Without Prior Stimulation, Tumor-associated Lymphocytes from Malignant Effusions Lyse Autologous Tumor Cells in the Presence of Bispecific Antibody HEA125xOKT3

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ABSTRACT

Women suffering from advanced stage ovarian or mammary carcinoma frequently develop malignant ascites or pleural effusions consisting of tumor cells and tumor-associated lymphocytes (TALs). Locoregional immunotherapy with bispecific antibodies (bsAbs), which retarget T cells to tumor cells and induce their lysis, has been applied as an adjuvant treatment in the late stage of the disease. Until now, most of these therapies use peripheral blood mononuclear cells (PBMCs) as effector cells that have been stimulated and expanded ex vivo and loaded with bsAb before reinjection. Here we investigated whether TALs derived from malignant ascites or pleural effusions can be used as bsAb-guided effector cells without prior in vitro stimulation.

For this we established a bsAb, HEA125xOKT3, which recognizes the epithelial antigen Egp34 on carcinoma cells and the CD3 molecule on T cells. BsAb HEA125xOKT3 induced lysis of various Egp34-expressing carcinoma lines by stimulated PBMCs. Optimal cytotoxicity was achieved at a bsAb concentration of 1 μg/ml. In three ovarian and two mammary carcinoma patients, we demonstrated efficient lytic activity of lymphocytes, isolated from malignant ascites or pleural effusion. Without prior stimulation, they lysed autologous tumor cells in the presence of bsAb HEA125xOKT3, indicating that they are already activated. Along this line, a subset of CD4+ and CD8+ unstimulated TALs expressed the early activation marker CD69. They are, however, negative for CD95, and only a small subpopulation of CD4+ TALs expresses CD25. OKT3/interleukin 2 stimulation of TALs increased the expression of activation markers on the CD4+ and CD8+ T-cell compartment. The activation markers CD69, CD25, CD95, and DR molecules are up-regulated on both T-cell types. However, lysis of autologous tumor cells by stimulated TALs is not significantly enhanced compared with unstimulated TALs. Our results may offer a novel and promising concept of adjuvant immunotherapy for ovarian and mammary carcinoma patients. Preactivation and expansion of PBMCs can be circumvented by exploring the cytotoxic capacity of unstimulated TALs in the presence of bsAbs in a locoregional therapeutic approach.

INTRODUCTION

Ovarian cancer is the leading cause of death among gynecological malignancies. At the moment, the best treatment results are obtained with cytoreductive surgery, followed by combination chemotherapy (1). However, <20% of the stage IV patients have a survival time of >5 years (2). Already in the early stage of the disease, carcinoma cells spread from the ovaries to the peritoneal cavity, giving rise to multiple metastases, including the formation of malignant ascites. Ascites formation is associated with severe symptoms such as anorexia, sensation of abdominal fullness, and dyspnea (3). Therefore, new treatment approaches have to be established to prolong survival time and improve quality of life. Several trials of i.p. injections of cytokines such as IFN-γ (4), tumor necrosis factor (5), IL-2-2 (6), or other biological response modifiers (7) have shown positive results in the treatment of malignant ascites. Antibodies directed against carcinoma cell surface markers offer an alternative therapeutic strategy. However, most of the antibodies developed against human tumors (8) have little therapeutic effects because they are unable to activate the cellular immune effector functions, such as complement fixation and antibody-dependent, cell-mediated cytotoxicity. Promising results are obtained in colorectal cancer after surgery using mAb 17-1A, which recognizes a surface glycoprotein of epithelial origin on human tumor cells (9).

To activate effector functions, bsAbs were developed that are able to retarget immunocompetent cells to tumor cells in an MHC-independent manner. This is achieved by the dual specificity of their antigen-binding arms, which facilitate the cross-linking of tumor cells via tumor-associated antigens and effector cells via binding to various cell surface markers such as CD3, CD2, CD5, or CD28 on immunocompetent cells. Promising results are reported in phase I trials of patients with non-Hodgkin’s lymphomas (10) or advanced breast and ovarian

