Non-specifically labelled cells that simulate bone marrow metastases in patients with non-metastatic breast cancer

M Lagrange, J M Ferrero, J L Lagrange, J C Machiavello, J Monticelli, C Bayle, A Creisson, M Namer, A Thyss, C Bourcier, J Gioanni, M Schneider

Abstract

Aims—To determine whether the presence of disseminated bone marrow tumour cells at diagnosis is a prognostic factor for breast cancer patients at high risk of recurrence or bone metastasis, and to assess their presence as a criterion for evaluation of the potential benefits of adjuvant chemotherapy.

Methods—Multiple bone marrow aspirates from 72 breast cancer patients free from metastasis were obtained during surgery at the time of diagnosis and were tested immunologically by alkaline phosphatase antialkaline phosphatase technique with a panel of three antipithelial monoclonal antibodies (MoAb) KL1, EMA, and HMFG1.

Results—In nine of 72 patients, with each MoAb tested, numerous strongly positive cells always isolated were observed. However, it was demonstrated that these cells were non-specifically labelled and could be found in normal controls.

Conclusions—There was no evidence of marrow tumour cells in 72 operable breast cancer patients. It is suggested that published results may be greatly overestimated and that non-specific labelling may be undetected. More specific MoAb should be found and a correlation with molecular biology should be performed if this criterion is to be considered as a prognostic factor.

(J Clin Pathol 1997;50:206-211)

Keywords: bone marrow tumour cells; breast cancer; immunocytochemistry.

Numerous studies suggest that breast cancer metastases at a very early stage. Although the prognosis for breast cancer patients without metastases (M0) at the time of initial disease staging (including isotopic scanning and biochemical tests) remains unknown, almost 50% of patients relapse within five years,1 and 25% of those who are node-negative at initial diagnosis relapse within 10 years.2 The skeleton and bone marrow are often the first sites of metastasis in breast cancer patients,3,4 yet risk assessment using conventional prognostic factors (number of involved nodes, tumour size, peritumoral vascular invasion, hormone receptor status) is disappointing.4-5

Sloane et al were the first to demonstrate successfully marrow tumour cells in breast cancer patients with metastases using an immunocytochemical technique, whereas malignant cells were not found in haematoxylin/eosin sections.6 Since then, more than 25 publications on the subject have concluded that occult bone marrow micrometastases are present at the time of earliest detection of non-metastatic breast carcinoma. The main studies concerning marrow samples from M0 patients during surgery are listed in table 1. Results are most diverse with respect to detection rate (mean 19%, range 0%-44%) and prognostic relevance. Some investigators have reported a correlation with conventional prognostic factors such as tumour size, lymph node status, and intratumoral vascular invasion,1,3 whereas others have failed to observe such relationships.1,5,6 The presence of marrow tumour cells is generally considered an essential prognostic factor for early recurrence,1,7 and relapse-free survival.1,8 Marrow tumour cells are also reportedly a strong independent prognostic factor for bone metastasis,1,9 and overall survival.10 Moreover, marrow tumour cell detection has thus been proposed as a replacement for axillary lymphadenectomy in cancer patients with clinically free axillary lymph nodes.10 However, several authors failed to find any positive cases4,6 while two others found only a low rate of positivity (table 1).1,11 This considerable variability in results is probably due to the various criteria used for identification of marrow tumour cells and the numerous differences in methodology. Studies differ not only by the immunological technique used, but also the number and type of antibodies, and whether controls are used to guarantee accurate interpretation (table 1). The feasibility of the technique is another decisive factor, because results may be used to select candidates, and in particular node-negative patients, for adjuvant therapy.1

In the present study, bone marrow aspirates from breast cancer patients free from metastases at time of initial diagnosis were studied immunocytoalogically with all known guarantees concerning methodology according to literature results: analysis of multiple bone marrow aspirates,1,9,11 use of alkaline phosphatase antialkaline phosphatase (APAAP) immunocytochemical technique4 on cytospins which allows much better identification of labelled cells than immunofluorescence,7 and
Table 1 Techniques used and results of major publications about the research of marrow tumour cells in patients without metastases at the time of primary surgery

