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New ways to optimize breast cancer treatment

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Purging of epithelial tumor cells from peripheral blood stem cells by means of the bispecific antibody BIS-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 PBSC is a potential source of relapse. Specific carcinoma cell kill 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 breast cancer patients PBSC, activation of T cells in PBSC and T cell retargeting by BIS-1 was studied.

PBSC, obtained by leucapheresis after chemotherapy and rhG-CSF, were cultured in the presence of PBS, IL-2, OKT3 or IL-2/OKT3 for induction of T cell activation. Subsequently, lysis of epithelial tumor cell lines by activated T cells of PBSC in the presence or absence of BIS-1, was assessed with the Cr⁵¹ release assay or immunocytochemical staining. The effect on PBSC hematopoietic colony formation (HCF) was evaluated by the CFU-GM assay.

Prior to activation, breast cancer patients PBSC contained higher levels of CD8+ T cells, compared to peripheral blood from healthy volunteers ($p < 0.05$). The potential of PBSC 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 72 h OKT3 activation of PBSC ($p < 0.0005$), 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 PBSC can be obtained in vitro by OKT3 activation and BIS-1 retargeting of T cells, without affecting HCF. Current studies evaluate this format for future clinical application.

Introduction

Peripheral blood stem cell (PBSC) 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 of methods to clear tumor cells from PBSC (purging) has 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 non-selective chemotherapeutic drugs was proven 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 PBSC, treatment with monoclonal antibodies has been studied. The use of antibodies was found to be effective and feasible in purging tumor cells from PBSC in hematological cancer patient studies (7-12), although the sole binding of a monoclonal antibody does not induce tumor cell lysis. In the systemic treatment of solid tumors, antibody-based treatment was shown to be beneficial in a setting of minimal residual disease (13). Compared to former disappointing anti-tumor effects of immunotherapy in patients with high tumor load (14-16), adjuvant administration of monoclonal antibodies was found to induce a survival benefit in colorectal carcinoma patients (17). Immunotherapy gained new interest as also 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 is eligible for this type of treatment, as HER2/neu expression in breast cancer is around 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, also the use of cytokines has been studied. Interleukin-2 (IL-2) incubation of PBSC induced up to 50% tumor cell kill in vitro (21), and it did not negatively affect stem cell engraftment in breast cancer patients (22). Additional effect of anti-CD3 antibody OKT3, next to IL-2, on tumor cell kill was seen in bone marrow of hematological patients (23). Also in the hematological setting, Kaneko et al. 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 (24). A bispecific antibody combines affinity to both target and cytotoxic 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 PBSC, which to our knowledge has not been described before. In our study, we have used the bispecific monoclonal antibody BIS-1, which is directed against the pan-carcinoma-associated membrane antigen epithelial glycoprotein-2 (EGP-2) and the CD3 complex present on T cells. EGP-2, also called Ep-CAM, is a 40-kDa membrane-bound glycoprotein, strongly expressed by most carcinomas and universally expressed in breast cancer specimens (reviewed in 26). As such, EGP-2 is a commonly used target antigen in many carcinoma-directed immunotherapeutical 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 kill 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 PBSC by BIS-1 retargeting of activated PBSC.

Materials and methods

PBSC (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, PBSC were mobilized after combination chemotherapy (5-fluorouracil, epirubicin and cyclophosphamide, FEC) and recombinant human granulocyte-colony stimulating growth factor (rhG-CSF), and collected by means of leucapheresis apparatus Cobe Spectra™ (Cobe Netherlands, Uden, The Netherlands). Briefly, from day 2 of the third course of FEC, s.c. rhG-CSF 263 µg was administered daily. On day 9, leucapheresis 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 leucapheresis procedures were required until at least $6 \cdot 10^6$ CD34+ cells/kg body weight were collected. PBSC samples for this study were cryopreserved in 10% dimethyl sulfoxide in a maximal final cell concentration of $200 \cdot 10^6$ cells/mL and stored in liquid nitrogen. Prior to experiments, PBSC were thawed rapidly, washed in newborn calf serum (NCS, Gibco Europe, Breda, The Netherlands), incubated for 15 min in 6 mL NCS to which 2000 U DNase I (Boehringer Mannheim), 0.2 mM magnesium sulfate and 1000 U heparin was added, and centrifuged 5 min at 591 g. Erylysis was performed on all samples (including whole blood control samples) with an ammonium chloride solution (155 mM NH₄Cl, 10 mM potassium hydrogen

