Influence of Adjuvant Hormone Therapy and Chemotherapy on the Immune System Analysed in the Bone Marrow of Patients with Breast Cancer1

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ABSTRACT

Purpose: Bone marrow is a special compartment for antitumor immunological memory in patients with breast cancer. Until now, the influence of adjuvant systemic therapy on the immune system has only been investigated in peripheral blood and not in bone marrow. In this study, we analyzed the effect of hormone therapy and chemotherapy on the immune activation status in bone marrow.

Experimental Design: In 34 patients with breast cancer, bone marrow was aspirated 24 months after primary surgery and adjuvant systemic therapy. The immune system of these patients was compared with that of patients at the time of primary surgery (n = 90). Three-color flow cytometry was used to identify the number and activation state of T cells, natural killer (NK) cells, monocytes/macrophages, and subsets by means of a panel of monoclonal antibodies.

Results: The proportion of all T cells was significantly lower in patients after adjuvant systemic therapy than in patients with primary breast cancer or normal healthy donors. Chemotherapy apparently had a particularly suppressive effect on naive CD4 T cells and, to a lesser extent, on memory CD4 T cells. Hormone therapy apparently had a significant suppressive effect on both naive and memory CD8 T cells. The numbers of NK cells (CD56) and of monocytes/macrophages (CD14) recovered rapidly after adjuvant chemotherapy. However, subpopulations with potential antitumor reactivity, such as activated NK and NK T cells, were reduced per long term after chemotherapy.

Conclusions: These findings suggest profound and long-lasting negative effects on the bone marrow immune system by present day adjuvant therapy in breast cancer.

INTRODUCTION

After surgery of the primary tumor and local radiotherapy, high-risk breast cancer patients receive systemic CHT1 or HT as standard adjuvant prophylaxis against disseminated tumor cells (1). BM is a favored compartment for breast cancer cell dissemination (2). Although the frequency of disseminated tumor cells detected in the BM of operated breast cancer patients is low (one tumor cell in $10^5$ to $10^6$ BM cells; Refs. 3–6), they may be involved in late metastasis ≤20 years after primary treatment (1). CHT has cytostatic or cytotoxic effects on rapidly dividing cells. Side effects of such treatment are, e.g., leucopenia attributable to BM suppression, as well as suppression of proliferation or function of lymphocyte subsets.

The immune system may play an important role in the control of tumor growth and metastasis (7, 8). Some studies demonstrated elevated levels of tumor antigen-specific autoantibodies in cancer patients (9, 10). Others showed strong infiltration of primary tumors and draining lymph nodes by tumor-specific cytotoxic T lymphocytes (11). Moreover, the direct antitumor activity of the immune system seems to be important for the prognosis of cancer patients. It has been demonstrated that cell-mediated immunity to tumor-associated antigens is a better predictor of survival than stage, grade, and lymph node status (8).

However, many investigators failed to detect functional tumor-specific lymphocytes in the peripheral blood of cancer patients (12, 13). This may be attributable to tumor immune-suppressive mechanisms leading to T-cell anergy, or such cells may reside in lymphoid organs and not in the peripheral blood. Apart from blood, other potential sources for lymphoid cells like spleen or nonmetastatic lymph nodes are usually not available from patients.

In contrast to spleen and lymph nodes, BM is easily accessible by iliac crest puncture. BM is a large lymphoid organ containing T and B lymphocytes, NK cells, and monocyte/macrophage populations (14). The removal of aspirates from BM for tumor cell detection offers the opportunity to analyze its immune cellular content and activation and memory status. We performed previously an analysis of BM-derived immune cell populations from nontreated primary breast cancer patients and found significantly elevated levels of memory T and NK T cells

3 The abbreviations used are: CHT, chemotherapy; HT, hormone therapy; BM, bone marrow; PG, primary group; PE, phycoerythrine; ND, normal donor; NK, natural killer; CK19, cytokeratin 19.
compared with NDs and to peripheral blood (14). These expressed markers of activation and proliferation indicated that the human immune system may not be ignorant of autochthonous cancer cells. Immunological parameters in BM were found to be stable and less variable than those of peripheral blood (14). We furthermore demonstrated that BM of such patients contained highly enriched numbers of tumor-reactive memory CD4 and CD8 T cells capable of specific IFN-γ secretion and tumor cell lysis after appropriate restimulation with tumor antigen-loaded dendritic cells (15).

