IN THE MATTER OF the Commission of Inquiry on Hormone Receptor Testing established under s. 3 of the *Public Inquiries Act, 2006* by Order dated July 3, 2007

REPLY OF THE MEMBERS OF THE BREAST CANCER TESTING CLASS ACTION

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TABLE OF CONTENTS

Reply to the Submission of the Cancer Society

1. In our Submission on behalf of the Members of the Breast Cancer Testing Class Action we put forward that the source of error in ER/PR testing was at the start up of testing in 1997. Dr. Khalifa made the assumption that Mr. Gulliver was looking after the technical performance of the test and Mr. Gulliver assumed Dr. Khalifa knew about the technical difficulties of the test and how to correct them. As we know from the evidence, neither had any conception of what was going on at the bench level. The absence of monitoring, documentation of controls, validation of antigen retrieval, and failure to use negative controls, led to repetitious errors for over a six year period (1997 to 2003) until the Ejeckam intervention.

2. Our focus, like that of Drs. Cook and Carter, was on false negatives which, according to the Predham data,[1] the error rate was 44% false negatives, which affected 319 patients before the Ejeckam intervention.

3. We also referred to a second source of error in para. 19 of our Submission related to confirmed negative cases. It was the evidence of Dr. Banerjee who opined: "no amount of antigen retrieval would have any effect if the protein (estrogen and progesterone receptors) had been completely lost in processing."[2] In other words, in the 512 confirmed negative by Mount Sinai on retesting, there are an unknown percentage of false negatives, ie. cases that were positive, but the receptors were lost in processing. Unfortunately, these cases are irretrievably lost to retesting.

[1] P-1852 and P-1853
[2] P-0046, p. 4

4. In para. 49, page 20 of the Members' Submission, we raised a third area where some patients may have been missed and who may have benefited from anti-hormone therapy. Dr. Dabbs discredited the use of a 30% cut point first instituted by Dr. Khalifa in 1997. This set the criteria for retesting at 30% from 1997 to 2000, when the cut point was lowered to 10%. The Members respectfully proposed that given the discrediting of the use of a 30% cut point, the same criteria be used for the 1014 cases in the 1997 to 2000 period as was used for testing beyond 2001, that is 10%. Anyone with access to the data could calculate the number of cases.

5. The Members propose that Dr. Reza or Dr. McDonald of the Centre for Health Information who know the dates of testing on the 1014 cases, identify the number of cases so affected and have them retested at Mount Sinai. The Members suspect the number is not great but given the high percentages of false negatives, it would not surprise us if there would be many. Even one case is important to the individual concerned.

6. Ms. Newbury, in her Submission on behalf of the Canadian Cancer Society, raises a fourth source of error – false positives. We did not include the possibility of false positives as a big issue in that only 4 cases out of 16 retests were false positives. The literature would support that it is theoretically possible and the experts Dabbs, Torlakovic, Banerjee and Mullen testified as to the rarity of false positives. Of course this is based on the assumption that testing was performed properly, which we know it was not. Again the focus was on false negatives and little thought given to false positives.

7. We support Ms. Newbury's proposal that the Commissioner give some thought to recommending an investigation of false positives. As an initial step, we support para. 273 of her Submission that 15 to 20% of randomly selected cases be retested. If there is a significant error detected, then some thought should be given to retesting all estrogen positive cases.

8. There is a practical side to this issue. We are informed by a pharmacy that Tamoxifen costs about $20 per month, aromatase inhibitors $200, and Herceptin $2,500-3,000 per month. Patients taking these drugs must have periodic checkups with liver function tests, calcium levels, bone x-ray, and regular gynecological checks for pelvic bleeding. All this costs money, whomever is paying. If a true false positive is found, then there are valuable cost savings to the government and others, in addition to the harm avoided by identifying patients who are being unnecessarily exposed to the adverse effects of anti-hormone therapy.

Reply to the Submission of Dr. Kara Laing, et al.

"Part II – The Pathology Perspective"

9. The Submission of Dr. Kara Laing et al. ("Laing Submission") discusses "what factors may have caused or contributed to the problem?" starting on p. 7. It reviews the evidence of Dr. Brendan Mullen and Dr. Banerjee.[3] Both pathologists describe artifacts that Dr. Mullen thought should be evident to pathologists. Dr. Banerjee did qualify his observations by stating that not every pathologist would recognize problems with fixation and tissue processing as it was conceivable that they became used to the presence of artifacts on slides and simply read through them, accepting it as part of the normal quality. This latter point was confirmed by Dr. Cook in his evidence.[4]

Paras. 23-25 – "Dr. Gershon Ejeckam"

10. The Laing Submission states that Dr. Cook "immediately recognized" Dr. Ejeckam as having an interest in IHC and asked him to act as a resource person in IHC. There was nothing immediate about this recognition. Dr. Ejeckam joined the staff at the General in September 2002 and was asked to act as a resource person to look into the complaints arising at the Tuesday/Wednesday sessions about erratic and unreliable staining. Dr. Ejeckam testified that this appointment or "task" started "at the tail end of 2002 and early 2003". The complaints by the pathologists over the seven months from September 2002

[3] Laing Submission, paras. 11-19
[4] Submission on Behalf of Members of the Breast Cancer Testing Class Action ("Members' Submission"), p. 41, para. 105

to April 2003 caused Dr. Ejeckam to shut down IHC testing on eight antibodies, including ER/PR.

Paras. 26-28 – "Dr. Donald Cook"

11. Dr. Cook denies observing any artifacts over the years and never received any complaints from anybody surrounding fixation/tissue processing for the slide quality. Over seven months, from September 2002 to April 2003, there were more than 50 Tuesday/ Wednesday group sessions where complaints were made by the pathologists present about the erratic and unreliable staining. Did Dr. Cook consider these complaints? If not, why did he appoint Dr. Ejeckam as a resource person?

12. Dr. Cook's response to Dr. Ejeckam's memo of April 2003 was a dereliction of his responsibilities. He knew there was something amiss in the IHC lab, Dr. Ejeckam made that quite clear in his memo; but he chose to do nothing. He did not even discuss the memo with Dr. Ejeckam. Dr. Cook thought it was just a quality assurance problem. The question begs, what quality assurance? There was none. There is a further discussion of Dr. Cook's reaction to the Ejeckam memos in the Members' Submission.[5]

[5] Members' Submission, p. 40, paras. 103-107

Page 74 – "(C) Were the testing protocols in place between 1997 and 2005 reasonable and appropriate?"

13. The Members' Submission put forward that the protocols were neither reasonable nor appropriate. In fact, there were no protocols, just a hodgepodge of manufacturers' specs followed by the two senior techs Mary Butler and Peggy Welsh, with no supervision by either Mr. Gulliver or Dr. Khalifa, who testified as to not having any in-depth knowledge of the bench procedures for ER/PR testing. The Laing Submission chooses to not cover this area. Instead its focus is on cut points of positivity for ER.

14. Dr. Khalifa was tasked by the chairman Dr. Haegert and the staff pathologist at the General Hospital to see that ER/PR testing was set up in the IHC lab to replace the biochemical method being performed in Biochemistry. Dr. Khalifa's only involvement was to read the external positive controls and validate the antibody titrations. The Laing Submission discussed the February 16, 1998 memo from Dr. Khalifa to all pathologists in Newfoundland and Labrador where he muses on what constitutes positivity and negativity in ER/PR results.[6] Essentially Dr. Khalifa says to report the degree of positivity and if it is 30% or less, to refer to the O'Keane publication advocating a 30% cut point. As the Laing Submission acknowledges, Dr. Dabbs trashed this publication as "apples and oranges", and the cut point in the mid 90's literature advocated a 5-10% cut point. Unfortunately, many oncologists and pathologists used a 30% cut point of positivity in their practice until the cut point was reduced to 10% in 2001. In fact, when it was decided to retest all negative ER cases in the summer of 2005, the oncologists,

[6] P-1850, Example 2

pathologists and lab representatives used the 30% cut point from 1997 to 2001 as the criteria for retesting.

15. Paragraph 199 on p. 77 of the Laing Submission suggests two publications as rebuttal to Dr. Dabbs' critical testimony of Dr. Khalifa's use of a 30% cut-off value. The importance of this issue justifies some discussion of the two publications. The first is irrelevant, and the second is merely a survey whose conclusions even the authors warn should be viewed "with caution".

16. The first publication by Santeusanio was published in *Allied Immunohistochemistry & Molecular Morphology*, December 2000. As this article is not in the exhibit list, we enclose a copy at Appendix A. Essentially, Santeusanio is saying that there are six receptor sites on the estrogen receptor protein, which he labels A, B, C, D, E and F. The antibody 1D5 only attaches to sites A and B. He postulates that even though a case is strongly positive for ER using 1D5, the other four sites can take up estrogen. This is why only 60-80% of ER positive cases respond to Tamoxifen. He proposes using a panel of four antibodies for ER, AER311, LH2, LH1 and 1D5, the combinations attaching to all six receptor sites. He compares his results to the results of the radioimmune biochemical assay (dextran-coated charcoal "DCC"), which Dabbs declares obsolete in the mid 90's, being replaced by IHC methods. He tabulates his findings on page 279, Table 1. By a complex statistical analysis, factoring in the clinical stability or progression of 46 cases of breast cancer and comparing the sensitivity and specificity of IHC and DCC methods and using cut points of 10 to 50%, he concludes that using a 30% cut point for each antibody used possesses the best sensitivity/specificity ratio.

17. Santeusanio proposes the most accurate results for assessing estrogen receptor status would be attained by using a panel of three antibodies AER311, ER 1D5 and LH2, for every case and using a 30% cut point.[7] While Santeusanio's proposal may be theoretically sound, the reality in the world of ER testing is that only one antibody is used – ER1D5 or 6F11 or in 2005 SP1 – and the literature of the mid 90's established 5-10% cut points using ER1D5. As an example, we enclose at Appendix B one of the publications of Pertschuk, who Dr. Dabbs suggested Dr. Khalifa should have used instead of the outdated O'Keane article when first setting up the ER/PR in 1997. Here, a 10% cut point is used.[8]

18. Santeusanio also cites the classical publication of Clarke, Harvey, Osbourne and Allred which proposes 1-10% positivity.[9] The publication of Santeusanio et al. is not relevant to the present Inquiry.

19. One thing apparent from the Santeusanio publication is how quality control is used relative to preparation of controls for ER/PR testing[10]:

    (a) A slide containing tumor and normal breast tissue is used as a positive internal control.

    (b) If there is no normal tissue in the specimen (ie. fine needle biopsies), then normal breast tissue is used from another patient as an internal positive control from a

[7] Santeusanio, p. 282, right column, 2nd paragraph
[8] Pertschuk, p. 2518, 1st paragraph, right column
[9] P-1750, Tab 5, p. 1, left hand column under results
[10] p. 277, 1st paragraph, left column

pre-menopausal patient. Post-menopausal patients tend to have low or no estrogen expression in their normal breast tissue.

(c) A negative external control from the patient containing tumor (omit the primary antibody application).

(d) A positive external control from a known strongly positive specimen of breast cancer from another patient.

20. As we know, at the Eastern Health IHC lab they were only using an external positive control with little or no emphasis on internal positive controls. Dr. Khalifa said when he set up the ER/PR testing, information on internal positive controls was not available in 1997-98. Dr. Dabbs said he was exposed to utilizing internal positive controls in the mid 80's.[11] In fact, most of the pathologists testifying admitted they were not aware of the importance of internal positive controls until the second Ejeckam memo of May 2003. In particular, the site chief Dr. Parai admitted he did not know about internal positive controls until he received the second Ejeckam memo.[12]

21. As stated, the only control used was an external positive control and that was rationed due to financial constraints.[13] As if it were a luxury to be dispensed with! As a compromise, Dr. Khalifa read the external positive controls per batch before sending out the patient slides for interpretation by the pathologist. Nobody seemed to object to this

[11] Members' Submission, p. 19, para. 44
[12] Members' Submission, pp. 39-40, para. 100, last line
[13] Laing Submission, para. 197

practice except the Peninsula Hospital in Clarenville, who discontinued using the IHC services in 1999 "because of poor quality and lack of controls".[14]

22. The Inquiry was not told what the cost would be to produce an external positive control for each case.

23. And finally, not using a negative external control is another example how ill planned the ER/PR testing was when started in 1997. Although there were financial constraints in 1997-98 somehow Mr. Gulliver, the manager, did find sufficient funds in March 1998 to purchase a Dako autostainer for $62,675 and signed a 5-year reagent and maintenance contract for $225,000.[15] Yet, they could not afford an external positive control for each case.

24. Paragraph 200 on p. 78 of Laing Submission refers to a publication of Dr. Lester J. Layfield, who is an acknowledged expert in clinical IHC. We enclose the publication at Appendix C and note the following:

    (a) The Layfield publication is a survey of current practice of ER/PR testing in the United States circa 2000. Questionnaires were sent to 300 pathology departments in the 50 states.

    (b) 200 were sent to four pathologists in each of the 50 states chosen randomly from the College of American Pathology (CAP) directory.

[14] P-2141
[15] P-1893

(c) 100 questionnaires were sent to 100 pathologists who had published on ER/PR or related issues.

(d) The questionnaire had questions on:

(i) size and type of hospital practice;
(ii) were ER/PR testing ordered routinely on all breast cancers;
(iii) assay in-house or referred out;
(iv) if in-house, what method (IHC, DCC, etc.);
(v) material used paraffin section, frozen sections, FNA;
(vi) type of antibody and dilution used;
(vii) method used by pathologist to assess staining %, H score, etc.;
(viii) controls – external positive, negative, internal positive;
(ix) enrolled in an external QA program;
(x) cut off point for positivity.

(e) Only 80 questionnaires were answered. Of the 80 respondents, only 45 answered all the criteria used to separate positive and negative results, which are listed in Table 4 of the article on p. 192.

25. The highest cut point used was 19% by four labs or 8% of the 45 labs surveyed. Four labs used the H scoring system or the St. Antonio score without giving the equivalence in percentages. 84% used 1%-10%. So it would seem that the vast majority of labs surveyed were using 1-10% as cut points.

26. A second survey was sent to 150 hematology/oncology programs and only 27 useful responses were received. Table 7, p. 193, summarizes the cut off thresholds used by the oncologists. If you eliminate the 7 (no response), the 4 (did not know), and the 1 (lab does it), you then have 15 useful responses. Of the 15 respondents, 3 used a 30% cut point (20%) and the remaining 12 (80%) used 1-10% cut points.

27. In his discussion on the hematology/oncology survey, the author cautions:

> Only 26 responses (17%) from our survey of 150 directors of hematology/oncology programs were received, meaning conclusions based on this small database should be made with caution.[16]

28. All these numbers prove is that the hematology/oncology groups surveyed who used 30% as a cut point were as poorly informed of the current literature of the day as all of the pathologists in St. John's, and some of the oncologists practicing at our Cancer Centre.

Para. 204 – "Dr. Carolyn Morris-Larkin"

29. In response to a series of questions from class action counsel on the issue of Dr. Khalifa's introduction of IHC for ER/PR, Dr. Morris-Larkin explained that the nature of pathology involved learning on the job. As such, she viewed IHC for ER/PR as falling within that context. She also felt that Dr. Khalifa provided some guidance and denied being over confident in reporting since a learning curve would be expected. However in hindsight, if IHC were introduced today it is likely that a more formal in-service training would occur in light of what has happened.

