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Dimitri Flieger · Peter Kufer · Imke Beier
Tilman Sauerbruch · Ingo G. H. Schmidt-Wolf

A bispecific single-chain antibody directed against EpCAM/CD3 in combination with the cytokines interferon α and interleukin-2 efficiently retargets T and $CD3^+CD56^+$ natural-killer-like T lymphocytes to EpCAM-expressing tumor cells

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Abstract Cytokine-induced killer cells (CIK), generated in vitro from peripheral blood mononuclear cells (PBMC) by addition of interferon γ (IFNγ), interleukin-2 (IL-2), IL-1 and a monoclonal antibody (mAb) against CD3, are highly efficient cytotoxic effector cells with the $CD3^+CD56^+$ phenotype. In this study, we evaluated whether the cytotoxicity of these natural-killer-like T lymphocytes against the colorectal tumor cell line HT29 can be enhanced by the addition of a bispecific single-chain antibody (bsAb) directed against EpCAM/CD3. For determination of bsAb-redirected cellular cytotoxicity we used a new flow-cytometric assay, which directly counts viable tumor cells and can assess long-term cytotoxicity. We found that this bsAb induced distinct cytotoxicity at a concentration above 100 ng/ml with both PBMC and CIK at an effector-to-target cell ratio as low as 1:1. CIK cells revealed higher bsAb-redirected cytotoxicity than PBMC. Cellular cytotoxicity appeared after 24 h whereas PBMC showed the highest bsAb-redirected cytotoxicity after 72 h. The addition of the cytokines IL-2 and IFNα but not granulocyte/macrophage-colony-stimulating factor enhanced bsAb-redirected cytotoxicity of both PBMC and CIK. When the bsAb was combined with the murine mAb BR55-2, which recognizes the $Lewis^y$ antigen, bsAb-redirected cytotoxicity was partly augmented, whereas murine mAb 17-1A, which binds to EpCAM as well, slightly suppressed bsAb-redirected cytotoxicity induced by the bsAb. We conclude that CIK generated in vitro or in vivo combined with this new EpCAM/CD3 bsAb and the cytokine IL-2 should be evaluated for the treatment of EpCAM-expressing tumors.

Key words Cytotoxicity · Bispecific antibody · CIK cells · Interleukin-2

D. Flieger (✉) · I. Beier · T. Sauerbruch
I. G. H. Schmidt-Wolf
Medizinische Klinik und Poliklinik I,
Allgemeine Innere Medizin, Universität Bonn,
Sigmund-Freud-Str. 25, D-53105 Bonn, Germany
e-mail: D.Flieger@uni-bonn.de
Tel.: +49-228-287-5489
Fax: +49-228-287-5849

P. Kufer
Institut für Immunologie der LMU-Universität München,
Goethestr. 31, 80336 München, Germany

Introduction

The major mechanisms responsible for mAb cytotoxicity are induction of apoptosis, complement-dependent cellular cytotoxicity and specific attack of tumor cells by immune cells, which is termed antibody-dependent cellular cytotoxicity (ADCC). However, because of their large size, mAb cannot penetrate into large tumors. Therefore, smaller effective molecules, which specifically bind to tumor cells, are highly desirable for tumor immunotherapy. Bispecific antibodies are such constructs, and can bridge cytotoxic effector cells with tumor cells by coligation of tumor-associated antigens with surface proteins on cytotoxic cells. A recently developed bispecific single-chain antibody (bsAb), which combines the binding specificities of anti-CD3 and EpCAM monoclonal antibodies, proved to be highly cytotoxic to tumor cell lines, even when used in 1000-fold lower concentrations than murine monoclonal antibodies [15]. The EpCAM tumor-associated antigen is highly expressed on many tumor cell types like gastrointestinal malignancies and non-small-cell lung cancer [7]. In a clinical trial with the murine mAb 17-1A, which recognizes EpCAM, a 30% decrease in the overall death rate was observed in patients with resected Dukes' C colorectal carcinoma and minimal residual disease after a mean follow-up of 7 years [29].