 Nevertheless, there have been only few investigations of the immune status in the BM of cancer patients. In addition, little attention has been paid to the potential influence of adjuvant systemic therapy on the immune system, in particular, to such a central compartment as the BM.

 In this study, we therefore performed immunophenotyping of BM aspirates from primary breast cancer patients, patients after systemic therapy, and NDs to analyze the effects of CHT and HT in breast cancer on the immune system in the BM.

PATIENTS AND METHODS

Patients. BM was taken from 124 breast cancer patients from the University of Heidelberg, Departments of Obstetrics and Gynecology. Exclusion criteria were malignant disease other than breast cancer, breast cancer recurrence, preoperative HT, or CHT; serious functional disorders of the liver or kidneys; and metabolic disease. Informed consent was obtained from all participants. Depending on the time of the BM aspiration and type of the adjuvant therapy, the following groups were constituted: (a) patients with BM aspiration at the time of primary surgery (PG; n = 90); (b) patients with BM aspiration 2 years (20–28 months) after primary surgery (n = 34); i) CHT group: patients who had undergone standard CHT (n = 19) and ii) HT group: patients who had undergone standard HT with tamoxifen (n = 15); and (c) ND group (10 healthy women, 26–57 years old).

Characteristics of the Patients. The characteristics of the PG and study group with HT or CHT are shown in Table 1. The patients in the HT group were treated with 20 mg/day tamoxifen. The patients in the CHT group were treated as follows: (a) six cycles of standard CHT with cyclophosphamide (500 mg/m²), methotrexate (40 mg/m²), and 5-fluorouracil (600 mg/m²; n = 10); (b) three cycles of standard CHT with cyclophosphamide (600 mg/m²), methotrexate (40 mg/m²), and 5-fluorouracil (600 mg/m²; n = 4); and (c) four cycles of cyclophosphamide (600 mg/m²) and epirubicin (60 mg/m²; n = 5). A loco-regional radiotherapy was done in 87 and 84% of the patients in the HT and CHT groups. BM aspiration was performed 24 months after the primary therapy (range 20–28 months). Fisher’s exact test showed that there were no significant differences in the distribution of the individual characteristics and prognostic factors between the primary and the study group (Table 1).

BM Preparation. BM samples (10 ml) were aspirated from each anterior iliac crest. The procedure was performed either immediately after surgery while the patient was still under anesthesia (PG) or local anesthesia (HT and CHT). The heparinized BM was subjected to Ficoll gradient centrifugation (Amersham Pharmacia, Uppsala, Sweden) in Leuко SEP tubes (Greiner, Frickenhausen, Germany). The cells in the interphase were used for immunophenotyping via flow cytometry. Mononuclear BM cells (5 × 10⁸) were blocked with human immunoglobulins (Endobulin; Immuno GmbH, Heidelberg, Germany) to avoid nonspecific binding of monoclonal antibodies to Fc receptors. Staining was then performed using the following directly conjugated mouse antihuman monoclonal antibodies: (a) for T cells: FITC-CD3 (clone HIT3a), PE- and CyChrome-CD4 (clone B159), PE- and CyChrome-CD8 (clone RPA-T8), FITC- and PE-CD45RA (clone HI100), and FITC- and PE-CD45RO (clone UCHL-1); (b) for macrophages/monocytes: PE-CD14 and for costimulatory molecules: FITC-CD80 (B7.1; clone MAB 104); and (c) for NK cells, PE-CD56 (NCAM; clone B159).

