Cancer Immunol Immunother (2002) 51: 621–629
DOI 10.1007/s00262-002-0326-y

ORIGINAL ARTICLE

**Christof Schweizer · Gudrun Strauss · Matthias Lindner
Alexander Marmé · Yashwant M. Deo
Gerhard Moldenhauer**

Efficient carcinoma cell killing by activated polymorphonuclear neutrophils targeted with an Ep-CAM×CD64 (HEA125×197) bispecific antibody

Received: 25 May 2002 / Accepted: 8 August 2002 / Published online: 8 October 2002

Abstract Bispecific antibodies (bsAb) have attracted much attention over the past several years as a mean to improve immunotherapy of cancer. Due to their dual specificity, bsAb are able to redirect effector cells against tumor targets. In this study, the development and preclinical testing of a new quadroma-derived bsAb, HEA125×197, recognizing the tumor-associated Ep-CAM antigen and the high affinity Fc receptor for IgG, CD64, is reported. Using granulocyte-colony stimulating factor (G-CSF) and interferon-gamma (IFN-γ)-stimulated polymorphonuclear neutrophils to induce CD64 expression, bsAb HEA125×197 elicited strong cytotoxic activity towards allogeneic and autologous ovarian carcinoma cells. The cytolytic efficiency of this antibody was comparable to that of a previously described bsAb, HEA125×OKT3, targeting preactivated T lymphocytes against Ep-CAM-carrying tumor cells. Based on the pan-carcinoma specificity and the stable expression of Ep-CAM, bsAb HEA125×197 may broaden the spectrum of bispecific reagents for the treatment of epithelial malignancies.

Keywords Bispecific antibody · Ep-CAM · Fc receptor · Immunotherapy · Ovarian carcinoma · Tumor targeting

C. Schweizer · G. Moldenhauer (✉)
Department of Molecular Immunology (G0400),
German Cancer Research Center,
Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
E-mail: g.moldenhauer@dkfz.de
Tel.: +49-6221-423750
Fax: +49-6221-401629

G. Strauss
Department of Obstetrics and Gynecology,
University of Heidelberg, Children's Hospital,
University of Ulm, Ulm, Germany

M. Lindner · A. Marmé
Heidelberg, Germany

Y.M. Deo
Medarex Inc., Annandale, NJ, USA

Introduction

Bispecific antibodies (bsAb) are artificial proteins which carry two different antigen binding sites. They can be produced either by chemical coupling of different immunoglobulin Fab' fragments at the hinge region or by cell fusion of two hybridoma cell lines, resulting in a quadroma cell that secretes amongst other immunoglobulin combinations bsAb [26]. Alternatively, genetic engineering allows the creation of a variety of bispecific molecules of different sizes and binding strengths [17]. By virtue of their dual specificity, bsAb can trigger effector cells via a membrane receptor and at the same time link them to a tumor cell. This interaction leads to the subsequent destruction of the tumor cell [34].

Most of the early studies investigating bsAb for therapy of malignancies have focused on T lymphocytes as effector cells. For this, T cell activation was achieved by ligation of the T cell receptor-associated CD3 epsilon chain. Such anti-T cell×anti-tumor cell bsAb have been used for the treatment of non-Hodgkin's lymphoma and solid tumors like ovarian and renal cell cancer [4, 7, 15]. To become fully activated, T cells require costimulatory signals via the CD28 receptor, making this approach not easy to perform. Unfortunately, the global activation of T cells in the circulation following intravenous bsAb application causes severe and dose-limiting side effects through the release of several cytokines.

More recently, cells of the myeloid lineage comprising granulocytes, monocytes, macrophages and dendritic cells (DC) have received attention as potentially better-suited effector cells. Polymorphonuclear neutrophils (PMN) are for instance the most abundant circulating white blood cells, and are able to elicit strong cytolytic and phagocytic activities. They release soluble chemotactic factors that recruit further nonspecific and specific immune effector cells [6]. Myeloid cells express constitutively or upon activation Fc receptors which represent exquisite trigger molecules for the induction of cytotoxicity [22]. Especially the high affinity receptor for

IgG, termed FcγRI or CD64, has been identified as an attractive target molecule for bsAb-based immunotherapy of cancer [5]. CD64 constitutes an activating Fc receptor on monocytes, macrophages and DC, whereas its expression can be induced on neutrophils and eosinophils by interferon-gamma (IFN-γ) or granulocyte-colony stimulating factor (G-CSF) treatment. Importantly, induction of FcγRI on granulocytes and substantial upregulation on macrophages can be easily achieved in patients by systemic application of the respective cytokine or growth factor [16].

Several bsAb recognizing the CD64 molecule in conjunction with an epithelial tumor-associated antigen (TAA) have been established. The tyrosine kinase receptor HER2/neu [12, 20, 21, 32, 33, 35], the EGF receptor [3, 30, 37] and the MUC1 antigen [1] are prominent examples of the second binding specificity for those bsAb being prepared or having already entered clinical testing. So far, most bsAb trials taking advantage of FcγRI targeting have demonstrated low toxicity and resulted in tumor regression in some patients [3]. Ep-CAM (also known as 17–1A, GA733–2 and EGP-2 antigen) represents another epithelial glycoprotein that is overexpressed on virtually all carcinoma cells [18, 19]. One striking feature of this antigen is its stable expression during malignant transformation; the molecule is retained even in highly de-differentiated carcinomas. In addition, Ep-CAM is not released into the blood of patients [2].

The present study demonstrates the cytotoxic efficacy of the newly developed bsAb HEA125×197 by redirecting activated polymorphonuclear granulocytes towards autologous ovarian carcinoma cells. The cytolytic potency of this reagent is throughout comparable to that of the recently described bsAb HEA125×OKT3 (Ep-CAM×CD3) in combination with preactivated T lymphocytes. To the best of our knowledge, this is the first report on the characterization of an Ep-CAM×CD64-reactive bsAb.

Materials and methods

Production and purification of bsAb HEA125×197

Bispecific antibody HEA125×197 (specificity Ep-CAM×CD64) was produced using the mouse hybrid-hybridoma technique. Briefly, bsAb was prepared by fusing the hybridoma cell lines HEA125 (IgG1, directed against the epithelial glycoprotein Ep-CAM) [18] and 197 (IgG2a, directed against an epitope distinct from the ligand binding site of the high-affinity FcγRI, CD64) [9]. After several rounds of subcloning and testing for the secretion of bi-isotypic antibodies a stable quadroma cell line was established. Quadroma cells were cultured in a Miniperm bioreactor (Vivascience, Hannover, Germany). Supernatant containing the antibody mixture was purified first by affinity chromatography on a protein A-Sepharose CL-4B column (Amersham Pharmacia Biotech Europe, Freiburg, Germany) to remove IgG1 parental antibodies. Subsequently, the eluant was subjected to HPLC purification on a Bakerbond ABx column (T.J. Baker, Philipsburg, N.J.) which allowed separation of bispecific antibody using a morpholinoethane sulfonic acid (MES)/sodium acetate gradient. The purity of eluted material was assessed by SDS-PAGE under reducing conditions. The establishment of the HEA125×OKT3 (specificity Ep-CAM×CD3) hybrid-hybridoma that was employed for comparative cytotoxicity studies has been described recently [29].