carbonate, 0.1 mM sodium ethylene diamine tetra acetate, EDTA). Cells were washed in RPMI medium (Boehringer Ingelheim) and resuspended in RPMI medium containing 5% heat inactivated human pooled serum (HPS), 60 µg/mL gentamycin (Biowithaker, Verviers, Belgium) and 2 mM glutamin, to a final concentration of 1.10^6 nucleated cells/mL.

Activation

PBSC were incubated for 0 h, 24 h and 72 h in the above described culture medium containing one of the following additives: 1) Phosphate buffered saline (PBS) solution (0.14 M NaCl, 2.7 mM KCl, 6.4 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.5 mM KH_2PO_4), pH 7.4, or 2) 100 U/mL IL-2 (aldesleukin, Chiron, Amsterdam, The Netherlands), or 3) 5 %v/v anti-CD3, (tissue culture supernatant containing 10 µg mL⁻¹ OKT3), or 4) 100 U/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: fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labelled anti-CD4, anti-CD8, anti-CD25, anti-CD69 and anti-HLA DR monoclonal antibodies (Immuno Quality Products, Groningen, The Netherlands). PBSC were incubated for 30 min at 4°C (5 µl antibody for 1.10^6 cells in 100 µL PBS with 1% HPS), washed once in PBS, resuspended in 150 µL PBS. Samples were analyzed on a Coulter Elite Cytometer (Coulter Electronics, Hilaleah, FL) using an argon laser (488 nm) for FITC and PE excitation.

Target cell lines

GLC-1 (EGP-2-negative parental cell line) and GLC-1M13 (EGP-2-positive subclone) are small-cell lung cancer (SCLC)-derived cell lines (29). These cell lines were cultured according to routine procedures in RPMI 1640 based medium supplemented with 14% heat inactivated fetal calf serum (FCS), 2 mM glutamine, 60 µg/mL gentamycin, 0.05 mM β-mercaptoethanol and 1 mM sodium pyruvate at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO₂). The EGP-2 positive (GLC1M13) and EGP-2 negative (GLC1) cell model of similar origin was used in the ⁵¹Cr-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% CO₂.

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 was performed as described earlier (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 G150 Sephadex gel filtration, and added to a 0.9% sodium chloride solution to obtain a final concentration of 0.2 mg/mL.

⁵¹Cr-release assay

⁵¹Cr-release assays were performed according to standard procedures to assess BIS-1 redirected T cell cytotoxicity (14). All determinations were executed in triplicate. $5 \cdot 10^6$ target cells (tumor cells GLC-1 or GLC-1M13) were suspended in 100 μ L of culture medium containing 3.7 MBq of [⁵¹Cr]sodium chromate (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) and incubated for 1 h at 37°C in a humidified, 5% CO₂-containing atmosphere. Unbound [⁵¹Cr]sodium chromate was removed by washing the tumor cells three times with medium. Subsequently, aliquots of 100 μ L medium containing 0, $2.5 \cdot 10^3$, $2.5 \cdot 10^4$ or $2.5 \cdot 10^5$ of PBSC (effector cells) after the above mentioned 24 h or 72 h with PBS, IL-2, OKT-3 or IL-2/OKT3, were added into a 96-well round bottom microtiter plate (Greiner no. 650180, Greiner, Alphen aan de Rijn, The Netherlands). To each well, also 50 μ L medium was added containing $2.5 \cdot 10^3$ [⁵¹Cr] labelled target tumor cells, resulting in effector:target ratios of 0, 1, 10 and 100 in a final volume of 200 μ L/well. Finally, a 50 μ L aliquot of medium with 0.4 μ g/mL BIS-1 F(ab')₂ (with a final concentration during the assay of 0.1 μ g/mL) or 50 μ L medium without BIS-1, was pipetted. The microtiter plates were centrifuged at 46 g, for 2 min and incubated at 37°C in 5% CO₂. After a 4 h incubation, the plates were centrifuged at 182 g, for 5 min and 100 μ L samples taken from the supernatant were counted in a γ counter (Wizard, EG&G/Wallac). Cell lysis was calculated from the percentage [⁵¹Cr] released, according to the formula: experimental release-spontaneous release divided by maximal release-spontaneous release x 100%. Maximal release was determined from a sample to which 100 μ L of 2% Triton X-100 solution was added instead of effector

