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 investigators 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 (r = 0.92; r = 0.919; P < 0.01) or by H-score calculations (r = 0.92; 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 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 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 intratumor 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 used 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 progesterone 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 fractured 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. 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 graduated ethanol baths to xylene. Slides were mounted in DPX and coverslipped. Comparability between interassay estimations of the overall percentage of tumor cells expressing receptor concentrations based on standard curves produced on established control specimens.

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. Immunocytochemically 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 X40 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 pixeling 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 pixeling levels until the nuclei (which are only counterstained and hence invisible) just began to appear on 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 cell 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. 1a illustrates a histogram obtained from a CAS ER-ICA positive breast specimen. The threshold levels set are depicted by the dotted vertical line 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. 1b, 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 = 0, weak staining but above background = 1, moderate = 2, strong/intense = 3). From these semiquantitative estimates of immunostaining, a score was derived according to a modifi-
cation of previously reported methods (19, 20), to give a histological

Consequently in sample b almost all the nuclear area is depicted to the left of the

stained section of the tumor. Shaded regions to the right of this threshold represent

and (ft) an ER-ICA-negative breast tumor. Vertical dotted lines represent threshold

levels for positivity as defined by analysis of the inappropriate control antibody

(21). However, since intranuclear staining appears relatively homoge-

ous for ER-ICA it is assumed to be equivalent to the proportion of

positive stain intensity data. Staining distributions for each

tumor is thus ER negative. CAS scores for these cases are superimposed onto the

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IMMUNOLOGICALLY LOCALIZED ESTROGEN RECEPTORS

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 \( (i.e., \text{between} \ 1 \text{and} \ 40\% \text{of cells} \text{stained}) \) were found to be responsive to therapy. In the high 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
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 proportionally 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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Automated Quantitation of Immunocytochemically Localized Estrogen Receptors in Human Breast Cancer

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