Cell lines

The Ep-CAM-expressing ovarian adenocarcinoma cell line SK-OV-3 and the promyelocytic leukemia line HL-60 were obtained from the American Type Culture Collection (Manassas, Va.). Cells were grown in RPMI 1640 medium (Life Technologies, Paisley, U.K.) supplemented with 10% heat-inactivated FCS (Conco, Wiesbaden, Germany), 2 mM L-glutamine, and 1 mM sodium pyruvate. The CiMe cell line was newly established from an HLA-A*0201-positive patient with ovarian carcinoma. Tumor cells were separated from ascitic fluid by density gradient centrifugation and cultured in DMEM medium (Life Technologies) containing 10% fetal calf serum (FCS), penicillin/streptomycin, 2 mM L-glutamine, and 1% insulin-transferrin-sodium selenite media supplement (Sigma, St. Louis, Mo.). The phenotype of CiMe cells was analyzed by flow cytometry and cytospin staining: they express cytokeratins 8 and 18, MHC class I, Ep-CAM, CD40 and CD95. The costimulatory molecules CD80 and CD86 are absent from the cell line.

Isolation of peripheral blood lymphocytes and polymorphonuclear cells

Peripheral blood lymphocytes (PBL) and PMN were isolated from buffy coat obtained from healthy donors via the blood bank of the University of Heidelberg or from heparinized peripheral blood of ovarian cancer patients. Isolation of PBL was carried out by density centrifugation of diluted (1:2 in PBS) buffy coat on Ficoll-Paque Plus (Amersham Pharmacia Biotech) at 400 *g* for 20 min at room temperature. The PBL interphase was washed twice in complete RPMI 1640 medium. For PMN isolation, buffy coat was mixed with an equal volume of dextran solution [3% dextran T-500 (Amersham Pharmacia Biotech) in 0.9% NaCl] and incubated in an upright position for 20–30 min at room temperature. The upper layer (leukocyte-rich plasma) was aspirated and centrifuged (10 min at 250 *g*, 5°C). The pellet was resuspended in 0.9% NaCl and overlaid with 10 ml Ficoll-Paque Plus. After centrifugation (40 min at 400 *g* at 20°C without brake), the neutrophil/RBC pellet was subjected to hypotonic lysis to remove residual erythrocytes. Culture of PBL and PMN was performed in RPMI 1640 supplemented with 2% heat-inactivated FCS, 2 mM L-glutamine, 1 mM pyruvate, and 0.05 mM 2-ME.

Effector cell activation

In order to activate resting T cells, PBL were cultured at a concentration of 2×10^6 cell/ml in medium containing OKT3 (5 μg/ml; the authors' own preparation) and recombinant human IL-2 (20 U/ml; Eurocetus, Frankfurt, Germany). After 3 days, cells were washed twice to remove remaining cell-bound antibody and cultured overnight in medium alone. On day 4, a cytotoxicity assay was performed. The PMN were activated by incubation in the presence of IFN-γ (150 U/ml; Roche Diagnostics, Mannheim, Germany) and G-CSF (300 U/ml; Neupogen) for 3 days.

Cytotoxicity assay

Cytotoxicity 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. Fc-receptor binding of the PMN was blocked by the addition of 2.5 mg/ml polyclonal human immunoglobulin (Endobulin; Immuno, Heidelberg, Germany). Cytolytic activity was assessed using a standard ^{51}Cr release assay. Target cells (2×10^6) were labeled with 200 μCi sodium $^{51}CrO_4$ (Amersham Pharmacia Biotech) followed by 4

washing cycles, and resuspended in medium at a concentration of 1×10^5/ml. Effector cells were adjusted to a concentration of 5×10^6/ml. Increasing numbers of effector cells in 100 µl were titrated to 5×10^3 target cells/well in 50 µl; 50 µl of antibody solution (10 µg/ml) was added to each well. The whole assay was set up in triplicate and incubated for 4 h at 37°C. One hundred microliters 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% sodium dodecyl sulphate (SDS), and spontaneous release was calculated from a sample to which 100 µl of medium was added instead of effector cells. The percentage of specific release was determined as:

$$\%\ \text{specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Flow cytometry

Target and effector cells were analyzed for surface markers by indirect immunofluorescence. Approximately 1×10^6 cells were stained with the following mAb: HEA125 (anti-Ep-CAM, IgG1), 197 (CD64, IgG2a) and HD20 (anti-idiotype, IgG1, serving as a negative control). Isotype-specific, FITC-labeled goat-anti mouse IgG1 and IgG2a antibodies (Southern Biotechnology Associates, Birmingham, Ala.) were used as second-step reagents. Dead cells were discriminated by propidium iodide staining (Sigma, Deisenhofen, Germany). To prevent mAb interaction with the natural ligand binding site of Fc receptors, all incubations were carried out in the presence of 2.5 mg/ml polyclonal human IgG (Endobulin). Analysis was performed on a FACScan cytometer (Becton Dickinson, Heidelberg, Germany) using CellQuest software.

Results

Establishment of the quadroma line, production and purification of bsAb HEA125×197

The quadroma cell line HEA125×197 was raised by fusion of the HGPRT-deficient hybridoma HEA125 (anti-Ep-CAM, IgG1 isotype) and the iodoacetamide-treated hybridoma 197 (anti-CD64, IgG2a isotype). After cell fusion, 39 clones grew out under selection in HAT medium. They were initially tested for the production of both parental immunoglobulin species by a double isotype ELISA and for reactivity with cells expressing the respective antigens using flow cytometry. The quadroma line showing the highest immunoglobulin secretion rate was subsequently cloned twice by limiting dilution and established as hybrid-hybridoma clone HEA125×197.

For medium-scale production of bsAb, a modular miniaturized bioreactor was employed. Since quadroma cell lines produce the desired bispecific antibody as well as different immunoglobulin missense combinations, the bsAb had to be purified by a 2-step method. The first step consisted of protein A affinity chromatography and the second step of HPLC separation of the protein A eluant on a Bakerbond ABx column. The HPLC elution profile using a sodium acetate gradient in MES is depicted in Fig. 1A. Samples of the eluted fractions were separated by SDS-PAGE and analyzed by flow cytometry to identify those fractions containing the bsAb.