<table>
<thead>
<tr>
<th>Authors</th>
<th>Specimens</th>
<th>Immunological technique</th>
<th>Negative control without primary antibody</th>
<th>Antibodies</th>
<th>Positive patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sloane*</td>
<td>BMA, Ficoll, inclusion</td>
<td>Alkaline phosphatase</td>
<td>Yes</td>
<td>Polyclonal EMA</td>
<td>0/28</td>
</tr>
<tr>
<td>Cote*</td>
<td>BMA, Ficoll, smears</td>
<td>Immunofluorescence</td>
<td>Not specified</td>
<td>Monoclonal C26*, T16*/AE1*</td>
<td>18/51</td>
</tr>
<tr>
<td>Porre*</td>
<td>Cell suspension from BMB</td>
<td>Indirect immunoglucone oxidase</td>
<td>Yes</td>
<td>Monoclonal MBRI/</td>
<td>25/159</td>
</tr>
<tr>
<td>Ellis*</td>
<td>BMA, Ficoll, drop air dried</td>
<td>Immunoperoxidase</td>
<td>Not specified</td>
<td>Monoclonal 35BH11t, 34 E1A1*</td>
<td>2/9</td>
</tr>
<tr>
<td>Giai*</td>
<td>BMA, inclusion</td>
<td>Peroxidase-antiperoxidase</td>
<td>Yes</td>
<td>Monoclonal AB3*</td>
<td>1/39</td>
</tr>
<tr>
<td>Mathieu*</td>
<td>BMB</td>
<td>Avindo-Biotin peroxidase</td>
<td>Not specified</td>
<td>Monoclonal LGR-10N M8*</td>
<td>1/50</td>
</tr>
<tr>
<td>Courtemanche*</td>
<td>BMB</td>
<td>Alkaline phosphatase</td>
<td>Yes</td>
<td>Polyclonal EMA</td>
<td>89/350</td>
</tr>
<tr>
<td>Mansi*</td>
<td>BMA, Ficoll, smears</td>
<td>Streptavidin alkaline phosphatase</td>
<td>Not specified</td>
<td>Monoclonal 2E112 (anti-TAG 12 protein)</td>
<td>114/260</td>
</tr>
<tr>
<td>Diet*</td>
<td>BMA, Ficoll, smears</td>
<td>Immunofluorescence</td>
<td>Not specified</td>
<td>Monoclonal C26*, T16*, AE1*</td>
<td>111/348</td>
</tr>
<tr>
<td>Osborne*</td>
<td>BMA, Ficoll, smears</td>
<td>APAAP</td>
<td>Not specified</td>
<td>Monoclonal EMA, EMA/</td>
<td>38/100</td>
</tr>
<tr>
<td>Pecorin*</td>
<td>BMB</td>
<td>APAAP</td>
<td>Not specified</td>
<td>Monoclonal MBRI*/ MBR8*/MOV8*/</td>
<td>32/197</td>
</tr>
<tr>
<td>Harbeck*</td>
<td>BMA, Ficoll, smears</td>
<td>APAAP</td>
<td>Not specified</td>
<td>MOV16*/ MLUC1*, CK2</td>
<td>31/197</td>
</tr>
</tbody>
</table>

BMA = bone marrow aspirate; BMB = bone marrow aspirate; APAAP = alkaline phosphatase antialkaline phosphatase; CK = cytokeratin.
\*Antiepithelial membrane antigens; \*Anti-ck; \*Antipolymorphic epithelial mucin.