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3 The abbreviations used are: IL, interleukin; bsAb, bispecific antibody; CD, cluster of differentiation; Egp, epithelial glycoprotein; PBMC, peripheral blood mononuclear cell; TAL, tumor-associated lymphocyte; PE, phycoerythrin; ICAM, intercellular adhesion molecule.
cancers. In the latter study, bsAbs were directed against the folate-binding protein, which is overexpressed in ~90% of ovarian carcinoma specimens (11) and the CD3 molecule on T cells (12). Other groups applied antibodies, binding the carcinoma-associated tumor antigen AMOC-31 combined with anti-CD3 reactivity (13). Valone et al. (14) and Weiner et al. (15) established bsAbs directed against the proto-oncogene product HER-2/neu expressed on ovarian and mammary carcinomas and further against the FcγRI receptor (CD64), found mainly on monocytes, macrophages, and neutrophils, or the FcγRIII receptor (CD16) on natural killer cells and differentiated macrophages. All these trials demonstrated low toxic side effects and resulted in tumor regression in several patients.

Egp34 represents another epithelial glycoprotein expressed on virtually all carcinoma cells (16–18). It is absent from non-epithelial tumor types, such as sarcomas, lymphomas, melanomas, and neurogenic tumors, and is not released into the serum of patients. We chose this antigen as the target molecule on carcinoma cells for bsAb-mediated T-cell cytotoxicity. Although different bsAbs cross-linking natural killer cells (19, 20) or neutrophils (21) to tumor cells have been described, the most common approach is still the targeting via the CD3 molecule on cytotoxic T cells (22, 23). Preactivation of the effector T cells is an important prerequisite for the establishment of an effective T-cell cytotoxicity (24). This activation can be achieved via stimulation of the CD3 complex or application of recombinant IL-2. Although systemic IL-2 therapy induces effective T-cell activation (25), severe side effects have been reported. To circumvent these disadvantages, several recent reports demonstrate that the induction of T cell cytotoxicity can also be reached by a combined stimulation of different accessory molecules on the effector cell such as CD3, CD5, and CD28 (26, 27). But even using these protocols, PBMC have to be stimulated and expanded in vitro before they are reinjected into the patient, procedures that go along with time-consuming cell culture work, high contamination risk, and an amplification process for every individual patient. However, not only PBMCs exhibit cytotoxic activity. Tumor-infiltrating lymphocytes or TALs isolated from malignant ascites or malignant effusions can also be used as effector cells. They can be immediately as effector cells or was stored in liquid nitrogen until treatment. BsAb HEA125xOKT3 was produced using the hybrid-hybridoma technique (23). Briefly, bsAb was prepared by fusing the murine hybridoma cell lines OKT3 (IgG2a, directed against the ε-chain of the CD3 molecule, obtained from the American Type Culture Collection, Rockville, MD) and HEA125 (IgG1, directed against the epithelial glycoprotein Egp34; Ref. 16). Monoclonal antibodies were obtained from the Miniaper system (Heraeus, Osterrode, Germany), and supernatant was purified further by affinity chromatography on a protein A-Sepharose CL-4B column (Pharmacia Fine Chemicals, Uppsala, Sweden); eluant was further subjected to HPLC purification on a Bakerbond ABx column (J.T. Baker, Inc., Philipsburg, NJ). The purity of eluted material was assessed by SDS-PAGE under reducing conditions.

**Cell Lines.** Three Egp34+ cell lines were used: the colon carcinoma cell line Colo205; the freshly established colon carcinoma cell line C-HD3 (a gift from Dr. P. Möller, Institute of Pathology, Ulm, Germany); and the mammary carcinoma line MCF-7. The melanoma cell line MZ-MEL2 is Egp34 negative. All lines were grown in RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% heat-inactivated FCS (Conco, Wiesbaden, Germany), 2 mM l-glutamine, and 1 mM sodium pyruvate at 37°C in a humidified atmosphere containing 7.5% CO₂.