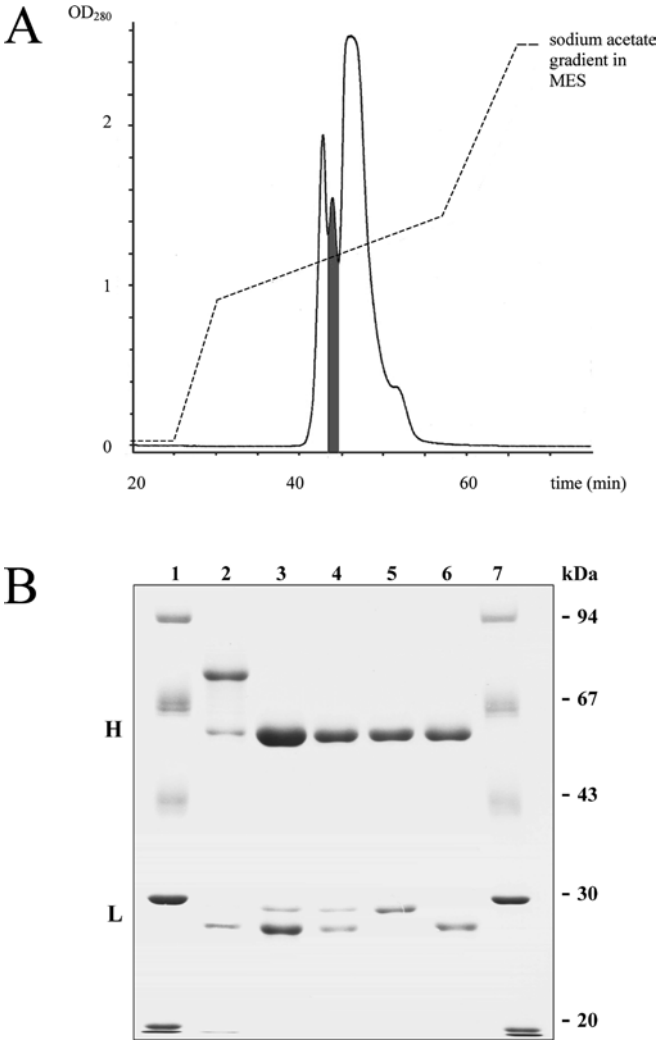

Fig. 1 **A** Purification of bsAb HEA125×197 by HPLC separation on a Bakerbond ABx column. Elution was performed using a sodium acetate gradient in morpholinoethane sulfonic acid (MES). The *shaded peak* contained the bispecific antibody. **B** SDS-PAGE analysis under reducing conditions of HEA125×197 preparations in comparison with parental mAb. *Lanes 1 and 7*, molecular weight marker; *lane 2*, crude bioreactor harvest; *lane 3*, protein A-Sepharose CL-4B purified material of harvest; *lane 4*, HPLC-purified bispecific antibody; *lane 5*, purified mAb HEA125; *lane 6*, purified mAb 197. The positions of immunoglobulin heavy (*H*) and light (*L*) chains are indicated

A representative SDS-PAGE under reducing conditions is displayed in Fig. 1B. Noteworthy, the apparent electrophoretic mobilities of the parental antibodies differed in the case of the light chains (lanes 5 and 6). This allows a rough estimate of the relative amounts of both parental immunoglobulins. After protein A purification of the crude bioreactor-derived supernatant (lane 2), a clear overrepresentation of IgG2a immunoglobulin species containing bsAb and parental antibody 197 was seen, which also reflects a loss of parental antibody HEA125. By a further purification procedure using a Bakerbond ABx column the parental 197

antibody was removed, resulting in a high enrichment of bsAb (lane 4) as indicated by the nearly equimolar representation of both immunoglobulin light chains. The yield of pure bsAb HEA125×197 was in the range of 10–20% of total immunoglobulins.

Immunoreactivity of the purified bsAb was evaluated by flow cytometry (Fig. 2). For this, the ovarian carcinoma cell line SK-OV-3 (Ep-CAM$^+$, CD64$^-$) and the myeloid cell line HL60 (CD64$^+$, Ep-CAM$^-$) served as target cells. Surface expression of the CD64 antigen on