Table 2 Pathological data on 72 operable breast cancer patients at initial diagnosis

<table>
<thead>
<tr>
<th>Parameters</th>
<th>No patients</th>
<th>% patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour type</td>
<td>Ductal</td>
<td>54</td>
</tr>
<tr>
<td>Lobular</td>
<td>10</td>
<td>13.8</td>
</tr>
<tr>
<td>Mixed</td>
<td>4</td>
<td>5.6</td>
</tr>
<tr>
<td>Other</td>
<td>4</td>
<td>5.6</td>
</tr>
<tr>
<td>Tumour size</td>
<td>T1</td>
<td>47</td>
</tr>
<tr>
<td>T2</td>
<td>19</td>
<td>26.4</td>
</tr>
<tr>
<td>T3</td>
<td>5</td>
<td>6.9</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>Tumour stage*</td>
<td>IA</td>
<td>33</td>
</tr>
<tr>
<td>IB</td>
<td>29</td>
<td>40.2</td>
</tr>
<tr>
<td>IIB</td>
<td>9</td>
<td>12.5</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>Tumour grade†</td>
<td>1-3</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>8.3</td>
</tr>
</tbody>
</table>

Cut-off for oestrogen and progesterone receptors: 10 fmol/mg protein.
*UICC classification; †Scarff-Bloom-Richardson.

Avoids interference of endogenous enzymes much better than the peroxidase technique. Moreover, we tested a panel of four antiepithelial monoclonal antibodies (MoAb), none of which is specific when used alone. Study of the sensitivity of the method, assessment of its feasibility using known tumoral epithelial cells and marrow samples from carcinoma free controls, negative control of the reaction by replacement of each primary antibody by its isotype identical IgG, were performed. Finally, systematic comparison of cytospins stained with May-Grünwald-Giemsa (MGG) and immunologically treated cytospins, using a meticulous morphological study in case of immuno-cytotoxic positivity was performed.

Materials and methods

PATIENTS

Between February 1993 and July 1994 six marrow aspirates were obtained during surgery for a primary breast tumour from 72 unselected, consecutive patients. They were aged 35 to 84 years (median 64 years); 12 premenopausal and 60 postmenopausal. Pathological details are given in table 2. Before surgery, each patient was screened for metastatic disease by clinical examination, chest radiography, liver function tests (plasma alkaline phosphatase, y glutamyl transferase) and liver ultrasound. All were free from metastases. Depending on tumour size and location, surgery comprised tumorectomy, quadrantectomy, or mastectomy, plus axillary lymph node dissection. There was a high prevalence of small tumours without lymph node involvement and frequent positive hormone receptor status suggesting a favourable prognosis (table 2).

MARROW SPECIMENS FROM BREAST CANCER PATIENTS

A mean volume of 6 ml of bone marrow pooled from two aspirations per site (sternum, right and left anterior iliac crests) was obtained in heparin-lithium anticoagulant. Bone marrow cells were separated on a Ficoll-Hypaque density gradient by centrifugation at 250 xg for 20 minutes. The mononuclear layer was extracted and washed twice in RPMI 1640 medium. The final suspension was adjusted to 3x10^6 cells/ml and cytospin onto 30 slides. Two slides were stained by the MGG technique for cytological examination; the other slides were air dried at room temperature for 24 to 48 hours, then stored at –20°C or fixed and treated for immuno-cytchemistry (three slides for each antibody). Bone marrow biopsy was not performed in this study.

POSITIVE CONTROL SPECIMENS

Sixteen samples containing breast tumour cells were studied: seven ascites, four pleural effusions, three bone marrow specimens, one nodal aspirate, and the CAL 51 breast cancer cell line.¹
CONTROL OF THE SPECIFICITY
Negative controls were performed for each experiment and each antibody by replacing the primary MoAb with PBS or irrelevant mouse IgG or IgG, immunoglobulin corresponding to the MoAb isotypes tested (table 3). The CAL 51 breast cancer cell line or a metastatic breast cancer effusion (pleural or peritoneal) was used as a positive control for each series of slides.