**Isolation of PBMCs, TALs, and Tumor Cells from Malignant Ascites and Pleural Effusions.** PBMCs were obtained from heparinized peripheral blood of healthy donors. Isolation was done by density centrifugation of diluted (1:3 in PBS) blood on Ficoll (Pharmacia Biotech AB, Uppsala, Sweden) at 1800 rpm for 20 min at room temperature. The PBMC interphase was washed twice in complete RPMI and used immediately as effector cells or was stored in liquid nitrogen until use. Culture of PBMCs was done in RPMI 1640 supplemented with 2% heat-inactivated FCS, 2 mM l-glutamine, 1 mM pyruvate, and 0.05 mM 2-ME. Heparinized ascites fluid or pleural exudates were centrifuged for 20 min at 1200 rpm to pellet tumor cells and mononuclear cells. Separation of tumor cells from mononuclear cells was performed by centrifugation on 18–40% discontinuous Percoll gradient. In brief, pelleted cells were placed at the top of the 18% gradient and overlaid over the 40% Percoll and centrifuged for 45 min at 2100 rpm at room temperature. Tumor cells were mostly enriched in the second interphase, whereas the lymphocytes formed a pellet at the bottom of the tube. Both fractions were collected and washed twice before culturing or storage in liquid nitrogen. TALs and tumor cells from patients were cultured in RPMI 1640, supplemented with 5% heat-inactivated human serum (Bio Wittaker, Walkersville, MD), 2 mM l-glutamine, 1 mM pyruvate, and 0.05 mM 2-ME.

**MATERIALS AND METHODS**

**Patients.** Malignant ascites or pleural effusions were obtained from patients with advanced stages of invasive ovarian or breast cancer. Most patients had received chemotherapy several months before the collection of the malignant effusions but at the time of collections were not treated with chemotherapy.

**Production and Purification of bsAb HEA125xOKT3.** BsAb HEA125xOKT3 was produced using the hybrid-hybridoma technique (23). Briefly, bsAb was prepared by fusing the murine hybridoma cell lines OKT3 (IgG2a, directed against the ε-chain of the CD3 molecule, obtained from the American Type Culture Collection, Rockville, MD) and HEA125 (IgG1, directed against the epithelial glycoprotein Egp34; Ref. 16). Quadranta cells were cultured in the Miniaper system (Heraeus, Osterrode, Germany), and supernatant was purified first by affinity chromatography on a protein A-Sepharose CL-4B column (Pharmacia Fine Chemicals, Uppsala, Sweden); eluant was further subjected to HPLC purification on a Bakerbond ABx column (J.T. Baker, Inc., Philipsburg, NJ). The purity of eluted material was assessed by SDS-PAGE under reducing conditions.

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**CTL Activation.** To obtain activated PBMCs or TALs, Ficoll or Percoll purified cells were cultured at a concentration of 2 × 10^6 cells/ml in medium containing OKT3 (5 μg/ml) and recombinant human IL-2 (20 units/ml; Eurocetus, Frankfurt, Germany). After 4 days, cells were washed twice to remove remaining antibodies and cultured overnight in medium alone. On day 5, a cytotoxicity assay was performed.

**Cytotoxicity Assay.** Cytotoxic T cell assays were carried out in RPMI 1640, supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 1 mM pyruvate, 0.05 mM 2-ME, and 1 mM HEPES. Cytolytic activity was assessed using a standard ^51^Cr release assay. Target cells (2 × 10^5) were labeled with 200 μCi sodium ^51^CrO_4 (Amersham-Buchler, Braunschweig, Germany), followed by four washing cycles and resuspended in medium at a concentration of 1 × 10^6/ml. Effector cells were adjusted to a concentration of 2.5 × 10^6/ml. Increasing numbers of effector cells in 100 μl were titrated to 5 × 10^3 target cells/well in 50 μl; 0.50 μl of antibody solution (1 μg/ml) was added to each well. The whole assay was set up in triplicates and incubated in medium at 37°C for 4 h. One hundred μl of supernatant were harvested and assayed for ^51^Cr release in a gamma counter (Cobra Auto Gamma; Canberra Packard, Dreieich, Germany). Maximum release was determined by incubation of labeled target cells in 100 μl of 10% SDS, and spontaneous release was calculated from a sample to which 100 μl of medium were added instead of effector cells. The percentage of specific release was determined as:

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\text{% specific release} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100
\]

**Flow Cytometry.** For analysis of T-cell activation markers and surface markers of tumor cells, 1 × 10^6 cells were stained with the following antibodies: W6/32-biotin (HLA-A, B, C; Ref. 37); L243-biotin (HLA-DR; Ref. 38); CD54-PE (ICAM-1; PharMingen, San Diego, CA); CD80-biotin (B7-1; PharMingen) or CD86-biotin (B7-2, PharMingen); CD40-FITC (PharMingen); HP2/6-biotin or FITC (CD4; Ref. 39); OKT8-biotin or FITC (CD8; Ref. 40); CD95-biotin (APO-1/Fas, generously provided by Dr. P. Krammer, Deutsches Krebsforschungszentrum, Heidelberg, Germany); CD25-PE (IL-2Rα; PharMingen); CD69-FITC (activation inducer molecule; PharMingen); Streptavidin-PE (Southern Biotechnology Associates, Inc. Birmingham, AL) was used as a second-step reagent. Dead cells were discriminated by propidium iodide staining (Sigma, Deisenhofen, Germany). Analysis was performed on a FACSscan cytometer (Becton Dickinson, Heidelberg, Germany) using CELLQUEST software.

