Eradication of Tumors from a Human Colon Cancer Cell Line and from Ovarian Cancer Metastases in Immunodeficient Mice by a Single-Chain Ep-CAM-/CD3-Bispecific Antibody Construct

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Abstract

Bispecific T-cell engager (BiTE) are a class of bispecific single-chain antibodies that can very effectively redirect cytotoxic T cells for killing of tumor target cells. Here, we have assessed the in vivo efficacy of one representative, called bsEp-CAMxCD3, with specificity for tumors overexpressing epithelial cell adhesion molecule (Ep-CAM) in human xenograft models. Cells of the human colon carcinoma line SW480 were mixed at a 1:1 ratio with unstimulated human peripheral blood mononuclear cells, s.c. injected in nonobese diabetes/severe combined immunodeficiency (NOD/SCID) mice, and animals were treated with bsEp-CAMxCD3. Five daily i.v. injections of as little as 100 ng per mouse of bsEp-CAMxCD3 completely prevented tumor outgrowth when treatment was started at the day of tumor cell inoculation. BscEp-CAMxCD3 was also efficacious when administered up to 8 days after xenograft injection. Established tumors could be eradicated in all animals by five 10 μg doses given between days 8 and 12 after tumor cell inoculation. To test the efficacy of bsEp-CAMxCD3 in a more physiologic model, pieces of primary metastatic tumor tissue from ovarian cancer patients were implanted in NOD/SCID mice. Partial tumor engraftment and growth was observed with four of six patient samples. Treatment of established tumors with daily 5 μg doses led to a significant reduction and, in some cases, eradication of human tumor tissue. These effects obviously relied on the tumor-resident T cells reactivated by bsEp-CAMxCD3. Our data show that the class of single-chain bispecific antibodies has very high antitumor efficacy in vivo and can use previously unstimulated T cells at low effector-to-target ratios. (Cancer Res 2005; 65(7): 2882-9)
Bispecific Antibody for Carcinoma Treatment

from man. All experiments showed a high biological activity of the BiTE molecule.

Here we have investigated another member of this class of bispecific antibodies, called bscEp-CAMxCD3, for its in vivo activity with respect to inhibition of tumor take and activity against established adenocarcinoma-derived tumors in immunodeficient NOD/SCID mouse models. BscEp-CAMxCD3 is specific for epithelial cell adhesion molecule (Ep-CAM), a target antigen that has been extensively used by various antibody-based therapies (6, 15–19). Like most other tumor-associated antigens, Ep-CAM is expressed on normal tissue. However, Ep-CAM expression on a wide variety of carcinomas does exceed expression and accessibility of the antigen on normal cells, thereby establishing a useful therapeutic window for a targeted antibody approach (20, 21). BscEp-CAMxCD3 is here shown to be highly efficacious in inhibition of tumor growth and in eradication of established tumors.

Materials and Methods

Antibodies and reagents. Single-chain bispecific antibody bscEp-CAMxCD3 with the internal code name MT102 was produced by Micromet AG (Munich, Germany). In brief, two single-chain Fv fragments directed against Ep-CAM and CD3, respectively, were joined by a flexible Gly-Ser linker. The single-chain Fv fragment binding to Ep-CAM was derived from the mouse hybridoma M79 (16), and the single-chain Fv fragment specific for human CD3 from the murine monoclonal antibody L2K. C-terminal hexahistidine-tagged bscEp-CAMxCD3 antibody was expressed in Chinese hamster ovary (CHO) cells and purified on a Ni-nitrilotriacetic acid affinity column (Qagen, Hilden, Germany). Monomeric and dimeric forms of bscEp-CAMxCD3, as naturally produced and secreted by CHO cells as a mixture, were separated by gel filtration and the dimeric form used for the experiments. For the present study, the dimeric form was selected because of its higher abundance. It was most likely formed by a diabody-like structure. A bispecific control antibody containing the same CD3 binding arm as bscEp-CAMxCD3 but with a different target specificity not recognizing SW480 cells was produced as described for bscEp-CAMxCD3, and the dimeric form of this bispecific antibody used for control experiments. This control antibody had a similar in vitro efficacy against a target cell line expressing its respective target as the Ep-CAM–specific BiTE against SW480 cells (data not shown).

Anti–Ep-CAM antibody BerP-4 was purchased from DAKO (Hamburg, Germany), anti-CD3 antibody UCHT-1 from Sigma-Aldrich (Taufkirchen, Germany), anti-CD4 antibody RPA-T4, and anti-CD8 antibody RPA-T8 from BD Biosciences (Heidelberg, Germany). Simultest IMK-Lymphocyte subpopulation differentiation kit was purchased from BD Biosciences and LSAB immunohistochemistry detection kit from DAKO. SW480 human colon carcinoma cell line was purchased from American Type Culture Collection (Manassas, VA). CHO cells stably expressing human Ep-CAM were generated at Micromet. Primary ovarian tumor specimens were kindly provided by Prof. Dr. R. Kimmig (University of Essen, Essen, Germany). All patients had an advanced ovarian cancer stage (Federation Internationale de Gynecologie et d’Obstetrique stage IIIC or IV). CHO cells stably expressing human Ep-CAM were generated at Micromet. Primary ovarian tumor specimens were kindly provided by Prof. Dr. R. Kimmig (University of Essen, Essen, Germany). All patients had an advanced ovarian cancer stage (Federation Internationale de Gynecologie et d’Obstetrique stage IIIC or IV). Histology mainly was serous papillary. Informed consent was obtained from each subject.