30. Unfortunately, all the pathologists from Newfoundland and Labrador who testified at the Inquiry had little or no experience in reading ER/PR stains. To Dr. Khalifa, Mr. Gulliver, and the senior techs this was just another stain. How wrong they were. None of the pathologists had done any literature search on IHC staining, otherwise they would have asked: Where are the external negative controls? Why are the external positive controls

[16] Layfield publication, p. 195, left column, para. 2

varying in staining from day to day? Why are the artifacts subsequently noted by Dr. Banerjee occurring?

31. The Members maintain that ER/PR testing is not just another special stain. It is used for therapeutic guidance and prognosis by the oncologist. As such, *there should be no learning curve allowed*.

32. Unfortunately, Dr. Cook, the clinical chief, had the same attitude: "Immuno stains can vary from day to day, can vary in intensity, can vary in staining characteristics". *Dr. Cook had gotten so used to a poor product, he thought this was the norm*.[17]

33. The Commissioner has heard evidence from the experts related to *sensitivity* (is the methodology used able to expose the target antigen to the antibody?) and *specificity* (is the antibody used specific enough to couple with the target antibody only and not couple with other proteins in the tumor, and give non-specific background staining in the cytoplasm of the cell or other proteins in the tumor?).

34. What we know for sure is that the peroxidase (PAP) methodology and the ER ID5 antibody has been used since the mid 90's and is still used worldwide today in 2008. It is noted that the two antibodies ER ID5 and 6F11, were the most commonly used ER antibodies for many years. These antibodies will probably be replaced by the new ER antibody SP1 (2005), where the manufacturer claims a 5-10% increased specificity.[18] So

[17] Members' Submission, p. 41, para. 105
[18] P-0049, p. 2, item 3

the methodology and antibody used by the Eastern Health labs were acceptable if performed properly.

35. What was not discussed in any detail was testing *reproducibility* (that is, if one is performing the testing properly, he should get the same results every time). In ER/PR testing the only way of getting reproducibility is that the known external positive control is just that, strongly positive every time. The negative control is negative every time, and the internal positive control is positive every time. Unfortunately, only an external positive control was used by the IHC lab and that is described by Dr. Banerjee, Dr. Mullen and even Dr. Cook as varying in positivity from day to day.

36. Having observed this variability for seven months at the Tuesday/Wednesday sessions, Dr. Ejeckam shut down testing of ER/PR for what he characterized as "erratic" and "unreliable" staining. It is a fundamental principle in any laboratory procedure using controls, that if they are not working then do not report the result of that test. Otherwise, why use a control. Unfortunately the IHC lab at Eastern Health did not use the two other levels of quality control – the mandatory negative control and no emphasis on the internal positive control.

Post-Submission Developments

37. Two important reports, bearing on issues touching the Terms of Reference of this Commission, have been made public since the date for filing of Submissions. These are the *Commissioner's Report: Commission of Inquiry into Pathology Services at the*

Miramichi Regional Health Authority, www.miramichicommission.ca, and *The Report of the Task Force on Adverse Health Events*, www.gov.nl.ca/ahe/pdf/AEHealth(web).pdf.

38. The Commissioner for the Miramichi Inquiry described the origin of his inquiry as "a perceived pattern of clinical diagnostic error of an elderly pathologist in a small laboratory at a small hospital in Northern New Brunswick."[19] By contrast, the present Inquiry examined into events at a tertiary health science complex associated with an academic centre. Nonetheless, some common themes emerge.

39. One common theme is the lack of quality assurance:

> It was self evident from the beginning that the matter of quality assurance and quality control was central to the problem under investigation.[20]

40. The following statement of the Miramichi Commission would apply equally to the Eastern Health situation:

> In the future, the laboratory … must be operated under a culture of patient safety, rather than a policy of risk management in a situation where a problem arises.[21]

41. The Miramichi Commission made numerous recommendations for improvements in pathology services and the Commissioner of the present Inquiry might derive benefit from a perusal of the findings.

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[19] Miramichi Commissioner's Report, pp. 1-5
[20] Miramichi Commissioner's Report, p. 125
[21] Miramichi Commissioner's Report, p. 126

42. The Task Force on Adverse Health Events was established to determine if the Newfoundland and Labrador healthcare system "responds appropriately to adverse events when they happen".[22] Unlike this Commission, it was not established to inquire into specific adverse events. There are at least two broad areas where the experience of the Commission may permit a recommendation which reflects on the work of the Task Force.

43. The first area is the tendency of a large and complex organization to ignore its own policies on disclosure. No witness could be found who consulted the Eastern Health disclosure policies as a guide to decision making, or knew of anyone who did. Therefore when on page 139 the Task Force urges training and education to orient new employees to occurrence reporting, and "targeted disclosure training across the organization", the Members feel this requires reinforcement.

44. The implementation of the occurrence reporting system envisioned by the Task Force will require in-service instruction at all levels, including quality assurance follow up to verify that the system is actually implemented and functioning as intended.

45. The second area is in the variety of changes to legislation recommended by the Task Force. All parties to this Inquiry and their counsel have acquired a level of expertise in the legislative framework of adverse events and occurrences, not just through the Commission hearings, but through hearings before Justice Dymond as to the issue of protection and privilege of proceedings of peer review committees and quality assurance committees. The Members therefore submit that the recommendations of the Task Force

[22] Task Force Report, p. IX

bearing on amendment to the *Regional Health Authorities Act* or the *Access to Information and Protection of Privacy Act* or section 8.1 of the *Evidence Act*, should they be adopted by government, should be provided to the parties to this Commission for comment, prior to being introduced in the legislature. The hope is that any legislative changes may benefit from the accumulated experience of the parties, acquired at public expense.

Conclusions

46. We maintain that in 1997-98 it was incumbent on the pathologists to read the literature on IHC staining for ER/PR. Standard surgical pathology textbooks are inadequate. Any pathologist could walk down the hall to the MUN Medical School Library which is adjacent to the pathology lab. There are hundreds of textbooks on pathology and laboratory medicine, including IHC. Most of the authoritative journals are available for reference or online. For example, the classic Rhodes article front page lists 957 articles on immunology and 664 articles on breast cancer.[23]

47. The Wegrynowski report of November 5, 2005[24] made literature suggestions and on page 20 under "textbooks", all three were available at the MUN Medical School Library, including the *Introduction to Immunocytochemistry*: see Appendix D.

[23] P-1851
[24] P-0047

48. Unfortunately, most of the pathologists except those in Clarenville, accepted the poor quality slides and trusted Dr. Khalifa for guidance. This trust was misplaced and led to disaster.

49. The overall tenor of the Laing and the Eastern Health Submissions is that a "systemic" failure occurred, for which no one is responsible.

50. The Members reply that three individuals are principally responsible for the testing debacle: Dr. Khalifa, who lacked the scientific, practical and managerial knowledge to establish the test competently; Mr. Gulliver, who was 10 years off the bench and who completely lacked practical or theoretical knowledge of the test; and Dr. Cook, who failed to educate himself in the test, acquiesced in poor quality material, ignored problems when they arose, and whose instincts were to withhold full disclosure when in 2005 the scope of the problem finally became impossible to ignore.

51. The testing failures, and the failures in disclosure, are fundamentally failures of the leadership of Eastern Health. However, responsibility was not only deep but broad. None of the pathologists in St. John's or the lab technical managers bothered to walk down the hall to access resources and instruct themselves. A culture of cover-up informed the leadership from Mr. Tilley down, but permeated the organization at all levels. The words of the Miramichi Commission define the task for Eastern Health:

> In the future, the laboratory … must be operated under a culture of patient safety, rather than a policy of risk management in a situation where a problem arises.[25]

[25] Miramichi Commissioner's Report, p. 126

Recommendations

52. The further recommendations which the Members would commend to the consideration of the Commissioner are as follows:

(1) That Eastern Health conduct an appropriate random sample of positive results.

(2) That Eastern Health and other healthcare boards adopt a rigorous system of in-service instruction at all levels and follow up quality assurance to ensure that any reporting system as recommended by the Task Force be implemented and fully functioning.

(3) That the Government consider providing proposed legislative amendments to counsel for the parties to the Inquiry for comment with a view to possible improvement.

RESPECTFULLY SUBMITTED this 15th day of December, 2008.

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APPENDIX A

Immunohistochemical Analysis of Estrogen Receptors in Breast Carcinomas Using Monoclonal Antibodies That Recognize Different Domains of the Receptor Molecule

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Estrogen receptor (ER) analysis was performed in 46 primary breast carcinomas using four monoclonal antibodies (MABs) to ER (AER311, ER1D5, LH1, and LH2), each of which recognizes a distinct domain of the receptor protein. ER was expressed as the percentage of positively stained tumor cells. Statistical analysis was performed using the SPSS/PC+ program to set the cut off of positivity and the prognostic value of each MAB. A positivity >30% for each MAB possessed the best sensitivity/specificity ratio and was used as the cut-off value. Multivariate discriminant analysis showed that MABs AER311, ER1D5, and LH2 had significant prognostic value. Fourteen tumors showed positivity for these three MABs; 17 were positive for one or two of the three MABs, and 15 were negative for all three MABs. Survival analysis showed that patients with tumors negative for all three of these MABs had progression of the disease within 8 years from the diagnosis of the tumor, whereas all patients with tumors positive for all three MABs were alive 13 years after surgery. A significant correlation ($P = 0.0006$) between tumor grading and ER status was found; 71% of the tumors that were positive for all three MABs were grade 1, whereas tumors negative for all three MABs were mostly grades 2 and 3. No significant relationship was observed between ER status and tumor size. A significant correlation ($P = 0.008$) between lymph node status and ER was found; breast tumors positive for all three MABs were in the majority (92.9%) of cases pN0, whereas 67% of tumors negative for all three MABs were pN1. Results from the present study suggest that the use of a panel of MABs that target distinct epitopes within domains of the ER protein could offer a better approach for assessing the ER status in breast cancer patients, because it enables the recognition of breast tumors with intact or structurally defective ER proteins.

Key Words: Estrogen receptors—Monoclonal antibodies—Immunohistochemistry—Breast cancer.

Applied Immunohistochemistry & Molecular Morphology 8(4): 275–284, 2000.

The availability of monoclonal antibodies (MABs) to estrogen receptors (ER) (1) has allowed the cloning and sequencing of the receptor protein leading to a better understanding of its structure and function (2). Human ER is a protein composed of 595 amino-acid residues, with a molecular weight of approximately 66 kd, that is divided into six distinct domains with specific functions denoted from the NH_2 to the COOH terminal regions by six letters from A to F (3). The amino terminal domain A/B is implicated in transactivating function (Taf1) and is involved in the activation of estrogen-responsive genes. C is the DNA-binding domain. The D domain is involved in the nuclear localization of receptors. E is the hormone binding domain and is also implicated in another transactivating function (Taf2). F is the carboxyl-terminus domain and appears to have a modulatory function in regulating the transcriptional response to estrogens and antiestrogens (4).

The availability of MABs to ER that target specific epitopes within domains over the entire length of the receptor protein offers the opportunity to study the expression of such domains in breast tumors (5). This ability is potentially very useful in that discordant results of ER status observed in some breast carcinomas by using

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MABs, which recognize different domains (6–19), seem owing to the presence of ER variants (6,20). Additionally, the failure of some ER-positive breast tumors to respond to endocrine therapy, the development of endocrine resistance, and progression in human breast cancer could be related to the presence of such altered receptors.

We performed a retrospective immunohistochemical study of ER in paraffin sections from 46 primary breast carcinomas using four MABs to ER, each of which recognizes a distinct domain of the receptor protein. The main purpose of this study was to ascertain whether this approach could provide information of the ER status beyond that obtained by using single MABs to ER.

MATERIAL AND METHODS

Materials

We used formalin-fixed, paraffin-embedded tumor specimens from 46 patients who had operable primary breast carcinoma. None of the patients had received prior adjuvant systemic therapy. All cases were selected adopting the following criteria: (a) all tumors were assayed for ER by the dextran-coated charcoal (DCC) method, (b) at least one paraffin block of the primary tumor and one paraffin block containing nonneoplastic breast tissue from the same patient were available, (c) all patients were treated by total mastectomy with axillary dissection followed by hormonal manipulation, which was based on original ER DCC results, with tamoxifen alone or combined with chemotherapy or radiotherapy, (d) follow-up was available.

Clinical data on all patients included patient age, tumor size, histologic classification, tumor grading (21), axillary lymph node status, pTNM (22), and ER status assessed by DCC analysis. Patient ages ranged from 35 to 79 years (seven premenopausal, four perimenopausal, 35 postmenopausal). All tumors used were primary, infiltrating carcinomas of the ductal type in 40 cases and of the lobular type in six cases. Nine patients had node-negative disease and 37 had node-positive disease. After surgery, ER status was assayed by the DCC ligand-binding method using a standard cut-off point of 5 fmol/mg cytosol protein to distinguish positive from negative tumors; 22 tumors were ER positive and 24 were ER negative by DCC assay. Adjuvant chemotherapy was given to node-positive patients, either pre- or postmenopausal, with negative ER status; postmenopausal patients with positive ER status received tamoxifen. The patients were followed-up from 6 months to 14 years. All patients had complete clinical follow-up at 6-month intervals for the first 36 months and at 1-year intervals thereafter. Before carrying out the present study, the follow-up of all cases was updated. Twenty-one patients (mean age, 56.61 ± 2.23) had progression of the disease (local recurrence, metastasis, or death) and 25 (mean age, 58.16 ± 1.81) were stable (alive and without local recurrence or metastasis at this time).

Immunohistochemistry

For each case, we sought one paraffin block containing both tumor and normal breast; when normal breast was absent, one paraffin block from the same breast specimen containing nonneoplastic breast tissue was used in parallel for positive control. Serial sections (4 μ thick) were cut from paraffin blocks, placed on silane-coated slides, and dried overnight at +60°C.

Antibodies

The following MABs were used according to the manufacturer's instructions: LH2, clone CC4-5 (YLEM, Avezzano, Italy), produced using as antigen for immunization a recombinant ER fusion protein—the location of the epitope recognized by this MAB is on the A/F domain (23); LH1, clone ER-P31 (YLEM), produced using as antigen for immunization a synthetic peptide (24)—the epitope recognized by this MAB is on the F domain of the C-terminal (23); ER1D5, clone 1D5 (YLEM), produced using as antigen for immunization a recombinant ER fusion protein—the location of the epitope recognized by this MAB is in the A/B domain at the N-terminal (25); this MAB reacts not only with wild-type (WT) ER but also with several truncated forms that are translated from splice variant mRNA. AER311, an antibody raised against the SDS-denatured calf uterus ER (kindly provided by Dr. Ciro Abbondanza, Cattedra di Patologia Generale, II Università di Napoli, Naples, Italy), which recognizes an epitope in the E/F domain at the C-terminal (5) and reacts only with WT ER, with the exception of the DNA-binding truncated protein (11).