**RESULTS**

**BsAb HEA125xOKT3 Induce Lysis of Egp34-expressing Carcinoma Cell Lines by Activated PBMCs.** To establish a bsAb that is able to retarget cytotoxic T cells to carcinoma cells, we produced the bsAb HEA125xOKT3 by fusion of the parental hybridoma lines HEA125 and OKT3. To test its biological function in vitro, we performed CTL assays. PBMCs from healthy donors were used as effector cells that were stimulated with soluble OKT3 antibody and IL-2. After 4 days, the cells were washed to remove remaining antibody and cultured in medium alone overnight. The Egp34 expressing cell lines Colo205 (colon carcinoma), C-HD3 (freshly established colon carcinoma), and MCF-7 (mammary carcinoma) were used as target cells. In the presence of bsAb HEA125xOKT3, a standard chromium release assay with increasing E:T cell ratios was carried out. A mixture of both parental antibodies (HEA125 and OKT3) or irrelevant bsAb HEA125xOKT3 (directed against the CD19 antigen on B cells and the CD3 molecule on T cells) served as negative control and determined background lysis. To demonstrate specific binding of HEA125xOKT3, the Egp34-negative melanoma cell line MZ-MEL2 was included as target cell. Fig. 1 illustrates that Egp34-expressing carcinoma lines are efficiently lysed in the presence of bsAb HEA125xOKT3. Specific lysis of the three target cell lines ranges between 50 and 70% at an E:T ratio of 50:1. Similar results were obtained in five independent experiments. However, these variations in cytotoxicity of distinct lines do not correspond to the amount of Egp34 antigen expressed on the cell surface. Addition of a mixture of parental antibodies or irrelevant bsAb yielded a background lysis of 10–20%. The Egp34-negative melanoma cell line MZ-MEL2 was not lysed at all. The addition of bivalent anti-CD28 mAb did not increase specific tumor cell lysis induced by HEA125xOKT3 (data not shown).

To determine the optimal concentration of HEA125xOKT3 for bsAb-induced cytotoxicity, we performed a chromium release assay with activated PBMCs (as described above) as effector cells and Colo205 as target cells at a fixed ratio of 50:1 (Fig. 2). For control, cells were incubated with a mixture of both bivalent parental mAbs. Lysis of target cells was already detected at a concentration of 1 ng/ml of bsAb and increases with increasing amounts, reaching a plateau at 10 μg/ml. For all additional experiments, a concentration of 1 μg/ml was used. These results indicate that HEA125xOKT3 is a useful bispecific reagent to focus CTLs against carcinoma cells in vitro.

**Phenotype of Tumor Cells from Malignant Ascites.** Women with advanced stages of ovarian or mammary carcinomas frequently develop malignant ascites or pleural effusions, from which tumor cells as well as tumor-associated lymphocytes can be isolated. Expression of cell surface antigens of ovarian tumor cells derived from malignant ascites was studied. Tumor cells and TALs were separated by discontinuous Percoll gradient centrifugation, but the tumor cell fraction always contained contaminating mesothelial cells, varying between 10 and 30% of the total cell number. Therefore, cells were double stained for HEA125 and various cell surface markers to exclude mesothelial cells, which do not express Egp34. Analysis was done by flow cytometry, showing the expression of HLA-A, HLA-B, HLA-C, HLA-DR, B7-1 (CD80), B7-2 (CD86), CD40, ICAM-1 (CD54), and APO-1/Fas antigen (CD95) on HEA125* tumor cells (Fig. 3). Nearly all tumor cells (90–98%) stain positive for MHC class I, HLA-DR, and ICAM-1 but negative for B7-1 and APO-1/Fas antigens. Only a small subpopulation (1–2%) expressed CD40 and B7-2 antigens. Analogous experiments using tumor cells from different patients were performed, revealing similar results with the exception of HLA-DR expression, which ranged from 30 to 80% for individual patients, and B7-2 expression, which was often not detectable.