All antibodies were from PharMingen (Hamburg, Germany) with the exception of anti-CD80, which was from Beckman-Coulter (Krefeld, Germany). Propidiumiodide-negative viable cells were analyzed by three-color flow cytometry using a FACSscan with CELLQuest software (Becton Dickinson). Typically, 20,000 events were collected, and the data were expressed as bivariate scatterplots.

Detection of Disseminated Tumor Cells in BM. BM cells (10⁶) were snap frozen in liquid nitrogen and kept at −70°C. Total RNA was extracted using the High Pure Isolation Kit (Roche, Mannheim, Germany). First-strand cDNA was synthesized in 20-µl reaction mixture from 2 µg of total RNA using a cDNA preamplification kit (Life Technologies, Inc., Eggenstein, Germany). cDNA (10 µl) was subjected to nested PCR using the CK19 primers already described (16). After an initial denaturing process for 10 min at 94°C, PCR analysis was performed in 30 cycles under the following conditions: (a) 65-s denaturation at 94°C; (b) 65-s annealing at 58°C; and (c) 2-min polymerization at 72°C. After the first amplification, an aliquot

| Table 1 Clinical and pathological features of the study group (n = 34), which received adjuvant therapy, in comparison with a PG (n = 90), which had not yet received adjuvant therapy |
|---------------------------------------------------------------|----------------|----------------|-----------|
| Prognostic marker | PG (n = 90) | Study group (HT or CHT) (n = 34) | p*        |
| Tumor size | | | |
| T1 | 49 (54%) | 19 (56%) | 1.0 |
| T2–4 | 41 (46%) | 15 (44%) | 0.404 |
| Nodal status | | | |
| N0 | 54 (61%) | 24 (70%) | 0.223 |
| N+ | 34 (39%) | 10 (30%) | |
| Estrogen receptor | | | |
| Positivea | 46 (61%) | 10 (45%) | 0.118 |
| Negative | 29 (39%) | 12 (50%) | |
| Progesterone receptor | | | |
| Positiveb | 48 (70%) | 10 (50%) | |
| Negative | 21 (30%) | 10 (50%) | |
| Menopausal status | | | |
| Pre | 32 (35%) | 13 (38%) | 0.836 |
| Post | 58 (65%) | 21 (62%) | |
| Grade (n = 86) | | | |
| I + II | 60 (66%) | 19 (70%) | 1.0 |
| III | 26 (34%) | 8 (30%) | |

* Fisher’s exact test.

** Positive ≥20 fmol/mg protein.
was diluted at 1:100, and 5 μl were submitted to the second amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested primers; the thermal cycle was similar except that the extension time was reduced to 55 s. Amplification with nested

Table 2 Phenotype analysis of T cell (CD3, CD4, and CD8), NK cell (CD56), and monocyte/macrophage (CD14) populations in BM of patients from the PG (n = 90), HT group (n = 15), CHT group (n = 19), and ND group (n = 10).

