Purging of Epithelial Tumor Cells from Peripheral Blood Stem Cells by Means of the Bispecific Antibody BIS-1[1]

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ABSTRACT

Peripheral blood stem cell (PBSC) support in breast cancer patients allows high-dose chemotherapy, but tumor cell contamination of the PBSCs is a potential source of relapse. Specific carcinoma cell killing can be obtained by retargeting activated T cells with bispecific antibody BIS-1, directed against epithelial glycoprotein-2 and CD3. To purge epithelial tumor cells from the PBSCs of breast cancer patients, activation of T cells in PBSCs and T-cell retargeting by BIS-1 was studied. PBSCs, obtained by leukapheresis after chemotherapy and recombinant human granulocyte colony-stimulating factor, were cultured in the presence of PBS, interleukin-2, OKT3, or interleukin-2/OKT3 for induction of T-cell activation. Subsequently, lysis of epithelial tumor cell lines by activated T cells of PBSCs in the presence or absence of BIS-1 was assessed with the ^{51}Cr-release assay or immunocytochemical staining. The effect on PBSC hematopoietic colony formation (HCF) was evaluated by the granulocyte macrophage colony-stimulating units assay. Prior to activation, PBSCs from breast cancer patients contained higher levels of CD8+ T cells than peripheral blood from healthy volunteers ($P < 0.05$). The potential of PBSCs to sustain tumor cell lysis was increased after all prior activations and was further enhanced by BIS-1. Maximal BIS-1 effect was observed after OKT3 activation of PBSCs for 72 h ($P < 0.0005$), inducing a >3 log depletion of tumor cells. HCF was not affected by prior OKT3 activation and/or BIS-1. In conclusion, specific tumor cell lysis by PBSCs can be obtained *in vitro* by OKT3 activation and BIS-1 retargeting of T cells, without affecting HCF. At present, studies are evaluating this format for future clinical application.

INTRODUCTION

PBSC[3] support in breast cancer patients allows high-dose chemotherapy, but tumor cell contamination is a potential source of relapse as was demonstrated in marker-gene studies in hematological and solid tumor types (1, 2). A number f methods to clear tumor cells from PBSCs (purging) have been described, including depletion of tumor cells and selection of stem cells from the graft (3, 4). Tumor cell depletion by means of treatment with nonselective chemotherapeutic drugs was shown to eliminate tumor cells, but hematopoietic colony formation was negatively affected as well (5). Stem cells selected through enrichment of CD34-positive cells still contained a number of tumor cells (6).

To obtain a more specific way to eliminate tumor cells from PBSCs, treatment with monoclonal antibodies has been studied. The use of antibodies was found to be effective and feasible in purging tumor cells from PBSCs in hematological cancer patient studies (7–12), although the binding of a monoclonal antibody alone does not induce tumor cell lysis. In the systemic treatment of solid tumors, antibody-based treatment has been shown to be beneficial in a setting of minimal residual disease (13). Compared with previous disappointing antitumor effects of immunotherapy in patients with high tumor loads (14–16), adjuvant administration of monoclonal antibodies was found to induce a survival benefit in colorectal carcinoma patients (17). Immunotherapy also gained new interest because a clinically beneficial effect was seen in disseminated breast cancer patients treated with the humanized anti-HER2 antibody Herceptin (18). However, only a minority of patients are eligible for this type of treatment because HER2/neu expression in breast cancer is ~25–30%. Elimination of breast cancer cells from bone marrow after antigen-binding by means of immunobeads and immunotoxins was shown to be effective *in vitro* (19, 20).

To increase cytotoxicity, the use of cytokines also has been studied. IL-2 incubation of PBSCs induced tumor cell killing *in vitro* up to 50% (21), and it did not negatively affect stem cell engraftment in breast cancer patients (22). An additional effect of anti-CD3 antibody OKT3, in conjunction with IL-2, on tumor cell killing was seen in the bone marrow of hematological patients (23). Also in the hematological setting, Kaneko *et al.* (24) described activation of peripheral blood mononuclear cells with IL-2 and OKT3, combined with bispecific antibodies, for *ex vivo* purging of leukemic cells from bone marrow. Adding bispecific antibodies clearly increased cytolysis in this study. A bispecific antibody combines affinity to both target and cyto-

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[3] The abbreviations used are: PBSC, peripheral blood stem cell; IL, interleukin; EGP-2, epithelial glycoprotein-2; BIS-1, bispecific antibody-1; NCS, newborn calf serum; HPS, human pooled serum; CFU-GM, granulocyte macrophage colony-forming unit.

toxic effector cells, thus allowing more efficient cell lysis than with a monoclonal antibody alone (25).