SENSITIVITY OF THE TEST SYSTEM
A sample of normal human bone marrow obtained on heparin-lithium was contaminated by CAL 51 breast cancer cells and maintained since October 1985 in Dulbecco's modified minimum essential medium with Earle's salts (DHEM, Boehringer, France SA) supplemented with 10% fetal calf serum. The cancer cells in culture were separated by trypsinisation and washed in RPMI medium. They were introduced in decreasing concentrations as aliquots of 5x10^6 normal marrow cells per 5000, 500, 50, 5, 0.5, 0.05 cancer cells to obtain final concentrations of 1:10^6, 1:10^5, 1:10^4, 1:10^3, and 1:10^2 normal marrow cells. Each cell preparation was treated in the same manner as the marrow samples from the breast cancer patients. KL, was used to test the sensitivity of the method because it was strongly positive on CAL 51 breast cancer cells and it is known to be the most specific and the most constantly positive MoAb for mammary adenocarcinomas.

RESULTS

SENSITIVITY OF THE METHOD
The test system, consisting of normal human bone marrow contaminated with the CAL 51 breast cancer cell line, revealed that the technique was sensitive enough to detect at least one cancer cell in 10^5 haematopoietic cells.

CONTROL DATA

Positive controls
As shown in table 4, KL, was positive in all 16 specimens, HMFG, in 15 of 16 and EMA in 8 of 16 specimens. CA 15-3, tested in 14 specimens, was strongly positive in 12 cases.

Negative control specimens
As shown in table 5, KL, EMA, and HMFG, non-specifically labelled only some well recognisable plasmocytes and stromal cells. CA 15-3 was positive in the myeloid stem cells; therefore, because of its non-specificity in this study it was given up, although it was an excellent marker for mammary cells.

PATIENTS
No evidence of marrow tumour cells was found in any of the 72 patients. Nevertheless, a granular but particularly intense positivity similar to that of tumoral cells was observed in nine of the 72 patients (12%) with the panel of three MoAb as shown in figure 1. Attention to their morphological characteristics and their frequency on cytospins permitted detection of these cells by careful examination of MGG stained slides (figs 2 and 3). Six of these nine
Non-specifically labelled cells that simulate bone marrow metastases in patients with non-metastatic breast cancer

patients were node negative and three were node positive; histologically, five patients were stage I, and four had stage II disease; four were grade I, three grade II, and two non-classified.

These cells were obviously not tumoral, but rather medullary stromal cells (probably histiocytes) for three reasons:

(1) Their cytological appearance: in MGG stained slides these cells look like histiocytes with their oval often irregular nucleus, thin chromatin forming a homogeneous network, rare small nucleoli, abundant cytoplasm with irregular outlines giving them a sea urchin appearance on cytospins, often submerged in the centre of a nidus of cells (figs 2 and 3), usually erythroblasts. No evidence of malignancy were found and they were always isolated.

(2) Non-specificity of immunological labelling: these cells remained positive even when the primary antibody was replaced by the IgG, or IgG2 isotype (fig 4).

(3) The same type of cells can be observed in all marrow cytospins stained with MGG even from normal controls (fig 5), but in these cases any immunological reactivity was not as intense as in marrow from some cancer patients.

To test the hypothesis that Fc receptors might be responsible for this non-specific fixation, slides were pretreated with aggregated human
immunoglobulins; however, this failed to inhibit the immunological reaction.

Finally, cytochemical testing revealed that these cells were strongly positive with butyrate esterase and diffusely positive with acid phosphatase, characteristics which suggest a histiocytic origin.

In addition to these cells, several strongly labelled plasmocytes with a lacy red cytoplasmic stain were sometimes observed. They were easily identified by their eccentric nucleus, dense chromatin and abundant cytoplasm, and their archoplasm was still visible in some cases (fig 6).

**FOLLOW UP**

During short term follow up (21 to 38 months, median 29 months), six of 72 (8.3%) patients had developed a metastatic disease, one of these six had false positive marrow cells (T2, node-positive, grade III).

