Evidence for a Graft-Versus-Tumor Effect in a Patient Treated With Marrow Ablative Chemotherapy and Allogeneic Bone Marrow Transplantation for Breast Cancer

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Graft-versus-leukemia (GvL) has been shown to be an important immune-mediated antitumor effect in hematologic malignancies. It is still unknown whether such an immune-mediated antitumor effect has clinical implications in patients with solid tumors. A 32-year-old woman with inflammatory breast cancer received a bone marrow transplant (BMT) from her HLA-identical sibling. During graft-versus-host disease (GvHD) cytotoxic T lymphocytes were grown and tested in a chromium-release assay against B and T lymphocytes of the patient and donor and against a panel of breast cancer cell lines. Resolution of liver metastases was observed simultaneously with clinical GvHD in the first weeks after transplant. In addition, minor histocompatibility antigen (MiHA)-specific and major histocompatibility complex (MHC) class I antigen-restricted cytotoxic T lymphocytes recognizing breast carcinoma target cells were isolated from the blood of the patient. Pretreatment of such target cells with tumor necrosis factor (TNF)-α but not with interferon (IFN)-α or IFN-γ increased susceptibility of these cells to lysis by cytotoxic T lymphocytes. Clinical course and in vitro results suggest that a graft-versus-tumor (GvT) effect might exist after allogeneic BMT for breast cancer. However, clinical experience on a larger scale would be required to determine the clinical efficacy of GvT effects in patients with solid tumors.

ALLOGENEIC BONE MARROW transplantation (BMT) offers not only the potential of applying marrow ablative doses of chemoradiotherapy, but also of inducing an immune-mediated antitumor effect referred to as graft-versus-leukemia (GvL).[1-3] Evidence for a substantial GvL effect is given by retrospective analysis of clinical BMT in hematologic malignancies and from experimental tumor models.[1,2,4-8] Isolation of donor cytotoxic T lymphocytes from patients with graft-versus-host disease (GvHD) capable of killing host leukemic cells has provided additional support and pathophysiologic insights into the mechanisms of GvL effects, permitting a search for target molecules of GvHD and GvL.[9-11] Until recently, little was known about the molecular nature or the biological properties of these antigens called minor histocompatibility antigens (MiHAs), mainly due to the lack of recognition by antibodies. Recent findings indicate that MiHAs are peptides derived from cellular proteins, which are presented to T cells by major histocompatibility complex (MHC) molecules in the same way as viral antigens and expressed on normal hematopoietic (eg, progenitors, B and T lymphocytes, monocytes), epithelial (eg, keratinocytes, fibroblasts, gut, liver), and malignant hematopoietic cells.[11-19] One of these peptides was recently isolated and biochemically characterized.[20] It is still unknown, however, whether such MiHAs are also expressed on malignant epithelial cells and, if so, whether this finding has any clinical implications with respect to a graft-versus-tumor (GvT) effect.

A clinically relevant antitumor effect for solid tumors in vivo has not been demonstrated so far. The low frequency of allogeneic BMT for solid tumors (30 patients in Europe during the last 5 years[21] and none with breast cancer) might well account for the lack of information about this important issue.

In this article we report on a patient with breast cancer transplanted with bone marrow from her HLA-identical sister. Disappearance of liver and bone metastases observed simultaneously with clinical GvHD on day +27 and the presence of MiHA-specific cytotoxic T lymphocytes recognizing breast carcinoma cell lines suggest that a GvT effect might also be operative in patients with breast cancer after allogeneic BMT.

MATERIALS AND METHODS

Isolation of peripheral blood mononuclear cells (PBMC). Heparinized peripheral blood was obtained from the donor and from the patient before transplantation and on day +27 during GvHD. PBMC were separated on Ficoll-Isopaque (Lymphoprep; Nyegaard, Oslo, Norway), washed three times, and stored in liquid nitrogen until use.