In view of the above, it seems reasonable to further evaluate the combination of activation of T cells present in breast cancer patient PBSC harvests and a bispecific antibody for purging of carcinoma cells from PBSCs, which to our knowledge has not been described previously. In our study, we used the bispecific monoclonal antibody BIS-1, which is directed against the pancarcinoma-associated membrane antigen EGP-2 and the CD3 complex present on T cells. EGP-2, also called epithelial cellular adhesion molecule, is a 40-kDa membrane-bound glycoprotein, strongly expressed by most carcinomas and universally expressed in breast cancer specimens (reviewed in Ref. 26). As such, EGP-2 is a commonly used target antigen in many carcinoma-directed immunotherapeutic approaches (17, 25, 26). The bispecific antibody BIS-1 creates functional cross-linking of the activated T cells and EGP-2-positive tumor cells, allowing the delivery of a tumor cell-specific lethal hit, and this T cell retargeting with BIS-1 induces specific epithelial tumor cell killing *in vitro* and *in vivo* (14, 27). The goal of this study was to examine *in vitro* activation of T cells present in PBSC harvests obtained from breast cancer patients for generation of cytotoxic effector cells and to study purging of epithelial tumor cells from PBSCs by BIS-1 retargeting of activated PBSCs.

MATERIALS AND METHODS

PBSCs (Effector Cells). Patients participated in a national randomized adjuvant breast carcinoma study (28), which was approved by the Medical Ethical Committee of the University Hospital Groningen. All patients gave informed consent. As part of this study, PBSCs were mobilized after combination chemotherapy (5-fluorouracil-epirubicin-cyclophosphamide) and recombinant human granulocyte colony-stimulating growth factor, and collected by means of a Cobe Spectra leukapheresis apparatus (Cobe Netherlands, Uden, the Netherlands). Briefly, from day 2 of the third course of 5-fluorouracil-epirubicin-cyclophosphamide, 263 μg of recombinant human granulocyte colony-stimulating growth factor were administered s.c. daily. On day 9, leukapheresis was started by means of continuous flow cell separation. The PBSC harvest consisted of a (nearly granulocyte free) mononucleated cell product. Usually two to three leukapheresis procedures were required until at least 6×10^6 CD34+ cells/kg body weight were collected. PBSC samples for this study were cryopreserved in 10% DMSO in a maximal final cell concentration of 200×10^6 cells/ml and stored in liquid nitrogen. Prior to experiments, PBSCs were thawed rapidly; washed in NCS (Gibco Europe, Breda, the Netherlands); incubated for 15 min in 6 ml of NCS to which 2000 units of DNase I (Boehringer Mannheim), 0.2 mM magnesium sulfate, and 1000 units of heparin were added; and centrifuged 5 min at $591 \times g$. Erylysis was performed on all samples (including whole blood control samples) with an ammonium chloride solution (155 mM NH_4Cl, 10 mM potassium hydrogen carbonate, 0.1 mM sodium EDTA). Cells were washed in RPMI (Boehringer Ingelheim) and resuspended in RPMI containing 5% heat-inactivated HPS, 60 μg/ml gentamicin (Biowhittaker, Verviers, Belgium) and 2 mM glutamine, to a final concentration of 1×10^6 nucleated cells/ml.

Activation. PBSCs were incubated for 0, 24, and 72 h in the above-described culture medium containing one of the following additives: (*a*) PBS solution [0.14 M NaCl, 2.7 mM KCl, 6.4 mM $Na_2HPO_4 \cdot 2\ H_2O$, 1.5 mM KH_2PO_4, (pH 7.4)]; (*b*) 100 units/ml IL-2 (aldesleukin; Chiron, Amsterdam, the Netherlands); (*c*) 5% (v/v) anti-CD3, (tissue culture supernatant containing 10 μg/ml OKT3); or (*d*) 100 units/ml IL-2 and 5% (v/v) OKT3. Prior to further use, cells were washed in the culture medium without activating additives.