In our study, we evaluated whether the bsAb can retarget a rare subset of $CD3^+CD56^+$ T lymphocytes in peripheral blood. These were originally described 1986 [18, 30] and further functionally characterized as mediating non-MHC-restricted lysis and not secreting interferon γ (IFNγ) after stimulation by interleukin-2 (IL-2),

which is a characteristic of $CD3^{-}CD56^{+}$ lymphocytes [28]. This $CD3^{+}CD56^{+}$ cell population can be highly expanded in vitro by the addition of the cytokines IFNγ, IL-1, IL-2 and a monoclonal antibody against CD3. They have been termed cytokine-induced killer cells (CIK cells) and it was shown that they possess enhanced cytotoxicity against various tumor cells and a higher proliferation rate than lymphokine-activated killer (LAK) cells [25, 26, 32–34].

Since the cytokines IL-2 [5, 23, 27], IFNα [3, 10] and granulocyte/macrophage-colony-stimulating factor (GM-CSF) [19, 22] can enhance ADCC, we also evaluated their impact on cytotoxicity induced by the bsAb. Moreover, we examined whether the murine mAb 17-1A, which is specific for EpCAM, and the murine mAb BR55-2, which binds to Lewis^{y}, can modulate bsAb-redirected cytotoxicity.

Materials and methods

Medium and cells

RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 200 µg/ml streptomycin, 200 IU/ml penicillin and 300 µg/ml L-glutamine (complete medium) was used throughout. The colon carcinoma cell line HT29 (ATCC) was kept in exponential growth conditions in 12.5 ml medium in plastic 75-cm^2 culture flasks (Greiner, Solingen, Germany). The cells were detached with a rubber policeman.

Cell separation and storage

Peripheral blood mononuclear cells (PBMC) were prepared from healthy donors' buffy coat diluted in phosphate-buffered saline (PBS) by Ficoll-Hypaque density gradient centrifugation, according to routine procedures [2]. After controlled freezing in medium containing 10% heat-inactivated FCS and 10% dimethylsulfoxide, aliquots were stored in liquid nitrogen.

Monoclonal antibodies and cytokines

The bsAb CD3/EpCAM was produced by fusion of two single-chain Fv fragments through a flexible Gly-Ser linker. One single-chain Fv fragment (directed against CD3) was derived from the TR66 hybridoma [38] and the other Fv fragment (directed against EpCAM) was obtained from the M79 hybridoma, which recognizes a distinct epitope on the EpCAM antigen as compared to mAb 17-1A [6, 7]. The construct was expressed in Chinese hamster ovary cells and purified via its C-terminal histidine tail on a nitrilotriacetic acid affinity column [15, 21]. Purified bsAb was kindly provided by Patrick Baeuerle (Micromet GmbH, Martinsried-München, Germany). IL-2 (1.8×10^7 U/mg) was obtained from Chiron (Ratingen, Germany). IFNα (1.8×10^9 IE/mg) was kindly provided by Hoffmann-La Roche (Grenzach-Wyhlen, Germany). GM-CSF (1.1×10^7 U/mg) was purchased from Novartis (Nuernberg, Germany), IFNγ and IL-1 from Boehringer-Mannheim (Mannheim, Germany) and anti-CD3 (OKT3) from Janssen-Cilag (Neuss, Germany).

Production of CIK cells

CIK cells were generated essentially as previously described [32, 34]. In brief, non-adherent Ficoll-separated human PBMC were prepared and grown in RPMI-1640 complete medium at 37 °C and 5% CO_2 in a humidified atmosphere. Human recombinant IFNγ (1000 U/ml) was added on day 0. After 24 h of incubation, 50 ng/ml mAb OKT3, 100 U/ml IL-1β and 300 U/ml IL-2 were added. Cells were subcultured on day 4 in fresh complete medium with 300 U/ml IL-2 at 3×10^6 cells/ml and incubated for a total of 7 days. After this period of time, an approximately tenfold increase of $CD3^{+}CD56^{+}$ lymphocytes was evident.