Establishment of cytotoxic T-cell line (CTL). CTL was established as previously described.[11] In short, 2×10^6/mL posttransplant recipient PBMC harvested during GvHD were stimulated with 2×10^6/mL irradiated (30 Gy) pretransplant recipient PBMC and cultured in a 24-well plate (2 mL/well; Costar, Cambridge, MA) using tissue culture medium consisting of RPMI 1640 (Biochrom, Berlin, Germany) containing 2 mmol/L L-glutamine (Biochrom), 100 U/mL penicillin (Biochemie, Vienna, Austria), and 20 U/mL streptomycin (Biochemie) supplemented with 10% human male AB pool serum at 37°C in a humidified 5% CO_2/95% air atmosphere. On day 6, cells were adjusted at a concentration of 2×10^5/mL and restimulated with 4×10^5/mL irradiated (50 Gy) Epstein Barr Virus (EBV)-transformed, pretransplant recipient PBMC in tissue culture medium supplemented with 2% high-purity Lymphokult-T (Biotest, Dreieich, Germany), final concentration as interleukin-2 source. Every third day, 50% of the medium was replaced with fresh medium containing 20% Lymphokult-T (Biotest) at final concentration. CTL obtained

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after 3 weeks was used as polyclonal effector cells in the cytotoxic assays and restimulated weekly with the same cell mixture as on day 6.

Breast cancer cell lines. The human breast cancer cell lines MCF-7,[22,23] T-47D,[24] 734-B,[22] HBL-100,[25] BT-20,[26,27] ZR 75.1,[28] MDA MB,[29] and SKBR-3[30] were cultured in minimal essential medium (Life Technologies, Vienna, Austria) supplemented with 4 mmol/L L-glutamine (Biochrom), 1% nonessential amino acids (Biochrom), 1% antibiotics and 10% fetal calf serum (FCS; Life Technologies). Cells were grown as monolayer in 75-cm^2 plastic flasks (Costar, Cambridge, MA) and fed every third day. Breast cancer cell lines were typed by extracting DNA, as previously described,[31] and performing polymerase chain reaction with sequence-specific-primers (PCR-SSP). The primers for HLA-A were kindly provided by the Department of Immunology at Heidelberg University (Heidelberg, Germany), and used as described.[32] Cell lines were also typed for HLA-A using the HLA-A "low resolution" SSP (Dynal, Oslo, Norway) providing the same results. Typing of HLA-B was performed by the Tissue Typing Laboratory of Oxford Transplant Centre, Churchill Hospital, Oxford, England with PCR using sequence-specific primers, as previously described.[33] HLA-DR typing was performed with commercially available kits (DR low resolution, Dynal and the Standard Inno-Lipa HLA-DR B test, Innogenetics, Zwijndrecht, Belgium).[34] The following typing results were obtained: MCF-7: HLA-A*02, B*44, B*18, DRB1*0301, DRB1*15; HBL-100: HLA-A*01, A*02, B*0801, B*4001, DRB1*0301, DRB1*15; 734-B: HLA-A*02, B*44, B*18, DRB1*0301, DRB1*15; T-47D: HLA-A*33, B*14, DRB1*01; MDA MB 231: HLA-A*02, B*41, B*4001, DRB1*1305, DRB1*0701; BT-20: HLA-A*24, B*38, B*62, DRB1*1301, DRB1*04; SKBR-3: HLA-A*02, A*03, B*14, B*4001, DRB1*1302, DRB1*0701; ZR 75.1: HLA-A*11, B*35, DRB1*01.

Establishment of EBV-transformed lymphoblastoid cell lines (LCL) and phytohemagglutinin (PHA) blasts. Pretransplant recipient and donor PBMC were resuspended in tissue culture medium supplemented with 10% FCS at a concentration of 2×10^6/mL and mixed with the same amount of a mycoplasma-free EBV producer line B95-8 supernatant and 200 ng/mL cyclosporine A (CsA, Sandimmun; Sandoz, Vienna, Austria). On day 6 and every third day thereafter, 50% of the culture medium was replaced with fresh medium. CsA was added to the culture until the third week.

PBMC (1×10^6/mL) from the host and the donor were incubated with 1% PHA (Difco, Detroit, MI) in a humidified 5% CO_2/95% air atmosphere at 37°C for 72 hours. For further growth, cells were cultured in tissue culture medium supplemented with 20 U/mL recombinant IL-2 (Hoffmann-La Roche, Basel, Switzerland).