Flow Cytometry. After PBSC activation as described above, phenotyping of T cells was assessed using: FITC- or phycoerythrin-labeled anti-CD4, anti-CD8, anti-CD25, anti-CD69, and anti-HLA DR monoclonal antibodies (Immuno Quality Products, Groningen, the Netherlands). PBSCs were incubated for 30 min at 4°C (5 μl of antibody for 1×10^6 cells in 100 μl of PBS containing 1% HPS), washed once in PBS, and resuspended in 150 μl of PBS. Samples were analyzed on a Coulter Elite Cytometer (Coulter Electronics, Hialeah, FL) using an argon laser (488 nm) for FITC and phycoerythrin excitation.

Target Cell Lines. GLC1 (EGP-2-negative parental cell line) and GLC1M13 (EGP-2-positive subclone) are small-cell lung cancer-derived cell lines (29). These cell lines were cultured according to routine procedures in RPMI 1640-based medium supplemented with 14% heat-inactivated FCS, 2 mM glutamine, 60 μg/ml gentamicin, 0.05 mM β-mercaptoethanol, and 1 mM sodium pyruvate at 37°C in a humidified atmosphere containing 5% CO_2. The EGP-2-positive (GLC1M13) and EGP-2 negative (GLC1) cell models of similar origin were used in the ^{51}Cr-release assay. For morphological reasons, the EGP-2-positive breast cancer-derived cell line MCF-7 was used in the log-depletion assay. MCF-7 was cultured according to routine procedures in RPMI-based medium supplemented with 10% FCS at 37°C in a humidified atmosphere containing 5% CO_2.

BIS-1. The BIS-1-producing quadroma was made in our department by fusion of the hybridomas RIV-9 and MOC-31, producing anti-CD3 (IgG3) and anti-EGP-2 (IgG1) antibodies, respectively, according to De Lau *et al.* (30). Preparation and purification were performed as described previously (14). Briefly, BIS-1 was produced on large scale by means of a hollow fiber culture system (Endotronics, Minneapolis, MN). Purification of the hybrid antibodies (IgG3/IgG1) from parental-type antibodies, also produced by the quadroma, was performed by protein A column chromatography. BIS-1 $F(ab')_2$ was then produced by means of digestion by pepsin followed by G-150 Sephadex gel filtration, and was added to a 0.9% sodium chloride solution to obtain a final concentration of 0.2 mg/ml.

^{51}Cr-Release Assay. ^{51}Cr-release assays were performed according to standard procedures to assess BIS-1-redirected T-cell cytotoxicity (14). All determinations were executed in triplicate. Target cells (5×10^6; tumor cells GLC1 or GLC1M13) were suspended in 100 μl of culture medium containing 3.7 MBq of [^{51}Cr]sodium chromate (Amersham Pharmacia Biotech Benelux, Roosendaal, the Netherlands) and incubated for 1 h at 37°C in a humidified 5% CO_2 atmosphere. Unbound [^{51}Cr]sodium chromate was removed by washing the tumor cells three times with medium. Subsequently, aliquots of 100 μl of medium containing 0, 2.5×10^3, 2.5×10^4, or 2.5×10^5 PBSCs (effector cells) after the above-mentioned 24 or 72 h

with PBS, IL-2, OKT3, or IL-2/OKT3 were added into a 96-well round-bottomed microtiter plate (Greiner no. 650180; Greiner, Alphen aan de Rijn, the Netherlands). To each well, 50 μl of medium containing 2.5×10^3 ^{51}Cr-labeled target tumor cells were also added, resulting in E:T ratios of 0, 1, 10, and 100 in a final volume of 200 μl/well. Finally, 50 μl of medium containing 0.4 μg/ml BIS-1 $F(ab')_2$ (final concentration during the assay, 0.1 μg/ml) or 50 μl of medium without BIS-1 were pipetted. The microtiter plates were centrifuged at $46 \times g$ for 2 min and incubated at 37°C in 5% CO_2. After a 4-h incubation, the plates were centrifuged at $182 \times g$ for 5 min, and 100-μl samples taken from the supernatant were counted in a gamma counter (Wizard; EG&G/Wallac). Cell lysis was calculated from the percentage of ^{51}Cr released, according to the formula: [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100%. Maximal release was determined from a sample to which 100 μl of 2% Triton X-100 solution were added instead of effector cells. Spontaneous release was determined from a sample to which 100 μl of medium were added instead of effector cells.