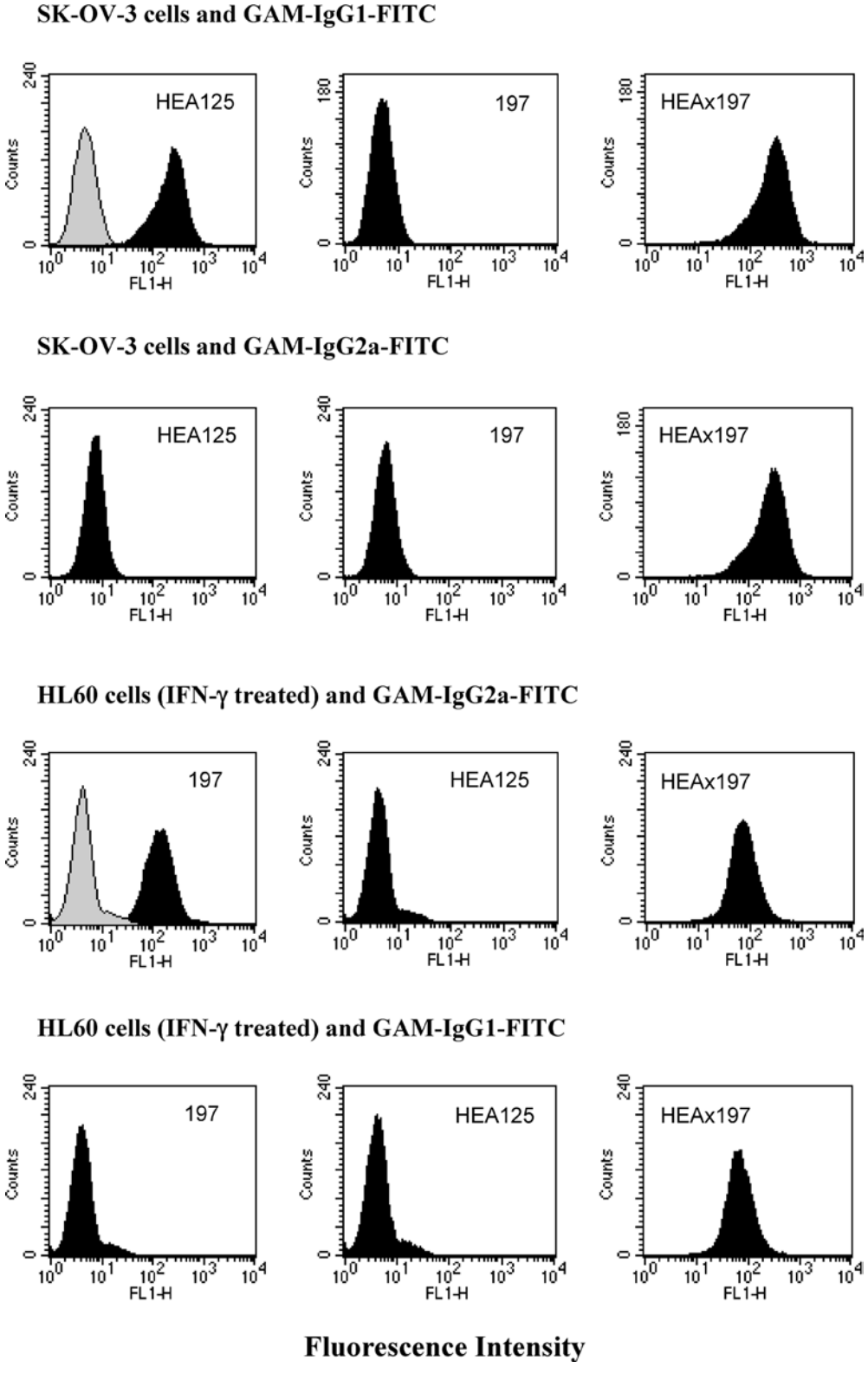

Fig. 2 Flow cytometric analysis of reactivity of bsAb HEA125×197. Binding of indicated mAb and bsAb to SK-OV-3 (Ep-CAM$^+$ and CD64$^-$) and HL60 (Ep-CAM$^-$ and CD64$^+$) target cells was detected with isotype-specific, FITC-labeled second-step reagents. CD64 expression on HL60 cells was induced by treatment with IFN-γ (150 U/ml). The *shaded histograms* represent the negative controls using the irrelevant mAb HD20

HL60 cells was up-regulated by culturing the cells in medium containing IFN-γ. The parental mAb HEA125 (IgG1) binds to its antigen on SK-OV-3 cells, and can solely be detected with FITC-labeled GAM-IgG1 antibodies. Since CD64 antigen is not expressed on SK-OV-3 cells, no staining was observed. By contrast, bsAb HEA125×197 interacted with the Ep-CAM antigen and was detected by both isotype-specific antibodies GAM-IgG1- and GAM-IgG2a-FITC. The corresponding staining was performed on HL60 cells. To minimize nonspecific Fc receptor-mediated binding, all incubations were carried out in medium containing 5% pooled human IgG. On myeloid HL60 cell line parental antibody 197 (IgG2a) bound to the CD64 antigen as detected by GAM-IGg2a-FITC second step reagent. mAb HEA125 did not show any specific reaction with HL60 cells that are devoid of the Ep-CAM antigen. Again, only bsAb HEA125×197 was reactive with HL60 cells using both GAM-IgG1- and GAM-IgG2a-FITC secondary antibodies. These results clearly demonstrated that the two binding sites of the bispecific antibody are functional in that they interact with both Ep-CAM and CD64 molecules.

bsAb HEA125×197 induce lysis of Ep-CAM-expressing ovarian carcinoma cells by activated allogeneic PMN: comparison with cytotoxicity elicited by T lymphocytes

The ability of bsAb HEA125×197 to direct allogeneic PMN against carcinoma cells was tested by a chromium release assay. Polymorphonuclear effector cells from healthy donors were isolated from buffy coat by dextran sedimentation followed by density gradient centrifugation. Stimulation of PMN was achieved by incubation for 3 days in medium containing IFN-γ and G-CSF. As target cells we used the cell line CiMe which was established recently from a patient with advanced ovarian carcinoma, and which was shown to express Ep-CAM. In the presence of 10 μg/ml bsAb HEA125×197, a standard chromium release assay with increasing effector:target (E:T) cell ratios was carried out. In previous titration experiments this bsAb concentration was found to be optimal (data not shown). The parental antibodies HEA125 and 197 – alone and as a mixture – at the same concentration as well as medium served as negative control.

The results of a representative experiment are shown in Fig. 3A. At an E:T cell ratio of 100:1, bsAb HEA125×197 yielded 43% specific lysis of CiMe cells. Addition of parental mAbs or a mixture of them resulted in background lysis of 10% or less. The specific cytotoxicity induced by the bsAb declined with the decrease of E:T ratios used (21% at 50:1, 10% at 25:1 and 4% at 12:1). In the respective controls background lysis was always below 5%. Cell line SK-OV-3 was even more susceptible to bsAb HEA125×197-induced lysis by PMN; 62% specific cytotoxicity was obtained at an E:T ratio of 100:1 (data not shown). To block binding of

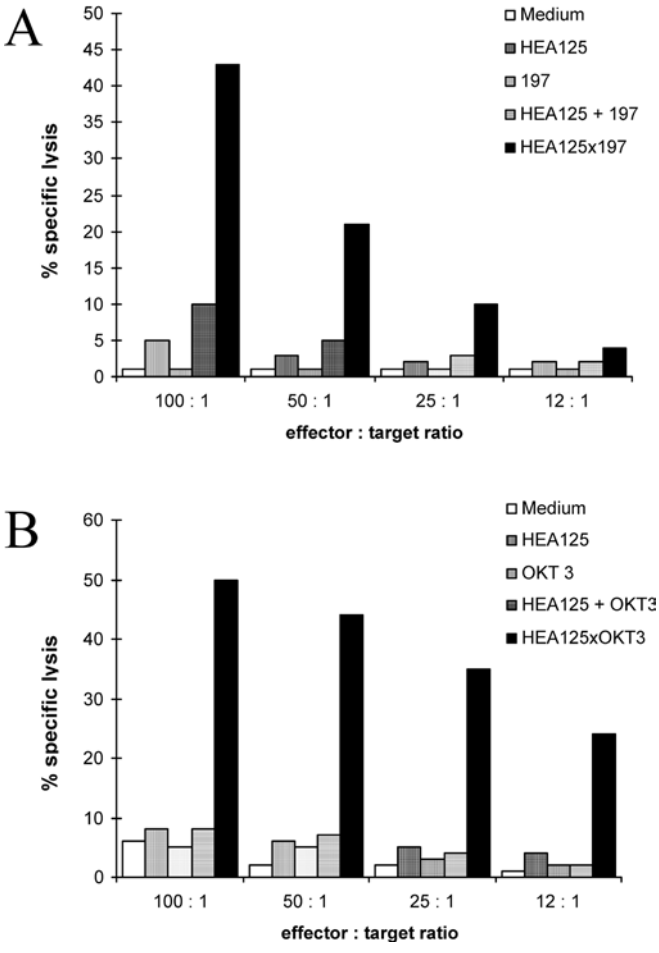

Fig. 3 **A** BsAb HEA125×197-induced cytotoxic activity of allogeneic PMN towards the ovarian carcinoma line CiMe. PMN were stimulated in vitro by INF-γ (150 U/ml) and G-CSF (300 U/ml). The indicated antibodies were added at a final concentration of 10 μg/ml. ^{51}Cr-labeled target cell were incubated with the effector cells at different E:T ratios for 4 h. **B** bsAb HEA125×OKT3-induced cytotoxicity of allogeneic PBL towards the CiMe target cells. PBL were stimulated in vitro by IL-2 (20 U/ml) and mAb OKT3 (5 μg/ml). The indicated antibodies were added at a final concentration of 10 μg/ml