<table>
<thead>
<tr>
<th>Phenotypea</th>
<th>PG (n = 100)</th>
<th>HT (n = 100)</th>
<th>CHT (n = 100)</th>
<th>ND (n = 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+ (t)</td>
<td>30.6 ± 12.7N</td>
<td>25.5 ± 8.5N</td>
<td>22.7 ± 6.4N</td>
<td>40.8 ± 10.9</td>
</tr>
<tr>
<td>CD4+ (t)</td>
<td>18.8 ± 8.5N</td>
<td>16.7 ± 6.6</td>
<td>13.7 ± 5.0N</td>
<td>22.9 ± 6.6</td>
</tr>
<tr>
<td>CD8+ (t)</td>
<td>10.7 ± 5.8N</td>
<td>7.4 ± 3.1N</td>
<td>8.4 ± 2.0N</td>
<td>14.7 ± 7.7</td>
</tr>
<tr>
<td>CD4+CD45RA+ (t)</td>
<td>97.5 ± 5.3N</td>
<td>8.1 ± 3.3N</td>
<td>5.8 ± 3.4N</td>
<td>13.4 ± 3.7</td>
</tr>
<tr>
<td>CD4+CD45RO+ (t)</td>
<td>13.5 ± 6.4N</td>
<td>10.4 ± 5.7</td>
<td>9.9 ± 3.5N</td>
<td>10.1 ± 4.8</td>
</tr>
<tr>
<td>CD8+CD45RA+ (t)</td>
<td>8.0 ± 4.9N</td>
<td>5.3 ± 2.4N</td>
<td>6.1 ± 2.2N</td>
<td>13.4 ± 6.9</td>
</tr>
<tr>
<td>CD8+CD45RO+ (t)</td>
<td>5.7 ± 3.3N</td>
<td>3.8 ± 1.7P</td>
<td>4.6 ± 2.5</td>
<td>5.8 ± 3.0</td>
</tr>
<tr>
<td>CD4+CD26+ (t)</td>
<td>14.9 ± 7.6N</td>
<td>11.5 ± 6.3P</td>
<td>10.4 ± 4.6P</td>
<td>14.4 ± 3.9</td>
</tr>
<tr>
<td>CD4+CD26+ (CD4)</td>
<td>78.8 ± 11.6N</td>
<td>67.5 ± 24.0P</td>
<td>75.3 ± 6.7N</td>
<td>62.8 ± 9.8</td>
</tr>
<tr>
<td>CD56+ (t)</td>
<td>8.2 ± 5.0</td>
<td>9.8 ± 4.3</td>
<td>9.0 ± 4.0</td>
<td>13.3 ± 2.5</td>
</tr>
<tr>
<td>CD14+ (t)</td>
<td>4.3 ± 3.0P</td>
<td>5.1 ± 3.1</td>
<td>5.3 ± 2.7</td>
<td>7.7 ± 4.6</td>
</tr>
<tr>
<td>CD14+CD4+ (CD14)</td>
<td>78.5 ± 15.7N</td>
<td>82.2 ± 16.2</td>
<td>89.3 ± 8.8P</td>
<td>87.0 ± 11.6</td>
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<tr>
<td>CD14+CD45RA+ (CD14)</td>
<td>46.5 ± 11.2N</td>
<td>43.9 ± 12.4</td>
<td>29.9 ± 8.5N</td>
<td>35.1 ± 9.0</td>
</tr>
</tbody>
</table>

a Fraction of single positive or double positive T and NK cells and monocytes relative to the total number (t) of BM-derived mononuclear cells or relative to the respective subset (CD4 or CD14).

b N represents a significant difference compared with the ND group, P represents a significant difference compared with the PG, C represents a significant difference compared with the CHT group, and H represents a significant difference compared with the HT group.

RESULTS

Immunophenotype of BM without Adjuvant Therapy.

The phenotype analysis of BM T lymphocytes, NK cells, and CD14 monocytes/macrophages is shown in Table 2. Immune cell populations of the treated HT or CHT groups were compared with corresponding populations of the nontreated PG and to healthy NDs.

The comparison of the PG with the ND group revealed similar results as a previous study with a smaller number of patients (15). The PG and ND groups differed with regard to numbers of total T cells. We detected in the PG a decrease of total naïve (CD45RA+) CD4 and CD8 T cells in the PG, which was accompanied by an increase of total CD4 memory (CD45RO) T cells. Correspondingly, the fraction of memory T cells in the CD4 and CD8 compartment was higher in the PG. We furthermore observed a higher fraction of activated (CD26+) CD4 T cells in the CD4 compartment of the PG.

No significant difference existed in the total NK (CD56+) cell numbers. However, we observed phenotypical changes within the CD56 compartment in primary operated tumor patients (Fig. 1). These were characterized by an increase of NK T cells (CD56+ CD3+ A and CD56+ CD8+ C) and activated NK cells (CD56+ CD45RO+ B) compared with ND (Fig. 1). Finally, the total amount of the monocyte/macrophage population was reduced in PG as compared with ND (Table 2).