cells. Spontaneous release was determined from a sample to which 100 μ L of medium was 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 granulocyte and macrophage-colony forming unit (CFU-GM) assay (31). Briefly, hematopoietic colony formation was assessed in 1 mL Dulbecco's Modified Eagle Medium (DMEM) including 1.1% methyl cellulose, 20% FCS, 1% deionized bovine serum albumin, 1.10⁻³% α -thioglycerol, and 10 ng/mL IL-3 and GM-CSF. PBSC ($2 \cdot 10^5$ cells, after the prior activations as mentioned above) were plated after 4 h incubation with or without BIS-1 (0.1 μ g in 200 μ L DMEM); with or without GLC1M13 at effector:target ratio 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 PBSC after 72 h prior activation with OKT3, under conditions as mentioned above. MCF-7 tumor (target) cells were added to (effector) PBSC in an effector: target ratio of 1.10⁴:1, in a total volume of 6 mL of RPMI 1640 medium (supplemented with HPS, gentamycin and glutamin, as mentioned above), in the presence or absence of BIS1 (with a final concentration during the assay of 0.1 μ g/mL). As a control, MCF-7 tumor cells were also added to 6 mL of RPMI 1640 medium without PBSC. After a 4 h incubation at 37°C in 5% CO₂, sedimentation of cells unto slides was performed. Cells

were stained with monoclonal antibody MOC31, directed against EGP-2, using indirect immunoperoxidase staining with horseradish peroxidase conjugated rabbit anti mouse as a second antibody and AEC as a substrate. Slides were routinely counterstained with hematoxilin-eosin.

Statistics

Cytotoxic cell lysis, hematopoietic colony formation and leukocyte phenotype were analyzed by means of the Student's t-test. A $p < 0.05$ was considered significant.

Results

Cytotoxic activity in PBSC

⁵¹Cr-release assay

To purge epithelial tumor cells from PBSC, we studied prior activation of T cells present in breast cancer patient PBSC harvests, combined with BIS-1 in the ⁵¹Cr-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 figure 1. Tumor cell lysis was increased by the addition of BIS-1, after all prior activations, compared to cell lysis without BIS-1. Maximal effect of BIS-1 was seen after 72 h of prior PBSC activation with OKT3 ($p < 0.0005$ compared to 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 to OKT3 alone. 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 to tumor cell lysis without BIS-1 (not shown).

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 also in the absence of BIS-1, when compared to the PBS control (maximum GLC1M13 lysis: $p < 0.0005$ compared to PBS after 72 h PBSC activation 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 to 24 h activation with OKT3 and IL-2/OKT3 ($p < 0.005$ and $p < 0.025$, respectively).

In figure 2, the effect of increasing ratios of effector: tumor cells is shown. After all PBSC activations, increasing E:T ratio coincided with increased BIS-1 redirected

Log depletion assay

At an effector: target ratio of $1 \cdot 10^4$ OKT3 activated PBSC to 1 MCF-7 tumor cell, in the presence of BIS-1, a >3 log depletion of MCF-7 tumor cells (mean 0.09% of total number of MCF-7 cells remaining) was observed, as compared to a control to which no effector PBSC was added (see also figure 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 PBSC. The sensitivity of tumor cell detection in these experiments was 1 MCF-7 tumor cell in the total number (e.g. $6 \cdot 10^7$) of PBSC screened, in line with refs. 32 and 33.