Hematopoietic Colony Formation. Toxicity of prior T-cell activation and subsequent BIS-1 treatment on hematopoietic stem cell recovery was studied with the CFU-GM assay (31). Briefly, hematopoietic colony formation was assessed in 1 ml of DMEM including 1.1% methyl cellulose, 20% FCS, 1% deionized BSA, 0.001% α-thioglycerol, and 10 ng/ml IL-3 and granulocyte macrophage-colony stimulating factor. PBSCs (2×10^5 cells, after the prior activations as mentioned above) were plated after a 4-h incubation with or without BIS-1 (0.1 μg in 200 μl DMEM), with or without GLC1M13 at an E:T ratio of 100:1. Cells were plated in 35-mm dishes and cultured for 14 days at 37°C. Hematopoietic colonies containing ≥40 cells were counted under an inverse microscope.

Log-Depletion Assay. To assess the log-depletion of tumor cells by activated PBSC, MCF-7 tumor cells were added to PBSCs after 72 h prior activation with OKT3 under the conditions mentioned above. MCF-7 tumor (target) cells were added to (effector) PBSCs in an E:T ratio of 1×10^4:1 in a total volume of 6 ml of RPMI 1640 (supplemented with HPS, gentamicin, and glutamine, as mentioned above), in the presence or absence of BIS1 (final concentration during the assay, 0.1 μg/ml). As a control, MCF-7 tumor cells were also added to 6 ml of RPMI 1640 without PBSCs. After a 4-h incubation at 37°C in 5% CO, sedimentation of cells onto slides was performed. Cells were stained with monoclonal antibody MOC31, directed against EGP-2, using indirect immunoperoxidase staining with horseradish peroxidase-conjugated rabbit antimouse as a second antibody and 3-amino-g-ethylcarbazol as a substrate. Slides were routinely counterstained with H&E.

Statistics. Cytotoxic cell lysis, hematopoietic colony formation, and leukocyte phenotype were analyzed by means of Student's *t* test. $P < 0.05$ was considered significant.

RESULTS

Cytotoxic Activity in PBSCs

^{51}Cr-Release Assay. To purge epithelial tumor cells from PBSCs, we studied prior activation of T cells present in PBSC harvests from breast cancer patients, combined with BIS-1 in the ^{51}Cr-release assay. Therefore, *in vitro* tumor cells were added to PBSC harvests after prior T-cell activation, in the presence or absence of BIS-1. The effect of BIS-1 and prior activation of PBSC on GLC1M13 (EGP-2 positive) tumor cell lysis is shown in Fig. 1. Tumor cell lysis was increased by the addition of BIS-1, after all prior activations, compared with cell lysis without BIS-1. The maximal effect of BIS-1 was seen after 72 h of prior PBSC activation with OKT3 ($P < 0.0005$ compared with lysis without BIS-1). Tumor cell lysis in the presence of BIS-1 was not significantly different after prior PBSC activation with IL-2/OKT3 compared with OKT3 alone. The addition of BIS-1 did not increase lysis of control GLC1 (the EGP-2-negative counterpart of GLC1M13 and therefore incapable of binding BIS-1) compared with tumor cell lysis without BIS-1 (not shown).

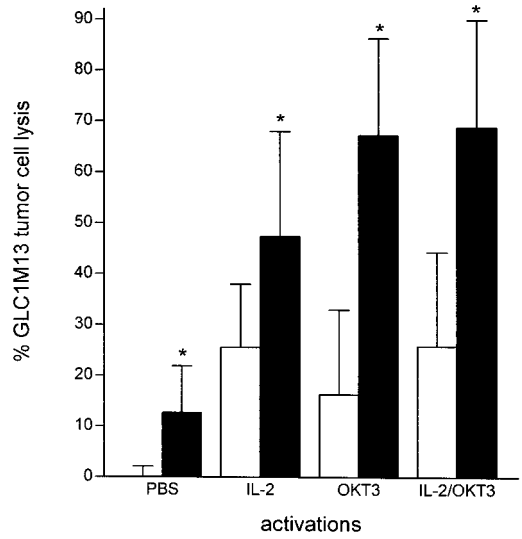

Fig. 1 GLC1M13 cell lysis by PBSCs with/without BIS-1. *Y axis*, percentage of GLC1M13 tumor cell lysis; *X axis*, activating agents. *Open columns* reflect activation without BIS-1; *filled columns* represent activation with subsequent BIS-1. Shown is the percentage of specific tumor cell lysis, determined in the ^{51}Cr-release assay (mean; *bars*, SD; $n = 6$) with E:T ratio of 100:1 after 72-h prior activation. * indicates significant difference compared with counterpart without BIS-1.