Pronounced Effect in the CHT Group on CD4 T and NKT Cells and Monocytes/Macrophages.

Compared with the PG as well as to the ND group, we observed a reduction of total CD3 T cells in the CHT group. This was attributable to a decrease in the CD4 T-cell compartment. The CD8 T-cell compartment in the CHT group was similar to the PG. The CD4:CD8 ratio (1.6) was significantly lower in the CHT group than in the HT group (2.3). Naïve and memory CD4 T-cell subpopulations were differentially affected by CHT. We found a more pronounced reduction of naïve (41%) than of memory T cells (27%). This led to an increase of the memory fraction in the CD4 T-cell population (75%) in comparison with the PG (69%) and to NDs (54%; Fig. 1E). Interestingly, CHT reduced the total number of activated CD4+CD26+ T cells compared with the PG and to ND group (Table 2). The fraction of activated CD4 T cells among the CD4 T cells [CD26+CD4+CD26+] was not reduced in comparison to the PG and was even higher than in NDs. This observation indicates a reductive effect of CHT mainly on nonactivated (CD26+) and on naïve (CD45RA+) CD4 T cells.

Although total NK (CD56+) cell numbers were not reduced by CHT, we detected a significant decrease of the initially elevated NK T-cell (CD56+ CD3+) subpopulation in comparison with normal levels. The proportion of activated NK cells (CD56+CD45RO+) was also lower after CHT than in the PG, whereas the CD8-coexpressing subpopulation was enhanced compared with the ND group (Fig. 2).

No change of total monocyte/macrophage numbers was found in the CHT group. However, there was an effect of CHT on subsets of the monocyte/macrophage population. There was an increase of the nonactivated CD14+CD4+ subpopulation and a decrease of the CD45RA+ subpopulation in comparison with the PG (Table 2).

Reduction of CD8 T and CD56+CD8+ NK Cells in the HT Group.

In the HT group, we observed reduced numbers of total CD3 T cells compared with the PG as well as to the ND group.
CD4 T cells, the activation status of such cells was reduced in compared with the PG. Although HT did not affect numbers of changes in the naïve CD56

−

not influenced, HT reduced significantly the proportion of the reduction of the proportion of activated CD56

−

pared with CHT. Although total CD56

−

/H11001

/H11001

/H11001

naive and memory CD8 T-cell numbers, HT affected both naïve and memory CD8 T cells similarly (reduction by 34% in both compartments). No changes in the naïve to memory T-cell ratio were observed compared with the PG. Although HT did not affect numbers of CD4 T cells, the activation status of such cells was reduced in comparison with the PG. Both the total amount of CD4

+/CD26

−

T cells as well as their proportion in the CD4 compartment were smaller influence of HT on total CD3 T-cell counts compared with CHT. In contrast to CHT, which reduced especially naïve CD4 T cells, whereas CD8 T cells were less affected. As a consequence, the CD4:CD8 ratio (2.3:1) was significantly higher than in the CHT group (1.6:1). Because the CD4 compartment is much larger than the CD8 compartment, we saw a significantly smaller influence of HT on total CD3 T-cell counts compared with CHT. In contrast to CHT, which reduced especially naïve CD4 T cells numbers, HT affected both naïve and memory CD8 T cells similarly (reduction by 34% in both compartments). No changes in the naïve to memory T-cell ratio were observed compared with the PG. Although HT did not affect numbers of CD4 T cells, the activation status of such cells was reduced in comparison with the PG. Both the total amount of CD4

+/CD26

−

T cells as well as their proportion in the CD4 compartment were significantly reduced (Table 2).