**CBS-1**

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TALs from Malignant Ascites and Pleural Effusion
Lyse Autologous Tumor Cells in the Presence of bsAb HEA125xOKT3.

To evaluate the lytic capacity of bsAb HEA125xOKT3 in an autologous setting, TALs and tumor cells from ovarian or mammary carcinoma patients were isolated by discontinuous Percoll gradient centrifugation. TALs were stimulated with soluble OKT3 and IL-2, washed after 4 days to remove remaining antibody, and cultured overnight in medium alone. Chromium-labeled autologous tumor cells were incubated with increasing amounts of effector cells in the presence of bsAb or a mixture of both parental antibodies. In Fig. 4, one experiment is displayed using malignant ascites-derived cells and a mixture of the parental antibodies HEA125 and OKT3 (C) as negative controls.

Previous work has suggested that malignant effusions sometimes contain inhibitors of cell-mediated cytotoxicity, e.g., transforming growth factor β and IL-10 (7). We have conducted chromium release assays in the presence of both autologous ascites and serum. However, an inhibition of bsAb-induced cytotoxicity was never observed.

Expression of Activation Markers on TALs from Malignant Ovarian Ascites.
As described above, cross-linking of preactivated TALs and tumor cells by bsAb HEA125xOKT3 induces the lytic activity of the lymphocytes, which are otherwise unreactive. We analyzed the activation markers of TALs obtained from malignant ovarian ascites and compared them to those of TALs from normal individuals. The results showed that bsAb HEA125xOKT3 efficiently induces the lytic activity of short term-stimulated TALs toward autologous tumor cells.

Three patients with malignant ovarian ascites and two patients with mammary pleural effusion were studied. TALs were stimulated as described above and used in a 4-h chromium release assay. Table 1 indicates that TALs induce efficient lysis of autologous tumor cells in the presence of bsAb HEA125xOKT3 in all cases. However, the effectiveness of TALs from individual patients ranged from 3 to 27 times specific lysis above background lysis. For all patients, the lytic capacity of CD4+ and CD8+ T-cell subsets was analyzed, revealing comparable results as depicted in Fig. 4. These findings indicate that bsAb HEA125xOKT3 efficiently induces the lytic activity of short term-stimulated TALs toward autologous tumor cells.

54% specific chromium release. Addition of parental antibodies HEA125 and OKT3 induce background lysis of 0–10%. These results suggest that in the presence of bsAb CD4+ and CD8+, TALs have similar antitumor reactivities. To rule out the possibility that cytotoxicity was induced via Fc receptor binding of the bispecific immunoglobulin molecule, F(ab′)2 fragments of bsAb HEA125xOKT3 were generated by pepsin digestion. The lytic capacity of the fragments compared favorably with that of the whole antibody molecule (data not shown). In addition, when cytotoxicity assays were performed in the presence of heat-denatured polyclonal human IgG to block Fcγ receptor-mediated killing, no decrease of specific lysis was observed.
with unstimulated TALs or unstimulated PBMCs from healthy donors. The TALs were divided into two fractions, one was cultured in medium alone and the other was stimulated with OKT3 (5 μg/ml) and IL-2 (20 units/ml). After 4 days, the stimulated cells were washed twice to remove remaining antibody and kept overnight in medium without additional stimulating reagents. The next day, double fluorescence analysis was carried out from stimulated and unstimulated TALs and unstimulated PBMCs to demonstrate CD25, CD69, HLA-DR, and APO-1/Fas antigen (CD95) expression on CD4 and CD8 T cells (Fig. 5). In the CD4 population of unstimulated TALs, ~15% express DR, 7% express CD25, and 8% express CD69 antigens; this population is negative for CD95. The CD8 population differs by high expression of the early activation marker CD69 (37%) but shows comparable staining as CD4 cells for the other surface markers. Enhancement of all activation markers appeared in both T-cell subpopulations after OKT3/IL-2 stimulation. The increase of CD95 expression is more pronounced in the CD4 population (from 0 to 22%) compared with the CD8 subset (from 0 to 2%), and more CD4 T cells expose CD25 (63%) than CD8 cells (36%), whereas CD69 expression on CD8 cells (35%) stays higher than on CD4 cells (18%) after OKT3/IL-2 stimulation. Unstimulated PBMCs show weak DR expression (10–15%) in both subsets, comparable with unstimulated TALs, but are negative for CD95 and CD69. Only a small subset of 5% of CD4 PBMCs stain positive for CD25 antigen. Analogous experiments were performed with TALs from three ovarian and two mammary carcinoma patients, yielding similar results. These phenotypic analyses indicate that ex vivo isolated TALs are already in an activated state, as clearly demonstrated by CD69 expression on CD4 and CD8 T-cell subsets. To exclude the possibility that the Percoll preparation used for isolation of TALs contained stimulatory contaminants like LPS, T lymphocytes from peripheral blood were isolated via Percoll under the same conditions. They displayed no signs of activation when analyzed by flow cytometry, documenting that this batch of Percoll was apparently free of stimulating agents.