Cytotoxicity assay

The cytotoxicity assay was performed as described previously with minor modifications [4]. Briefly, effector cells (PBMC or CIK cells) were incubated with the red membrane dye PKH-26 (Sigma Chemicals, St. Louis, Mo.) at 2×10^6 mol/l for 4 min; during this time the reaction tubes were agitated slightly at room temperature. HT29 tumor cells were harvested with a rubber policeman, washed in PBS and incubated with PKH-2 (green fluorescence) at 2×10^6 mol/l for 10 min. Staining was stopped by addition of FCS and the labeled cells were washed separately three times with medium. Viability (above 95%) and cell counts were determined with trypan blue in a Neubauer chamber and uniform cell labeling was ascertained by UV fluorescence microscopy. Labeled HT29 tumor cells and effector cells were seeded in 96-well flat-bottom microtiter plates (Nunc, Denmark) at an appropriate effector-to-target (E:T) ratio, as indicated. bsAb, mAb and cytokines were then added, as indicated. The final volume was always 200 µl and triplicates were used throughout. After an incubation period of 3 days, if not otherwise indicated, at 37 °C and in 5% CO_2 in a humidified atmosphere, the plates were washed with PBS, treated with 50 µl/well warm 0.02% EDTA and 0.05% trypsin in PBS for 10 min and agitated on a plate shaker for 1 min. Thereafter, 200 µl PBS containing 45% FCS for trypsin blocking, propidium iodide (12.5 µg/ml) for labeling of dead cells, and fluorescein-isothiocyanate-labeled chronic lymphocytic leukemia lymphocytes (150 000 cells/ml, i.e. 30 000 cells/200 µl), as standards for determination of cell counts, were added to the cells. All samples were analyzed by flow cytometry in a FACScan (Becton Dickinson, San Jose, Calif., USA) flow cytometer using identical gates and instrument settings. Gates were set for the chronic lymphocytic leukemia standards and for viable tumor cells, as described [4]. One example of a flow-cytometry histogram and the formula for calculating cellular cytotoxicity is depicted in Fig. 1.

Statistical analysis

Significant differences between triplicates were calculated using Student's t-test; $P < 0.05$ was regarded as significant.

Results

Cytokine-induced killer cells (CIK cells) were derived from T cells after stimulation with IFNγ, IL-2, IL-1 and a monoclonal antibody (mAb) against CD3. These effector cells are known to mediate effective cytotoxicity against neoplastic cell lines derived from hematological malignancies. However, the cytotoxicity against cell lines derived from solid tumors is modest. Therefore, we investigated in this study whether a bsAb with anti-EpCAM and anti-CD3 specificity can enhance the cytotoxicity of CIK cells that express CD3 by bridging their CD3 with EpCAM on tumor cells. For the determination of cytotoxicity we used a recently developed flow-cytometric assay, which directly counts viable cells and has distinct advantages over conventional radioactivity assays [4]. As target cells, we used the colorectal tumor cell line HT29, which expresses EpCAM and, as

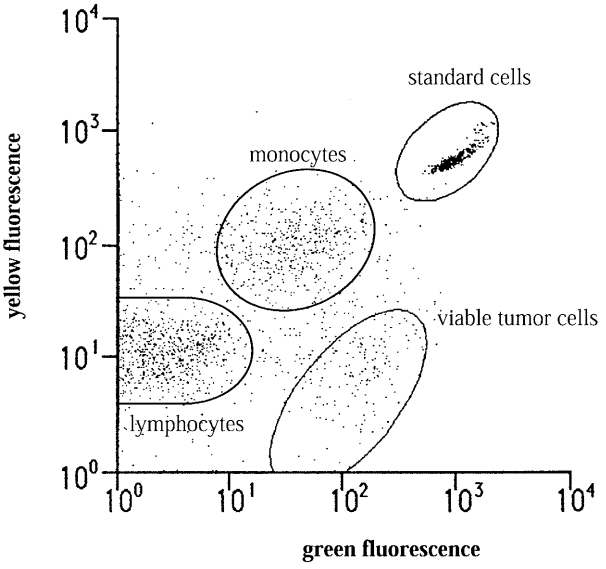


Fig. 1 Example of assessment of cellular cytotoxicity by flow cytometry. Effector and tumor cells together with bispecific antibody and/or cytokines were incubated for up to 3 days and then disaggregated by trypsin/EDTA. Thereafter, 30 000 standard cells were added to each well of the microtiter plate. The absolute number of viable tumor cells in each well was calculated by using the formula: 30 000 × (events in tumor cell gate)/(events in standard cell gate)