Cell-mediated lympholysis (CML) assay. CML assays were performed as previously described.[35] In short, 2×10^6 target cells (both donor and recipient PHA blasts, LCL and breast cancer cell lines pretreated for 72 hours with the following cytokines: 0, 10, 100, 1,000 U/mL interferon [IFN]-γ, 1,000 U/mL IFN-α and 1,000 U/mL tumor necrosis factor [TNF]-α were labeled with 250 μCi $Na_2Cr^{51}O_4$ (specific activity 300 to 500 Ci/g chromate; NEN, Dreieich, Germany) for 2 hours at 37°C, washed four times, and resuspended at a concentration of 5×10^4 cells/mL. Of the target cells, 100 μL was incubated in U-shaped microtiter plates (Falcon; Becton Dickinson, Lincoln Park, NJ) with 100 μL of various amounts of effector cells with E:T ratios ranging from 50:1 to 6:1. After 4 hours incubation at 37°C in a humidified 5% CO_2/95% air atmosphere, 100 μL of the culture supernatant was counted with a gamma-scintillation counter (LKB, Stockholm, Sweden). Results are expressed as percentage of specific lysis according to the formula:

$$\frac{\text{Experimental Release} - \text{Spontaneous Release}}{\text{Maximum Release} - \text{Spontaneous Release}} \times 100$$

Experimental release represents cpm released from the target cells incubated with the effector cells; spontaneous release is that from target cells cultivated without effector cells; maximum release is cpm released from target cells after 4 hours incubation with 2% Triton X-100 (Sigma, Deisenhofen, Germany).

Blocking experiments. The following monoclonal antibodies (MoAbs) were used to block the CML assay: anti–HLA-AB (MAS 1532, clone W6/32; Seralab, Crawley Down, Sussex, UK) and anti–HLA-DR (clone L 243; Becton Dickinson) MoAb against common determinants on class I and class II MHC molecules, respectively; BB 7.2 directed against HLA-A2.[36] Target cells (5,000/well) resuspended in 50 μL tissue culture medium were incubated with 50 μL MoAb (10 μg/mL) for 30 minutes at room temperature. Effector cells were then added to the target cells as described above. Treatment with the mouse-irrelevant MoAb TIB 216 (clone MAR 18.5; American Type Culture Collection, Rockville, MD) was performed as isotype control.

Flow cytometric analysis. Expression of various surface antigens on target cells was measured on a FACStar flow cytometer (Becton Dickinson) by direct or indirect immunofluorescence and flow cytometry. Using previously published standard techniques[37] the same MoAb used for the blocking experiments as well as MoAb ICAM-1 (CD 54), LFA-1 (CD 11a), LFA-3 (CD 58) (Immunotech SA, Marseille, France) and CD2 (Becton Dickinson) against adhesion molecules were applied. Treatment with the irrelevant MoAb TIB 216 (clone MAR 18.5; ATCC) and fluorescein isothiocyanate-conjugated sheep antimouse antibodies (An der Grub, Bio Research, Kaumberg, Austria) served as an isotype control. Data were processed with Consort 20 software (Becton Dickinson).

Statistical evaluation. Results are expressed as mean ± standard error (SE). Student's t test was used to analyze statistical differences.

CASE REPORT AND RESULTS

A 32-year-old pregnant woman presented with inflammatory ductal breast cancer grade III, and was estrogen receptor negative and progesterone receptor negative in November 1993. After premature termination of her pregnancy in December 1993, neoadjuvant polychemotherapy with cyclophosphamide (650 mg/m^2 intravenously [IV]) and epidoxorubicin (100 mg/m^2 IV) was administered three times from December 1993 to January 1994. Mastectomy was performed on March 2nd, 1994 and a multicentric ductal breast cancer grade III with disease stage pT4, pN1(8/13), M1 (liver metastases; see Fig 1A), and lymphangiosis carcinomatosa was diagnosed (Stage IIIB). Despite two further chemotherapy cycles with cyclophosphamide and epidoxorubicin in April 1994, liver metastases progressed (Fig 1B) and bone metastases (spine and iliac crest) appeared in May. The patient was referred for autologous BMT. Since the results of high-dose therapy with stem cell rescue in this advanced stage are poor and an HLA-identical (HLA-A2, A23, B44, B62, DR3, DR7), mixed lymphocyte culture-negative sibling donor was available, the patient was given the option of undergoing autologous or allogeneic BMT. The patient opted for allogeneic BMT after weighing the high relapse rate of autologous BMT against the lack of experience with allogeneic BMT in patients with breast cancer.