Immunostaining

Paraffin sections were deparaffinized, rehydrated in graded alcohol to distilled water, and then immersed in 0.01 mol/L citrate buffer (pH 6) solution and subjected to antigen retrieval (AR), irradiated using a standard microwave oven (Miele Supratronic M750, Italy) at 700 W for 15 minutes (3 × 5 minutes). Microwaved sections were then incubated for another 20 minutes in the hot buffer before processing for immunocytochemistry. Four serial sections from each paraffin block were incubated with MABs to ER and immunostained with the streptavidin-biotin-peroxidase (YLEM) technique followed by diaminobenzidine (DAB) chromogen solution. All steps of the immunostaining procedure were 30 minutes long and were followed by washes in buffered solution. Finally, sections were weakly counterstained with hematoxylin

and mounted on Eukit. In all cases, positive, negative, and internal controls and controls for general tissue reactivity were used; in fact, a paraffin section of a breast tumor known to be ER positive was used as positive control, whereas a negative control was obtained by omitting the primary antibody. Moreover, as internal control, we looked for the occurrence of immunostaining in the nonneoplastic breast tissue; if this was not available, we used sections of nonneoplastic breast tissue from the same case. Finally, MABs to cytokeratin AE1/AE3 (YLEM) and vimentin (clone V9, YLEM) were used as overall indicators of breast tissue immune reactivity.

Interpretation of Immunostaining Results

For each case, ER results were first examined by simultaneously viewing the immunostained slides on a dual-head microscope using a ×10 objective to select the same neoplastic areas that were localized with a permanent pen for the counting of positive nuclei. Positive cells were counted in adjacent fields in the areas showing neoplastic infiltration; the stained sections were scored independently by two pathologists (G.S. and F.L.) using a Nikon E600 Eclipse microscope (Nikon, Italy), by counting an average of 3,500 neoplastic cells at ×40 magnification. Variations in staining intensity were observed between tumor cells and between different areas within the same section, a phenomenon that might be attributed to the heterogeneity of the tumor-cell population. Although the intensity of staining was noted, it was not incorporated into the final score, because there was disagreement between the two observers.

Statistical Analysis

The statistical analysis was performed using the SPSS/PC + program (26). The positivity for each MAB was evaluated by analyzing different values of positivity, ranging from 10 to 50%, with 10% increases. For each value of positivity, the specificity and sensitivity were analyzed according to the following indices of Galen and Gambino (27):

$$\text{Sensitivity (positivity in disease)} = [(TP)/(TP + FN) \times 100]$$

$$\text{Specificity (negativity in health)} = [(TN)/(TN + FP) \times 100]$$

where TP is true positive, TN is true negative, FP are false positive, and FN is false negative.

Moreover, multivariate discriminant analysis, including stepwise analysis with the Wilks' lambda method (26), was applied to include variables that contained information concerning group differences. The χ^2 test was used to evaluate the relationship between ER results and histopathologic features (26). Last, survival analysis was performed according to the Kaplan-Meier method (26); differences between survival curves were analyzed by log-rank method (26). For all statistical analyses, a P value < 0.05 was considered to be significant.

RESULTS

Positive immunostaining was seen in the nuclei of both malignant and nonmalignant epithelial breast cells; a small percentage of cases showed scanty and focal cytoplasmic immunostaining with all MABs used (Figs. 1 and 2). In the nonneoplastic breast tissue surrounding the tumor, the percentage of positive nuclei ranged from 30 to 100% for all MABs. The percentage varied depending on the presence of normal ducts and lobules, duct ectasia, cysts, and intraductal hyperplasia. In normal ducts and lobules, the percentage of positivity ranged from 30 to 100%; however, differences in the percentages of positivity were often found in the same breast specimen; in fact, some ducts and lobules exhibited lesser or greater positivity percentages. In some breast tissue from postmenopausal patients, a marked reduction in the percentage of positivity in the lobules and ducts was observed, whereas there was greater positivity in the ducts with intraductal hyperplasia. Last, in ectasic ducts and even more so in cysts with apocrine metaplasia, we observed greater reduction and, at times, absence of nuclear positivity. In general, there were no marked differences in staining intensity between nonneoplastic, hyperplastic, and neoplastic breast tissue; however, in some elderly patients, the carcinomatous cells often exhibited greater staining intensity compared with nonneoplastic cells.

Table 1 provides a summary of ER immunostainings and histologic grading, size of the tumors, lymph node status, and follow-up. Some tumors showed differences of percentage of positivity for each MAB used; moreover, <5% of neoplastic cells were positive in 18 cases immunostained with AER311 MAB, in 13 cases with LH2 MAB, in 10 cases with ER1D5 MAB, and in 13 cases with LH1 MAB.

As shown in Tables 2–5, a positivity >30% for each MAB possessed the best sensitivity/specificity ratio. Using as cut off of positivity the value >30%, AER311 and ER1D5 MABs were positive in 23 tumors and negative in 23; LH2 and LH1 MABs were positive in 22 tumors and negative in 24. The multivariate discriminant analysis has shown that MABs AER311, ER1D5, and LH2 had significant prognostic value (Table 6) in that they discriminated patients with disease progression from those with stable disease; on the contrary, LH1 and ER DCC assays did not show significant prognostic value. Of all MABs, AER311 had the highest discriminant

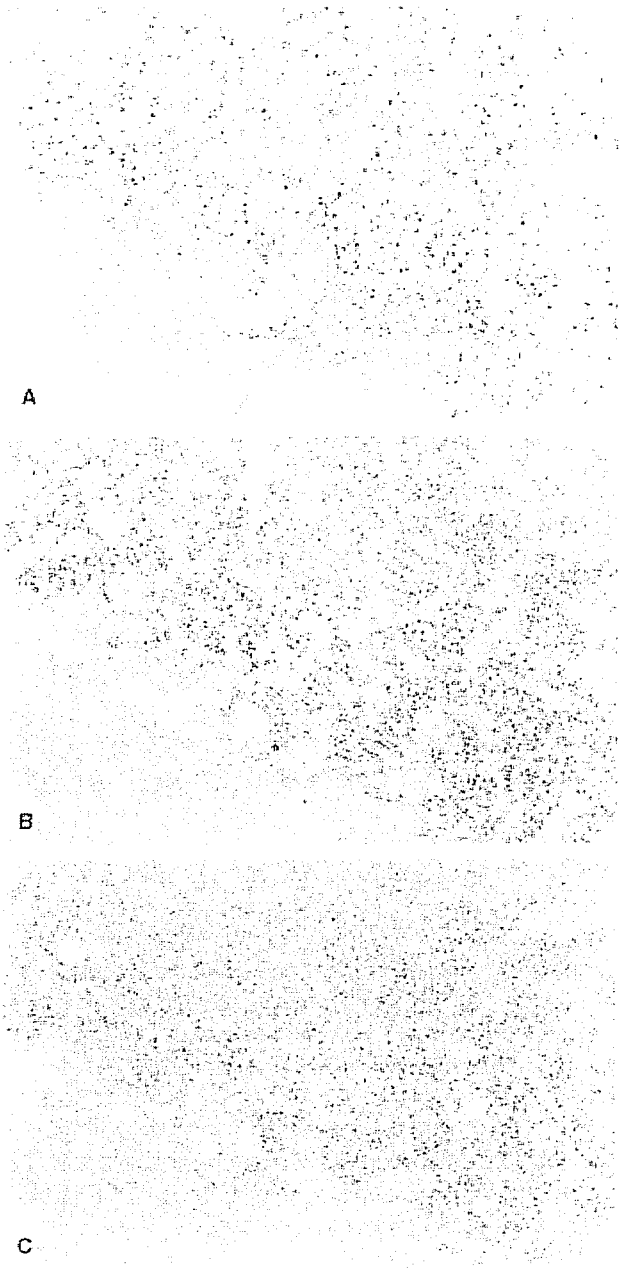

FIG. 1. Infiltrating breast carcinoma immmunostained with MABs to ER AER311 **(A)**, ER1D5 **(B)**, and LH2 **(C)**. Strepatavidin-biotin-peroxidase, DAB chromogen, hematoxylin counterstain, original magnification ×20.

value in that it had the highest Wilks lambda coefficient. At the cut-off value of positivity >30%, there were 14 tumors showing positivity for these three MABs that had significant prognostic value, whereas 17 tumors were positive for one or two of three MABs, and 15 were tumors negative for all three MABs. Within the group of tumors positive for one or two MABs, eight were positive for two MABs (four for AER311 and ER1D5, three for AER311 and LH2, one for LH2 and ER1D5) and nine were positive for only one MAB (two for AER311, four for LH2, and three for ER1D5).

A significant correlation ($P = 0.0001$) was also observed between ER results and the outcome of disease; all 14 patients with tumors positive for all three prognostically significant MABs were stable; on the contrary, all 15 patients with tumors negative for these three MABs had progression of the disease. It is worth noting that in the group of patients with tumors positive for one or two MABs, the presence of positivity for two MABs in a tumor indicates a tendency toward stabilization of the disease compared with tumors positive for only one

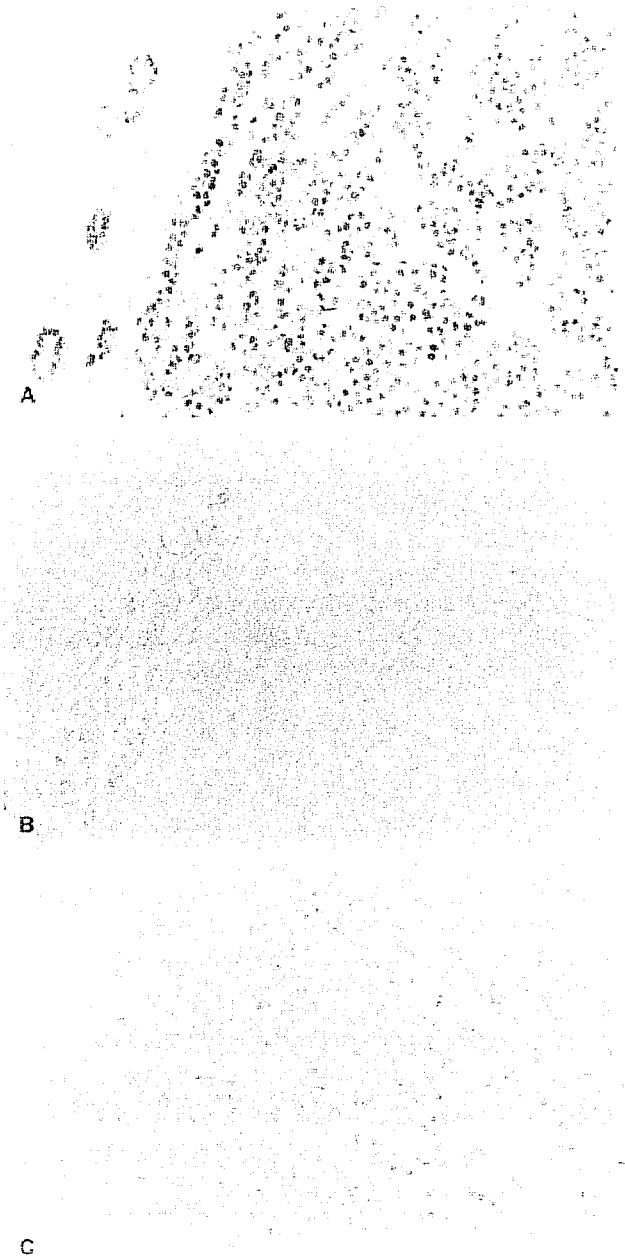

FIG. 2. Infiltrating breast carcinoma immunostained with MABs to ER AER311 **(A)**, ER1D5 **(B)**, and LH2 **(C)**. Streptavidin-biotin-peroxidase, DAB chromogen, hematoxylin counterstain, original magnification ×40.

TABLE 1. *Relationship between histologic grading, tumor size, lymph node status, outcome, ER by DCC, and ER by MABs*

Case no.	Age (y)	Histologic grading	pT	pN	Outcome	Follow-up (y)	ER-DCC (fmoles)	AER311 (% + cells)	LH2 (% + cells)	ER1D5 (% + cells)	LH1 (% + cells)
1	56	3	1	1	P	1	<5.0	<5	29	<5	<5
2	44	2	3	1	P	1	<5.0	<5	18	<5	5
3	36	2	2	1	P	1	<5.0	<5	28	<5	<5
4	58	3	1	0	P	1	95.6	72	<5	5	<5
5	63	3	2	1	P	1	<5.0	<5	<5	9	8
6	51	3	1	0	P	2	91.6	65	26	5	<5
7	57	2	3	1	P	2	<5.0	<5	<5	<5	<5
8	40	3	2	1	P	2	<5.0	<5	26	<5	<5
9	61	2	1	1	P	2	11.2	27	<5	18	5
10	64	2	1	1	P	2	<5.0	<5	5	5	<5
11	60	3	2	1	P	2	<5.0	<5	<5	5	5
12	65	2	1	0	P	3	<5.0	<5	38	8	<5
13	58	3	1	0	P	3	<5.0	<5	<5	28	5
14	57	2	1	1	P	3	<5.0	<5	8	<5	<5
15	42	2	1	0	P	3	<5.0	<5	36	5	5
16	79	2	1	0	P	3	<5.0	<5	<5	<5	5
17	64	3	1	0	P	4	<5.0	<5	<5	5	5
18	69	3	3	1	P	6	24.2	28	<5	36	48
19	59	3	2	0	P	7	<5.0	<5	<5	30	19
20	58	1	2	0	P	8	7.5	<5	17	20	<5
21	48	3	4	1	P	9	24.2	16	40	48	40
22	58	1	1	0	S	13	40.4	52	10	72	65
23	61	1	2	0	S	13	<5.0	39	78	65	80
24	39	1	1	1	S	13	11.2	56	71	95	86
25	59	2	1	1	S	13	29.2	18	39	<5	<5
26	64	2	1	0	S	13	166.7	51	86	84	71
27	63	2	1	1	S	13	108.7	<5	<5	47	38
28	69	2	2	0	S	13	149.7	<5	<5	38	37
29	50	1	2	0	S	13	<5.0	52	77	10	10
30	59	1	1	0	S	13	<5.0	52	82	72	77
31	35	1	1	0	S	13	<5.0	61	85	94	75
32	40	2	1	0	S	13	25.5	57	20	75	90
33	68	2	2	0	S	13	<5.0	66	75	72	81
34	70	1	2	0	S	13	12.4	53	83	91	75
35	59	2	1	1	S	13	110	38	<5	37	47
36	47	2	2	0	S	13	135.5	71	48	<5	<5
37	57	1	1	0	S	13	150.5	58	74	84	87
38	62	1	1	0	S	13	<5.0	54	71	91	81
39	61	1	2	0	S	13	<5.0	55	76	83	71
40	60	2	2	0	S	13	<5.0	38	71	86	85
41	63	2	2	0	S	13	21.5	54	78	82	77
42	59	1	1	0	S	13	35.5	58	81	75	82
43	60	2	1	0	S	13	60.5	51	9	71	65
44	61	2	1	1	S	13	35.6	29	40	<5	<5
45	63	1	2	0	S	13	<5.0	53	66	73	73
46	66	2	1	0	S	13	15.6	56	72	10	8

P, progression; S, stable; y, years; CC4-5 (clone LH2), ER1D5 (clone 1D5), LH1 (clone ER-P31).