antibodies via the ligand binding site of Fc receptors, polyclonal human IgG was applied in all cytotoxicity assays.

Having established the cytotoxic capacity of bsAb HEA125×197-directed PMN, we aimed to compare this with the lytic potential of cytotoxic T lymphocytes. For this, PBL of healthy volunteers were isolated from buffy coat by Ficoll Plus separation and were stimulated with soluble OKT3 antibody and IL-2. Effector cell targeting to ^{51}Cr-labeled CiMe cells was achieved by bsAb HEA125×OKT3 recognizing the epsilon-chain of the CD3 molecule on T lymphocytes. At the 100:1 E:T ratio, 50% of the target cells were lysed specifically (Fig. 3B). Specific killing dropped to 35% at an E:T ratio of 25:1 and to 24% at an E:T ratio of 12:1. In this particular experiment, background cytotoxicity in control samples never exceeded 8%. Both cytotoxicity assays were

performed with the identical target cell line and under the same incubation conditions. Together, the maximum specific lysis induced by HEA125×197-redirected PMN was comparably high as that induced by HEA125×CD3-redirected T cells. At lower E:T ratios, however, the cytotoxicity caused by PMN exhausted faster than that of T cells.

Activated PMN kill autologous carcinoma cells in the presence of bsAb HEA125×197

An important issue concerns the question whether not only allogeneic but also autologous PMN could be directed towards ovarian carcinoma cells. For this purpose, we isolated PMN from the peripheral blood of the same patient that the cell line CiMe was established from. The purified PMN were stimulated with IFN-γ and G-CSF for 3 days to enhance surface expression of the CD64 molecule. The effect of cytokine stimulation was monitored by flow cytometry (Fig. 4A). The activated PMN stained positive for both mAb 197 and bsAb HEA125×197 indicative for the expression of CD64. No specific binding was detected with the parental mAb HEA125 because the Ep-CAM antigen is not expressed on the PMN.

The cytotoxic capacity of the activated PMN redirected by the bsAb HEA125×197 was tested in a standard chromium release assay. The result of the assay is depicted in Fig. 4B. The addition of bsAb HEA125×197 to ^{51}Cr-labeled CiMe cells and preactivated PMN was compared with the effect of the parental antibodies (as single agent and as a mixture) and with medium alone. The maximum specific lysis induced by the bsAb was 47% at the E:T ratio 100:1. The effect ceased with decreasing amount of effector cells at the lower E:T ratios. The controls reached a maximal background lysis of 11% at an E:T ratio of 100:1 using the mixture of both parental antibodies. This experiment clearly demonstrated the efficacy of activated autologous PMN derived from a patient suffering from late stage ovarian carcinoma to induce a vigorous cytotoxic response in the