Pretransplant conditioning consisted of thiothepa 125 mg/m^2/d from day −7 to −4, carboplatin 200 mg/m^2/d from day −7 to −4, and high-dose cyclophosphamide 3 g/m^2/d on days −3 and −2. Prophylactic immunosuppression with CsA was started on day −1 according to a previously published

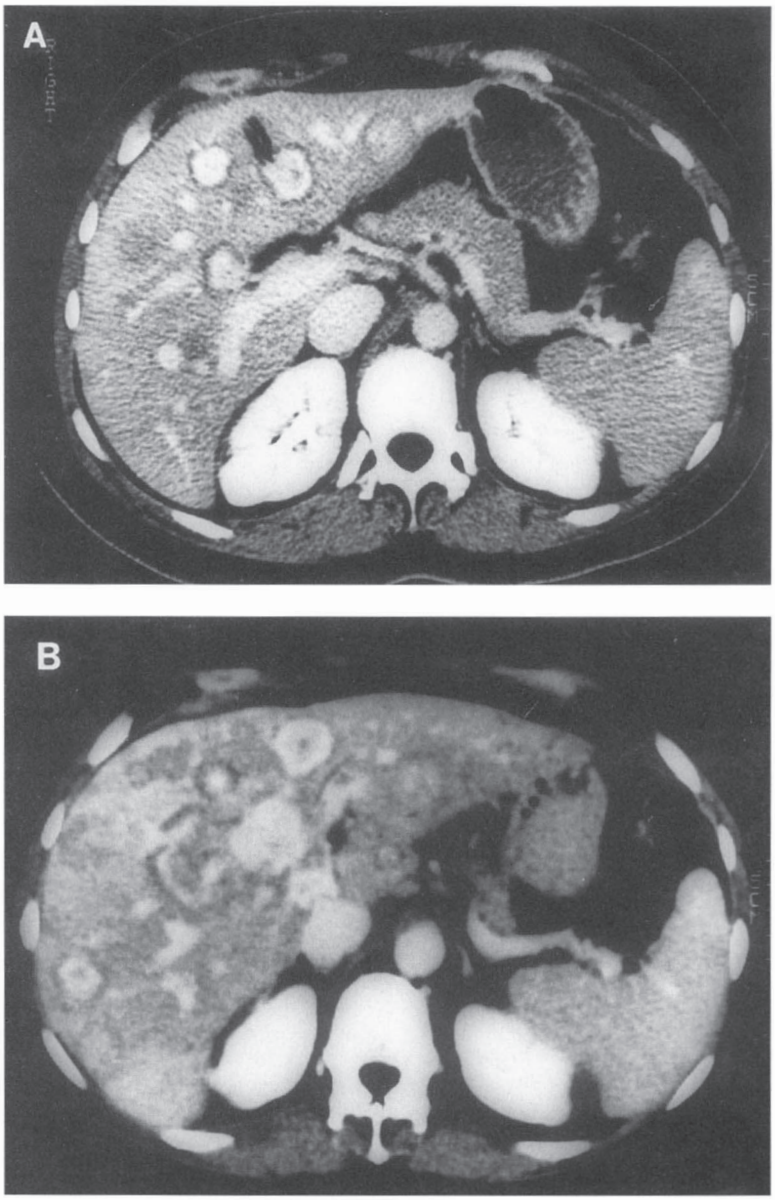

Fig 1. CT scan of the liver at mastectomy (A), before BMT (B), on day +27 (C), and on day +94 (D) after BMT.

regimen.[38] Successful engraftment, defined as the first of two consecutive days with more than 1,000 leukocytes/μL and self-sustaining hematopoiesis, was achieved on day +13 and the patient was discharged on day +18. Complete hematopoietic chimerism was documented 3 weeks after BMT by isoenzyme analysis in peripheral blood mononuclear cells.

The further posttransplant course was uneventful until day +27, when a biopsy-proven acute GvHD grade ++/+++ of the skin developed. Treatment with methylprednisolone was started for progressive GvHD at a dosage of 1 mg/kg body weight. On the same day, CT scan (Fig 1C) documented complete resolution of the liver metastases. Methylprednisolone was discontinued after GvHD had resolved. On day +58 the patient was readmitted to the hospital because of fever of unknown origin, which 4 days later was documented as being due to bacterial meningitis caused by Listeria monocytogenes. The infection resolved after appropriate antibiotic treatment. CsA was tapered and discontinued on day 80. Two weeks later, metastases in the liver (Fig 1D) were documented on CT scan and the patient was discharged. The patient died on day 110 after BMT due to progressive liver metastases. As confirmed by autopsy, no further metastases were detected and no bone metastases were found in the spine.