Composition of PBSC and activation markers on T lymphocytes

During the three consecutive days of the leucapheresis procedure (day 9, 10 and 11 since 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 percentage of CD4+ and CD8+ T cells remained the same. The lymphocyte percentage CD8+ T cells in PBSC harvests before activation (mean 28% CD8+ T cells, SD 10%; $n=4$), was higher compared to 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 to peripheral blood. The percentage of CD4+ or CD8+ T cells bearing activation markers CD69 and CD25, increased during the three consecutive days (day 9, 10 and 11) of the leucapheresis procedure (fig. 4).

Further in vitro activation of PBSC 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 (to mean 75% and 82% respectively, both $p < 0.0005$ compared to 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 (to mean 53% and 73% respectively, both $p < 0.0005$). 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 PBSC tended to rise during in vitro activation of PBSC with OKT3 and IL-2/OKT3, no significant difference was observed after 24 or 72 h activation as compared to 0 h. No difference in the total number of PBSC was found after 0, 24 or 72 h in the PBS control. No effect on the total number of PBSC was found after 24 and 72 h prior activation, compared to the PBS control. Also, no effect on lymphocyte and T cell subsets was observed after prior activation, as reflected in table 1.

Hematopoietic colony formation

Figure 5 shows the effect of prior PBSC activation on the ability of the hematopoietic stem cells to form hematopoietic colonies, measured as CFU-GM numbers. No effect of 24 h of prior activation was seen, when compared to the PBS control. The PBS control was not different after 24 or 72 h (mean 70 vs 67 CFU-GM, $n=3$, N.S.). 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 PBSC 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 PBSC 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 PBSC was a prerequisite for effective BIS-1 mediated cell lysis, which is compatible with studies showing that T cells need prior activation in order to gain cytolytic potential (25 for review). By employing OKT3 activation of PBSC and subsequent BIS-1, within the four hours of the assay a tumor cell depletion of more than 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 kill. 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 only for a minority of CD8⁺ T cells. Effector: target ratios in the clinical setting (i.e. the ratio of potential cytotoxic effector cells to tumor cells in tumor contaminated PBSC) are likely to be more than 1.10²:1 (as used in the ⁵¹Cr-release assay in this study) or 1.10⁴:1 (log-depletion assay). This is generally the case since highly sensitive methods, including immunocytochemistry (32, 33) and reverse transcriptase-polymerase chain reaction

(RT-PCR) (34), are required to detect single tumor cells in 1.10^6 to 1.10^7 PBSC. 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 haematologic recovery after stem cell transplantation (35), was used as a functional evaluation of haematopoietic 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 kill 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), while 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 used for induction of anti-tumor immunomodulation (38). The fact that we did not see a negative effect on PBSC of breast cancer patients of prior activation with OKT3 alone, is consistent with these findings. In vitro IL-2 incubation of breast cancer patient derived PBSC did not negatively affect hematopoietic colony formation in three studies (21, 22, 39). Our data confirm and extend these findings. In spite of 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, as the degree of T cell activation after prior treatment with IL-2/OKT3 was indeed higher compared to the other treatments (for instance 73% CD8+ T cells also positive for HLA DR after 72 h IL-2/OKT3 activation, compared to 53% after OKT3 activation) in this study.

In search of purging methods both efficient in eliminating tumor cells and maintaining sufficient hemapoiesis, non-selective 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, for instance 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 to be difficult, at least when compared to 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, as 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 (PBSC) 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 PBSC, i.e. immunocytochemistry and a quantitative RT-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 anti-tumor activity, also concerning possible residual disease in the patient (3). Long-term follow-up analyses after CD34+ stem cell selection of PBSC grafts (which do not include the immunocompetent natural killer cells or T cells) may shed more light on the impact of immunocompetence of the graft. At this point, data on small numbers of patients are

available after a short follow-up, not allowing conclusions on disease free or overall survival as yet (40, 41). If indeed 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 toxicity of OKT3 and BIS-1 is 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 (own observation). Therefore, we are currently investigating the possibility to perform a clinical study including the use of OKT3 for T cell activation and retargeting by BIS-1 for purging epithelial tumor cells from PBSC.