**Discussion**

In this study, marrow cells from nine of 72 (12%) M0 breast cancer patients labelled with three anti-epithelial antibodies were shown to be of non-tumoural nature. CA 15.3 which labelled myeloid stem cells was given up as it cannot be recommended for bone marrow investigations. Our results differ from those of most authors,3 5 7 8 13 20 24 but agree with those of five other investigators (table 1).2 3 9 11 12

Certain cells, whose immunological labelling was similar to that of cancer cells, were labelled non-specifically. These cells corresponded to stromal cells, which are also found in normal marrow and fix the linking antibody in the absence of primary antibody, or to plasmocytes, which have a characteristic morphology and are known to react with EMA and HMFG,20 25 as well as with cytokeratin.21 These cells cannot correspond to a skin contamination while they are non-specifically labelled.

Meticulous comparison of immunologically-treated cytospins and MGG stained cytospins was required to identify these stromal cells. Such comparisons of immunological and cytological results remain mandatory. Although the APAAP technique is known to be superior to immunofluorescence for preservation of cellular morphology, it alters cell structures sufficiently so that it becomes difficult, even for experienced cytofugists, to affirm the epithelial and tumoral nature of isolated immunologically-labelled cells. Such comparisons are not always mentioned in the literature, and few photographs have been published. In addition, controls of specificity with replacement of the primary antibody by its IgG isotype, are not performed routinely, even though they are required (table 1). Finally, while the non-specificity of the antibodies used separately requires a panel or cocktail of MoAb, several publications report use of only a single antibody (table 1).

The sensitivity of our technique is not responsible for the differences between our findings and published results: we detected one cancer cell in 106 marrow cells with the APAAP technique considered superior to other methods,13 21 25 and we used the optimum number of aspirations: six aspirations from three sites determined by several investigators.1 5 13 The high prevalence of favourable prognostic factors in our patients could in part explain our negative results as only six of 72 patients had more than three involved lymph nodes, none of them had any labelled cells. The short term follow up does not support any conclusion.

Two points remain unclear. First, the stromal cells of 12% of the breast cancer patients were strongly labelled yet they were always weakly labelled in normal marrow. Patient and control samples were assessed in the same way and at the same time and slides were reviewed when necessary. One can concur with Rabkin et al that reactivity with EMA may be a marker of histicye activation.26 Nevertheless this hypothesis requires other proofs of histicye activation in breast cancer patients. In the present study, these positive stromal cells were found with the three MoAb both in stage I and stage II disease, and in node-positive as well as negative patients. Second, while plasmocytes were relatively frequent on the marrow cytospins from breast cancer patients, only a small number were immunologically labelled by the three antibodies and always in intense manner.

While the possibility of marrow tumour cells in breast cancer patients classed M0 at the time of diagnosis cannot be ruled out, we suggest that literature results can be overestimated owing to antibody non-specificity and the difficulties encountered in interpretation of immunoreactive non-tumoral cells versus tumoral cells in the absence of any characteristic clumps. Proof of the tumoral nature of isolated cells is often inconclusive, and although immunocytochemistry and immunofluorescence are reference techniques for the detection of residual disease by cytophesis prior to autotransplantation,27 28 they may be suitable methods for marrow tumour cell detection only if more specific MoAb are found. It seems to be the case for cytokeratin 19 which does not label any myeloid cells. Molecular biology techniques may be promising, faster, and
probably more reliable and the two techniques should be compared.20-31 Confirmation of the presence of marrow tumour cells in node-negative operable breast cancer patients, associated with demonstration of their clonogenic capacity, is indispensable if this criterion is to serve as the basis for adjuvant therapy or become a major prognostic factor.

This work was supported by La Ligue Nationale Francaise contre le Cancer (Comité du Département de l'Yonne) et le Ministère de la Santé. The authors thank N Rameau for translation of the manuscript.