TABLE 2. *Sensitivity and specificity of MAB AER311 using various cut-off points of positivity*

Cut off[a]	Cases positive			Cases negative			Sensibility	Specificity
	Total	Stable	Progression	Total	Stable	Progression		
>10%	28	23 (82.1%)	5 (17.9%)	18	2 (11.1%)	16 (88.9%)	92.0	76.2
>20%	26	22 (84.6%)	4 (15.4%)	20	3 (15.0%)	17 (85.0%)	88.0	81.0
>30%	23	21 (91.3%)	2 (8.7%)	23	4 (17.4%)	19 (82.6%)	84.0	90.5
>40%	20	18 (90.0%)	2 (10.0%)	26	7 (26.9%)	19 (73.1%)	72.0	90.5
>50%	5	3 (60.0%)	2 (40.0%)	41	22 (53.7%)	19 (46.3%)	12.0	90.5

[a] Percentage of positive cells.

TABLE 3. *Sensitivity and specificity of MAB LH2 using various cut-off points of positivity*

	Cases positive			Cases negative				
Cut off[a]	Total	Stable	Progression	Total	Stable	Progression	Sensibitility	Specificity
>10%	29	20 (69.0%)	9 (31.0%)	17	5 (29.4%)	12 (70.6%)	80.0	57.1
>20%	26	19 (73.1%)	7 (26.9%)	20	6 (30.0%)	14 (70.0%)	76.0	66.7
>30%	22	19 (86.4%)	3 (13.6%)	24	6 (25.0%)	18 (75.0%)	76.0	85.7
>40%	17	17 (100%)	0	29	8 (27.6%)	21 (72.4%)	68.0	100
>50%	16	16 (100%)	0	30	9 (30.0%)	21 (70.0%)	64.0	100

[a] Percentage of positive cells.

MAB. Seven (87%) of 8 patients with tumors positive for two MABs (four tumors were positive for AER311 and ER1D5 and three tumors were positive for AER311 and LH2) were stable, whereas 4 (44.4%) of nine patients with tumors positive for one MAB (two tumors were positive for ER1D5 and two for LH2) had stabilization of the disease. Survival analysis shows that patients with tumors negative for all three of these MABs had progression of the disease within 8 years from the original diagnosis, whereas all patients with positive tumors for all three MABs were alive 13 years after surgery (Fig. 3).

Table 7 shows the relationship between ER results obtained with AER311, ER1D5, and LH2 MABs and tumor grading, tumor size, and lymph node metastases. A significant correlation ($P = 0.0006$) between tumor grading and ER status was found; tumors that were positive for all three MABs were grade 1 in 71% of cases, whereas tumors negative for all three MABs were mostly grades 2 and 3. Furthermore, no significant relationship was observed between ER status and tumor size. Last, a significant correlation ($P = 0.008$) between lymph node status and ER was found; in fact, breast tumors positive for all MABs were pN0 in the majority (92.9%) of cases, whereas ER-negative tumors were pN1 in the majority (66.7%) of cases.

DISCUSSION

In the present study of 46 primary breast carcinomas, the analysis of ER status using MABs against different domains of the ER protein showed differences in the percentage of staining for each antibody used; these findings support the notion that some domains of the ER protein were missing. Similar results were previously noted in studies in which two MABs that recognize different domains in the NH_2 or the COOH terminal regions of the ER protein were compared (6–19). Moreover, in the study performed by Elias et al. (6), the discrepancy observed between reactivity of ER1D5 MAB (which targets an epitope in the A/B region) and H222 MABs (which targets an epitope in the E region) in breast tumors was interpreted as the presence of "outlaw variants." Furthermore, Saccani-Jotti et al. (15) found some breast tumors strongly positive for ER1D5 MAB and negative for H222 MAB, suggesting that MABs directed against different parts of the receptor protein may also be of value in the investigation of variant forms of ER. Also, Traish et al. (28), who developed MABs to specific domains of ER protein, demonstrated the presence of structurally defective ER in human breast, suggesting that MABs to ER domains could provide a means of identification of dysfunctional ER in breast tumor biopsies.

In the present study, <5% of neoplastic cells were positive in 18 cases immunostained with AER311 MAB, in 13 cases with LH2 MAB, in 10 cases with ER1D5 MAB, and in 13 cases with LH1 MAB. These results document the occurrence of the lack of the carboxyl and amino terminal domains in some breast carcinomas and confirm the results of Hori et al. (16), who found a number of breast cancers lacking the carboxyl terminal of ER protein.

Because we used MABs that target different domains of the ER protein, we hypothesize that alterations of the receptor protein could have resulted in absence of the domain recognized by MABs. This alteration may be a

TABLE 4. *Sensitivity and specificity of MAB ER1D5 using various cut-off points of positivity*

	Cases positive			Cases negative				
Cut off[a]	Total	Stable	Progression	Total	Stable	Progression	Sensibility	Specificity
>10%	26	20 (76.9%)	6 (23.1%)	20	5 (25.0%)	15 (75.0%)	80.0	71.4
>20%	24	20 (83.3%)	4 (16.7%)	22	5 (22.7%)	17 (77.3%)	80.0	81.0
>30%	23	20 (87.0%)	3 (13.0%)	23	5 (21.7%)	18 (78.3%)	80.0	85.7
>40%	19	18 (94.7%)	1 (5.3%)	27	7 (25.9%)	20 (74.1%)	72.0	95.2
>50%	17	17 (100%)	0	29	8 (27.6%)	21 (72.4%)	68.0	100

[a] Percentage of positive cells.

TABLE 5. *Sensitivity and specificity of MAB LH1 using various cut-off points of positivity*

	Cases positive			Cases negative				
Cut off[a]	Total	Stable	Progression	Total	Stable	Progression	Sensibility	Specificity
>10%	23	20 (87.0%)	3 (13.0%)	23	5 (21.7%)	18 (78.3%)	80.0	85.7
>20%	22	20 (90.9%)	2 (9.1%)	24	5 (20.8%)	19 (79.2%)	80.0	90.5
>30%	22	20 (90.9%)	2 (9.1%)	24	5 (20.8%)	19 (79.2%)	80.0	90.5
>40%	19	18 (94.7%)	1 (5.3%)	27	7 (25.9%)	20 (74.1%)	72.0	95.2
>50%	17	17 (100%)	0	29	8 (27.6%)	21 (72.4%)	68.0	100

[a] Percentage of positive cells.

consequence of abnormalities of receptor protein at DNA or mRNAs level, rather than intratumoral heterogeneity of ER expression, different antibody affinities, loss of a portion of the protein molecule owing to proteolysis, or masking of the epitope target by either protein conformation changes or by binding of heat-shock protein. This hypothesis is also sustained by studies of Huang et al. (11), who also reported discordant results using ER1D5 and AER311 MABs in assessing the ER status in breast tumors. They also found significant correlation between expression of certain ER variant mRNAs, encoding truncated ER proteins assessed by polymerase chain reaction analysis, and inconsistent ER results in breast carcinomas assessed by immunohistochemistry using ER1D5 and AER311 MABs (20). This observation supports a growing body of evidence suggesting that ER variant mRNAs may be translated in vivo into altered ER-like proteins (29,30), thus determining the discrepant results of ER analysis when two or more MABs that recognize different domains of ER are used. The difference in target epitope specificity of MABs to ER may be important in view of the many different ER mRNA variants that have been described in normal and human breast cancer biopsy samples and cell lines (31,32), any of which may be translated into a variety of truncated or abnormal proteins (33–35) that may fail to produce any biologic function. The lack of response to endocrine therapy, for example, the development of tamoxifen resistance, in some patients with ER-positive tumors could be attributed to the presence of structural alteration of the ER protein.

The use of a panel of MABs that target distinct epitopes within domains of the ER protein, including AER311, which targets an epitope within the E/F hormone-binding domain, and ER1D5, which targets an epitope in the A/B of estrogen-responsive genes domain, could offer a better approach for assessing the ER status in breast cancer patients. This approach would allow the recognition of breast tumors with structurally defective ER proteins and would be helpful in cases that are initially negative for ER. Using a single MAB that recognizes a restricted domain within the ER protein may give negative results in tumors with defective domains with disappearance or distortion of their transcriptional function. These observations may have great clinical relevance, permitting the identification of that group of ER-positive tumors that exhibit no response to hormonal therapy, thereby reducing the proportion of apparently ER-negative responders. Therefore, in the evaluation of ER status in breast cancer specimens, if the "intact" ER is the predominant receptor species, then the tumor should be considered positive. However, using MABs that react with different domains of ER protein, any discrepancy in positivity observed should be considered as indicative of the possible presence of ER variants and should be further investigated using antibodies to progesterone receptors (PR) and pS2.

TABLE 6. *Multivariate discriminant analysis: discriminant coefficient (Wilks lamba) and relative statistical significance of the three MABs (AER311, ER1D5, and LH2) that had significant prognostic value*

Discriminant variables	Wilks' lambdas	*P*
AER311	0.45	<0.0001
LH2	0.29	<0.0001
ER1D5	0.35	<0.0001

Canonical discriminant function: $P < 0.0001$.

Although immunohistochemistry, compared with biochemical methods, offers greater advantages in evaluating ER status, it is influenced by a number of factors that must be considered if results are to be reliable. Among these factors, fixation and tissue processing are most important. Furthermore, the MABs used with particular attention to the ER domain recognized, the method of AR, and the sensitivity of the detection system are also key factors (36,37). Moreover, in evaluating staining results, the scoring method and the cut-off point for positivity that is chosen to define a tumor as ER positive are also important factors. At present, there is neither a standard scoring method for evaluating immunostaining results nor a uniformly agreed cut-off value that defines ER positivity and that can be considered as the standard for all histopathology laboratories (38–40).

In the present study, we observed that a cut-off value of positivity >30% for all MABs used best separates patients with tumor stability from those with tumor progression. Moreover, multivariate discriminant analysis revealed that among MABs and DCC assay, AER311, LH2, and ER1D5 MABs have independent prognostic

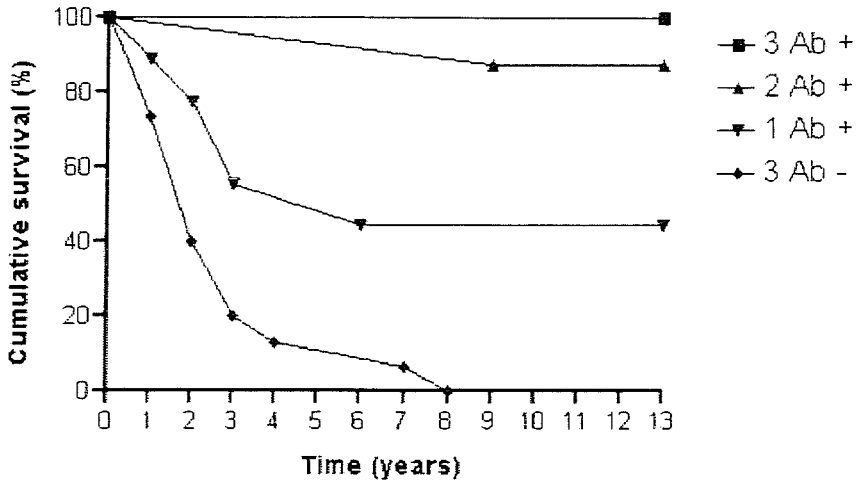

FIG. 3. Survival analysis according to the Kaplan-Meier method. Patients with tumors negative for all three MABs had progression of the disease within 8 years from the diagnosis of the tumor; all patients with tumors positive for all three MABs were alive 13 years after surgery.

value, and among these three MABs, AER311 has the highest discriminating power. At the positivity value of >30%, 14 tumors were positive for three MABs; this group of tumors should represent the estrogen-dependent phenotype tumors. In addition, 15 tumors were negative for these three MABs; these tumors, which show lack of both amino and carboxyl terminals, appear to have an hormone-independent phenotype. From a clinical point of view, recognition of "true" ER-negative tumors is most important in that aggressive therapies could be considered for patients with ER-negative tumors. Seventeen tumors that were positive for one or two MABs may represent that group of tumors in which there are ER variants.

Our results also indicate that ER-negative tumors are mostly grades 2 and 3 and more commonly have lymph node metastases. All patients with tumors negative for all three MABs showed progression of the disease within 8 years from the time of the diagnosis of the tumor, whereas all patients with tumors positive for all three MABs were alive 13 years after surgery. It has been hypothesized that the expression of variants of ER mRNAs may have a role in the development of endocrine resistance and progression of human breast cancer (33,41), but in the present study the presence of ER variants does not seem predictive of tumor progression into more aggressive stages.

Results from this study indicate that MABs that target different domains of the ER protein such as AER311, ER1D5, and LH2 should be used along with immunocytochemistry to detect ER in paraffin sections from breast tumors in that they improve the assessment of receptor status. This approach, at the cut-off positivity value of >30% of tumor cells, appears to offer better accuracy in the evaluation of receptor status, because MABs directed toward certain ER domains may give false negative results, if changes in the ER protein resulted in absence or conformational alteration of the domain targeted.

The present study deals only with specimens from primary tumors and not from recurrences and does not include PR analysis of the patient population. Further studies including primary and recurrent tumor specimens

TABLE 7. *Relationship between ER results (using MABs AER311, ER1D5, LH2) and tumor grading (G), tumor size (pT), and lymph node status (pN)*

Histopathologic features	Total cases	Immunohistochemical results: 3 MABs positive (14 cases)	2 MABs positive (8 cases)	1 MAB positive (9 cases)	3 MAB negative (15 cases)	*P*
Grade						
1	13	10 (71.4%)	2 (25.0%)	0	1 (6.7%)	
2	22	4 (28.6%)	5 (62.5%)	6 (66.7%)	7 (46.7%)	0.0006
3	11	0	1 (12.5%)	3 (33.3%)	7 (46.7%)	
Tumor size						
pT1	26	7 (50%)	5 (62.5%)	7 (77.8%)	7 (46.7%)	
pT2	16	7 (50%)	2 (25.0%)	1 (11.1%)	6 (40.0%)	0.25
pT3	3	0	0	1 (11.1%)	2 (13.3%)	
pT4	1	0	1 (12.5%)	0	0	
Lymph node status						
pN0	29	13 (92.9%)	6 (75.0%)	5 (55.6%)	5 (33.3%)	0.008
pN1	17	1 (7.1%)	2 (25.0%)	4 (44.4%)	10 (66.7%)	

$P < 0.05$ is statistically significant.

along with PR evaluation will be necessary to confirm whether breast tumors in which the "intact" ER is the predominant receptor type are likely to be estrogen dependent and tamoxifen sensitive and whether the presence of ER variants may contribute to tamoxifen resistance and to progression of human breast cancer. □

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APPENDIX B

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

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BACKGROUND. Historically, estrogen receptor (ER) determinations have been made by the ligand-binding assay of tumor homogenates, primarily by the dextran-coated charcoal method (DCC). Immunocytochemical assays (ICA) for ER are more recent and have been executed mostly on frozen sections with the monoclonal antibody H222Spγ (H222). Lately, new monoclonal antibodies derived by recombinant ER technology have been developed that work well on paraffin embedded, formalin fixed tissue sections. However, there is little information as to whether such assays prognosticate endocrine response.