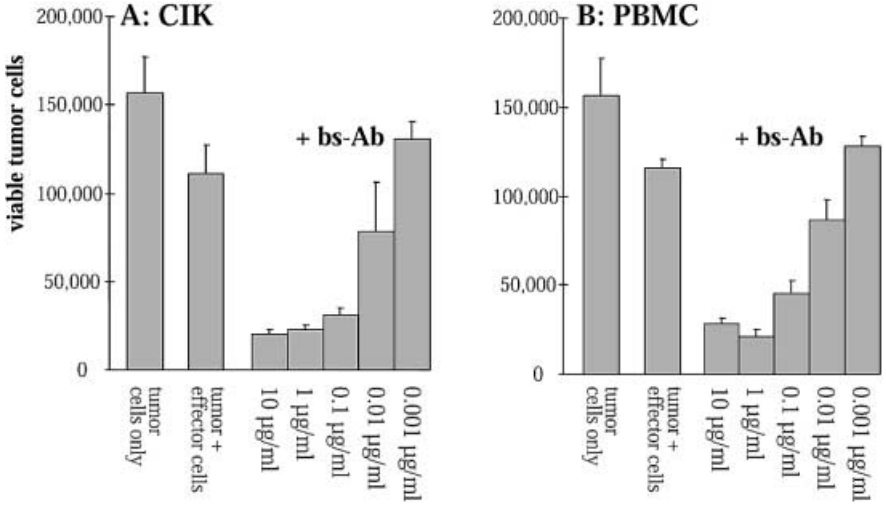
effector cells, both CIK cells and PBMC were used. First, we performed titration experiments with the bsAb in order to evaluate its minimal effective concentration and found that it induced a profound bsAb-redirected cytotoxicity above a concentration of 100 ng/ml with both PBMC and CIK cells (Fig. 2). Moreover, bsAb-redirected cytotoxicity was also evident at the lowest tested effector-to-target ratio of 1.1:1, increasing gradually when higher effector-to-target ratios were used (Fig. 3).

Next we performed kinetic experiments with both PBMC and CIK cells. Cytotoxicity induced by the bsAb appeared after 24 h and further increased after 72 h of culture, whereas for PBMC bsAb-redirected cytotoxicity was evident after 72 h (Fig. 4). Similar results were obtained with both effector-to-target ratios tested (e.g. 9:1 and 4.5:1), with higher cytotoxicity when more effector cells were used. We always evaluated cytotoxicity in microtiter plates by fluorescence microscopy and could observe cytotoxicity with abundant tumor cell debris when CIK cells were used as effector cells and additional phagocytosis of tumor cells by monocytes when PBMC were the effector cells. However, in the low effector-to-target concentrations, tumor cells that had escaped killing began again to proliferate, resulting in overall tumor cell growth inhibition as the net effect.

In order to evaluate whether cytokines may further enhance the cytotoxicity of the bsAb we combined the bsAb with the cytokines IL-2, IFNα and GM-CSF, which have been repeatedly reported to enhance ADCC of monoclonal antibodies. Both IL-2 and IFNα significantly enhanced bsAb-redirected cytotoxicity induced by both PBMC and CIK cells whereas GM-CSF slightly augmented the bsAb-redirected cytotoxicity of CIK cells only (Fig. 5). Interestingly, cytotoxicity induced by the bsAb was always higher when CIK cells were used as effector cells as than when PBMC were used (Fig. 3). However, when bsAb was combined with IL-2, equal cytotoxicity was reached with both effector cell populations (Table 1).

We next asked whether combining the bsAb with other monoclonal antibodies might yield more cytotoxicity than the bsAb alone. First we evaluated the

Fig. 2A, B Effect of antibody concentration on bispecific-antibody(*bsAb*)-redirected cytotoxicity. The colorectal carcinoma cell line HT-29 was incubated with the peripheral blood mononuclear cells (*PBMC*, **B**) or the cytokine-induced killer cells (*CIK*, **A**), obtained from two different healthy donors, at an effector-to-target ratio of 270 000 to 30 000 (9:1) for 3 days. Then viable tumor cells were determined as described in Materials and methods. *Bars* the means of viable tumor cells obtained in triplicate experiments with standard deviation. The right-hand side of each diagram depicts the effect of different concentrations of the bsAb (one representative experiment of three)