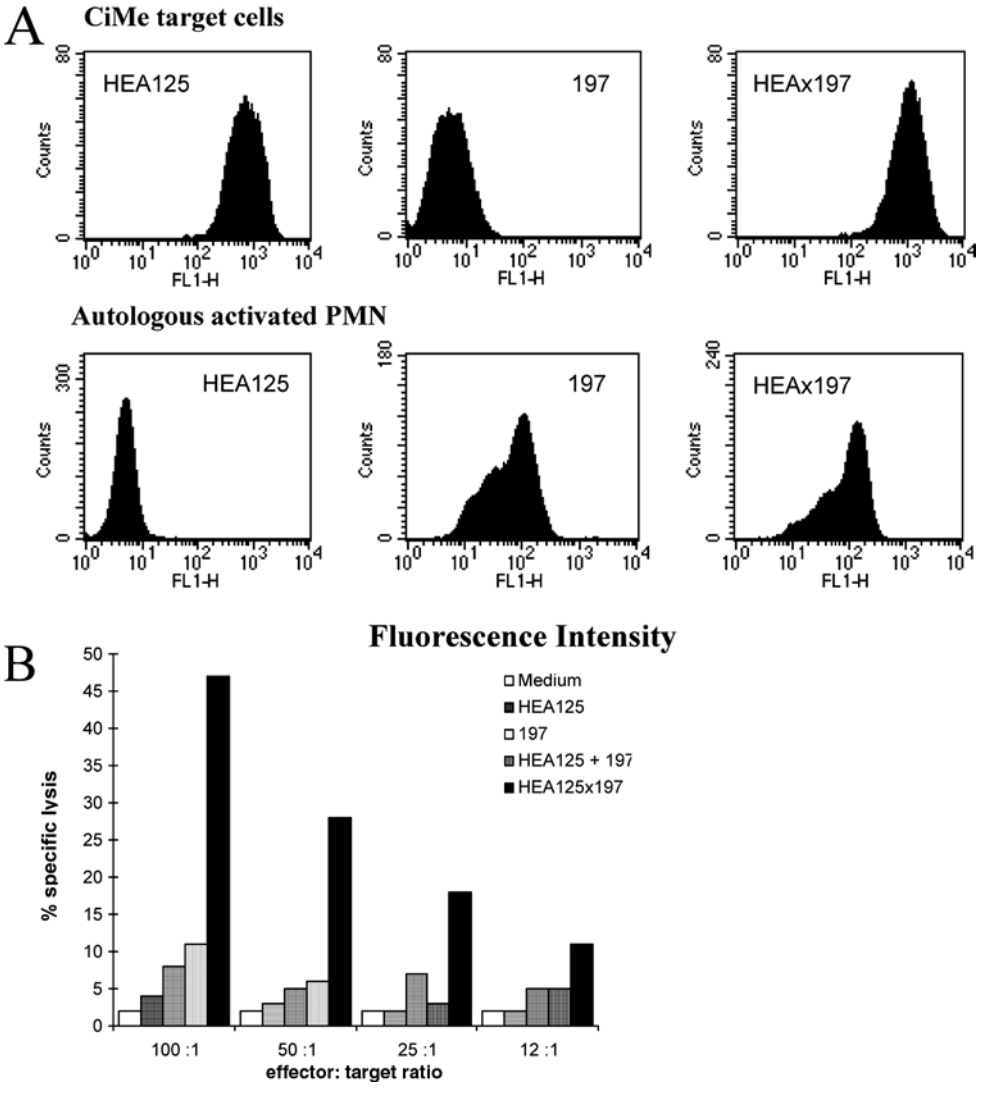

Fig. 4 A Phenotypic analysis of ovarian carcinoma line CiMe and of autologous PMN both isolated from the same patient. The effector cells were activated in vitro with INF-γ (150 U/ml) and G-CSF (300 U/ml). Binding of indicated antibodies was detected with FITC-labeled goat anti-mouse IgG/Fc reagent. **B** bsAb HEA125×197-induced cytolysis of CiMe carcinoma cells by autologous preactivated PMN. The indicated antibodies were added at a final concentration of 10 μg/ml

presence of bsAb HEA125×197. The maximum lysis observed in case of autologous effector cells was entirely comparable with the lysis achieved by allogeneic effector cells from healthy donors.

Discussion

In the present study, we investigated the cytolytic activity of polymorphonuclear neutrophils targeted towards ovarian carcinoma cells with a newly established bsAb, HEA125×197. This antibody is directed against the epithelial glycoprotein Ep-CAM on carcinoma cells and the high-affinity FcγRI receptor, CD64, on myeloid cells. The pan carcinoma marker Ep-CAM appears to be specially suited for immunotherapeutic approaches since its expression is rather stable even in de-differentiated epithelial neoplasms [19]. The quadroma line HEA125×197 was generated by cell fusion of the parental hybridoma lines. Larger quantities of bsAb were produced in a miniaturized bioreactor and subsequently purified by a 2-step method. We demonstrated that the bsAb efficiently induced the lysis of carcinoma cells by preactivated PMN. This proof of principle was obtained with an antibody of mouse origin. For future therapeutic application in patients a humanized version of the Ep-CAM×CD64 reagent appears desirable to prevent adverse effects based on the formation of a human anti-mouse antibody (HAMA) response.

We compared the lytic capacity of the HEA125×197 bsAb with that of the previously described bispecific HEA125×OKT3 reagent. Tumor cell killing mediated by HEA125×OKT3 is induced by CD3-positive T lymphocytes stimulated in vitro with OKT3 mAb and low-dose IL-2 29]. Maximum specific cytotoxicity of bsAb HEA125×197 and preactivated PMN (43% at an E:T ratio of 100:1) compared favorably with that achieved with bsAb HEA125×OKT3 and preactivated T cells (50% also at an E:T ratio of 100:1). At lower E:T ratios, however, the cytotoxicity caused by PMN exhausted faster than that of T cells. This may indicate that for a sustained cytotoxic anti-tumor response a higher number of bsAb-directed PMN are required. Further on, bsAb HEA125×197 was evaluated in an autologous setting. This was achieved by the use of PMN from the same ovarian carcinoma patient that the target cell line CiMe was established from. Interestingly, the maximum lysis of 47% reached at an E:T ratio of 100:1 with autologous effector cells was even slightly higher than that obtained with allogeneic effector cells from healthy donors.

Several bsAb have already been described containing one specificity against the CD64 molecule to redirect myeloid effector towards epithelial tumor-associated antigens. Russoniello et al. developed the bsAb HCC49×H22 for the immunotherapy of TAG-72-expressing malignancies. They demonstrated that IFN-γ-treated monocytes induced a specific lysis of TAG-72-positive KLEB cells of about 30% [24]. The high molecular weight glycoprotein MUC1 (CD227) was used as target antigen on breast cancer cells by Akewanlop et al. Their bsAb DF3xH22 solely induced phagocytosis but not cytolysis of tumor cells by macrophages stimulated by GM-CSF with or without IFN-γ [1]. Valerius et al. used bispecific immunoglobulins reacting with the epidermal growth factor receptor (EGFR) which is, besides other malignancies, overexpressed on the majority of renal cell carcinomas. In preclinical studies they compared several bsAb, all recognizing the EGF-receptor but different Fc receptors (CD16, CD64, CD89) on human leukocytes. This study provided evidence that myeloid effector cells from patients treated with G-CSF or GM-CSF in combination with an EGFR×CD89 antibody achieved the strongest tumor cell killing [8]. MDX-447 represents a chemically coupled bsAb that reacts with the EGF receptor and CD64. It has been shown to direct macrophage-activated killer cells against glioma and epidermoid carcinoma cells [37]. In a phase I/II dose escalation trial, this reagent was employed with and without G-CSF to treat refractory patients with different EGFR-positive tumors [3].

The most extensively studied bispecific construct binding to CD64 and an epithelial antigen represents the murine bsAb MDX-210 and its humanized version, MDX-H210. These antibodies recognize the HER-2/neu protooncogene product that is overexpressed on a variety of adenocarcinomas such as breast cancer, ovarian cancer colon cancer, renal cancer, and prostate cancer. In numerous preclinical studies MDX-210 efficiently redirected CD64-positive effector cells such as neutrophils and macrophages against HER2-carrying carcinoma cells [14, 25, 28, 31, 38]. Cytokine stimulation of effector cells elicited different effects on bsAb-mediated tumor cell elimination. Culturing of macrophages with IFN-γ enhanced both tumor cell lysis and phagocytosis, whereas stimulation with M-CSF or GM-CSF solely increased the phagocytic capacity [14]. Although treatment of patients with GM-CSF and G-CSF significantly increased neutrophil counts, only the application of G-CSF induced the surface expression of CD64 on neutrophils and augmented bsAb-based cytotoxicity [27].