Prior PBSC activation with IL-2, IL-2/OKT3, or OKT3 alone, but without subsequent BIS-1, increased GLC1M13 as well as GLC1 tumor cell lysis in the absence of BIS-1 when compared with the PBS control (maximum GLC1M13 lysis, $P < 0.0005$ compared with PBS after 72-h activation of PBSCs with IL-2).

Tumor cell lysis of GLC1M13 in the presence of BIS-1 was increased nearly 100% after 72 h of PBSC activation compared with 24 h of activation with OKT3 and IL-2/OKT3 ($P < 0.005$ and $P < 0.025$, respectively).

In Fig. 2, the effect of increasing E:T ratios is shown. After all PBSC activations, increasing E:T ratio coincided with increased BIS-1 redirected cytotoxicity (maximal GLC1M13 lysis after OKT3 activation, $P < 0.0005$).

Log-Depletion Assay. At an E:T ratio of 1×10^4 OKT3-activated PBSCs to 1 MCF-7 tumor cell in the presence of BIS-1, a >3 log depletion of MCF-7 tumor cells (mean, 0.09%

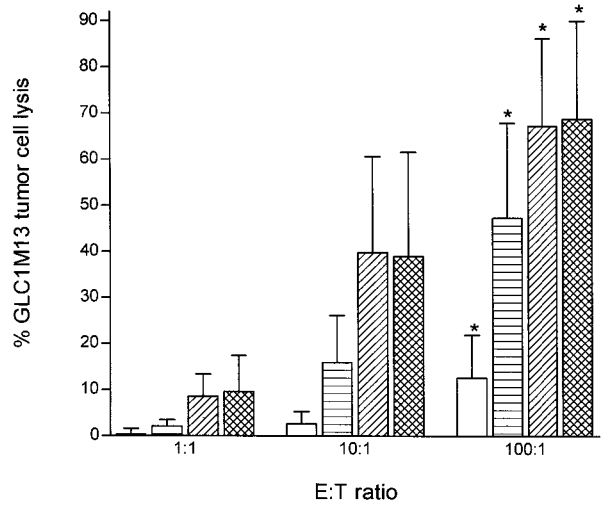

Fig. 2 Effect of E:T ratio. *Y axis*, percentage GLC1M13 tumor cell lysis; *X axis*, E:T ratios (1:1, 10:1, 100:1, representing PBSC:tumor cell ratio). Shown is the percentage of GLC1M13 cell lysis (mean; *bars*, SD; $n = 6$) by PBSC + BIS-1 after 72-h activation. □, PBS; ▤, IL-2; ▨, OKT3; ▩, IL-2/OKT3). ∗ indicates significant difference compared with E:T ratios of 1:1 and 10:1.

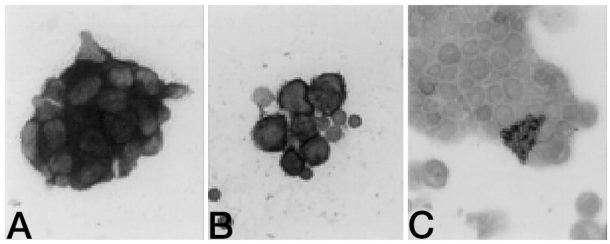

Fig. 3 Effect of activated PBSCs with BIS-1 on MCF-7 cells; 20 × 10 enlargement. *A*, MCF-7 tumor cells without PBSCs; *B*, MCF-7 tumor cells with OKT3-activated PBSCs, without BIS-1 (viable appearance); *C*; MCF-7 tumor cells with OKT3-activated PBSCs, with BIS-1 (nonviable appearance).

of total number of MCF-7 cells remaining) was observed, as compared with a control to which no effector PBSCs were added (see also Fig. 3). In the absence of BIS-1, only >1 log depletion of MCF-7 tumor cells (mean, 6% MCF-7 cells remaining) was observed with OKT3-activated PBSCs. The sensitivity of tumor cell detection in these experiments was 1 MCF-7 tumor cell in the total number (*e.g.*, 6×10^7) of PBSCs screened, in line with results obtained by Ross *et al.* (32) and Brügger *et al.* (33).