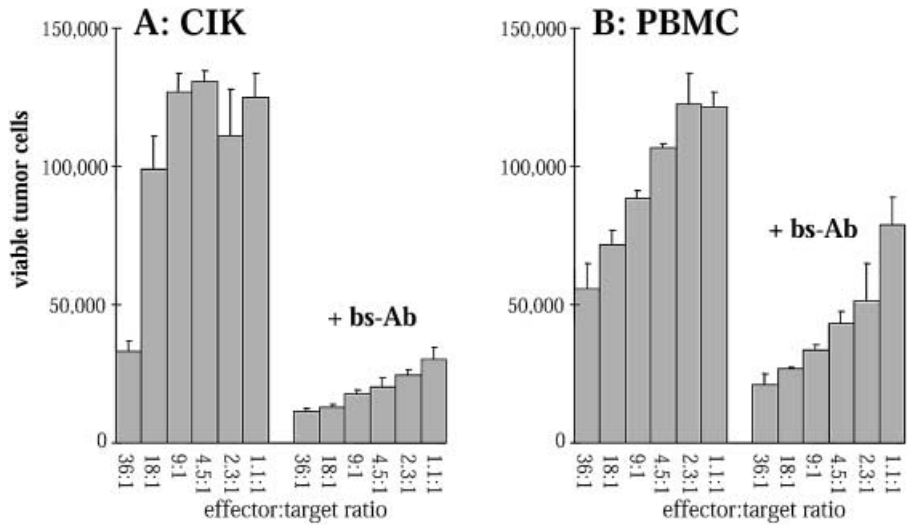


Fig. 3A, B Effect of effector-to-target ratio on bsAb-redirected cytotoxicity. 30 000 cells of the colorectal carcinoma cell line HT-29 were incubated with the PBMC (**B**) or the CIK cells (**A**) obtained from one healthy donor at the indicated effector-to-target ratio for 3 days. The bsAb was used at a concentration of 200 ng/ml. Viable tumor cells were than determined as described in Materials and methods. *Bars* the means of viable tumor cells obtained in triplicate experiments with standard deviation. The left-hand side of each diagram displays the effect of effector cells without the bsAb whereas the right-hand side depicts the effect of the bsAb (one representative experiment of three)

combination with the murine mAb 17-1A, which also specifically binds to EpCAM. Both the bsAb and the mAb 17-1A, with or without the addition of IL-2, induced distinct cytotoxicity in PBMC. However, when the two antibodies were combined, 17-1A slightly suppressed the cytotoxicity induced by the bsAb (Fig. 6). In contrast, the murine mAb BR55-2, which recognizes another tumor-associated antigen on tumor cells, termed the Lewis^y antigen, yielded heterogeneous results with effector cells from different normal cell donors, partly enhancing or having no influence on cytotoxicity induced by the bsAb (data not shown).

Discussion

When PBMC are stimulated in vitro by recombinant IFNγ on day 0 and then by mAb OKT3, IL-1β and IL-2, cells of the $CD3^+CD56^+$ phenotype increase up to 6000-fold [34]. These cells have been found to express the α, β T cell receptor, co-express the CD5 and CD8 antigens and not to express the CD16 antigen [33]. Potent antitumor activity was observed in a severe combined immunodeficiency mouse/human lymphoma model [20, 32] and in an in vitro model with autologous

