CORRESPONDENCE

Re: Micrometastatic Breast Cancer Cells in Bone Marrow at Primary Surgery: Prognostic Value in Comparison With Nodal Status

Early occult spread of tumor cells must be regarded as a major cause for the development of metastatic disease in patients with completely resected breast cancer. Immunocytochemical assays with monoclonal antibodies directed against epithelial differentiation antigens have allowed the detection of micrometastatic carcinoma cells in bone marrow. Most of the studies were performed with monoclonal antibodies either against cytokeratin (a major constituent of the cytoskeleton in epithelial cells) or against membrane-bound mucins (e.g., epithelial membrane antigen, human milk fat globule, and tumor-associated glycoprotein-12 [TAG12]).

Results from a recent study on more than 700 breast cancer patients (1) suggested that the presence of TAG12-positive cells in bone marrow, as detected with monoclonal antibody 2E11, could be used as an independent prognostic indicator of a clinical relapse (1). We were, therefore, interested in investigating in more detail the nature of the TAG12-positive cells. Thus, we analyzed bone marrow samples from 165 control patients without carcinoma with the use of monoclonal antibody 2E11 and simultaneously with the use of monoclonal antibody A45-B/B3 directed against a common epitope present on several cytokeratin peptides, including heterodimers of cytokeratin polypeptides 8 and 18 as well as 8 and 19 (2).

As described previously (3,4), the antibody reaction was developed with the alkaline phosphatase-anti-alkaline phosphatase technique, and we examined $2 \times 10^6$ mononucleated cells separated by Ficoll density centrifugation (900g for 20 minutes at room temperature) from each aspirate. As shown in Table 1, staining of bone marrow from these 165 control donors with antibody A45-B/B3 identified as positive two control cases only in which one and three positive cells, respectively, were found. In one case, these aspirates had been taken from a woman with acute exacerbation of a chronic mastitis; chronic inflammations are known to induce aberrant cytokeratin expression in hematopoietic cells (5). In contrast, antibody 2E11 stained bone marrow cells in 66 (62.9%) of 105 control aspirates (Table 1); 30 of these specimens (28.6%) presented with five or more TAG12-positive cells. To exclude the increased sensitivity of our assay as the cause of the difference between our findings and those of the previous study (1), we followed the published protocol even more closely. Sixty aspirates were analyzed with the described avidin–biotin–alkaline phosphatase complex technique (1); 35 of these specimens (58.3%) turned out to contain stained cells (Table 1).

The reactivity of antibody 2E11 with autochthonous bone marrow cells was further investigated by simultaneous double labeling of 60 aspirates from control patients without carcinoma by use of antibodies A45-B/B3 and 2E11 in parallel. To avoid interference of the two labeling systems, we used direct antibody conjugates consisting of fluorescein isothiocyanate or cyanine-2 bound to Fab fragments of antibody A45-B/B3. These conjugates lack the Fc portion of the A45-B/B3 antibody and therefore do not carry the risk of nonspecific binding to the abundant Fc receptor-expressing cells (e.g., monocytes). In 35 (58.3%) of these samples, all of the detectable TAG12-positive cells were negative for A45-B/B3 antibody staining, suggesting that the TAG12-positive cells lacked the epithelial cytoskeleton (data not shown). Some of the immunostained cells could be clearly identified as erythroblasts, while others could not be easily distinguished from tumor cells by morphologic criteria. Moreover, the double labeling of bone marrow from 11 breast cancer patients revealed the consistent presence of TAG12-positive cells coexpressing the common leukocyte antigen CD45.

In previous studies (3,4), the CK2 antibody directed against cytokeratin 18 had been shown to be a specific probe for bone marrow micrometastases that were detected in patients with adenocarcinomas of various origins. Recently, however, isolated down-regulation of cytokeratin 18 has been observed in some breast cancer cells (3,6); therefore,

Table 1. Specificity and sensitivity of monoclonal antibodies applied for detection of bone marrow micrometastases in breast cancer patients*

<table>
<thead>
<tr>
<th>Patients</th>
<th>Monoclonal antibody</th>
<th>Antigen</th>
<th>Staining technique</th>
<th>No. of patients</th>
<th>Fraction of BM samples with ≥1 immunoreactive cells (% of total)</th>
<th>Fraction of BM samples with ≥5 immunoreactive cells (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>A45-B/B3</td>
<td>CKs</td>
<td>APAAP</td>
<td>165</td>
<td>2 (1.2)†</td>
<td>0 (0.0)†</td>
</tr>
<tr>
<td></td>
<td>CK2†</td>
<td>CK18</td>
<td>APAAP</td>
<td>215</td>
<td>6 (2.8)†</td>
<td>0 (0.0)†</td>
</tr>
<tr>
<td></td>
<td>2E11</td>
<td>TAG12</td>
<td>APAAP</td>
<td>105</td>
<td>66 (62.9)</td>
<td>30 (28.6)</td>
</tr>
<tr>
<td></td>
<td>2E11</td>
<td>TAG12</td>
<td>ABC</td>
<td>60</td>
<td>35 (58.3)</td>
<td>8 (13.3)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>CK2</td>
<td>CK18</td>
<td>APAAP</td>
<td>185</td>
<td>60 (32.4)</td>
<td>16 (8.6)</td>
</tr>
<tr>
<td></td>
<td>A45-B/B3</td>
<td>CKs</td>
<td>APAAP</td>
<td>185</td>
<td>86 (46.5)§</td>
<td>37 (20.0)§</td>
</tr>
</tbody>
</table>