Composition of PBSCs and Activation Markers on T Lymphocytes

During the 3 consecutive days of the leukapheresis procedure (days 9, 10, and 11 after chemotherapy), in the PBSC harvest the percentage of CD34+ cells increased ($P < 0.05$) and lymphocyte levels decreased ($P < 0.05$), but within the lymphocyte compartment the percentages of CD4+ and CD8+ T cells remained the same. The lymphocyte percentage of CD8+ T cells in PBSC harvests before activation (mean, 28% CD8+ T cells; SD, 10%; $n = 4$) was higher compared with the peripheral blood of healthy volunteers (15%; SD, 1.6%; $n = 4$; $P < 0.05$). The lymphocyte percentage of CD4+ T cells was not different in PBSC harvests compared with peripheral blood. The percentage of CD4+ or CD8+ T cells bearing activation markers CD69 and CD25 increased during the 3 consecutive days (days 9, 10, and 11) of the leukapheresis procedure (Fig. 4).

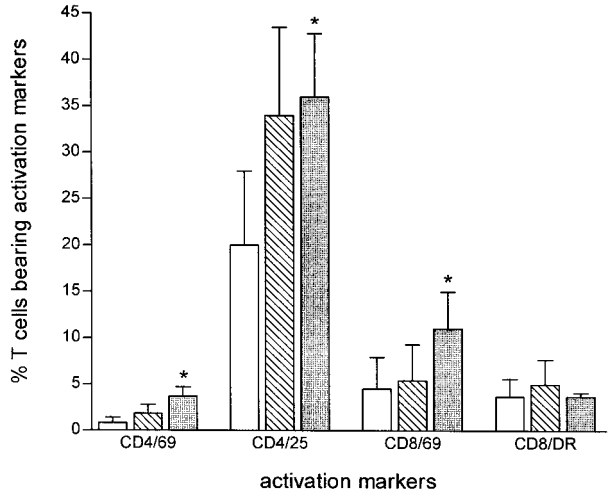

Fig. 4 Activation markers on CD4+ and 8+ T cells in PBSCs, during the 3 consecutive days of the leukapheresis procedure. *Y axis*, percentage of T cells bearing activation markers; *X axis*, activation markers on CD4+ and CD8+ T cells. Shown is the percentage of T cells in PBSCs with markers (mean; *bars*, SD; $n = 3$) on consecutive leukapheresis days: *open columns*, day 9 of the course; *hatched columns*, day 10 of the course; *gray columns*, day 11 of the course. ∗ indicates significant difference compared with first value on day 9.

Further *in vitro* activation of PBSCs induced a marked increase in the expression of activation markers on CD8+ T cells. After 24 h of prior *in vitro* activation with OKT3 and IL-2/OKT3, the percentage of CD8+ T cells also positive for early activation marker CD69 was increased (mean, 75 and 82%, respectively; $P < 0.0005$ for both compared with the PBS control), whereas after 72 h, the percentage of CD8+ T cells also expressing the late activation marker HLA DR was shown to be augmented (mean, 53 and 73%, respectively; $P < 0.0005$ for both). In the PBS control, no differences in activation markers was found after 0, 24, or 72 h. Although the percentage of CD8+ T cells in PBSCs tended to rise during *in vitro* activation of PBSCs with OKT3 and IL-2/OKT3, no significant difference was observed after 24 or 72 h of activation compared with 0 h. No difference in the total number of PBSCs was found after 0, 24, or 72 h in the PBS control. No effect on the total number of PBSCs was found after 24 and 72 h of prior activation compared with the PBS control. In addition, no effect on lymphocyte and T-cell subsets was observed after prior activation, as reflected in Table 1.

Hematopoietic Colony Formation

The effect of prior PBSC activation on the ability of the hematopoietic stem cells to form hematopoietic colonies, meas-

Table 1 Effect of processing on total PBSC numbers, lymphocytes, and T-cell subset fractions

Numbers prior to processing ($t = 0$) and after 72 h of prior PBSC activation with PBS (control), OKT3, or IL-2/OKT3 are shown ($n = 4$). Results given as mean (SD). Results not significantly different from $t = 0$, except where indicated.