At the time of GvHD (day +27), cytotoxic T lymphocytes recognizing host pretransplant cells (T cells and LCL) but not HLA-identical donor cells and therefore defined as MiHA-specific were grown from the peripheral blood of the patient (Fig 2). Recognition was MHC class I antigen-restricted, as shown by blocking experiments with MoAb against common determinants on MHC class I and II antigens.

Since a breast cancer cell line of the patient could not be established, CTL were tested against a panel of eight breast

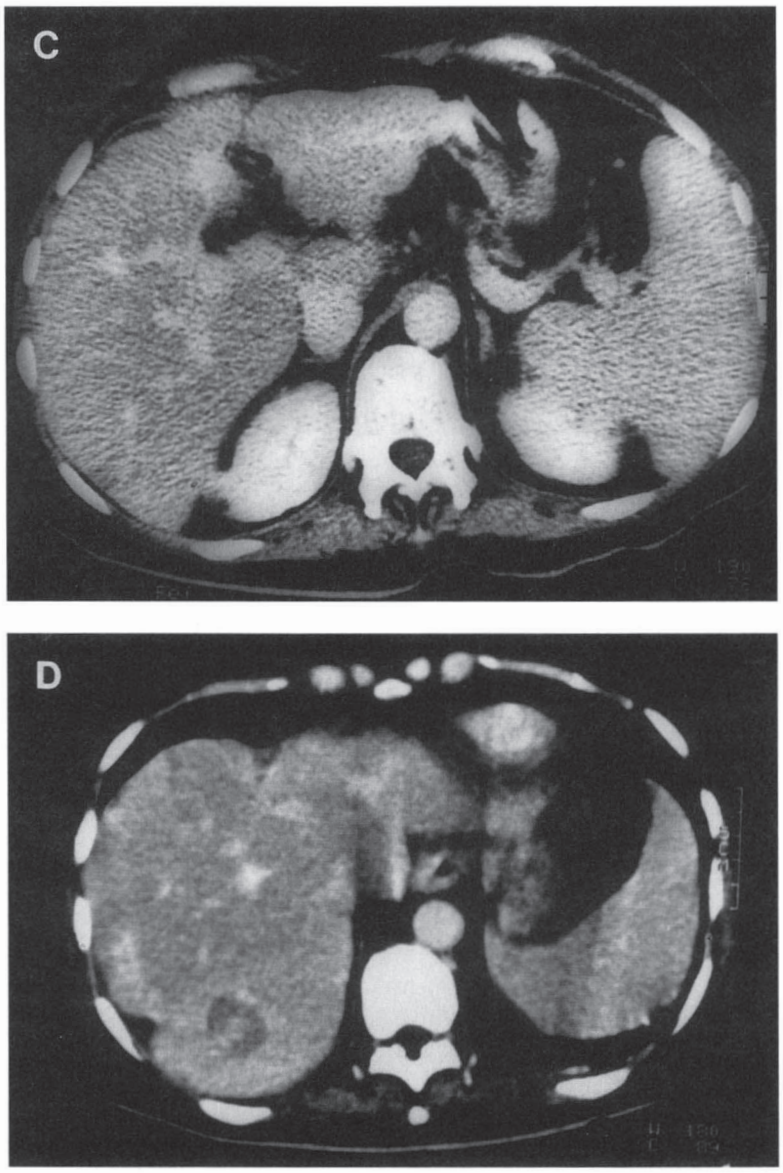

Fig 1 (cont'd).

cancer cell lines (MCF-7, T-47D, 734-B, HBL-100, BT-20, ZR 75.1, MDA MB, and SKBR-3). Four of these carcinoma cell lines (HBL-100, T-47D, MCF-7, 734-B) were recognized by the CTL and three of them shared the HLA-antigens HLA-A2, B44, and DR1 with the effector cells (Fig 3). HLA-A2 was the predominant restricting element since lysis was blocked by a MoAb against HLA-A2 (Fig 4). Despite expressing HLA-A2, MDA MB, and SKBR-3 cell lines were not recognized by the CTL.