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.

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Table 1:

The effect of processing on total PBSC numbers, lymphocytes and T cell subset fractions

	t=0	t=72 h: PBS	OKT3	IL-2/OKT3
Total no. PBSC <i>Per flask (10⁶)</i>	10	10.8 (SD 3)	16.6 (SD 3)	15.5 (SD 2.2)
Lymphocytes <i>(% of total no. PBSC)</i>	47 % (SD 19)	53 % (SD 10)	60% (SD 12)	58% (SD 16)
CD 8 T cells <i>(% of lymphocytes)</i>	28 % (SD 10)	13 % (SD 8) (*)	24% (SD 12)	28% (SD 13)
CD4 T cells <i>(% of lymphocytes)</i>	50 % (SD 13)	40 % (SD 13)	37% (SD 13)	37% (SD 13)

Numbers prior to processing (t=0) and after 72 h prior PBSC activation with PBS (control), OKT3 or IL-2/OKT3 are reflected in this table (n=4). Numbers were not significantly different compared to t=0, except (*): p<0.05.

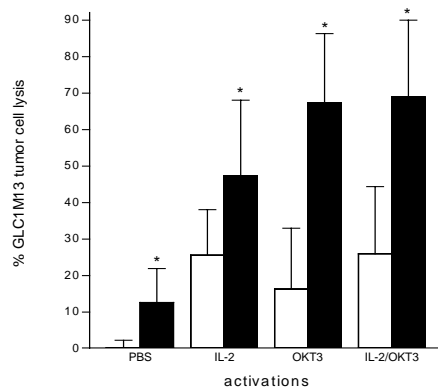


Figure 1: GLC1M13 cell lysis by PBSC with/without BIS-1

- On the Y-axis: % GLC1m13 tumor cell lysis; on the X-axis: activating agents. Open bars reflect activations without BIS-1; black bars represent activation with subsequent BIS-1.
- Shown is the % specific tumor cell lysis, determined in ^{51}Cr release assay (mean and SD, n=6) with E:T ratio 100:1, after 72 h prior activation. An * indicates significant difference compared to counterpart without BIS-1.

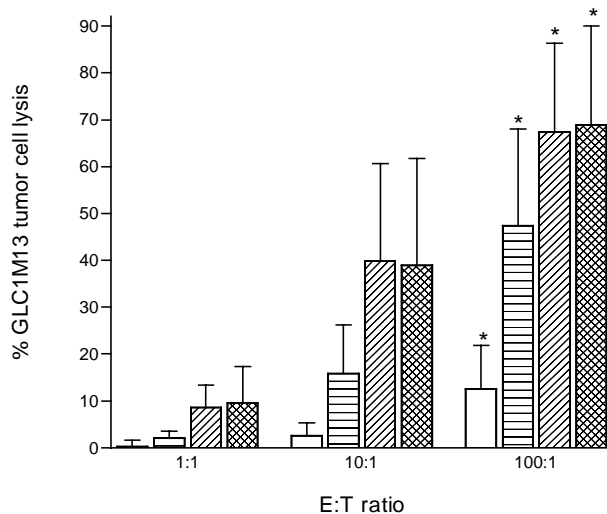


Figure 2: Effect of E:T ratio

- On the Y-axis: % GLC1M13 tumor cell lysis; on the X-axis: effector:target ratios 1:1, 10:1, 100:1 (=PBSC: tumor cell ratio).
- Shown is the % GLC1M13 cell lysis (mean, n=6) by PBSC + BIS-1 after 72h activation (PBS: open bar, IL-2: horizontal stripes, OKT3: oblique stripes, IL-2/OKT3: checkered bar). An * indicates significant difference compared to E:T ratios 1:1 and 10:1.