	$t = 0$	PBS ($t = 72$ h)	OKT3 ($t = 72$ h)	IL-2/OKT3 ($t = 72$ h)
Total PBSC cell number per flask (10^6)	10	10.8 (3)	16.6 (3)	15.5 (2.2)
Lymphocytes (% of total number PBSCs)	47 (19)	53 (10)	60 (12)	58 (16)
CD8 T cells (% of lymphocytes)	28 (10)	13 (8)[a]	24 (12)	28 (13)
CD4 T cells (% of lymphocytes)	50 (13)	40 (13)	37 (13)	37 (13)

[a] Significantly different compared with $t = 0$: $P < 0.05$.

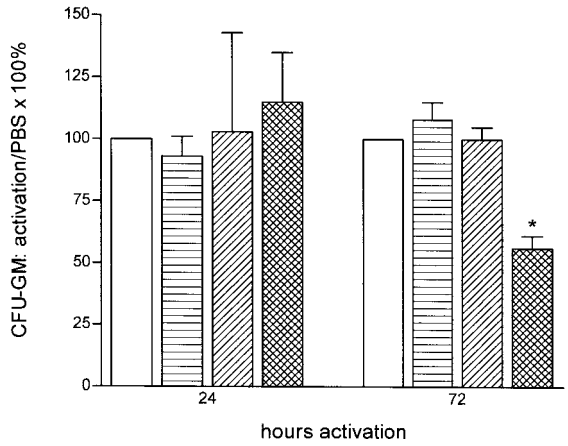

Fig. 5 Hematopoietic colony formation after activation of PBSCs. *Y axis*, percentage of hematopoietic colonies (*CFU-GM*) relative to PBS control (set at 100%); *X axis*, 24- and 72-h activation. Shown is percentage of hematopoietic colonies (mean; *bars*, SD; $n = 3$) after 24 or 72 h of PBSC activation with PBS (□), IL-2 (▤), OKT3 (▨) or IL-2/OKT3 (▩). * indicates a significant difference compared with PBS control.

ured as CFU-GM numbers, is shown in Fig. 5. No effect of 24 h of prior activation was seen when compared with the PBS control. The PBS control was not different after 24 or 72 h (mean, 70 *versus* 67 CFU-GM; $n = 3$; not significant). Also after 72 h, no effect of prior PBSC activation with IL-2 or OKT3 was seen. However, after 72 h of IL-2/OKT3 activation, CFU-GM numbers were decreased (mean, 39; $n = 3$; $P < 0.0005$). No negative effect of BIS-1 alone, or BIS-1 and GLC1M13 tumor cells, on CFU-GM numbers was observed after any of the prior PBSC activations (data not shown).

DISCUSSION

In this study, we examined the possibility to use activation and retargeting of PBSCs *in vitro* for purging of epithelial tumor cells from the PBSC isolate. As shown here, PBSC harvests from breast cancer patients appear to be intrinsically suitable for sustaining immunological purging procedures because they contain high levels of potential cytotoxic effector cells. This was also observed by Verma *et al.* (21). In our study, the capability of PBSCs to lyse epithelial tumor cell was increased after *in vitro* activation with IL-2, OKT3, or IL-2/OKT3, and this was further augmented by the addition of the bispecific antibody BIS-1 (see Fig. 1). Activation of PBSCs was a prerequisite for effective BIS-1-mediated cell lysis, which is compatible with studies showing that T cells need prior activation to gain cytolytic potential [see Kroesen *et al.* (25 for review]. When we activated PBSCs with OKT3 and then subsequently with BIS-1, within the 4 h of the assay tumor cell depletion of >3 logs was observed.

It can be argued that an even higher purging efficiency can be expected with this format in the clinical setting for a number of reasons. PBSC harvests, without further purification except for erythrocyte lysis, were used for T-cell activation and tumor cell killing. Selection by means of a density gradient was considered to be less desirable in view of the actual clinical situation. Thus, the "effector cells" consisted of only a minority of CD8+ T cells. E:T ratios in the clinical setting (*i.e.*, the ratio of potential cytotoxic effector cells to tumor cells in tumor-contaminated PBSCs) are likely to be $>1 \times 10^2$:1 (as used in the ^{51}Cr-release assay in this study) or 1×10^4:1 (log-depletion assay). This is generally the case because highly sensitive methods, including immunocytochemistry (32, 33) and reverse transcriptase-PCR (34), are required to detect single tumor cells in 1×10^6–1×10^7 PBSCs. Tumor cell lysis clearly increased with increasing E:T ratios, and therefore a high purging efficiency may be expected in the clinical setting.