**Unstimulated TALs Lysed Autologous Tumor Cells in the Presence of bsAb HEA125xOKT3.** In consideration of the pre-activated state of TALs, we tested their lytic activity in the presence of bsAb HEA125xOKT3 toward autologous tumor cells. After separation of TALs and tumor cells from malignant ovarian ascites, the lymphocytes were divided into two parts. One was stimulated for 4 days with OKT3/IL-2, and the other half was cultured in medium alone. To make sure that a 5-day culture of TALs did not change their activation state, we compared the expression of activation markers directly after Percoll gradient separation or after 5 days of culture by flow cytometry. No phenotypic differences could be detected within the two populations (data not shown). Exclusively OKT3/IL-2 treatment up-regulates the activation markers, as expected and depicted in Fig. 5.

Fig. 3 Surface marker expression of ovarian carcinoma cells. Carcinoma cells were isolated from malignant ascites and were double-stained for Egp34 expression (HEA125-FITC or -biotin) and for HLA-A, HLA-B, HLA-C (W6/32-biotin), HLA-DR (L243-biotin), CD54-PE, B7–1-biotin, B7–2-biotin, or CD40-FITC, followed by second-step reagent streptavidin-PE or streptavidin-FITC. Expression of the indicated surface markers is displayed on Egp34+ cells.
At day 5, a standard chromium release assay was performed. Increasing amounts of both effector populations were titrated to chromium-labeled tumor target cells in the presence of bsAb HEA125xOKT3. Unstimulated TALs induce efficient lysis of autologous target cells as illustrated in Fig. 6A. At an E:T ratio of 50:1, ~30% specific lysis was reached, whereas the addition of a mixture of both parental antibodies HEA125 and OKT3 achieved background lysis of <1%. Interestingly, unstimulated TALs show a remarkable cytotoxic response, although they were not prestimulated in vitro. Their lytic activity is in agreement with the expression of early activation marker CD69 on unstimulated TALs and confirms that these T cells exist in a preactivated state. Up to an E:T cell ratio of 1.5:1 specific lysis of unstimulated TALs exceeds background lysis.

We compared the lytic capacity of unstimulated and OKT3/IL-2-stimulated PBMCs from the same patient toward her tumor cells in the presence of bsAb HEA125xOKT3. PBMCs were stimulated for 4 days with soluble OKT3 and IL-2 or were cultured in medium alone. On day 5, both effector cell populations were titrated with increasing amounts to chromium-labeled autologous tumor cells in the presence of bsAb HEA125xOKT3 or a mixture of both parental antibodies. In contrast to unstimulated TALs, the addition of HEA125xOKT3 does not induce the lytic capacity of PBMCs, which have not been stimulated in vitro (Fig. 6B and Table 1). OKT3/IL-2-stimulated PBMCs, however, lyse 19% autologous tumor cells at an E:T ratio of 50:1.

A compilation of specific lysis of carcinoma cells either induced by autologous TALs or PBMCs with and without stimulation is given in Table 1. In three ovarian and two mammary carcinoma patients, we found an efficient cytotoxic activity of unstimulated TALs toward autologous tumor cells in the

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**Table 1** Specific lysis of carcinoma cells by autologous TALs and PBMCs

<table>
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<th>Patient</th>
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<th>Stimulation</th>
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<th>HEA125 × OKT3 (1 μg/ml)</th>
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<td>J. B.</td>
<td>TALs</td>
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* Data are given as mean values of triplicates at an E:T ratio of 50:1.
presence of bsAb HEA125xOKT3. This always correlated to the expression of CD69 on the effector cells. Induction of cytotoxicity by unstimulated TALs was 3–25-fold above unspecific lysis, achieved by the addition of both parental antibodies HEA125 and OKT3. Our data suggest that TALs from malignant ovarian ascites or mammary pleural effusions are preactivated in situ and that they can fulfill effective lytic activity, if they are retargeted to autologous tumor cells by bsAb HEA125xOKT3. In contrast to PBMCs, OKT3/IL-2 stimulation solely increases the cytotoxicity of TALs but is not essential for their effector function.