In previous publications we indicated the key role of cytokines in regulating susceptibility of GvHD (keratinocytes) and hematopoietic cell targets[11,37,39] and showed that IFN-γ was able to increase lysis of keratinocytes by its capacity to increase MHC class I antigen expression.[11,37] In order to investigate the mechanisms involved in carcinoma cell lysis the four recognized carcinoma lines were used as targets in the CML assay after pretreatment with IFN-γ, IFN-α and TNF-α. Interestingly, only pretreatment with TNF-α (1,000 U/mL), but not with IFN-γ or IFN-α, increased lysis (Fig 5). In order to elucidate the mechanisms of these findings in more detail, flow cytometric analyses of the cytokine-pretreated and untreated breast cancer target cell lines were performed with MoAb against MHC antigens and adhesion molecules. As shown in Table 1, all three cytokines except TNF-α were able to increase MHC class I antigen expression. IFN-γ was the only cytokine that induced de novo MHC class II antigen expression. TNF-α was the only cytokine increasing LFA-3 and CD2 expression. IFN-γ and TNF-α induced ICAM-1 expression, but none of the cytokines induced LFA-1 expression.

DISCUSSION

During recent years, high-dose chemotherapy and autologous stem cell support have increasingly been used for the

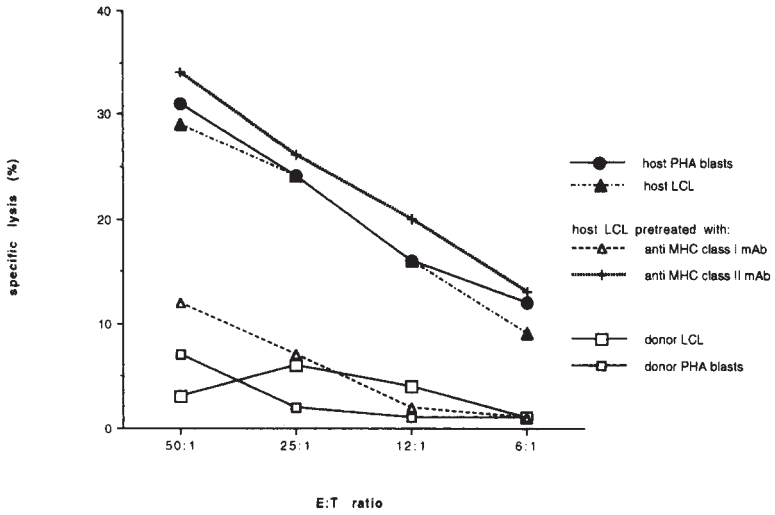

Fig 2. Lysis of hematopoietic target cells of the host and HLA-matched sibling as well as MHC class I and class II MoAb blocking experiments. One representative experiment is given.

treatment of patients with metastatic solid tumors or as consolidation in high-risk patients.[40-42] While treatment-related mortality is low, relapse of the disease remains the major obstacle.[40,43-45] Uncontrolled phase I and II studies have suggested that 15% to 20% of women with metastatic breast cancer will achieve durable complete remissions. The majority of patients with metastatic breast cancer, however, will relapse and the published data suggest that the median time to relapse or death is not greatly delayed by the addition of high-dose consolidation therapy.[46-49] Even total eradication of metastases by surgical means (resection or organ replacement) and treatment of the minimal residual disease by autologous BMT did not cure such patients.[50,51] In the search for other approaches and considering an antitumor effect of GvHD and of donor lymphocyte infusions after relapse following allogeneic BMT for hematologic diseases, adoptive immunotherapy resulting from allogeneic BMT may represent a potential tool for more effective therapy for metastatic breast cancer patients. In one patient refractory to chemotherapy and with no other curative option, we investigated the possibility of a GvT effect by analyzing her clinical course and the specificity and antitumor activity of circulating CTL during GvHD. In this patient a complete disappearance of liver metastases concomitant with severe GvHD of the skin was observed 27 days after BMT. Disappearance of liver and bone metastases could theoretically be due to high-dose chemotherapy alone. The following evidence, however, argues in favor of a possible GvT effect: first, progression of liver and appearance of bone metastases during chemotherapy before BMT; second, lack of such dramatic responses in patients with refractory metastatic breast cancer after autologous BMT; and, third, the presence of circulating MiHA-specific CTL, recognizing breast carcinoma target cells at the time of metastases regression.