METHODS. Using antigen retrieval, the immunoglobulin G_1 monoclonal antibody ER1D5, and the streptavidin-biotin detection system, 74 patients with breast cancer in whom endocrine response was known were assayed and the results compared with ER by DCC and ER by ICA in frozen section with H222.

RESULTS. ER1D5 in paraffin provided the highest correlation with endocrine response (Kendall's tau |r| = 0.57; $P < 0.001$) whereas ER by DCC failed to correlate ($r = -0.002$; $P < 0.99$). ER1D5 in paraffin correlated weakly though significantly with DCC (Kappa Statistic |K| = 0.204; $P < 0.02$). H222 in frozen sections also correlated moderately with endocrine response ($r = 0.34$; $P < 0.001$).

CONCLUSIONS. ER can be detected in routine tissue sections processed with antigen retrieval and ER1D5, and can be relied upon to provide accurate prognostic information regarding response to endocrine therapies in breast cancer patients. *Cancer* **1996; 77:2514–9.** © *1996 American Cancer Society.*

KEYWORDS: estrogen receptor, immunohistochemistry, paraffin sections, endocrine response.

It is well known that estrogen receptor (ER) determination is important in that it enables prediction of endocrine response in breast cancer.[1] Initially, steroid receptors were measured by cytosol-based ligand-binding techniques, predominantly the dextran-coated charcoal (DCC) and sucrose gradient assays. In the past few years, it has become evident that ER detection by immunocytochemical assay (ICA) is at least as accurate a prognosticator of endocrine response when performed on frozen tissue.[2] Much of the latter work utilized the monoclonal antibody H222Spγ (H222), which recognizes an epitope on the N terminus of the receptor molecule.[3,4] Recently, other anti-ER antibodies have become available, such as ER1D5, which perform well in formalin fixed paraffin embedded sections in combina-

TABLE 1
Types of Hormonal Therapies Administered

Therapy	No. of patients
Ovariectomy	6
Tamoxifen	36
Megestrol acetate	3
Fluoxymesterone	2
Aminoglutethimide	3
Tamoxifen and megestrol acetate	14
Tamoxifen and fluoxymesterone	4
Other endocrine therapies	6

tion with antigen retrieval techniques. In a survey by the College of American Pathologists,[5] 49% of 276 laboratories reported employing such methods. Nonetheless, there remains a lack of clinical information concerning their value in predicting endocrine response (the prime reason for measuring ER in the first place), and there is no agreed uniformity in processing methodology.[6,7]

To determine the most accurate method for detecting ER, 74 breast cancer patients were studied. In all cases, hormone response was correlated with ER by DCC, with H222 on fresh frozen tissue, and with ER1D5 in routinely prepared formalin fixed, paraffin embedded sections.

MATERIALS AND METHODS

The patients studied all satisfied the following criteria: 1) formalin fixed, paraffin embedded tumor tissue was available; 2) the tumor was initially assayed for ER by DCC in the laboratory of the late Dr. William L. McGuire at the University of Texas Health Science Center at San Antonio; 3) fresh frozen tissue had been assayed, as described previously, employing the monoclonal anti-ER antibody H222[2]; 4) primary treatment was with hormonal manipulation; and 5) endocrine response was documented according to the criteria of the Cooperative Breast Cancer Group.[8] Furthermore, patients had to have received endocrine therapy for a minimum of 3 months and any favorable response must have been maintained for an additional 3 months. No other selection criteria were employed.

Seventy-four women fulfilled these criteria, all with Stage IV disease (American Joint Committee on Cancer, 1983). Thirty-eight were white, 31 were African American, 4 were Hispanic, and 1 was Asian. Patient ages ranged from 26.8 to 88 years (mean, 62.6 years). At the time of diagnosis, 10 patients were premenopausal, 5 were perimenopausal (within 1 year of the last menses), and 59 were postmenopausal. The type(s) of endocrine therapy administered are shown in Table 1. Sixteen patients achieved a complete or partial endocrine response, and 10 women had their disease stabilized, whereas 48 women progressed on hormonal therapy.

Paraffin sections from each case were cut onto silanated slides, dewaxed in xylene, dehydrated in graded ethanols, and microwaved at high intensity in citrate buffer (pH 6) for 15 minutes.[9] The citrate buffer was replenished as needed. After cooling, the sections were processed for ER using the antibody ER1D5 (Dako Laboratories, Carpenteria, CA). This mouse immunoglobulin G_1 monoclonal antibody reacts with the A/B region of the N terminal domain of ER. It also reacts with the 67-kilodalton polypeptide chain of ER obtained by transformation of *Escherichia coli* and transfection with COS cells with plasmid vectors expressing ER.[10]

Each section was incubated with blocking antibody (10% goat serum) for 5 minutes to inhibit nonspecific binding and then with ER1D5 for 1 hour at ambient temperature. Immunoreactivity was detected employing the streptavidin-biotin technique as recommended by the manufacturer (DAKO LSAB2 kit; Dako Laboratories, Carpenteria, CA). Two 5-minute Tris phosphate-buffered saline washes were given between each incubation.

Parallel sections were incubated with nonimmune mouse immunoglobulin G (IgG) in lieu of specific antibody and a positive substrate control was run parallel with each batch of tissues. The latter was comprised of a mammary carcinoma known to be strongly ER positive by both DCC and, when frozen, with H222. A specimen was considered ER positive by ICA when nuclear staining was apparent in 10% or more of the constituent tumor cells, as estimated by visual inspection. The substrate control had to be positive with specific antibody whereas sections exposed to control antibody were negative. These criteria were the same as those previously utilized in evaluating H222 in frozen preparations, having been found to produce the best correlations with endocrine response.[2] Tumors with an ER content of ≥ 3 fmol/mg protein by DCC were considered biochemically ER positive because this was the cutoff employed in Dr. McGuire's laboratory.

Each prepared slide was read independently by two pathologists (Y.D.K. and F.S.). Neither examiner had any knowledge of, or access to, original ICA or ER by DCC results, or information as to the patients' clinical endocrine response. In two cases, there was a discrepancy in assay interpretation. These discrepancies were in tumors displaying circa 10% stained tumor nuclei, and which were designated as positive after examination by a third pathologist (L.P.P.).

TABLE 2
Relationship between Assay Results

A. Correlation between H222 (Frozen) and ER1D5 (Paraffin)

	ER1D5 (paraffin)		
H222 (frozen)	Negative	Positive	Total
Negative	24	7	31
Positive	14	29	43
Total	38	36	74

Kappa = 0.44 ± 0.10; $P = 0.001$

B. Correlation between ER1D5 in paraffin section and ER by DCC

	ER1D5 (paraffin)		
ER by DCC[a]	Negative	Positive	Total
Negative	10	2	12
Positive	28	34	62
Total	38	36	74

Kappa = 0.204 ± 0.082; $P < 0.02$

C. Correlation between H222 in frozen section and ER by DCC

	H222 in frozen section		
ER by DCC[a]	Negative	Positive	Total
Negative	8	4	12
Positive	23	39	52
Total	31	43	74

Kappa = 0.18 ± 0.10; $P < 0.057$

ER: estrogen receptor; DCC: dextran-coated charcoal.
[a] Assuming the cutoff value for estrogen receptor positivity is ≥3 fmol/mg protein.

Statistical Methods

Agreement was assessed with the Kappa statistic (K) when examining two variables, both with nominal outcomes (e.g., H222 vs. ER by DCC). Kendall's tau (r), a measure of correlation best used when the outcome is grouped frequency data, was utilized to compare associations of the assays with endocrine response. Exact probabilities were determined for K and r using the Exact tests module of SPSS for Windows.[11] Assay sensitivity, specificity, and predictive values were determined comparing disease progression versus stabilized and responding patients combined.

RESULTS

A total of 36 cases in paraffin were designated positive with ER1D5 (49%). There was no significant ethnic difference in the frequency of positivity in the paraffin embedded specimens. Nineteen white women had positive tumors and an equal number were negative, and 15 African American women had positive neoplasms whereas 16 were negative. Three of the four Hispanic women had negative tumors and the 1 Asian patient was positive.

Table 2 shows the relationship between the various assay results. Although all techniques produced statistically significant agreements with each other, the best concordance was between H222 in frozen tissue and ER1D5 in paraffin embedded tissue (K = 0.44 ± 0.10; $P < 0.001$). The second best correlation was between ER1D5 in paraffin and ER by DCC (K = 0.204 ± 0.082, $P < 0.02$).

Correlations between assay results and clinical endocrine response are shown in Table 3. ER1D5 in paraffin was the best predictor (r = 0.57; 95% confidence interval [CI], 0.51–0.73; $P < 0.001$). H222 in frozen tissue was also a significant predictor (r = 0.34; 95% CI, 0.16–0.51; $P < 0.001$). However, ER by DCC did not correlate in this series of cases (r = −0.002; 95% CI, −0.23–+.23; $P < 0.99$; Table 3B). Sensitivity, specificity, and predictive values were the highest for ER1D5 in paraffin (Table 3C). These results did not materially change if stabilized patients were included among patients with treatment failures rather than with the responders. Increasing the cutoff point for ER positivity by DCC to 10 fmol/mg protein produced better concordance with H222 (K = 0.404) and ER1D5 (K = 0.265), but only moderately improved agreement with hormonal response (r = 0.06).

There were 21 patients in whom there was discordance between ER1D5 in paraffin versus H222 in frozen sections. Seven samples that were ER1D5 positive were H222 negative when fresh. Of these patients, three regressed or became stabilized on endocrine therapies. An additional 14 women were ER1D5 negative and H222 positive. Thirteen of the H222 positive patients progressed and 1 was stabilized. ER1D5 in paraffin thus correctly predicted endocrine response in 16 of the 21 discordant cases ($P < 0.02$). There were 30 cases in which there was disagreement between ER1D5 in paraffin and ER by DCC. Two patients were ER1D5 positive and DCC negative. One patient regressed and the other was stabilized. Twenty-eight patients were ER1D5 negative and DCC positive, of whom 27 progressed. Therefore, in these 30 discordant cases, ER1D5 in paraffin correctly predicted hormone response in 29 patients ($P < 0.001$).

There was no difference in the frequency of ER positivity in the paraffin blocks from each of the four participating hospitals. Thus, the technique employed allowed successful identification of ER despite a lack of uniformity in paraffin block preparation.

DISCUSSION

With advances in methods of diagnosis and increased patient awareness, more and more breast cancers are

TABLE 3
Correlations between Assay Results and Clinical Endocrine Response

A. ER assay results and endocrine response

	R1D5 (paraffin)		H222 (frozen)		ER by DCC[a]	
Response	Negative	Positive	Negative	Positive	Negative	Positive
Disease regression:	1	15	2	14	3	13
Disease stable:	2	8	3	7	1	9
Disease progression:	35	13	26	22	8	40

B. Associations between endocrine response and ER assay results

	Kendall's tau	*P* value
ER1D5 (paraffin)	0.57 ± 0.08	0.001
H222 (frozen)	0.34 ± 0.09	0.001
ER by DCC[a]	−0.002 ± 0.114	0.99

C. A comparison of assay sensitivities, specificities, and predictive values

	ER1D5 (paraffin)	H222 (frozen)	ER by DCC[a]
Sensitivity	23/26 (89%)	21/26 (81%)	22/26 (85%)
Specificity	25/48 (73%)	26/48 (54%)	8/48 (17%)
+ Predictive value	23/36 (64%)	21/43 (49%)	22/62 (16%)
- Predictive value	35/38 (92%)	26/31 (84%)	8/12 (67%)

ER: estrogen receptor; DCC: dextran-coated charcoal.
[a] Assumes cutoff point for estrogen receptor positivity = ≥3 fmol/mg protein.

discovered at an early stage. As a consequence, many tumors are less than 1 cm in diameter and the entire specimen is required for pathologic analysis. Therefore, there is a real need for methods that enable determination of breast cancer prognostic markers, including ER, in formalin fixed, paraffin embedded tissue, which is often the only sample available. The current study shows that the antibody and methodology described permits an accurate estimation of ER, which is most efficient in prognosticating endocrine response. The technique can readily be performed and interpreted at the community hospital level. An added benefit is the optimal preservation of specimen morphology.

Logistic difficulties in performing ER by ICA on frozen tissue as well as ER by DCC include the need for sufficiently large specimens so as to be able to process partly for ER and partly for histology. Biochemistry requires homogenization, ultracentrifugation, radiolabeled probes, scintillation counting, and Scatchard plot analysis. Histochemistry on frozen tissue requires immediate ultracold refrigeration/freezing and storage. Institutions that do not perform their own assays need to ship specimens in dry ice to reference laboratories by air express. This entails extra expense and provides a further source for error in the event of a thaw.

Reasons for discordance between H222 on frozen sections and ER by DCC are well known.[12] Because specimens for DCC require homogenization, there is no way to detect the source of any receptor that may be measured. The receptor might equally be from benign epithelium and not from tumor cells. With ICA, a few positive cells among thousands can readily be detected whereas so few cells might not contribute enough ER to a cytosol to be discerned by DCC. The question arises, however, as to why there should be so much discordance between H222 in frozen sections and ER1D5 in paraffin sections.

In this study, some cases that were ER negative when frozen became ER positive in paraffin. The most likely explanation for this discrepancy has to do with the temperature lability of the receptor. There may have been a delay in initial freezing that allowed the receptor to degrade, or degradation occurred due to repeated freezing and thawing. When the biopsy is placed directly into formalin, however, receptor integrity is maintained until the epitopes are exposed during antigen retrieval. It is not clear why specimens that were H222 positive when frozen become ER1D5 negative in paraffin. One possible explanation is the different target for each monoclonal antibody. Although both are directed against the ER N terminal

domain, they do not recognize the same epitopes. Our patients whose tumors were ER1D5 negative in paraffin nearly all failed therapy. Therefore, the epitope recognized by this antibody may be more important to receptor function. Another reason for discordant results may lie in ER heterogenity. Because different blocks of tissue were studied, it is possible that only one contained ER positive tumor cells.

In prior unpublished work from this laboratory, 232 fresh frozen breast cancer specimens were subjected to ICA employing 2 different antiestrophilin antibodies: H222 and D75P3γ. Although both antibodies are directed against epitopes on the N terminus of ER,[3,4] concordance was only attained in 176 cases (76%). D75P3γ failed to detect ER in 15% of cases positive with H222, whereas H222 failed to detect ER in 26% of cases positive with D75P3γ. In addition, in some cancers, there were marked differences in the percentage of positive cells per tissue section. These results clearly show that different antibodies directed against the N terminal domain do not necessarily result in the same findings when employed for ICA.

In the past, many authors reported techniques designed to detect ER in routinely processed tissue.[13-22] To a large extent, these methods employed enzyme pretreatments. Not uncommonly, the proteolytic activity of enzymes is variable from batch to batch. As a consequence, reproduceability in other laboratories is difficult. Still other methods required fixatives and processing techniques differing substantially from those customarily used. Furthermore, correlations were primarily with H222 on frozen section or with ER by DCC. Only in one such study were results correlated with endocrine response.[23] Assay sensitivity and negative predictive values were satisfactory but specificity and positive predictive values were low.