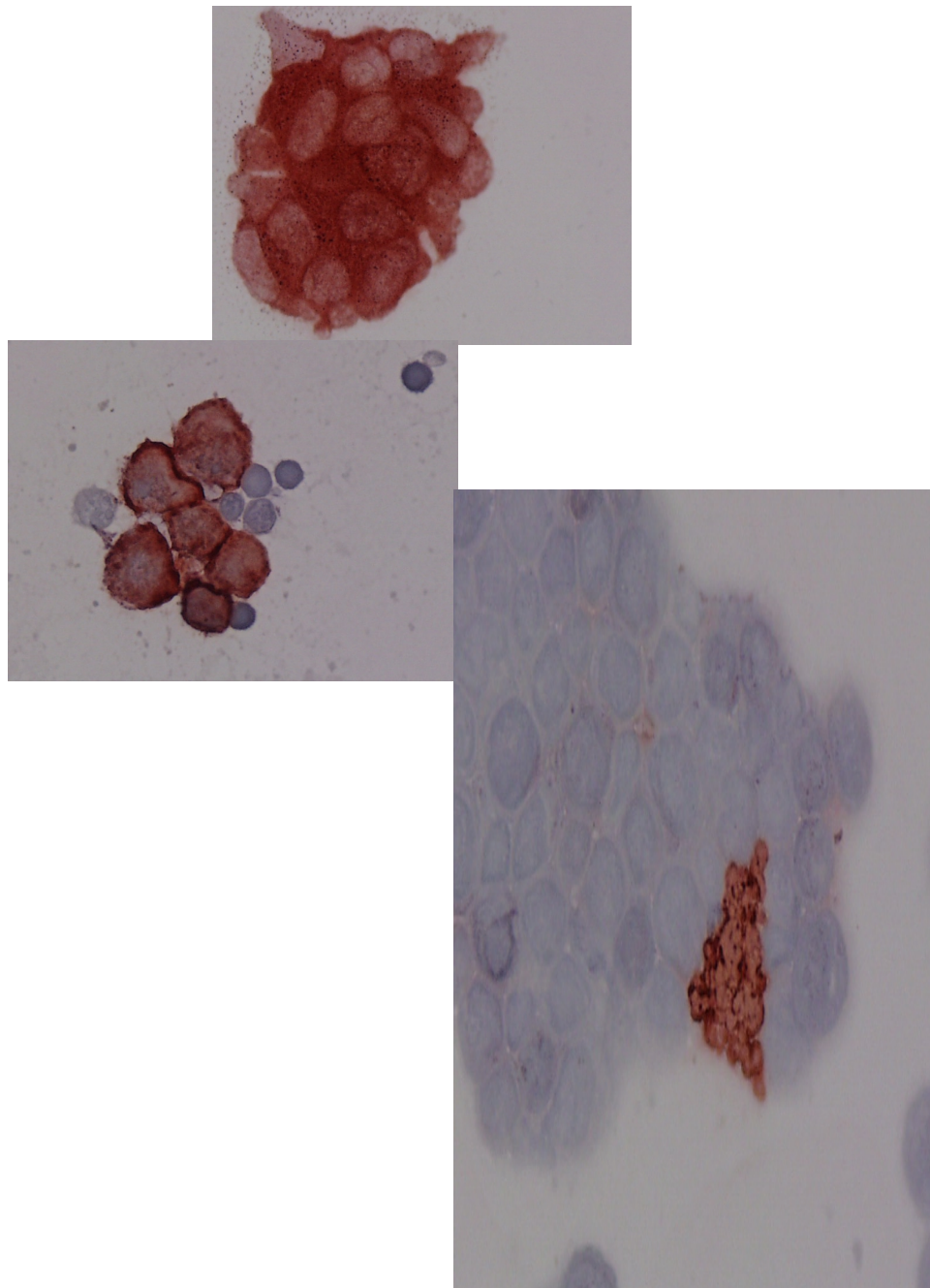


Figure 3: The effect of activated PBSC with BIS-1, on MCF-7

20x10 enlargement

- A: MCF-7 tumor cells without PBSC
- B: MCF-7 tumor cells with OKT3 activated PBSC, without BIS-1: viable appearance
- C: MCF-7 tumor cell with OKT3 activated PBSC, with BIS-1: non-viable appearance