DISCUSSION

In the present study, we investigated the cytolytic activity of TALs from malignant ovarian ascites or mammary pleural effusions.
Cytotoxicity of Unstimulated TAL by HEA125xOKT3

Specific lysis of target lines reached 50–70% in a standard chromium release assay. Unspecific lysis induced by the addition of a mixture of parental Abs HEA125 and OKT3 or an irrelevant unspecific bsAb was <20%. Titration of the bsAb HEA125xOKT3 indicated that concentrations as low as 1 ng/ml are sufficient for the induction of cytotoxicity; maximum lysis is, however, reached at a concentration of 1–10 μg/ml. Determination of the effective antibody concentration is important with regard to future clinical application. Moreover, the yield of bsAb, isolated from the hybrid-hybridoma supernatant, varies between 5 and 10% of total immunoglobulin content and requires costly purification processes.

Some Phase I studies (12–14) showed already the successful application of bsAbs for the treatment of ovarian carcinoma. Canevari et al. (12) conducted a trial with bsAb OC/TR, which is directed against the folate binding protein on carcinoma cells as well as the CD3 molecule on T cells. They demonstrated that locoregional immunotherapy of ovarian cancer using prestimulated PBMCs retargeted by bsAb can result in tumor regression without severe side effects. The study by Kroesen et al. (13) indicated that bsAb therapy in patients with malignant ascites or pleural exudates induces antitumor activity and a strong local inflammatory reaction. Their bsAb was directed against tumor-associated antigen AMOC-31 on carcinomas and the CD3 complex on T cells. These clinical studies used in vitro preactivated, expanded PBMCs loaded with bsAb as effector cells. The process of effector cell stimulation and expansion is laborious and time-consuming and requires exhaustive cell culture work for each individual patient. It was reported that lymphocytes isolated from ovarian malignant ascites exhibit cytotoxicity against autologous tumor cells after stimulation and expansion by IL-2 and irradiated autologous tumor cells (36). Therefore, we tested whether TALs from malignant ascites or pleural effusion could be used as effector cells for bsAb-directed immunotherapy.

TALs from three patients with malignant ovarian ascites and two patients with mammary pleural effusions were examined. Cells were stimulated by OKT3 antibody and IL-2 in a short-term (4-day) culture and tested for the induction of lysis toward tumor cells in the presence of bsAb HEA125xOKT3. A strong cytotoxic response ranging from 28 to 57% specific lysis was obtained in case of autologous target cells exceeding unspecific lysis 3–27-fold. Stimulated TALs elicit preferential killing of autologous tumor cells as compared with allogeneic tumor cells (data not shown), supporting the results published by Ioannides et al. (36). Our findings clearly indicate that both the CD3+CD4+ and the CD3+CD8+ lymphocyte subsets exhibit comparable cytolytic activity, although the lytic activity of the CD3+CD8+ population outnumbers that of the CD3+CD4+ subset to a minor extent.

One important issue concerns the question of whether TALs are already preactivated in situ via continuous stimulation by autologous tumor cells. To test this hypothesis, activation markers exposed on TALs freshly isolated from ascites or pleural effusion were analyzed by flow cytometry and compared

time-consuming activation and expansion of effector cells for each individual patient in vitro.

In an allogeneic test system, we demonstrated that bsAb HEA125xOKT3 can efficiently retarget PBMCs toward various Egp34-expressing tumor cells. Specific lysis of target lines reached 50–70% in a standard chromium release assay. Unspecific lysis induced by the addition of a mixture of parental Abs HEA125 and OKT3 or an irrelevant unspecific bsAb was <20%. Titration of the bsAb HEA125xOKT3 indicated that concentrations as low as 1 ng/ml are sufficient for the induction of cytotoxicity; maximum lysis is, however, reached at a concentration of 1–10 μg/ml. Determination of the effective antibody concentration is important with regard to future clinical application. Moreover, the yield of bsAb, isolated from the hybrid-hybridoma supernatant, varies between 5 and 10% of total immunoglobulin content and requires costly purification processes.