To investigate the in vivo effects of bsAb against CD64 more closely, Heijnen et al. developed a human FcγRI receptor transgenic mouse [10]. The animals express the transgene under control of endogenous regulatory elements. Treatment of mice with murine G-CSF increased the neutrophil counts and human FcγRI expression on PMN. Isolated PMN efficiently killed human breast cancer cells in the presence of a bsAb with anti-CD64 and anti-HER2 specificity in vitro. After intravenous administration, the bsAb coated the mouse PMN and remained bound during their migration. PMN armed in vivo with bsAb were able to kill tumor cells in vitro without prior stimulation [11]. Wallace et al. studied the in vitro phagocytic capacity of PMN and macrophages isolated from healthy donors and patients enrolled in a phase I clinical trial receiving

MDX-H210 and IFN-γ [36]. PMN were not able to induce bsAb-mediated phagocytosis prior to treatment, but were activated by in vivo administration of IFN-γ to kill tumor cells in a chromium release assay. By contrast, monocytes displayed a high phagocytic capacity regardless of IFN-γ-treatment of the patient.

bsAb MDX-210 and MDX-H210 have been evaluated in a series of clinical trials of patients suffering from breast, ovarian, colorectal and prostate cancer overexpressing the HER2 receptor [13, 21, 23, 32, 33, 35, 37]. These studies comprise phase I, phase I/II and phase II trials in which patients received intravenous bsAb infusions, frequently in conjunction with the administration of G-CSF, GM-CSF and IFN-γ. It was demonstrated that MDX-210 and MDX-H210 are generally well tolerated and that they can elicit biological effects such as elevation of cytokine levels in blood (e.g. TNF-α and IL-6). Although these responses proved its immunological activity, sustained anti-tumor responses occurred only in few patients. However, in some patients with prostate cancer a clinically significant decline of PSA levels was noted after MDX-H210 bsAb therapy, indicating a reduction of tumor mass [12].

One major limitation in the use of HER2 as target molecule for immunotherapeutic approaches is its overexpression in only a subset of carcinomas. This makes a laborious testing by immunohistology and/or western blotting necessary to confirm the level of expression prior to treatment. In addition, since carcinomas were frequently found to be heterogenous with respect to antigen decoration, loss variants may occur under therapy. These variant tumor cells might escape antibody attack and give rise to a therapy-resistant cell clone. Due to its pan-carcinoma specificity and its stable expression even in the neoplastic state, HEA125×197 antibody may broaden the spectrum of bispecific reagents for the treatment of epithelial malignancies. It is anticipated that targeting of the Ep-CAM antigen may allow the total eradication of tumor cells. The promising preclinical characteristics of bsAb HEA125×197 warrant further testing in clinical studies.

Acknowledgements We thank Elvira Hallauer for excellent technical assistance and Reinhard Schwartz-Albiez for critical reading of the manuscript. This work was supported by the Tumorzentrum Heidelberg/Mannheim.

References

1. Akewanlop C, Watanabe M, Singh B, Walker M, Kufe DW, Hayes DF (2001) Phagocytosis of breast cancer cells mediated by anti-MUC-1 monoclonal antibody, DF3, and its bispecific antibody. Cancer Res 61:4061
2. Balzar M, Winter MJ, de Boer CJ, Litvinov SV (1999) The biology of the 17–1A antigen (Ep-CAM). J Mol Med 77:699
3. Curnow RT (1997) Clinical experience with CD64-directed immunotherapy. An overview. Cancer Immunol Immunother 45:210
4. De Gast GC, Haagen IA, van Houten AA, Klein SC, Duits AJ, de Weger RA, Vroom TM, Clark M, Philips J, van Dijk AJG, de Lau WBM, Bast BJ (1995) CD8 T cell activation after intravenous administration of CD3×CD19 bispecific antibody in patients with non-Hodgkin lymphoma. Cancer Immunol Immunother 40:390
5. Deo YM, Graziano RF, Repp R, van de Winkel JGJ (1997) Clinical significance of IgG Fc receptors and FcγR-directed immunotherapies. Immunol Today 18:127
6. Di Carlo E, Forni G, Lollini PL, Colombo MP, Modesti A, Musiani P (2001) The intriguing role of polymorphonuclear neutrophils in antitumor reactions. Blood 97:339
7. Canevari S, Stoter G, Arienti F, Bolis G, Colnaghi MI, Di Re EM, Eggermont AM, Goey SH, Gratama JW, Lamers CH, Nooy MA, Parmiani G, Raspagliesi F, Ravagnani F, Scarfone G, Trimbos JB, Warnaar SO, Bolhuis RLH (1995) Regression of advanced ovarian carcinoma by intraperitoneal treatment with autologous T lymphocytes retargeted by a bispecific monoclonal antibody. J Natl Cancer Inst 87:1463
8. Elsässer D, Stadick H, Stark S, van de Winkel JGJ, Gramatzki M, Schrott KM, Valerius T, Schafhauser W (1999) Preclinical studies combining bispecific antibodies with cytokine-stimulated effector cells for immunotherapy of renal cell carcinoma. Anticancer Res 19:1525
9. Guyre PM, Graziano RF, Vance BA, Morganelli PM, Fanger MW (1989) Monoclonal antibodies that bind to distinct epitopes on FcγRI are able to trigger receptor function. J Immunol 143:1650
10. Heijnen IAFM, van de Winkel JGJ (1995) A human FcγRI/CD64 transgenic model for in vivo analysis of (bispecific) antibody therapeutics. J Hematother 4:351
11. Heijnen IAFM, Rijks LJM, Schiel A, Stockmeyer B, van Oijk HH, Dechant M, Valerius T, Keler T, Tutt AL, Glennie MJ, van Royen EA, Capel PJA, van de Winkel JGJ (1997) Generation of HER-2/neu-specific cytotoxic neutrophils in vivo: efficient arming of neutrophils by combined administration of granulocyte colony-stimulating factor and Fcγ receptor I bispecific antibodies. J Immunol 159:5629
12. James ND, Atherton PJ, Jones J, Howie AJ, Tchekmedyian S, Curnow RT (2001) A phase II study of the bispecific antibody MDX-H210 (anti-HER2×CD64) with GM-CSF in HER2$^+$ advanced prostate cancer. Br J Cancer 85: 152
13. Kaufman PA, Guyre PM, Lewis LD, Valone FH, Memoli VA, Wells WAA, Deo YM, Ernstoff MS, Fisher J, Mrozek-Orlowski M, Phipps K, Fanger MW (1996) HER-2/neu targeted immunotherapy: a pilot study of multidose MDX-210 in patients with breast or ovarian cancers that overexpress HER-2/neu and a report of an increased incidence of HER-2/neu overexpression in metastatic breast cancer. Tumor Targeting 2:17
14. Keler T, Graziano RF, Mandal A, Wallace PK, Fisher J, Guyre PM, Fanger MW, Deo YM (1997) Bispecific antibody-dependent cellular cytotoxicity of HER2/neu-overexpressing tumor cells by Fcγ receptor type I-expressing effector cells. Cancer Res 57:4008
15. Kroesen BJ, Bakker A, van Lier RA, The HT, de Leij L (1995) Bispecific antibody-mediated target cell-specific costimulation of resting T cells via CD5 and CD28. Cancer Res 55:4409
16. Lewis LD, Cole BF, Wallace PK, Fisher JL, Waugh M, Guyre PM, Fanger MW, Curnow RT, Kaufman PA, Ernstoff MS (2001) Pharmacokinetic-pharmacodynamic relationships of the bispecific antibody MDX-H210 when administered in combination with interferon-gamma: a multiple-dose phase-I study in patients with advanced cancer which overexpresses HER-2/neu. J Immunol Methods 248:149
17. Little M, Kipriyanov SM, Le Gall F, Moldenhauer G (2000) Of mice and men: hybridoma and recombinant antibodies. Immunol Today 21:364
18. Moldenhauer G, Momburg F, Möller P, Schwartz R, Hämmerling GJ (1987) Epithelium-specific surface glycoprotein of M_r 34,000 is a widely distributed human carcinoma marker. Br J Cancer 56:714
19. Momburg F, Moldenhauer G, Hämmerling GJ, Möller P (1987) Immunohistochemical study of the expression of a M_r