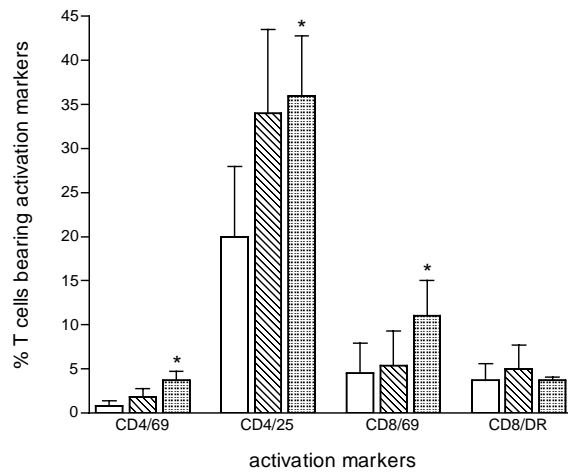


Figure 4: Activation markers on CD4+ and 8+ T cells in PBSC, during the three consecutive days of the leucapheresis procedure.

- On the Y-axis: % T cells with mentioned activation markers; on the X-axis: activation markers on CD4+ and CD8+ T cells.
- Shown is the % T cells in PBSC with marker (mean, n=3) on consecutive leucapheresis days: day 9 of the course: open bar, day 10 of the course: striped bar, day 11 of the course: checkered bar. An * indicates significant difference compared to first value on day 9.

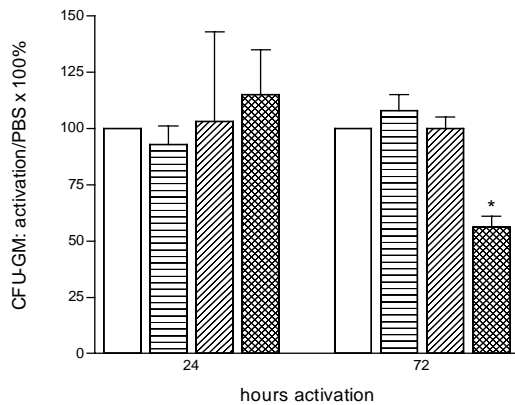


Figure 5: Hematopoietic colony formation after activation of PBSC.

- On the Y-axis: % hematopoietic colonies (CFU-GM) relative to PBS control (set at 100%); on the X-axis: 24 and 72 h activation.
- Shown is % hematopoietic colonies (mean and SD, n=3) after 24 or 72 h PBSC activation with PBS (open bar), IL-2 (horizontal stripes), OKT3 (oblique stripes) or IL-2/OKT3 (checkered bar). An * indicates a significant difference compared to PBS control.

List of abbreviations

PBSC:	peripheral blood stem cells
EGP-2:	epithelial glycoprotein-2
BIS-1:	bispecific antibody-1
IL-2:	interleukin-2
OKT3:	anti-CD3 antibody
CFU-GM:	granulocyte and macrophage-colony forming unit
PBS:	phosphate buffered saline solution
rhG-CSF:	recombinant human granulocyte-colony stimulating factor
FEC:	5-fluorouracil, epirubicin and cyclophosphamide
EDTA:	ethylene diamine tetra acetate
FCS:	fetal calf serum
NCS:	newborn calf serum
HPS:	human pooled serum
DMEM:	Dulbecco's Modified Eagle Medium
PE:	phycoerythrin
FITC:	fluorescein isothiocyanate
SCLC:	small cell lung carcinoma

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