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with those from TALs that were stimulated with a mixture of OKT3 and IL-2. A striking difference was observed in the expression of the CD69 differentiation antigen. CD69 has been reported as an early activation marker (41) because it appears on the cell surface of T cells as soon as 3–24 h after activation within the CD4 and CD8 subset. We found that 35% of the CD8+ and 18% of CD4+ TALs are CD69 positive, even after 4 days of stimulation. CD25, CD95, and HLA-DR are also upregulated on CD4+ and CD8+ T cells. Interestingly, the comparison with unstimulated TALs revealed that although the expression of activation markers increases after stimulation, they are not absent from the unstimulated population. Eight% of the CD4+ TALs and 37% of the CD8+ unstimulated TALs stain positive for CD69, indicating that these cells are already preactivated.

Along this line, the lytic capacity of unstimulated TALs in comparison with OKT3/IL-2-stimulated TALs was tested. For five carcinoma patients, we could demonstrate that unstimulated, autologous TALs targeted to tumor cells by bsAb HEA125xOKT3 induced specific lysis of 16–29%, indicating that reasonable portions of TALs are not anergic and can be rendered cytotoxic in the presence of bsAb HEA125xOKT3. The lytic ability of unstimulated TALs seems to be dependent on the exposure to autologous tumor cells in vivo, because unstimulated PBMCs from the same patient are not able to lyse the autologous tumor cells in the presence of HEA125xOKT3. This is also reflected by the differential expression of activation markers on unstimulated TALs and PBMCs. Only CD25 is expressed on a small population (5%) of CD4+ PBMCs, whereas CD4+ as well as CD8+ TALs express both activation antigens CD69 and CD25. Nevertheless, we cannot formally exclude the possibility that activation of TALs is caused by the isolation procedure itself. T cells isolated by Percoll density gradient centrifugation from peripheral blood using the identical separation method as for TALs show no expression of the early activation antigen CD69 speaking against artificial preactivation of TALs.

Although the lytic capacity of OKT3/IL-2-stimulated TALs exceeds that of unstimulated TALs about 1.5–2 times, it is questionable whether this difference will be of importance for local application of bsAb to ovarian and mammary carcinoma patients. Mack et al. (42) have demonstrated that CD8+ T cells reach maximum tumor cytotoxicity within 4 h, whereas CD4+ T cells need about 20 h to reach similar levels (42), indicating that T-cell cytotoxicity is maintained over a certain time period. Taking into account that lymphocytes recycle their lytic activity (43), one can envision that several target cells are subsequently lysed by one cytotoxic T cell.

Recent reports discussed alternative methods to circumvent ex vivo stimulation. Kroesen et al. (26) induced activation of PBMCs by co-incubation with three different bsAbs, one specificity of which is directed against the pan-carcinoma-associated antigen EGP-2, the other recognizing either CD3, CD28, or CD5 molecules on T cells. Complete T-cell stimulation was gained by simultaneous ligation of CD3, CD28, and CD5 and could not be augmented further by the addition of IL-2. A combination of bsAbs might result in enhanced local efficacy of CTLs, including tumor-specific homing and less systemic toxic side effects, because application of IL-2 can be avoided. Mazzoni et al. (27) could show that the cross-linking of tumor and effector cells by a combination of two bsAbs directed against the folate binding protein on carcinoma cells as well as CD3 and CD28 molecules on T cells induce T-cell activation and antitumor activity in <24 h. This indicates that costimulatory signals delivered via CD28 in combination with TCR triggering via CD3 will reduce the time required for activation, increase self-sustained proliferation, and thereby improve bsAb-mediated immunotherapy.

In conclusion, our results offer a novel modality of bsAb-directed tumor cell lysis. Ex vivo preactivation of effector cells can be omitted, taking advantage of the preactivated state of TALs. After this therapeutic strategy, local injection of bsAb HEA125xOKT3 might render the lymphocytes in malignant ascites or pleural effusion cytotoxic toward autologous tumor cells. Because the development of malignant ascites is common to several end-stage malignancies, we are going to extend our preclinical investigations to other tumors as well.

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Without Prior Stimulation, Tumor-associated Lymphocytes from Malignant Effusions Lyse Autologous Tumor Cells in the Presence of Bispecific Antibody HEA125xOKT3

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