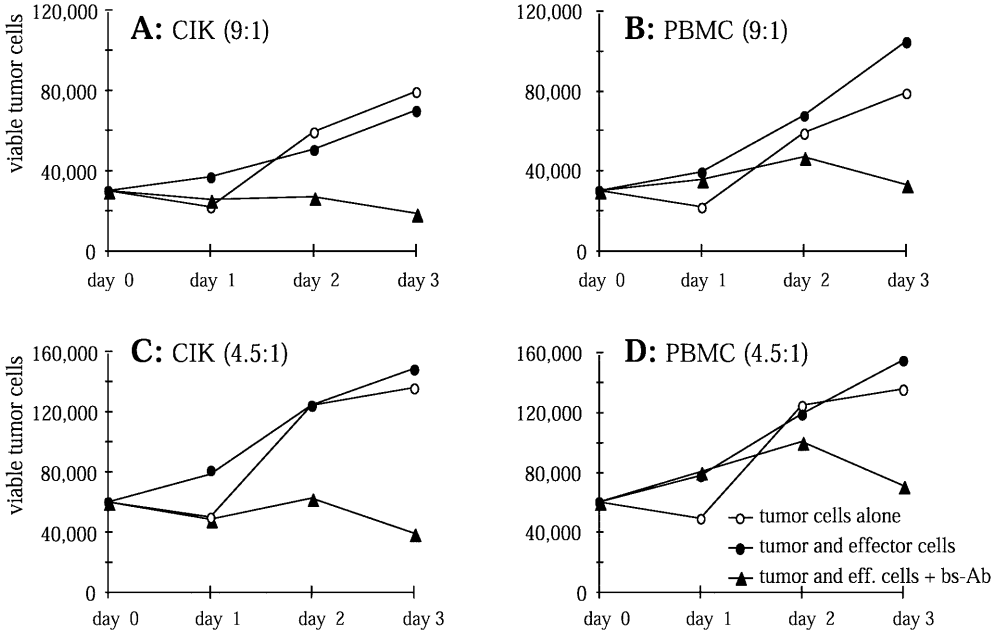


Fig. 4A–D Kinetics of bsAb-redirected cytotoxicity. 30 000 (**A**, **B**) or 60 000 (**C**, **D**) cells of the colorectal carcinoma cell line HT-29 were incubated with 270 000 PBMC (**B**, **D**) or CIK cells (**A**, **C**) obtained from one healthy donor at an effector-to-target ratio of 9:1 (**A**, **B**) or 4.5:1 (**C**, **D**), for 1–3 days. The bsAb was used at a concentration of 200 ng/ml. The viable tumor cells were then determined as described in Materials and methods (one representative experiment of four)

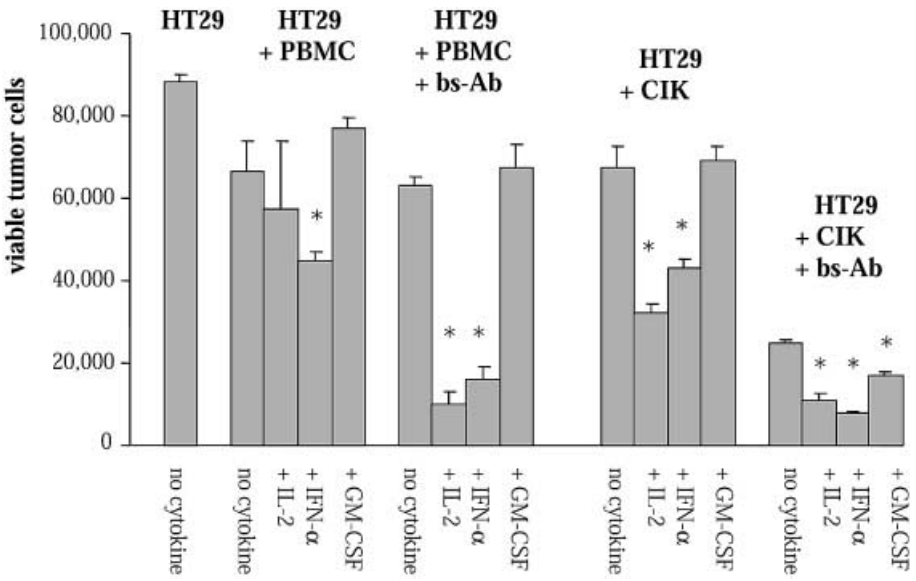


Fig. 5 Effect of different cytokines on bsAb-redirected cytotoxicity induced by the bsAb against EpCAM/CD3. 30 000 cells of the colorectal carcinoma cell line HT-29 were incubated with 270 000 PBMC or CIK cells obtained from one healthy donor at an effector-to-target ratio of 9:1 for 3 days. bsAb at a concentration of 200 ng/ml, interleukin-2 (*IL-2*) at 20 ng/ml, interferon α (*IFNα*) at 2 ng/ml and granulocyte/macrophage-colony-stimulating factor (*GM-CSF*) at 30 ng/ml were added as indicated. The viable tumor cells were then determined as described in Materials and methods. * Significant effect of cytokine as compared to control without cytokine (one representative experiment of three)