34,000 human epithelium-specific surface glycoprotein in normal and malignant tissues. Cancer Res 47:2883
20. Posey JA, Raspet R, Verma U, Deo YM, Keller T, Marshall JL, Hodgson J, Mazumder A, Hawkins MJ (1999) A pilot trial of GM-CSF and MDX-H210 in patients with erbB-2-positive advanced malignancies. J Immunother 22:371
21. Pullarkat V, Deo Y, Link J, Spears L, Marty V, Curnow R, Groshen S, Gee C, Weber JS (1999) A phase I study of a HER2/neu bispecific antibody with granulocyte-colony-stimulating factor in patients with metastatic breast cancer that overexpresses HER2/neu. Cancer Immunol Immunother 48:9
22. Ravetch JV, Bolland S (2001) IgG Fc receptors. Annu Rev Immunol 19:275
23. Repp R, Valerius T, Wieland G, Becker W, Steininger H, Deo Y, Helm G, Gramatzki M, van de Winkel JG, Lang N, Kalden JR (1995) G-CSF-stimulated PMN in immunotherapy of breast cancer with a bispecific antibody to Fc gamma RI and to HER-2/neu (MDX-210). J Hematother 4:415
24. Russoniello C, Somasundaram C, Schlom J, Deo YM, Keler T (1998) Characterization of a novel bispecific antibody that mediates Fcγ receptor type I-dependent killing of tumor-associated glycoprotein-72-expressing tumor cells. Clin Cancer Res 4:2237
25. Schwaab T, Lewis LD, Cole BF, Deo Y, Fanger MW, Wallace P, Guyre PM, Kaufman PA, Heaney JA, Schned AR, Harris RD, Ernstoff MS (2001) Phase I pilot trial of the bispecific antibody MDXH210 (anti-Fc gamma RI X anti-HER-2/neu) in patients whose prostate cancer overexpresses HER-2/neu. J Immunother 24:79
26. Segal DM, Weiner GJ, Weiner LM (1999) Bispecific antibodies in cancer therapy. Curr Opin Immunol 11:558
27. Stockmeyer B, Elsässer D, Dechant M, Repp R, Gramatzki M, Glennie MJ, van de Winkel JG, Valerius T (2001) Mechanisms of G-CSF- or GM-CSF-stimulated tumor cell killing by Fc receptor-directed bispecific antibodies. J Immunol Methods 248:103
28. Stockmeyer B, Valerius T, Repp R, Heijnen IAFM, Bühring H-J, Deo YM, Kalden JR, Gramatzki M, van de Winkel JGJ (1997) Preclinical studies with FcγR bispecific antibodies and granulocyte colony-stimulating factor-primed neutrophils as effector cells against HER-2/neu overexpressing breast cancer. Cancer Res 57:696
29. Strauss G, Gückel B, Wallwiener D, Moldenhauer G (1999) Without prior stimulation, tumor-associated lymphocytes from malignant effusions lyse autologous tumor cells in the presence of bispecific antibody HEA125×OKT3. Clin Cancer Res 5:171
30. Somasundaram C, Sundarapandiyan K, Keler T, Deo YM, Graziano RF (1999) Development of a trispecific antibody conjugate that directs two distinct tumor-associated antigens to CD64 on myeloid effector cells. Hum Antibodies 9:47
31. Valerius T, Repp R, de Wit TPM, Berthold S, Platzer E, Kalden JR, Gramatzki M, van de Winkel JGJ (1993) Involvement of the high-affinity receptor for IgG (FcγRI; CD64) in enhanced tumor cell cytotoxicity of neutrophils during granulocyte colony-stimulating factor therapy. Blood 82:931
32. Valone FH, Kaufman PA, Guyre PM, Lewis LD, Memoli V, Deo Y, Graziano R, Fisher JL, Meyer L, Mrozek-Orlowski M, Wardwell K, Guyre V, Morley TL, Arvizu C, Fanger MW (1995) Phase Ia/Ib trial of bispecific antibody MDX-210 in patients with advanced breast or ovarian cancer that overexpresses the proto-oncogene HER-2/neu. J Clin Oncol 13:2281
33. Valone FH, Kaufman PA, Guyre PM, Lewis LD, Memoli V, Ernstoff MS, Wells W, Barth R, Deo Y, Fisher J, et al (1995) Clinical trials of bispecific antibody MDX-210 in women with advanced breast or ovarian cancer that overexpresses HER-2/neu. J Hematother 4:471
34. Van Spriel AB, van Ojik HH, van de Winkel JGJ (2000) Immunotherapeutic perspective for bispecific antibodies. Immunol Today 21:391
35. Van Ojik HH, Repp R, Groenewegen G, Valerius T, van de Winkel JGJ (1997) Clinical evaluation of the bispecific antibody MDX-H210 (anti-FcγRI×anti HER-2/neu) in combination with granulocyte-colony-stimulating factor (Filgrastim) for treatment of advanced breast cancer. Cancer Immunol Immunother 45:207
36. Wallace PK, Kaufman PA, Lewis LD, Keler T, Givan AL, Fisher JL, Waugh MG, Wahner AE, Guyre PM, Fanger MW, Ernstoff MS (2001) Bispecific antibody-targeted phagocytosis of HER-2/neu expressing tumor cells by myeloid cells activated in vivo. J Immunol Methods 248:167
37. Wallace PK, Romet-Lemonne JL, Chokri M, Kasper LH, Fanger MW, Fadul CE (2000) Production of macrophage-activated killer cells for targeting of glioblastoma cells with bispecific antibody to Fc gamma RI and the epidermal growth factor receptor. Cancer Immunol Immunother 49:493
38. Watanabe M, Wallace PK, Keler T, Deo YM, Akewanlop C, Hayes DF (1999) Antibody-dependent cellular phagocytosis (ADCP) and antibody dependent cellular cytotoxicity (ADCC) of breast cancer cells mediated by bispecific antibody, MDX-210. Breast Cancer Res Treat 53:199