The CFU-GM assay, which has predictive value for hematological recovery after stem cell transplantation (35), was used as a functional evaluation of hematopoietic colony formation after the purging procedure in this study. CFU-GM numbers were not affected by PBSC treatment with OKT3, BIS-1, or even by tumor cell killing during the course of the cytotoxicity assay. In a number of studies, the use of antibodies for purging purposes was also found not to affect hematopoietic colony formation *in vitro* (20) or engraftment in patients (7–9, 11). PBSC treatment with OKT3 was found to suppress hematopoietic colony formation in hematological malignancies (36), whereas normal control bone marrow was not affected (37). Furthermore, no adverse effect on hemopoiesis was seen *in vivo* when patients were treated i.v. with low-dose OKT3, as is used for induction of antitumor immunomodulation (38). The fact that we did not see a negative effect of prior activation with OKT3 alone on PBSCs of breast cancer patients is consistent with these findings. *In vitro* IL-2 incubation of breast cancer patient-derived PBSCs did not negatively affect hematopoietic colony formation in three studies (21, 22, 39). Our data confirm and extend these findings. Despite this, the combination of OKT3 and IL-2 stimulation appeared to have a clear negative effect on hematopoietic colony formation in our study (Fig. 5).

In hematological malignancies, it was suggested that activated T cells could suppress hematopoietic colony formation (36). This might possibly explain our findings because the degree of T-cell activation after prior treatment with IL-2/OKT3 was indeed higher compared with the other treatments (*e.g.,* 73% of CD8+ T cells were also positive for HLA DR after 72 h of IL-2/OKT3 activation compared with 53% after OKT3 activation) in this study.

In search of purging methods efficient in both eliminating tumor cells and maintaining sufficient hemopoiesis, nonselective purging methods using chemotherapy failed to prove useful because of the negative effect on hematopoietic colony formation (5). As an alternative procedure, *in vitro* stem cell selection through enrichment of CD34-positive cells has been used, but tumor cells may not be completely eliminated this way (6). Antibody-based purging methods, *e.g.,* with immunotoxins, proved efficient in eliminating tumor cells (3–4 log depletion, compatible with our results) but were shown to have varying effects on hematopoietic stem cells (19, 20). To find a universally expressed epitope in solid tumors is considered difficult, at least when compared with the situation in hematological malignancies (3). However, antibody-based therapy using epitopes that are not universally expressed (15, 16, 18, 20) is obviously of little clinical significance. The method presented here may offer a good possibility for antibody-based tumor elimination from hematopoietic stem cell harvests because the EGP-2 transmembrane marker is not shed into the circulation, is frequently present and overexpressed in carcinoma cells, and is absent from bone marrow cells (26). The use of the patient material (PBSCs) itself to eliminate tumor cells is an additional asset of this method. Furthermore, highly sensitive methods for detection of tumor cells in peripheral blood and PBSCs, *i.e.,* immunocytochemistry and a quantitative reverse transcriptase-PCR based on EGP-2 expression, have been developed in our institute (34). This may allow us to evaluate our purging efficiency in clinically relevant patient samples, which may otherwise be potentially difficult.

It has been stated that an immunocompetent graft may provide antitumor activity, which may also affect possible residual disease in the patient (3). Long-term follow-up analyses after CD34+ stem cell selection of PBSC grafts (which do not include immunocompetent natural killer cells or T cells) may shed more light on the impact of the immunocompetence of the graft. At this point, data on small numbers of patients are available after a short follow-up, not yet allowing conclusions on disease-free or overall survival (40, 41). If indeed the immunocompetence of the graft should play a role, the purging method with BIS-1 described here is likely of interest because immunocompetent cells remain in the graft. Both OKT3 and BIS-1 are used clinically, and the toxicities of OKT3 and BIS-1 are well known *in vitro* (25–27) as well as *in vivo* (14, 27, 38). Autologous patient serum could replace NCS or HPS in this setting.[4] Therefore, we are investigating the possibility of performing a clinical study including the use of OKT3 for T-cell activation and retargeting by BIS-1 for purging epithelial tumor cells from PBSCs.

The results of the present *in vitro* study indicate that specific purging of epithelial cancer cells by means of bispecific antibody BIS-1 is feasible and effective *in vitro*.

[4] C. P. Schröder, personal observation.

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