Several studies have described ER detection in paraffin utilizing antigen retrieval but, with one exception, have not correlated results with any clinical parameter.[24-27] Goulding et al reported on 90 tamoxifen treated patients using antigen retrieval and ER1D5. Assay sensitivity was 90% but specificity was low (51%).[28] It is not presently clear why specificity was lower than in the current study (73%) but this difference could be associated with the method of scoring utilized: a histoscore. This is a semiquantitative approach based upon ER heterogeneity and staining intensity and would tend to produce more positive cases. For example, a tumor with 5% cells showing a 4+ staining intensity would have a histoscore of 20 (4 × 5) and be considered positive, whereas with the qualitative method, the score would be 5 and the tumor designated negative. In our experience, a positively stained cell is best considered positive regardless of the staining intensity, and a cutoff point of 10% positive cells produces the best clinical discriminations.

These findings support the concept that results of ER by ICA with ER1D5 performed on routinely prepared sections can be accepted by clinicians with confidence, assuming use of the antibody and methodology described. Such results are significantly more predictive of endocrine response than those of ER by DCC and/or H222 on frozen tissue.

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APPENDIX C

Assessment of Tissue Estrogen and Progesterone Receptor Levels: A Survey of Current Practice, Techniques, and Quantitation Methods

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■ **Abstract:** The assessment of steroid hormone receptors in resected breast carcinoma tissue is currently the standard of practice. The traditional method for assessment of receptor status is the ligand binding assay. More recently, immunohistochemistry (IHC) has become a popular method for such testing. Despite the widespread use of IHC and the availability of many antibodies, standardization of quantitative IHC for assessment of estrogen and progesterone receptors has not been achieved. While the College of American Pathologists (CAP) offers a Quality Assurance (QA) program for IHC quantitation of estrogen receptor (ER) and progesterone receptor (PgR), no universal standard is currently recognized in assessment of ER and PgR by IHC. We surveyed 300 laboratories within the United States for their current practices regarding the assessment of ER and PgR status in breast cancer tissue specimens. Eighty usable responses were received. Forty-nine (61%) laboratories performed the assay in-house, while the remainder sent the material out for assay. All responding laboratories performing their steroid receptor analysis in-house used the IHC technique. Forty-three (80%) laboratories answering the question on material accepted for analysis performed the assay only on paraffin-embedded material, three (6%) used either paraffin block or frozen material, and two (4%) used only frozen material. Eighty-eight percent of laboratories performing steroid receptor analysis in-house used a manual quantitation technique. Four (8%) used computer-assisted image analysis, and a single laboratory used laser scanning cytometry. Eight different antibodies were used among the 44 laboratories documenting the antibody supplier, and for any given commercially prepared antibody a wide variety of dilutions were used, with the exception of the standard solution used with the Ventana antibody. Of the laboratories using manual estimation techniques, 61% simply estimated the percentage of positive cells, 29% evaluated both the intensity of staining and percentage of nuclei staining, 6% used formal H-score analysis, 2% evaluated only intensity of nuclear staining, and 2% mainly counted the percentage of nuclei staining for ER but used a formal H score in the assessment of PgR. Cutoff points for the separation of positive and negative results varied widely, with some laboratories assessing any demonstrable positivity as a positive result, while others required as many as 19% of the nuclei to stain before a specimen was declared positive. Standardization techniques differed considerably among laboratories. Eighty-six percent used the CAP program for QA. While all laboratories utilized some form of intralaboratory control for assessment of ER and PgR, the nature of that control varied from laboratory to laboratory. Our survey indicates that a majority of laboratories perform their steroid hormone receptor

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analysis in-house using IHC. There is considerable variability in the antibodies utilized, the dilutions applied, and the quantitation method and level of expression used to dichotomize specimens into positive and negative groups. Finally, no universal control for interlaboratory standardization appears to exist. ■

Key Words: breast carcinoma, estrogen receptor, progesterone receptor, steroid hormone assay

Estrogen receptor (ER) and progesterone receptor (PgR), by their interaction with their respective steroid hormones play important roles in regulating the proliferation and differentiation of normal breast epithelium (1). The level of steroid hormone receptor expression in breast carcinoma cells is believed to be associated with the responsiveness of the neoplastic cells to circulating estrogen and progesterone. During the past quarter century, many studies have measured tissue levels of ER and PgR by biochemical methods and correlated them with both prognosis and response to hormone therapy (2–5). Within the past decade, a variety of antibodies against both the ERs and PgRs have become available on a commercial basis. Many studies have compared the results of immunohistochemically determined steroid receptor values with those obtained by ligand binding analysis. In general, the correlation has been good (6–11). Despite agreement of results derived from individual antibodies used in immunohistochemistry (IHC) determinations and the ligand binding technique, significant variability has been documented in the results obtained by IHC using different commercially available antibodies (12,13). While variability between the results achieved by different antibodies may exist, the overall value of IHC-determined ER and PgR levels for the prediction of response to hormonal therapy and overall prognosis appears high (9,14–16). Some studies have documented IHC determination of ER to be superior to the ligand binding assay for the prediction of response to adjuvant endocrine therapy in breast cancer (17).

Despite the documented value and accuracy of ER and PgR assayed by IHC, a wide variety of antibodies and quantitation techniques are currently in use. The extent to which these variabilities in technique and material affect the predictive value and standardization of IHC determination of ER and PgR is unknown. Of significance, there is no universally accepted control for standardization of the assays of ER and PgR by IHC. Hence interlaboratory comparisons of steroid receptors as determined by IHC may not be entirely valid. The authors are aware of only a single quality assurance/quality control (QA/QC) program within the United States for IHC determination of steroid receptors, again bringing into question the overall consistency of results obtainable between laboratories. Despite such problems, the overall robustness of the method appears to compensate for the lack of precision associated with the technique. Several laboratories are working to refine protocols and standardization methods and Riera et al. (18) have recently proposed tissue cell culture lines as a universal control.

In order to clarify the current status of ER and PgR level determinations, we surveyed 300 laboratories to determine their methods and interpretative approach for the estimation of steroid hormone receptors in breast tissue and whether they take part in a regional or national QA/QC program in this field. Herein we report the results of that survey.

MATERIALS AND METHODS

Questionnaires were mailed with return addressed and stamped envelopes to 300 hospital pathology departments. The first 200 questionnaire recipients were drawn from the College of American Pathologists (CAP) directory by randomly selecting four pathologists from each state. An additional 100 pathologists with interest and expertise in the area of breast pathology were selected on the basis of a literature search for publications concerning estrogen and progesterone receptors.

The questionnaire contains questions relating to size and type of hospital practice. The questionnaire asked if they routinely ordered ER and PgR assays on newly diagnosed breast carcinomas and whether this analysis was done in-house or if it was sent out. If they performed in-house analysis, questions about the method used [IHC, dextran-coated charcoal (DCC) assay, polymerase chain reaction (PCR), or flow cytometry], type of material accepted [paraffin embedded, frozen tissue, or fine needle aspiration (FNA)], type of antibody, and dilution employed were included. The pathologists were also asked how quantitation was performed (image analysis, manually calculating the nuclei staining percentage, estimating the intensity of staining, H score), what the cutoff point was for differentiating positive and negative results, what protocol was used for standardization, and what controls were used. Lastly, the laboratories were asked whether they participated in any of the interinstitutional QA programs and, if so, which one.

In addition, 150 questionnaires were mailed with return addressed and stamped envelopes to hematology/

oncology departments in all 50 states. One hundred and ten oncologists were program directors at teaching hospitals. The remaining were randomly selected oncologists based at community hospitals. The questionnaire contains questions relating to the method used by the laboratory for the analysis of ER and PgR, the quantitation method, and the cutoff point used to separate positive and negative results. The questionnaire asked whether their treatment approach changed following a switch from DCC to IHC, whether they equated immunohistochemical expression of ER and PgR to specific femtomol values, and whether they required quantitation or merely positive and negative results. The oncologists were also asked whether they were influenced in choice of therapy by PgR status, or whether they gave tamoxifen regardless of steroid hormone receptor status to all postmenopausal patients. They were also asked if ER and PgR status had ever changed during treatment. Finally, their opinion regarding current recommendations (19,20) on chemotherapy and endocrine therapy and the duration of such treatment (21) (2 years versus 5 years) was sought.

Following mailing of the survey questionnaire, 3 months were allowed to pass before closure of the data collection period, allowing for adequate response time. The responses were entered on a spreadsheet program (Excel 7.0, Microsoft, Redmond, WA) and analyzed.

RESULTS

Responses were received from 80 of the 300 questionnaire recipients, geographically representing 35 states. Thirty (38%) were obtained from institutions describing themselves as community/general hospitals, seven from nonacademic tertiary care centers, 41 from academic tertiary care centers, 1 from a reference laboratory, and 1 did not indicate the type of institution. Respondents were almost equally distributed between hospitals with fewer than 250 beds (22), hospitals between 250 and 500 beds (26), and hospitals with more than 500 beds (29). Three respondents did not state the size of their institution.

Seventy-seven of the 80 (96%) responding practices routinely performed ER and PgR analysis on both in situ and invasive carcinoma of the breast. Two performed it only on invasive carcinoma, and a single respondent did not indicate his practice pattern. Both institutions performing ER and PgR analysis only on invasive carcinomas were community hospitals. Forty-nine of the 80 (61%) respondents performed steroid receptor hormone analysis in their own laboratories, 30 routinely sent tissue out for such analysis, and 1 laboratory performed in-house IHC but sent out tissue to have DCC ligand binding assay performed on some specimens. Table 1 shows the distribution of institutions performing steroid receptor analysis by hospital type. Academic tertiary care hospitals were most likely to perform the assays within their institution. Similarly, hospitals with more than 500 beds were more likely to perform steroid receptor analysis in their own laboratories. Community/general hospitals were most likely to send out ER and PgR analyses (23; 77%). A wide variety of academic tertiary care medical centers and specialty commercial laboratories were used for referral of tissue for steroid hormone analysis.

All institutions performing ER and PgR analysis in-house used immunohistochemistry. A single institution performed in-house IHC but sent out material for ligand binding assay in selected cases. Forty-three (80%) of the institutions answering the question on tissue acceptable for analysis performed ER/PgR analysis only on paraffin tissue, 6 (11%) performed the analysis on paraffin-embedded and FNA material, 2 (4%) performed the assay on paraffin-embedded, frozen, and FNA material, while 2 (4%) performed the assay only on frozen material. One (2%) respondent performed the analysis on paraffin-embedded and frozen material.

Methods of quantitation varied among the laboratories responding to our survey. Forty-two (88%) used a manual counting method for quantitation, 4 (8%) used computer-aided image analysis, and 1 (2%) used laser scanning cytometry. One additional laboratory (2%) used computer-assisted image analysis between 1988 and 1998 but recently changed to a manual technique. Of the laboratories utilizing a manual counting method for quantitation of ER and PgR levels, 30 (61%) manually counted the number of tumor cell nuclei staining positively and calculated a staining percentage. Fourteen respondents (29%) used both the percentage of positive nuclei and the intensity of staining. Three laboratories (6%) used formal H-score analysis. One laboratory (2%) measured only the intensity of staining. A single

Table 1. Hospital Type

Type	Number	Percentage
Community/general	30	37.5
Nonacademic tertiary	7	8.75
Academic tertiary	41	51.25
Reference laboratory	1	1.25
No response	1	1.25
Total	80	100

Table 2. Suppliers of Antibodies Used for ER Analysis by Responding Laboratories

Type	Number	Percentage
Dako	17	39
Ventana	15	34
Novacastra	3	7
AMAC	2	5
Immunotech	4	9
Abbott	1	2
Biogenex	1	2
Zymed	1	2
Total	44	100

Table 3. Range of Dilutions Used

ER	
Dako	5–2,000
Ventana	Predilution
AMAC	50–500
Novacastra	40–200
Immunotech	50–400
PR	
Dako	20–160
Biogenex	25–350
Novacastra	50–100

laboratory manually counted the nuclei staining percentage for ER but employed the H-score technique for PgR analysis.

Table 2 shows the different commercial suppliers of antibodies used for IHC analysis of ER and PgR. Antibodies were supplied by eight different companies, and antibody dilutions varied considerably, as indicated in Table 3. Cutoff points for separation of positive and negative results varied widely among laboratories. Even when the H-score system was used, the cutoff point was not uniform. Table 4 shows the cutoff points reported by the respondents. Some laboratories accepted any visually detectable staining as indicative of positive ER while others required the nuclei staining percentage to be as high as 20% before a tissue specimen was considered positive. Two laboratories (4%) did not interpret the results as either positive or negative, but simply estimated the nuclei staining percentage and intensity of staining present, leaving interpretation to the clinicians.

Protocols for standardization are listed in Table 5. There was considerable variation, with reliance on manufacturer protocols, DCC validation, or CAP survey. The controls used for standardization were also inconsistent among laboratories, with the majority of institutions (42; 74%) using known positive and negative cases as their controls. Table 6 shows the types of controls used by the responding institutions. Sixty-nine of the 80 institutions responding (86%) took part in the CAP program. The remaining 11 institutions did not take part in any QA/QC program.

Only 26 (17%) useful responses were received from the 150 questionnaires sent out to directors of hematology/oncology programs (representing 17 states). All 26 respondents routinely ordered steroid hormone receptor analysis on all newly diagnosed breast carcinomas. One of these did not routinely request such an analysis on intraductal carcinoma. Twenty-five respondents believed the technique used for the analysis was IHC, while one received information from the ligand binding (DCC)

Table 4. Criteria Used to Separate Positive and Negative Results

	Number	Percentage
10% positive cells	15	34
5% positive cells	12	28
Any positivity	4	9
Combination formula (number of cells and intensity)	3	7
<9% = negative, 9–19% = borderline, >19% = positive	2	4
Different criteria for ER and PgR	2	4
20% ER, 5% PgR		
20% ER, 10% PgR		
Number and intensity supplied for clinician interpretation	2	4
Combination of number and intensity		
10% positive with ≥2 (scale of 1–4)	1	2
H score of 50	1	2
H score of 10	1	2
San Antonio score		
1–2 = negative, 3 = borderline, 4–8 = positive	1	2
Intensity (SI 0–3), PP = percentage 0–4, IS = SI × PP		
0–1 = negative, 2 or more = positive	1	2
Total	45	100

Table 5. Protocols Used for Standardization

Protocol	Number
Positive and negative controls	3
DCC validation	3
Ventana automated stainer	2
Dako protocol	2
Do not know (DNK)	1
In-house standardized protocol (Techmate Instrumentation) and standardized commercial reagents	1
Parallel testing for new kits and new antibody lot	1
CAP survey	1
Modified Techmate	1
All slides reviewed by the director	1
Ventana/microwave antigen retrieval	1
No protocol	1
DCC and reference laboratories	1
HIER target antigen retrieval (Dako)	1
CAS 2000	1
Manufacturers' guidelines/journals/textbooks	1

analysis. Of the 25 individuals experiencing a shift in analytic technique from DCC to IHC, only one changed their treatment approach because of the modification in technique. Thirteen of 25 (52%) hematologists/oncologists equated negative or low IHC values of ER and PgR with specific femtomol values. The other 12 did not directly correlate IHC results with femtomol levels.