*Abbreviations used: BM, bone marrow; TAG12, tumor-associated glycoprotein-12; CKs, heterodimers of cytokeratin peptides 8 and 18 and 8 and 19; APAAP, alkaline phosphatase anti-alkaline phosphatase; ABC, avidin–biotin–alkaline phosphatase complex.

†P < .0001 compared with immunostaining with monoclonal antibody 2E11 (two-sided chi-squared test).

‡ Data taken from (4).

§P = .0056 compared with immunostaining with the CK2 antibody (two-sided chi-squared test).

||P = .0018 compared with immunostaining with the CK2 antibody (two-sided chi-squared test).
false-negative results may be obtained with immunoassays based on this particular peptide alone. To compare the rate of tumor cells detected with the A45-B/B3 antibody or the CK2 antibody, we simultaneously analyzed bone marrow samples from 185 breast cancer patients (Table 1), with the majority (94.1%) showing no clinical signs of metastasis [stage M0 according to tumor–node–metastasis (TNM) classification (7)]. Antibody 2E11 was excluded from this analysis because of its low specificity. We found that a statistically significant correlation existed between the results obtained with both anti-cytokeratin antibodies (Spearman’s correlation coefficient r = .918; 95% confidence interval = .892–.938). The inclination of the regression line (y = 0.697x), together with the strong correlation coefficient, however, indicated an increased sensitivity of the pan-cytokeratin A45-B/B3 over the monoclonal antibody CK2 antibody (P<.0001; z test). This assumption is supported by the considerable shift toward higher numbers of positive cells that were detected with antibody A45-B/B3 compared with antibody CK2 (Table 1).

In conclusion, the specificity of the antibody used for tumor cell identification is one of the most critical variables for the reliable detection of micrometastases in bone marrow. Thus, the development of standardized protocols deserves the highest priority, while meta-analysis of extremely heterogeneous and thus incomparable sets of data (8) appears to be rather premature at present. Moreover, a cautionary note for too early interpretation of prognostic factors appears to be appropriate, since occult metastatic cells may disclose their influence on survival at 10 years or later.

**References**


**Notes**

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Supported by grants from the Dr. Mildred-Scheel-Foundation, Bonn; the Foundation “Freunde der Maistrasse,” Munich; the Curt-Bohnewand-Foundation, Munich; and the Friedrich-Baur-Foundation, Munich.

**Response**

In their letter Braun et al. argue that the monoclonal antibody 2E11 used by our group (1,2) has a low specificity and that it is therefore not suitable for the analysis of tumor cell detection in bone marrow of breast cancer patients. Their results, however, address only the specificity of the antibody, without trying to test its sensitivity in breast cancer patients. We think that the data presented by Braun et al. provide additional information to a method (i.e., false-positive results in bone marrow of patients without carcinoma). Since they have slightly modified the staining method, the direct comparison of the data is difficult. Until 1995, we have used bone marrow smears; we started to use cytopsin preparations in 1996. For that reason, our staining protocol has changed over time. Currently, we are comparing the two staining protocols to find out the reasons for the discrepancies in the results between our group and the Munich group. Moreover, we are comparing anti-cytokeratin antibodies with mucin-specific antibodies and are trying to improve the specificity and sensitivity of the detection system.

The discussion on sensitivity versus specificity reminds us of the debate about some serum tumor markers such as human chorionic gonadotropin (HCG). It is well known that HCG is not specific for gestational trophoblastic disease, since healthy pregnant individuals and patients with some lung cancers also have elevated HCG values. Nevertheless, once gestational trophoblastic disease has occurred, the sensitivity of HCG is near to 100%.

At present in Germany there are five other groups who are testing the 2E11 antibody in breast cancer. Their detection rate is in the range of 35%–45%, which is in concordance with our data. We have also encouraged other groups to use our detection system to confirm our clinical findings. But any statistical analysis requires a considerable number of breast cancer patients, and no group has yet investigated an appropriate number of patients to be able to compare it with our cohort of more than 1300 patients. To our knowledge, there is only one other group that has tested tumor cell detection in more than 200 breast cancer patients with long-term follow-up [using anti-epithelial membrane antigen-antibody; (3)].

An improvement in sensitivity can lead to a loss of specificity. However, as long as Braun and his group could not demonstrate that their tested sensitivity with 2E11 is at the same level