HT showed a different effect on NK cell populations compared with CHT. Although total CD56

−

NK cell numbers were not influenced, HT reduced significantly the proportion of the CD56

−

xCD8

−

subpopulation (Fig. 1C) that was elevated in the PG as compared with the ND group. In contrast to CHT, no reduction of the proportion of activated CD56

−

CD45RO

−

NK cells could be detected after HT (Fig. 1B). Effects on the monocyte/macrophage population could not be seen (Table 2).

Some of the treatment effects were characterized by a reversal back to normal levels, e.g., CD4

+/CD45RO

−

(t), CD56

−

xCD3

−

(CD56), and CD56

−

CD45RO

−

(CD56) in the CHT group and CD4

−

CD26

−

(CD4) and CD56

−

CD8

−

(CD56) in the HT group. Such changes could be explained both by direct influences of the treatment on distinct cell populations as well as by elimination of residual tumor cells with restoration of a normal situation in the patients. We therefore analyzed BM samples of 17 patients in the study groups for infiltration by disseminated tumor cells via PCR detection of CK19 mRNA as tumor marker. Tumor cells were found in 10 of the 17 BM samples. We could not detect any differences in immune cell populations with regard to the CK19 status (data not shown).

DISCUSSION

In this study, we found negative effects of adjuvant therapy on the immune system in the BM of breast cancer patients. CHT consisted of cyclophosphamide, methotrexate, and 5-fluourouracil as adjuvant standard treatment. Some effects of these drugs on the immune system have been described already. Cyclophosphamide induces a decrease in lymphocyte counts with a pronounced reduction of B cells (17) and inhibition of antibody responses (18), as well as a selective loss of NK cell activity (17). However, cyclophosphamide does not reduce proliferation of mitogen-activated lymphocytes (17). In contrast, methotrexate selectively induces apoptosis in activated and proliferating, but not in resting, T cells (19, 20). 5−fluourouracil also exerts cytotoxic effects on rapidly proliferating cells. It inhibits proliferation of mitogen-activated T cells (17, 21) and selectively reduces activation of NK cells (17).

Several studies investigated the long-term effects of combined CHT. In some studies, the proportion of naïve CD45RA T cells in the CD4 population of peripheral blood dropped initially and increased gradually during the first year after CHT. Even after 1 year, the number of CD4CD45RA cells was still one-third lower than the pretreatment level. The generation of new naïve CD4CD45RA cells appears to contribute little to CD4 recovery in the first year. Expansion of memory CD4CD45RO T cells was responsible for an early rise in CD4 T-cell counts after CHT (22, 23). The memory CD4CD45RO T cells increased rapidly after CHT, but this expansion was transient and fell steadily 3−6 months post-CHT. Recovery of CD4 T cells in blood was therefore delayed for >1 year after CHT (22). In blood, CD8 T cells were also suppressed, but they recovered faster than CD4 T cells and had reached 86% of the baseline values 12 months after CHT (22, 24). The delayed recovery of CD4 T cells compared with CD8 T cells was likely attributable to an increased susceptibility to apoptosis after CHT (22). The depression of CD4 T cells plays an important role in the immune deficiency after CHT and may explain the increased susceptibility to opportunistic infections (23, 25).

NK (CD 56) cells and monocytes/macrophages (CD 14) in BM and peripheral blood recover more rapidly after CHT than CD4 T cells. The levels of post-CHT were comparable with baseline (26, 27), but despite recovery of the total counts of these cells, their potential for direct antitumor reactivity appeared to be reduced.

The influence of hormone treatment on immune cell pop-
ulations has been scarcely investigated. Several studies demonstrated reduced alloreactivity after tamoxifen treatment in vitro. This was attributable to reduced cytokine production (tumor necrosis factor-α and interleukin-12) by the monocyte/macrophage population, as well as to blockade of IFN-γ secretion by T cells (27, 28). In a murine model of lupus erythematodes, tamoxifen prevented production and deposition of autoantibodies in kidneys by reduction of B-cell counts and tumor necrosis factor-α secretion (29). Direct effects on CD8 T cells may occur by blockade of FasL expression (30). However, comparison of total peripheral cell counts of primary breast cancer patients treated with CHT or CHT plus tamoxifen revealed attenuation of cytoreduction of CD4 and CD8 T-cell populations in the tamoxifen-treated group possibly attributable to drug interactions (31).