Table 1 Comparison of cytotoxicity induced by peripheral blood mononuclear cells (*PBMC*) and cytokine-induced killer cells (*CIK*). Mean and standard deviation of remaining viable tumor cells as a percentage of the control (tumor cells with effector cells) obtained from three experiments with three different donors. 30 000 cells of the colorectal carcinoma cell line HT-29 were incubated with 270 000 PBMC or CIK at an effector-to-target ratio of 9:1 for 3 days. Bispecific antibody (*bsAb*) at a concentration of 200 ng/ml and 20 ng/ml interleukin-2 (*IL-2*) were added as indicated. *P* was calculated by Student's *t*-test with $P < 0.05$ regarded as being significant. Bold numbers indicate significant differences between cells and PBMC

Effector cells	Viable tumor cells remaining (%)		*P*
	PBMC	CIK	
Effector + target	100	92 ± 23	0.476
Effector + target + IL-2	54 ± 37	29 ± 17	0.114
Effector + target + bsAb	96 ± 13	**29 ± 15**	0.0000001
Effector + target + bsAb + IL-2	15 ± 6	14 ± 4	0.675

chronic myelogenous leukemia blasts [11]. The cytotoxic effect of CIK cells against tumor targets is blocked by antibodies directed against the lymphocyte-function-associated antigen and its counter receptor, intercellular adhesion molecule 1 [33]. The cytotoxicity of CIK cells against acute myeloid leukemia cells was reported to be augmented when anti-CD3 × anti-CD13 bsAb was added [12].

bsAb are increasingly used to retarget immune effector cells against tumor cells. Hybrid hybridomas generated by fusion of two myeloma cells (quadroma) or chemical linkage of an mAb were first employed for their production. However, recently smaller bispecific single-chain antibodies have been constructed that consist of two different single-chain Fv fragments and have distinct advantages [35]. By displaying anti-CD3 specificity, such constructs can redirect cytotoxic T cells to attack tumor cells [16, 21].

Since antibody cytotoxicity against solid tumor cells can be missed by conventional radioactive assays, as demonstrated for monoclonal antibodies because of phagocytosis of the radioactive label [24] and for bsAb as well [1], we used a new flow-cytometric cytotoxicity assay that we had recently developed, which can also accurately assess long-term cytotoxicity or inhibition of tumor cell growth by counting the remaining viable tumor cells after a defined period of time [4]. Indeed, the bsAb proved able to induce a marked cytotoxicity at concentrations above 100 ng/ml with effector-to-target ratios as low as 1:1. Similar results were obtained with the same bsAb in another study with human T cells as effector cells against EpCAM-positive tumor cells [15]. Moreover, we could demonstrate that CIK cells, when used as effector cells, induced a higher level of cytotoxicity than did PBMC.

There is evidence that IL-2 enhances ADCC mediated by monoclonal antibodies such as 17-1A [3, 5] and cytotoxicity mediated by bsAb with CD3 specificity as well [8, 36]. Therefore we examined the effect of three different cytokines on bsAb-redirected cytotoxicity mediated by the bsAb. In our experiments IL-2 and IFNα but not GM-CSF enhanced bsAb-redirected cytotoxicity mediated by both CIK cells and PBMC. IL-2 has also been reported to increase ADCC by hybrid-hybridoma(quadroma)-produced bsAb with an intact Fc portion and EpCAM/CD3 specificity [13]. This led to a clinical trial with intravenous application of the $F(ab')_2$ portion of the bsAb and subcutaneous treatment with IL-2 [14]. However, treatment was limited because of considerable toxicity probably caused by induction of secondary cytokines, like tumor necrosis factor α and IFNγ, by the bsAb. Indeed, Zeidler et al. reported that a quadroma-produced whole-chain bsAb against EpCAM/CD3 effectively induced the production of