MiHAs have been shown to play a major role in GvHD and GvL effect after allogeneic BMT and their tissue distribution has been shown to be heterogeneous with either ubiquitous or a more cell-type confined expression.[11,12,15] While ubiquitous MiHAs are expressed on cells of different origins (eg, hematopoietic and epithelial), the latter are expressed

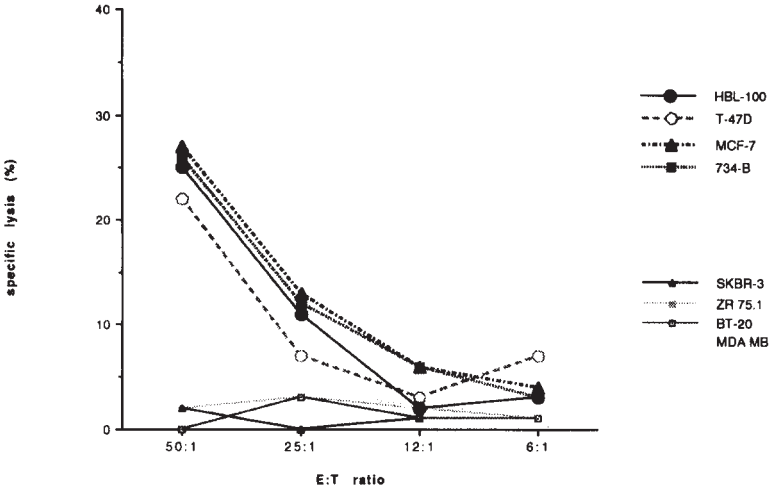

Fig 3. Specific lysis of breast cancer cell lines by CTL from the patient with GvHD. One representative experiment is given.

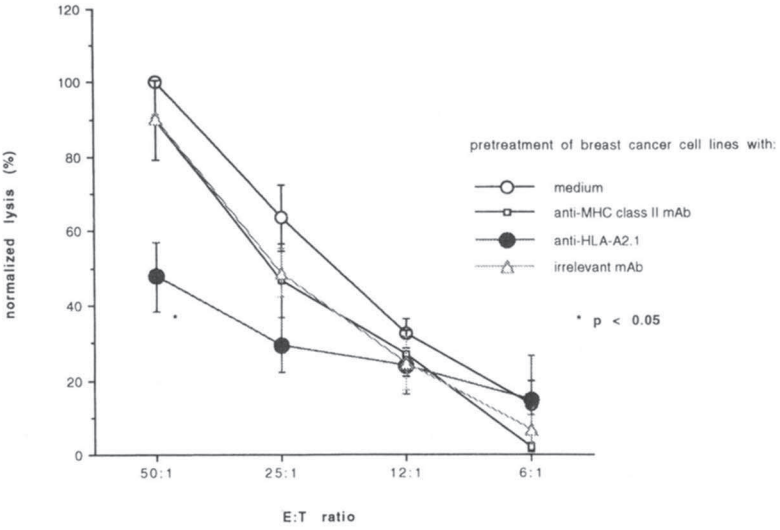

Fig 4. MoAb blocking of lysis of three breast carcinoma cell lines (HBL-100, MCF-7, 734-B) by TCL. Values (means ± SE) are normalized to lysis of untreated targets at an E:T ratio of 50:1. Specific lysis in percentage of carcinoma cells pretreated with medium was: 32% ± 10%, 22% ± 8%, 11% ± 4%, and 6% ± 3% for an E:T ratio of 50:1, 25:1, 12:1, and 6:1, respectively.

only on one cell type (eg, only on hematopoietic cells).[15] Lymphocytes recognizing both types of MiHAs have been isolated from patients with GvHD, providing an experimental background for the GvHD-dependent and GvHD-independent GvL effect.[7]

For the first time we are able to demonstrate that breast cancer cell lines express such MHC class I antigen-restricted, ubiquitous MiHA and that cytotoxic MiHA-specific T cells recognizing breast cancer cells were present in the patient after allogeneic BMT. In previous publications we and others have shown that recognition of MiHA-positive target cells by CTL on hematopoietic and epithelial cells is dependent on the density of the restricting MHC elements on their surface and controlled by IFN-γ and its capacity to increase MHC class I antigen expression.[11,37,40] Recognition of breast cancer cells was MHC class I-restricted, but cytokine-induced increase of their expression did not result in higher lysis by CTL, showing that other surface molecules might be primarily involved in controlling breast cancer cell lysis. LFA-3 density distribution might be one of these surface molecules, since its increased expression was exclusively seen after TNF-α pretreatment and paralleled enhanced susceptibility of these cells to CTL.

