assessments, however, are concluded to be more representative despite lower mean scores, since variations within a test group showed much reduced inter-assessor ranges of estimates for positive cases.

Bacus et al. (18) have previously described the validity of the CAS 100 system in interpreting ER-ICA-stained slides and record similar relationships between manually and automated analysis results. Such similarities, despite less experience with the system in our case, add credence to reports of the reproducibility and simplicity of operation of the machine. Other papers concerning automated procedures for assessing ER-stained sections using an alternative system have also been published (24, 30, 31) and have shown good results.

A measure of the true value of any method of ER assessment is its ability to enable the correct selection of candidates for successful endocrine therapy. This is the first report to undertake such a study. By making the assumption that successful endocrine measures (antiestrogen therapy) require the presence of estrogen receptors in tumor cells, it is possible to relate ER content of untreated lesions to the subsequent response of the patient who has a recurrence of her disease and obtain data relating to the predictive ability of the test. Response to treatment was observed only within the CAS ER-ICA defined positive group of tumors, and a proportionately higher response rate was observed with increased CAS score and percentage positivity.

Computer-assisted image analysis by CAS 100 with its appropriate software for interpreting nuclear steroid receptor staining is shown to be an effective method for quantifying immunocytochemical preparations. It shows comparability with manual procedures in qualitative terms and may be better at reliable quantification. Furthermore it enables good prediction of outcome of endocrine therapy potential and may therefore have value in the clinical laboratory.

Schwartz et al. (21) have recently described the value of the CAS 100 system in digital image analysis of Ki67 antibody-stained non-Hodgkin's lymphomas. Research using CAS 100 in assessing Ki67-localized antigens in breast cancer is currently in progress. Future studies with this instrument should also help to assess the relative importance of tumor cell heterogeneity with respect to steroid receptors, in relation to the failure of many, seemingly estrogen receptor-rich tumors, to respond to endocrine therapy.

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