In agreement with the results of the survey of pathologists, there was considerable variation in the cutoff points used by hematologists and oncologists to separate positive from negative ER results. These values ranged from 1 to 30%. Four hematologists/oncologists (18%) did not know the value used for stratifying ER results into positive and negative. Table 7 shows the distribution of cutoff points used by the responding hematologists and oncologists. Eleven respondents required quantitation in their practice, while 16 required only a statement of positive or negative. Thirteen of 25 respondents agreed with the recommendations for treatment of early breast cancer as stated in the *British Journal of Cancer* (19) and in the *Review of Seminars in Oncology* (20).

Table 7. Distribution of Thresholds for Establishing ER Positivity Used by Hematologists/Oncologists

Threshold	Number	Percentage
No response	7	26
10%	5	19
Do not know	4	15
10 fmol	4	15
5%	2	7
30%	2	7
1%	1	4
20% = negative, <30% = borderline, >30% = positive	1	4
Laboratory does it	1	3
Total	27	100

Sixteen of 25 (64%) responding hematologists/oncologists would not treat an ER-negative carcinoma in a postmenopausal patient with tamoxifen. Twenty-three of 27 responding hematologists/oncologists stated that they were influenced in their treatment decisions by the presence or absence of PgR positivity in the neoplasm. Twenty-five of 26 hematologists/oncologists stated that they had modified their treatment plans based on the ER or PgR status of the patient. Finally, 20 of 27 responding hematologists/oncologists stated that they had not changed the duration of treatment after publication of the Swedish Breast Cancer Cooperative Group results in *Journal of the National Cancer Institute* (21).

DISCUSSION

Since the recognition of the relationship between ER and PgR levels in breast carcinoma and patient prognosis and response to hormonal therapy, the assessment of steroid hormone receptors has become a widely accepted component in the examination of breast carcinomas (23). Initially assessment of ER and PgR levels was performed using a ligand binding technology (DCC) (2–5). More recently, ER and PgR assessment by IHC has become popular, if not the predominant technique. Many

Table 6. Type of Controls Used for ER and PgR Analysis

Control	Number	Percentage
Known positive and negative cases	42	71
Internal and external breast tissue control	9	17
EIA assayed breast cancers	1	2
Endometrium	1	2
ER, breast; PgR, endometrium	1	2
Abbott ER-ICA, PgR-ICA control slides for frozen and cytology	1	2
Control cases with $H < 10$, 10–100, and >100	1	2
Multitumor blocks	1	2
Total	57	100

studies have correlated the results of IHC with those obtained by the ligand binding method (6–12), and have confirmed the relationship of ER and PgR with patient prognosis and response to hormonal therapy (1,14–17). Despite the near uniformity in finding a high correlation between IHC and ligand binding assay results and good predictive value for IHC, these studies have used a variety of antibodies, antibody dilutions, staining and quantitation techniques, and cutoff points (23). The variation within the literature is reflected within community practice. Many of the issues relating to IHC determination of steroid hormone receptor levels reflect basic issues in quantitative IHC as discussed by a panel of experts during a recent meeting of the European Section of the International Academy of Pathology (Nice, France, October 1998).

In order to assess the current status of ER and PgR assay methods in the United States, we undertook a mail survey investigating the methodologies, controls, quantitation techniques, and cutoff points utilized by a variety of academic and nonacademic laboratories. Our study found a wide variation in the antibodies, dilutions, quantitation techniques, and cutoff points used by the respondents. While these variations do not in and of themselves negate the clinical significance of steroid hormone receptor analysis, the existence of such variations raises the potential for clinically significant discordance in reported steroid hormone receptor values between laboratories.

Commercial and large academic medical center laboratories perform approximately 38% of all ER and PgR assays, but the majority of such assays are performed in-house by local laboratories. Nearly all of these laboratories report using IHC for the assessment of ER and PgR. A number of observations can be made on the basis of this study. First, a confounding variable for interlaboratory comparisons of steroid hormone receptor results is the variety of materials accepted by various laboratories. Eighty-one percent of laboratories accept only paraffin-embedded material for analysis, but 11% used only frozen tissue or both frozen and paraffin-embedded tissue for analysis. In addition, 4% also accepted FNA specimens. Second, various antibodies were used. The laboratories in our study employed antibodies supplied by eight different manufacturers. Seventy-three percent of institutions used an antibody supplied by either Dako (Carpinteria, CA) or Ventana (Tucson, AZ). As shown in Table 3, even when using the same antibody, various institutions employed widely different antibody dilutions for their assays. These differences in antibodies and dilutions may have significant impact on the quantitative assessment of ER and PgR by IHC.

Third, methods for the quantitation of IHC results varied considerably between respondents. The majority (88%) of laboratories completing the survey questionnaire used various manual techniques for quantitation in which estimates of nuclei staining percentage were made. Multiple manual quantitation methods exist in addition to simply estimating the nuclei staining percentage. Twenty-nine percent of laboratories using a manual quantitation technique employed a technique where both the number of positive cells and the intensity with which the cell nuclei stained were estimated. Six percent used formal H-score analysis (6). We did not obtain information on the threshold of staining intensity used to accept a nucleus as positive for quantitation purposes. Neither did we obtain information on cell selection techniques. Clearly differences in counting technique can affect whether a neoplasm is designated positive or negative for ER and PgR. Recommendations for cell counting have been published (11).

Fourth, the cutoff points used for the assignment of breast cancer cell populations as positive for ER or PgR differ between laboratories. Our survey documented at least a fourfold variation in the nuclei staining percentage used by laboratories for the assignment of positivity. Twelve laboratories (27%) used a 5% cutoff point to designate a specimen as positive and four laboratories (9%) used a value of 20% as their cutoff point. Fifteen laboratories (33%) used a 10% nuclear positivity rate as an indicator of a positive ER level. Variability in cutoff point results in discordance of results even when methodologic aspects of the assay are identical. Such variability in threshold for positivity highlights the need for laboratories to include cutoff points in their reports. Thresholds used with other quantitation methods (H score) also varied. A few laboratories simply assessed the number of positively staining cells and the intensity of staining present, allowing the clinicians to interpret the data. Thus wide variability in practice exists and such variability may have a significant impact on the documentation of the presence or absence of clinically significant levels of ER and PgR.

Standardization protocols varied widely among the respondents to our survey. Only 22 respondents (28%) answered our query concerning their standardization protocol. Of these, three used DCC validation as their standardization technique, while a majority relied on manufacturer's guidelines, previously tested positive and negative intralaboratory controls, or simply review

of all slides by the laboratory director. Controls for the standardization of ER and PgR assays varied among the laboratories. The majority (52%) used previously assayed tissue blocks known to be positive and negative. This offered a degree of intralaboratory consistency but did not allow external verification of laboratory assay levels. Nine laboratories utilized internal breast tissue controls for the assessment of steroid hormone receptors in associated neoplastic tissue. Occasionally laboratories utilized endometrium as a positive control. As stated by Riera et al. (18), no universal control exists for external validation of steroid hormone receptor assays by IHC. The lack of such a control complicates comparisons between laboratories using different antibodies, dilutions of antibodies, and modifications of the IHC technique. Recently, cultured cells have been suggested as a control for quantitative immunocytochemical analysis of ER levels (18). Widespread utilization of such a standard control should increase the comparability of ER results performed at different laboratories.

Only 26 responses (17%) from our survey of 150 directors of hematology/oncology programs were received, meaning conclusions based on this small dataset should be made with caution. However, certain trends were noted. First, the respondents routinely ordered ER and PgR analysis on all newly diagnosed breast carcinomas, implying general acceptance by oncologists for measurement of ER and PgR. The majority of responding oncologists did not alter their treatment approach when their laboratory switched from the DCC to the IHC methodology. Variability existed among clinicians in how they equated IHC expression to femtomol values. Approximately half of the respondents did not equate negative or low IHC values with specific femtomol values.

In agreement with our laboratory survey findings, the threshold for calling a result positive varied widely among oncologists. Values associated with a positive result by IHC varied from 1 to 30%. Of equal importance, 59% of the responding hematologists/oncologists treating breast cancer patients did not require quantitative data but merely desired a statement by the laboratory as to whether the assay was positive or negative. This finding is of particular interest in light of a recent study showing that very high levels of ER are associated with an unfavorable prognosis (22). Simply dividing ER values into positive and negative may yield incomplete and misleading information. The reporting of femtomol equivalents, nuclei staining percentage, or the stratification of results into negative, borderline, intermediate, and high levels may be more clinically useful.

There does not appear to be uniformity in approach to the interpretation and utilization of ER and PgR data by oncologists. Only 13 respondents (48%) agreed with recently published recommendations concerning the use of endocrinology and chemotherapy in patients with breast cancer (19,20). The majority of oncologists responding to our questionnaire acknowledged that ER or PgR status had modified their treatment of patient's with breast cancer (92%). Despite this reliance on steroid hormone receptor assay results, fully one-third of responding oncologists would treat a postmenopausal patient whose carcinoma was ER negative with tamoxifen. The results of the Swedish BCCG study (21) appeared to change the treatment approach of only a minority of oncologists responding to our survey (26%).

The oncologists varied significantly in the threshold they used to classify specimens as positive or negative. Reported cutoff points for positivity varied from any staining to a cutoff point of at least 30% of nuclei staining. When specific percentages were given, there was a sixfold variation in the cutoff point (5–30%). Such variability in interpretative thresholds renders interlaboratory comparisons of ER and PgR results difficult if only positive and negative assessments are reported. Interlaboratory comparisons are more easily achieved if laboratories record the nuclei staining percentage, cutoff point used, and interpretation of the results rather than simply reporting the specimen as positive or negative.

The CAP QA program is commonly used by laboratories assessing ER and PgR in breast tissue, but its level of success in ensuring interlaboratory uniformity was not assessed by this survey. Further studies into the effectiveness of this program would be of value both to pathology laboratories performing steroid hormone receptor analysis as well as to oncologists interpreting the results.

Steroid hormone receptor assay by IHC appears robust enough to maintain the correlation with prognosis established by DCC, despite the many variations discussed. This interpretation is supported by the observation that the majority of studies in the literature using variable techniques, antibodies, titers, and cutoff points still report good correlation of the IHC results with DCC assays and demonstrate good predictive and prognostic value for the test (1,6–17). As in many areas of surgical pathology, reliability may exceed reproducibility (24). This may lead some clinicians and pathologists to adopt a nihilistic approach to standardization. However, it may be that important prognostic information associated with steroid hormone receptor levels is being

concealed by the imprecision of current IHC methods. The development of a universal control and improved standardization methods should improve the validity of interlaboratory comparison of the results of ER and PgR measurement by the IHC technique.

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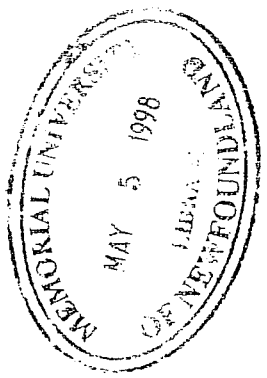
APPENDIX D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Introduction to Immunocytochemistry

SECOND EDITION

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Contents

Sections from pre-fixed frozen blocks and acetone- or alcohol-fixed cell culture preparations can be stored in the same way after drying on the slides or, for a shorter time, at 4°C. Cytospin preparations or cultured cells that have been fixed in alcohol can also be kept in that solution at room temperature or 4°C for at least some days, and possibly indefinitely, without destroying fixed antigens.

Paraffin blocks and sections. Antigens in paraffin blocks and even in sections stored for many years at room temperature seem to retain their antigenicity indefinitely, allowing full use of archive material.

3.1.8 Adherence of sections and cell preparations to slides

Paraffin sections for immunostaining should never be 'baked' on a hot-plate, as this degrades the antigenicity of many substances. They should be thoroughly dried for several hours or overnight at 37°C and, if necessary, heated in a 60°C oven for 10–15 min.

For the simpler techniques, sections will adhere well to clean slides with no coating, particularly if they have been well dried; but to ensure that sections and cells will not detach from slides during the longer procedures of immunocytochemical staining, an adhesive is usually applied to the slide before the section is picked up or the cells are allowed to settle. This is an esssential precaution if antigen retrieval methods have to be used (see Section 3.2.2).

Among the many slide coatings that have been suggested, a universally applicable one is *poly*-L-lysine (Huang *et al.*, 1983). This imparts a negative charge to the slide which attracts positively charged tissue elements. An alternative is to use slides dipped in aminopropyltriethoxysilane (APES), which provides an even surer adhesive for methods involving heat-mediated antigen retrieval. Slide coating methods are decribed in the Appendix (Section A.3).

Many of the commercial companies concerned with immunocytochemistry supply ready-to-use slides either pre-coated or with a charge incorporated in the glass. These are expensive but convenient (e.g. Vectabond from Vector Laboratories, Polysine from Merck).

3.2 Antigen retrieval in fixed tissues

The process now known as antigen retrieval is applied to aldehyde-fixed tissues in which antigenicity has been reduced by the formation of hydroxy-methylene bridges between components of the amino acid chains of proteins. In many instances, immunoreactivity can be restored without compromising the structure of the tissues.

3.2.1 Washing

The simplest form of reversing the effects of formalin is to wash the tissue well before processing, but this is not usually possible in histopathology laboratories, where rapid turnover of specimens is required.

3.2.2 Protease treatment

A little understood but practical way of revealing strongly cross-linked proteins is to treat the (de-waxed, hydrated) sections with a protease such as trypsin or pronase before immunostaining (*Figure 3.1*) (Huang *et al.*, 1976). It is thought that the enzyme breaks the cross-linking bonds of the fixative with the protein to reveal antigenic sites. A few antigens may be adversely affected and there is also a possibility that large protein molecules (e.g. precursors of bioactive peptides) may be cleaved by the enzyme to smaller molecules (Ravazzola and Orci, 1980).

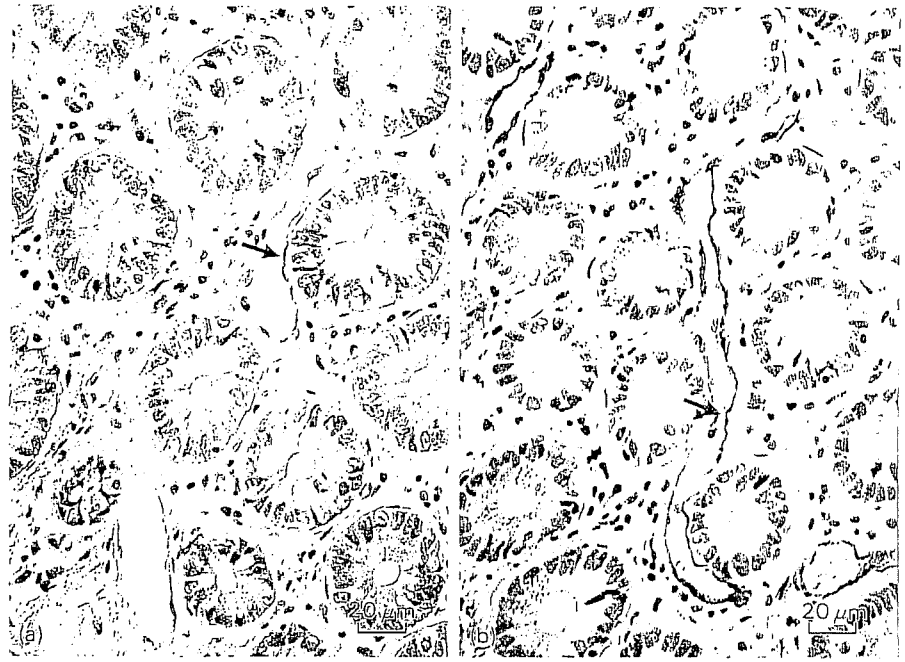

Figure 3.1: Human duodenum immunostained for Factor VIII-related protein, a marker for endothelial cells, by the PAP method using a rabbit antibody to Factor VIII followed by unconjugated goat anti-rabbit IgG, then rabbit PAP complex. The section shown in (a) was stained without pretreatment; the adjacent section in (b) was stained after treatment at 37°C with 0.1% trypsin in 0.1% calcium chloride at pH 7.8. Note the intensification of the reaction in the vessel walls in (b) (arrow). Tissue preparation: formalin-fixed, paraffin-embedded section; haematoxylin counterstain. Reproduced from Polak, J.M. and Van Noorden, S. (1987) *An Introduction to Immunocytochemistry: current techniques and problems*, Microscopy Handbook 11 (revised edition), Oxford University Press, with permission from the Royal Microscopical Society (Figure 2, p. 11).