All described effects of systemic cytostatic treatment have been investigated with circulating mononuclear cells from peripheral blood. However, immune cells from lymphoid organs may differ from circulating cells with regard to proportion, phenotype, migratory capacity, activation status, and their functional relevance in malignant disease. We demonstrated previously that immunological parameters in BM were more stable and less variable than those of peripheral blood. BM-derived immune cell populations from nontreated primary breast cancer patients contained elevated levels of memory T and NK T cells and higher numbers of activated or proliferating cells (14). Tumor-reactive memory CD4 and CD8 T cells capable of specific IFN-γ secretion and tumor cell lysis after appropriate restimulation were also highly enriched compared with peripheral blood (15).

The reasons for the observed differences between peripheral blood and BM are still unclear. BM provides a spongioid microenvironment with stromal cells, adhesion molecules, and antigen-presenting cells similar to other lymphoid organs (32–36). This organ microenvironment may favor local enrichment of reactive T cells, encounter of antigen, and expansion of specific T-cell clones.

Moreover, BM seems to be a compartment particularly prone for metastases in solid tumors (2). Particularly, breast cancer cells are known to develop disseminated BM micrometastases. Some disseminated tumor cells may be eliminated as a result of adjuvant systemic therapy. Other disseminated tumor cells may be kept under local control by a chronically activated immune system resulting in the so-called tumor dormancy status. In animal experiments, it has been demonstrated that persistence of dormant tumor cells in the BM of mice vaccinated with tumor cells correlated with long-term immunological pro-

**Fig. 2** Comparison of CD4 and CD8 T cells (A and B), CD45RO memory CD4 and CD8 T cells (C and D), and the fraction of CD45RO memory CD4 and CD8 T cells in the respective T-cell subpopulation in BM of patients from the PG (n = 90), HT group (n = 15), CHT group (n = 19), and ND group (n = 10). Means and SDs are represented as a percentage of subpopulations among mononuclear cells (A–D) or among CD4 and CD8 T cells (E and F, respectively). N, P, C, and H represent a significant P (P < 0.05) compared with the ND, PG, CHT, and HT groups, respectively.
Breakdown of such a control can lead to metastasis formation.

We here demonstrate influences of systemic cytostatic treatment on various BM mononuclear cell populations. The numbers of cells with potential antitumor cytotoxic activity, such as NK T cells (CD56+CD3+), activated NK cells (CD56+CD45RO+), and activated CD4 T cells (CD4+CD26+), remained lower after CHT. The proportions of nonactivated NK cells [CD56CD45RA+CD56− (CD56); data not shown] and of nonactivated monocytes/macrophages [CD14+CD4+ (CD14)] were increased. Both naive (CD4+CD45RA+) and memory (CD4+CD45RO+) CD4 T cells in BM were lower 18 months after CHT than in the PG. These findings could mean that the direct antitumor potential of the immune system is reduced after CHT in all three compartments: (a) T cells; (b) NK cells; and (c) monocytes/macrophages. However, naïve CD4 T cells were much more reduced than memory T cells, a finding most likely attributable to a faster regeneration of the memory T-cell pool (22, 23). Yet, it is not clear if such memory expansion is attributable to encounter of residual tumor antigens or the result of unspecific homeostasis mechanisms. Although total counts were reduced, the proportion of activated CD4+CD26+ T cells among total CD4 T cells was conserved after CHT. This finding implies a possible preservation of the tumor-reactive memory T-cell pool in the BM of breast cancer patients that was described previously (15).