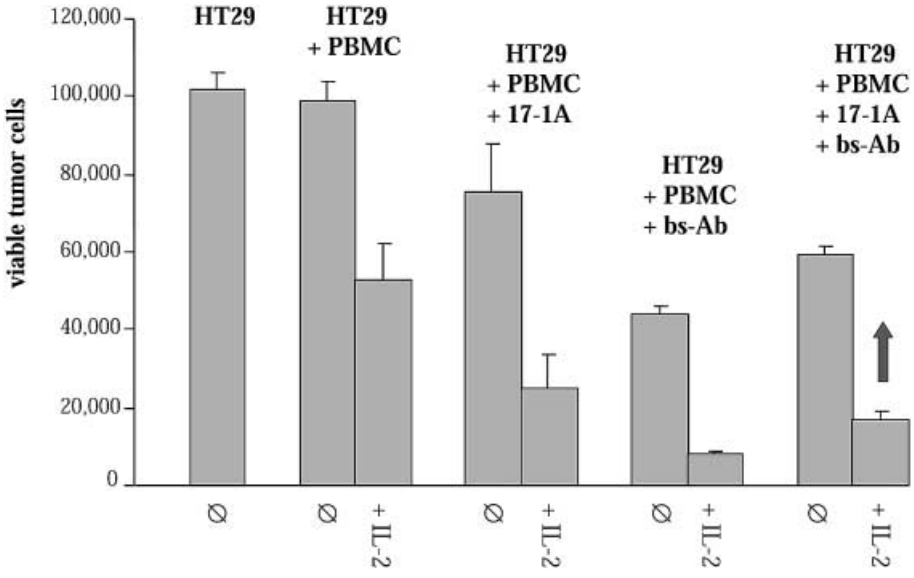


Fig. 6 Combination of bsAb with monoclonal antibody 17-1A and IL-2. 30 000 cells of the colorectal carcinoma cell line HT-29 were incubated with 270 000 PBMC from one healthy donor at an effector-to-target ratio of 9:1 for 3 days. bsAb at a concentration of 200 ng/ml, monoclonal antibody 17-1A at 100 µg/ml and IL-2 at 20 ng/ml were added as indicated. The viable tumor cells were then determined as described in Materials and methods. *Arrow* significant suppression of bsAb-induced cytotoxicity by 17-1A (one representative experiment of four)

secondary cytokines like IL-1β, IL-2, IL-6 and IL-12 by coligation of lymphocytes, monocytes and tumor cells [40]. Another research group, which also examined the effects of another quadroma-produced whole-chain bsAb against EpCAM/CD3, could demonstrate that effective cytotoxicity was obtained without prior stimulation of effector cells [37]. Nevertheless, whether single-chain bsAb will produce fewer side-effects than quadroma-produced bsAb is not known. Clinical trials with this single-chain bsAb or similar bsAb described by others [9] may help to resolve this issue.

Monoclonal antibodies recognizing different epitopes on the EpCAM antigen can enhance cytotoxicity against colorectal tumor cells [6]. However, in our study, mAb 17-1A, which recognizes a different epitope on the EpCAM antigen from that recognised by the bsAb, tended to suppress bsAb-redirected cytotoxicity. One may assume that CD3 lymphocytes involved in bsAb-redirected cytotoxicity and both monocytes and natural killer cells, which are the major effector cells performing Fc-receptor-mediated ADCC, do not cooperate with each other to increase cytotoxicity. The Lewis^y mAb provided inconsistent results, with partly increased cytotoxicity or no significant enhancement of bsAb-redirected cytotoxicity. Interestingly, cytotoxicity induced by the bsAb was obvious at concentrations as low as 100 ng/ml whereas, for mAb 17-1A, antibody concentrations 10^3 times higher were required to obtain a similar level of cytotoxicity, as already demonstrated [3, 39].

In a phase I clinical study, CIK cells transfected with the IL-2 gene were given without serious side-effects to 10 patients with metastatic renal cancer, colorectal cancer and lymphoma, demonstrating clinical activity with 1 complete clinical response in a patient suffering from lymphoma and 3 cases in which the disease was stabilized [31]. Since EpCAM is expressed on the surface of many epithelial tumors, specific retargeting of CIK cells by means of the bsAb may lead to more effective cytotoxicity. We therefore suggest that ex vivo or in vivo production of CIK cells and combination with the bsAb against EpCAM/CD3 may be a valuable therapeutic protocol to be tested for the treatment of EpCAM-expressing tumors. An in vitro approach similar to ours was elaborated in 1992 by Bolhuis et al., demonstrating that blood lymphocytes can be expanded ex vivo with phytohemagglutinin and armed with a bsAb recognizing CD3 and an anti-folate-binding protein (MOv18) [17]. When this approach was used in a phase I/II trial for patients with ovarian carcinoma, an interesting 27% clinical response rate (complete or partial intraperitoneal responses) was obtained in 7 out of 26 patients treated. Since the CD3/EpCAM bsAb has not yet been evaluated in the clinic for treating EpCAM-expressing carcinomas, a dose-escalation feasibility trial is now planned at our institution.

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