If these peptide molecules display antigenic sites that were not available on the precursor, a false positive reaction for the peptide may occur. In practice, this rarely happens, and protease pretreatment should be tried routinely whenever preliminary immunostaining is found to be inadequate (Curran and Gregory, 1977; Mepham *et al.*, 1979; Finlay and Petrusz, 1982).

Several different enzymes have been advocated and it is a question of trial or individual preference as to which is used. We use routinely a crude and relatively inexpensive form of trypsin from porcine pancreas which contains some chymotrypsin. In fact, chymotrypsin may be the active ingredient, since purified trypsin gave poor results, and chymotrypsin alone can be used (Brozman, 1980). A few antigens are preferentially revealed by other enzymes, for example IgE by protease XXIV. The enzymes must be used at their optimal pH for a time determined by experiment in the laboratory. Methods for several are given in the Appendix (Section A.5).

The time for which enzyme treatment is carried out may be critical within 1 or 2 min if optimal revelation is to be combined with acceptable tissue structure. Over-digestion can lead to destruction of the tissue matrix. It is important that everyone in the laboratory uses a standard method, so that 'a 10 min treatment with trypsin' is understood to mean, for instance, that the slides are (or are not) warmed to 37°C before treatment and that the enzyme solution has been freshly prepared in a prewarmed (or cold) solvent. Results are more likely to be uniform when a rack of slides is immersed in a large volume, but when expensive enzymes such as protease XXIV have to be used, it is more economical simply to cover the preparations with drops of solution, staggering the application times to give the correct incubation period for each.

The term 'correct' is really only applicable when tissues of uniform size have been fixed for a standard time. The longer a tissue sample is left in formalin, the greater will be the degree of cross-linking and the longer the digestion time needed to reveal a given antigen. Different proteins require different digestion times, even after standard fixation, and different batches of enzyme may vary in effectiveness; so it is necesary for each laboratory to establish the treatment time required by each antigen, to be prepared to try a range of digestion times for samples received from other laboratories, and to test each new batch of enzyme received from the supplier if its composition is different from that of the previous batch. We have found also that the same antigen in different locations may require different digestion times. For instance, immunoglobulin deposits in basement membranes of skin or renal glomeruli are optimally revealed only after 1.5 h in trypsin, compared with 10 min for the same molecules in plasma cells. This may be partly due to the increased visibility of the immunostained deposits after the plasma in renal glomerular loops has been digested away. This process can be monitored microscopically (Howie *et al.*, 1990).

3.2.3 Heat-mediated antigen retrieval

A major step forward in immunocytochemistry was made with the discovery that some antigens previously unreactive in formalin-fixed, paraffin-embedded tissue, even after protease treatment, could be 'retrieved' by heating sections in a solution of a heavy metal salt in a microwave oven without deleterious effects on the structure of the tissue (Shi *et al.*, 1991). Subsequently, it was shown that the rather toxic heavy metal salts could be replaced by simple buffers such as citrate buffer at pH 6.0 (Cattoretti *et al.*, 1993). It was shown that heat, rather than microwaves *per se,* is important in the retrieval process, since boiling the sections in a pressure cooker (Norton *et al.*, 1994) or autoclaving them (Bankfalvi *et al.*, 1994) in the buffer solution achieved the same effect. A possible explanation of the process has been put forward by Morgan *et al.* (1994), who suggested that heating provides the energy not only to rupture the hydroxyl bonds formed by the fixative with the protein antigen, freeing some antigens, but also to release tissue-bound calcium ions which contribute to tighter bonds with the fixative. They showed that the salt solutions in which the sections are heated are, in fact, all able to chelate or precipitate calcium to varying degrees and thus remove released calcium from the sections, breaking fixative bonds permanently and revealing antigens. The most effective solutions (EDTA, EGTA) were also the best calcium chelators. The acidity of the buffer may also play a part in antigen retrieval (Shi *et al.*, 1993).

Examples of diagnostically important antigens that are revealed by this method are the oestrogen and progesterone receptor antigens in breast carcinomas (*Plate 3*, p. 36) and the CD 30 antigen in Reed–Sternberg cells in Hodgkins' lymphoma. Published lists of antigens and their preferred retrieval methods abound and are of some help in deciding on the 'correct' method to use (Cuevas *et al.*, 1994; Werner *et al.*, 1996); but again, it is probably necessary for each laboratory to optimize its antigen retrieval method for each antigen–antibody combination, as separate epitopes on the same antigen may respond differently, so that identification with monoclonal antibodies other than the ones cited may require different conditions.

It seems that antigens are more tolerant of heat-mediated than of enzyme-mediated retrieval methods, in that antigens and tissues survive heating longer than the minimum time required to reveal the antigen. However, some antigens require a longer period of heating than others, the time usually falling between 2 and 30 min. Some laboratories give all their sections a standard time, equating to the longest required, which could help to overcome vagaries of variable fixation time, while others prefer to treat each antigen individually. As with the enzyme digestion methods, it is important for standard procedures to be set up. The method used in our laboratories is given in the Appendix (Section A.6). It is of great importance that sections are firmly attached to slides for methods involving heating in buffer solutions (see Section 3.1.8).

Heat-mediated antigen retrieval increases the sensitivity of immunoreactions to such an extent that, for antigens on which it confers improved immunoreactivity, it is usually necessary to dilute a primary antibody considerably further than for the standard, non-retrieval method. Retrieval sometimes also reveals unwanted background reactivity, which can be a nuisance and require further treatment to block it (see Section 3.4 and Chapter 5).

There is still no universal antigen retrieval method. Although very many antigens respond to heat-mediated methods and can be used as markers in histopathology, some are still preferentially revealed by enzyme pre-treatment; and it must be remembered that some are unaffected by formalin fixation and are quite adequately demonstrated without any pre-treatment which may, indeed, destroy their immunoreactivity. Antigen retrieval methods have been reviewed by Taylor *et al.* (1996) and microwaving by Cattoretti and Suurjmeijer (1995).

3.3 Visualizing the end-product of reaction

In order for an immunocytochemical reaction to be seen in the microscope, a component of the reaction must carry a label. The first label to be attached to an antibody was a coloured dye (Marrack, 1934) but the resulting intensity was too low for visualization. Nearly all labels that have been used subsequently require additional steps to enhance them to the point of visibility. Fluorescent compounds, the first practical labels, require excitation with light of a specific wavelength to make them emit visible light. Enzymes must react with a substrate and chromogen to produce a visible deposit. Radioactive labels require autoradiographic development. Biotin must itself be labelled or reacted with labelled avidin. Colloidal gold provides an electron-dense label for electron microscopical immunocytochemistry and can be seen in the light microscope if enough is applied, but it is more visible after enhancement with metallic silver.

The methods of attaching labels to antibodies are beyond the scope of this book and will be discussed only briefly. For details, see the work of Sternberger (1986) and Johnstone and Thorpe (1996). The ratio of label to antibody is important, since overloading the antibody with label will reduce its immunoreactivity, but it is also important to ensure that all available antibody is labelled optimally, for maximum efficiency.

Labelling an antibody with fluorescein isothiocyanate (FITC) is done quite simply. Briefly, the method consists of:

1. isolation of IgG from the antiserum;
2. reaction of the IgG with FITC in the right proportions at alkaline pH;
3. separation of antibody from unconjugated FITC on a Sephadex G25 column; and

4. separation of labelled from unlabelled IgG on a DEAE cellulose column.

Labelling with an enzyme requires an additional large molecule such as glutaraldehyde to cross-link the enzyme to the antibody, unless an antigen–antibody reaction is used, as in the preparation of the peroxidase–anti-peroxidase (PAP) complex (Sternberger, 1986). A radioactive label (e.g. $Na^{125}I$) may be conjugated to an antibody via another molecule (Johnstone and Thorpe, 1996) or incorporated within a monoclonal antibody during its production, thus avoiding the conjugation process (Cuello *et al.*, 1982). Antibodies are easily labelled with biotin and kits for doing this are available commercially. Antibodies are attached to colloidal gold particles by non-covalent adsorption (Beesley, 1989).

3.3.1 Fluorescent labels

Advantages. Fluorescence provides an instantly visible label with excellent contrast when seen against a dark, non-fluorescent background. It is usually used on frozen sections or fresh whole-cell preparations because formalin-fixed tissue tends to be autofluorescent or even, if catecholamines are present, to emit formaldehyde-induced specific fluorescence of a colour approaching that of fluorescein compounds (Falck *et al.*, 1962; Hökfelt and Ljungdhal, 1972). In an enzyme-labelled preparation, frozen sections show structural imperfections, but with immunofluorescence these can be ignored, as the background tissue should only be visible enough to set the specifically labelled structures in context.

Disadvantages. A special microscope is necessary, preferably with epi-illumination, so that neither exciting nor emitted light is lost by passing through the specimen. Different filter sets are required for the different fluorescent markers to prevent transmission of extraneous light. Preparations are not permanent because fluorescent labels are not resistant to dehydration and solvent-based mountants, so aqueous mountants must be used. In addition, fluorescence tends to fade, particularly under exposure to the excitation light; photography is therefore difficult, although mountants incorporating fluorescence preservers are now available. These are recommended, particularly those that harden so that the danger of damaging the preparation by moving the coverslip is removed (e.g. Permafluor from Immunotech). Fluorescent dyes other than fluorescein have a longer life (see below).

Fluorescent counterstains. A fluorescent tissue counterstain such as Pontamine Sky Blue (Cowen *et al.*,1985) which fluoresces red at the excitation wavelength for fluorescein, can provide a useful background to

tion of staining. Positive control tissue should be tested alongside the experimental tissue to ensure that the absorption is effective.

5.3 Remedies for non-specificity due to tissue factors

5.3.1 Blocking binding sites with normal serum

This has been described in Chapter 3 (Section 3.4.1) and should remove binding to charged or hydrophobic tissue sites and to Fc receptors.

5.3.2 Absorption with tissue powder

Non-specific attachment of immunoglobulins to common tissue components can also be prevented by absorption of the antiserum with a tissue powder (e.g. acetone-dried liver) from the species in which the staining is to be done, provided it is certain that the tissue powder does not contain the antigen to be investigated. After reaction with the tissue powder, the mixture is centrifuged and the supernatant used as the antibody. It may be necessary to re-establish the optimal dilution.

5.4 Remedies for non-specificity due to heterogeneity of the antibody

5.4.1 Dilution

High dilution of the antiserum can reduce the amount of unwanted antibody compared with the amount of wanted, specific antibody in the serum to the point where its effects become negligible.

5.4.2 Affinity purification

Antisera can be 'purified' by immunoabsorption, with the specific antigen bound to a solid phase such as sepharose beads: the antibody is subsequently eluted. However, the most useful antibodies for immunocytochemistry are very avid and it may be difficult to elute them from the antigen used for absorption. Thus some antibody may be lost and the eluted antibody, though pure, may be of low avidity.

If the contaminating antibody is known, it may be possible to remove it from solution by solid phase absorption with the contaminant, the specific antibody passing unaffected through the column.

5.5 Remedies for non-specificity due to cross-reactivity

Genuine cross-reactivity is a difficult problem. In a polyclonal antiserum, when relationships between families of antigen molecules are known, it may be possible to remove populations of cross-reacting antibodies by absorption with the related antigens, leaving some non-cross-reacting antibodies still available in the serum. The initial production of discriminating 'region-specific' antibodies requires immunization with unshared fragments of the respective molecules.

5.6. Controls

5.6.1 Negative controls

In order to check that a tissue sample is being immunostained specifically, a negative control must be performed for every tissue block or sample stained, consisting of substitution of the primary antiserum with non-immune serum or, for a monoclonal primary, an inappropriate monoclonal antibody or simply culture medium or antibody diluent. All other conditions must be identical with those for the test. If any staining occurs on the negative control, preferably the cause should be investigated and overcome, but at the very least, the result must be mentally subtracted from the result of the test.

5.6.2 Positive control

It is essential that a positive control sample, known to contain the antigen in question, is included every time an immunostain is performed. Without such a control, a negative result on the test material will be meaningless, because there is no guarantee that the reagents are in good working condition and have been applied in the correct order and at the correct dilutions. If the positive control is satisfactory, it is a reasonable assumption that the correct method was carried out on the test material too. If the control is weaker than usual or unstained, then it is likely that something has gone wrong and the test result is unreliable.

5.6.3 Experimental controls

When an unknown tissue is being tested with an unknown antibody to show an antigen in an unknown location, seemingly positive results must be accepted with extreme caution. Absorption controls with the

antigen are essential, and preferably with related and unrelated antigens and basic amino acids (see Sections 5.2.2 and 5.2.4) as well. If several different antibodies to the same antigen localize it in the same place, this constitutes confirmatory evidence of its presence.

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6 Enhancement of Standard Methods

The impact of immunocytochemical stains comes from the contrast between the colour of the end-product and the unstained or counterstained background. Whether the purpose is to emphasise the presence of a small amount of antigen, to provide good conditions for photography or image analysis, or merely for aesthetic reasons, there are numerous methods for enhancing the intensity of the result, the aim being to achieve more marker at the site of reaction or to darken an existing product. All require the initial reaction to have little or no background staining, since this, too, will be intensified. The positive and negative controls should also be enhanced if comparison is needed. The immunogold reaction with silver intensification is one of the most sensitive methods. It has been described in Chapter 3 (Section 3.3.3).

6.1 Build-up methods

The greater sensitivity of three-layer methods over two-layer methods in achieving more label at the site of the original antigen–antibody reaction has already been shown in Chapter 4. Any immunoglobulin can act as a bridging antigen for another antibody, and thus the layers of antibody and labelled antibody can be continued. Adding to a rabbit PAP reaction with a further layer of anti-rabbit Ig and another rabbit PAP triples the amount of peroxidase available (*Figure 6.1*). If the anti-rabbit Ig is peroxidase-conjugated, even more peroxidase molecules are added to the site.

Build-up by doubling the application of the second and third layers has been recommended as routine for APAAP staining (Cordell *et al.*, 1984; Mason, 1985).

In a two-layer indirect method, after the labelled secondary antibody has been added, normal serum containing immunoglobulin from the species providing the primary antibody or another layer of the primary