Since tumor-specific antigens (eg, MAGE-1) recognized by cytotoxic lymphocytes have also been found on breast tumors, we cannot exclude the possibility that a GvHD-independent GvT effect also operates in patients with breast cancer.[52] In vitro experiments investigating this aspect are in progress.

Despite evidence for a graft-versus-breast cancer effect, the patient relapsed after BMT. Reasons for this failure may include local factors such as site of metastases and encapsulation, where CTL might not penetrate, and also the large tumor burden present before BMT. Treatment of acute GvHD with steroids might have hampered the GvT effect as well.[53] Similar observations have also been made in pa-

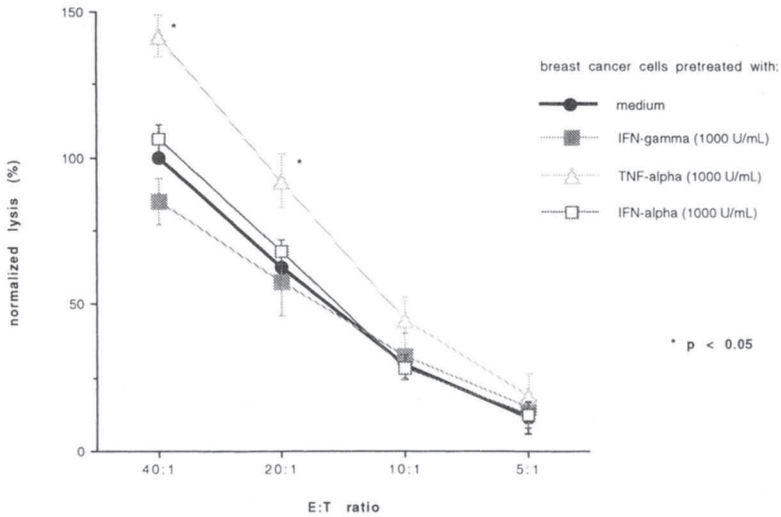

Fig 5. Lysis of untreated and cytokine- (IFN-γ, IFN-α, and TNF-α 1,000 U/mL) treated breast cancer cell lines (HBL-100, MCF-7, 734-B, T-47D). Values (means ± SE; n = 4) are normalized to lysis of targets without cytokine pretreatment at an E:T ratio of 40:1. Specific lysis in percentage for carcinoma cells pretreated with medium was: 30% ± 7%, 20% ± 6%, 9% ± 3%, and 4% ± 2% for an E:T ratio of 40:1, 20:1, 10:1, and 5:1, respectively.

Table 1. Flow Cytometric Analysis of Untreated or IFN-γ, IFN-α, or TNF-α–Pretreated Carcinoma Cell Lines (HBL-100, MCF-7, 734-B, T-47D)

Pretreatment	MHC Class I	P*	MHC Class II	P*	CD 2	P*	LFA-3	P*	ICAM-1	P*	LFA-1	P*
Untreated	247.6 ± 179.0		4.1 ± 0.7		2.7 ± 0.3		55.2 ± 13.4		29.2 ± 6.1		3.5 ± 0.3	
IFN-γ (1,000 U/mL)	1,154.4 ± 228.0	<.01	130.2 ± 55.6	<.05	2.7 ± 0.2	NS	55.4 ± 12.3	NS	62.7 ± 15.6	<.05	3.4 ± 0.3	NS
TNF-α (1,000 U/mL)	483.7 ± 310.8	NS	6.4 ± 2.5	NS	3.2 ± 0.5	<.05	90.0 ± 11.0	<.05	64.9 ± 9.1	<.05	4.1 ± 0.5	NS
IFN-α (1,000 U/mL)	709.0 ± 296.4	<.05	5.3 ± 1.6	NS	3.0 ± 0.3	NS	58.5 ± 12.4	NS	34.2 ± 7.7	NS	4.0 ± 0.2	NS

Results are expressed as mean channel ± SE; n = 4.
Abbreviation: NS, not significant.
*Student's *t*-test against untreated cells.

tients with advanced hematologic diseases, where, despite GvHD, relapse rates of more than 50% are seen in patients with large tumor burden before transplant.[54]

These data show that a slightly modified STAMP V conditioning regimen, recently used only in autologous BMT, permits allogeneic engraftment.[55] They further suggest that a GvT effect might exist after allogeneic BMT for breast cancer. As with leukemia, larger numbers of patients receiving allogeneic BMT for breast cancer would be required to determine whether a GvT effect produces a clinically relevant response.

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