This might be of importance, because the antitumor reactivity influences the prognosis of patients. McCoy et al. (8) demonstrated in 2000 that the cell-mediated immunity to tumor-associated antigens is a better predictor of survival than stage, grade, and lymph node status. A better cell-mediated immunity may offer a protective effect against metastatic development. Survival in breast cancer patients is favorably influenced by a natural humoral immune response to polymorphic epithelial mucin (7). The important role of immune system activation in the metastatic process is underlined by the fact that most metastases are diagnosed in a period of reduced antitumor activity of the immune system.

In a previous analysis of primary operable breast cancer patients, we found higher numbers of CD4+CD45RO+ memory T cells and CD56+CD8+ NK T cell subsets in patients with CK19+ tumor cells in their BM (14). Interestingly, in our present study, these subsets were found to be reduced to normal levels after systemic adjuvant therapy. Importantly, CD4+ memory T cells were only reduced after CHT, whereas CD8+CD56+ NK T cells were reduced exclusively after HT. It is unlikely that this effect is exclusively caused by a reversal of a normal situation, because the absolute numbers of total CD4 T cells were clearly reduced compared with NDs, so that the proportion of memory to naïve T cells was strongly elevated. This demonstrates clear differences between the normal and post-CHT situation with regard to T cells. Moreover, if an elimination of tumor cells caused the normalization of both subsets in the treated patients, it cannot be explained why these subsets were differently affected by the two kinds of treatment.

We did not detect differences in BM T-cell populations after CHT between patients containing CK19+ cells in their BM and patients without CK19+ cells in their BM. This finding might imply that the direct influence of cytostatic treatment is much stronger than possible effects of tumor cells. However, the number of analyzed samples from the study group (17) was limited, so some influences cannot be excluded.

Interestingly, CHT did not significantly affect BM CD8 T-cell numbers. This might be attributable to the described elevated sensitivity to apoptosis induction of CD4 T cells by CHT (24). CHT neither affected BM CD56+CD8+ NK T cells as much as activated (CD56+CD45RO+) or total (CD56+CD3+) NK T cells, a finding consistent with previous results (17).

In contrast to CHT, tamoxifen treatment mainly affected the CD8 T-cell population that was reduced to ~50% of normal levels. No difference between reduction of naïve or memory cells could be detected after HT. There was a severe reduction in the tumor-specific CD8 memory T-cell pool. It is not clear if such reduction is caused by treatment alone or also caused by elimination of tumor antigens. Although we did not detect differences between patients bearing dormant tumor cells in their BM (CK19+ in BM) and tumor-free patients (CK19− in BM), the number of analyzed samples was limited. Additional studies may clarify this point. It is known that proliferation of CD8 T cells often depends on CD4 T-cell help. Interestingly, although T-cell counts were not significantly reduced by hormone treatment, we observed a significant loss in the proportion of activated (CD26) CD4 T helper cells. This might, besides direct effects, exert a negative influence on the CD8 T-cell pool. Similar to the selective reduction of CD8 T cells, we detected a selective reduction in the proportion of CD8 NK T cells among total NK cells after tamoxifen treatment. Total NK T cells as well as activated NK T cells were not reduced.

In this study, we demonstrate for the first time profound long-term effects of systemic cytostatic treatment on BM immune cell populations of breast cancer patients. CHT reduced selectively CD4 T cells with a pronounced effect on naïve cells, whereas proportions of CD4− or CD8−activated and memory T cells were not reduced compared with a normal group. In contrast, HT reduced both naïve as well as memory CD8 T-cell numbers significantly below normal levels. Although total NK cell numbers were not affected, the different treatments influenced NK T cells similarly to their T-cell counterparts.

This may be of importance, because tumor-specific memory T and NK T cells in the BM may play a role in local or systemic long-term immune control of residual tumor cells. It should therefore be taken into consideration that adjuvant CHT and HT might have negative impacts on the struggle between the immune system and residual tumor cells. The functional significance of our observations remains to be elucidated by further follow-up analysis of the patients’ outcome.

REFERENCES