Effector and target cell preparation. SW480 cells were grown in RPMI (Life Technologies, Karlsruhe, Germany) supplemented with 10% FCS (Life Technologies, Karlsruhe, Germany) and acidified (pH 4.0) drinking water ad libitum. NOD/SCID mice were tested for leukosis of immunodeficiency and only mice with IgG levels below 100 ng/mL were used. All experiments were done according to the German Animal Protection Law with permission from the responsible local authorities. In compliance with the Animal Protection Law, mice had to be euthanized when tumor volumes exceeded 10% of their body weight. Statistical analysis of the mean tumor volume of the corresponding treatment groups versus the vehicle control group was done by the nonparametric Mann-Whitney U test.

SW480 colon carcinoma model. For each animal, 2.5 × 10^5 or 5 × 10^5 human PBMC were mixed with 5 × 10^6 SW480 colon carcinoma cells in a final volume of 0.2 mL PBS. The PBMC effector-target cell mixture (1:2 or 1:1) was s.c. injected into the right flank of each NOD/SCID mouse. Two different variants of the tumor model (early treatment and established tumor model) were used. For the early treatment model, four to six animals per group were i.v. treated with bscEp-CAMxCD3, PBS control vehicle, or bispecific antibody control starting 1 hour after SW480/PBMC inoculation at the indicated doses and treatment was repeated for 4 consecutive days. In the established tumor model, initiation of treatment was delayed until s.c. growing SW480 tumors had developed (50-200 mm³). Six animals per group were treated starting at days 4, 8, and 12, respectively, and treatment was repeated for 4 consecutive days. Tumors were measured on the
was tested in a FACS-based cytotoxicity assay using CD3-positive cells. The percentage of positively stained cells was assessed. Between 200 and 400 cells in at least four microscopic views were measured on the indicated days with a caliper in two perpendicular dimensions and tumor volumes calculated according to the following formula: tumor volume = [(width^2 × length) / 2].

**Primary ovarian human xenograft model.** Following surgical resection of peritoneal metastasis of histologically proven ovarian cancer patients, primary tumor specimens were cut into 50 to 100 mm^3 cubes and s.c. implanted into the right flank of six female 6- to 10-week-old NOD/SCID mice per group. Animals were i.v. treated with 5 μg bscEp-CAMxCD3 injection or PBS control vehicle for 5 consecutive days at the indicated time points post tumor implantation. Tumor sizes were measured on the indicated days with a caliper in two perpendicular dimensions and tumor volumes calculated according to the following formula: tumor volume = [(width^2 × length) / 2].

In parallel, at least four representative pieces of the individual ovarian tumor specimens were subjected to immunohistochemistry analysis to determine the level of Ep-CAM expression and presence of T cells. In brief, cryo-slides of the primary ovarian carcinomas were fixed with glutaraldehyde. Endogenous peroxidase activity was quenched with freshly prepared 3% hydrogen peroxide for 5 minutes at room temperature. Non-specific antibody binding was blocked with bovine serum albumin (BSA) for 30 minutes at room temperature. Sections were then incubated with the primary anti-human antibodies: Ep-CAM (BerEP4/FITC, DAKO), CD3 (clone UCHT-1, Sigma), CD4 (clone RPA-T4, BioScience), and CD8a (clone RPA-T8, BioScience) for 30 minutes at room temperature. Secondary anti-mouse or anti-rabbit antibodies and the streptavidin peroxidase conjugate (DAKO LSAB kit) were used for staining. Between 200 and 400 cells in at least four microscopic views were semiquantitatively evaluated. The percentage of positively stained cells was assessed.

**Pharmacokinetic analysis of bscEp-CAMxCD3.** Twenty C57BL/6 mice were i.v. injected with 100 μg dimeric bscEp-CAMxCD3 and allocated to four different groups of five mice each. Different groups were alternatingly bled at different time points after bscEp-CAMxCD3 injection (pre-dose, 2, 15, and 30 minutes and 2, 4, 6, and 24 hours) with a maximum of two bleeds per animal. BscEp-CAMxCD3 serum concentrations were quantified by a bscEp-CAMxCD3–specific ELISA.

ELISA plates (Nunc, Wiesbaden, Germany) were coated with 100 μL anti-His antibody (Penta-His; Qiagen) diluted in PBS to a final concentration of 2 μg/mL and incubated overnight at 4°C. Plates were blocked by PBS/1% BSA for 60 minutes at 25°C and washed thrice. Test samples were incubated in PBS/10% mouse serum pool, 100 μL added per well, and incubated for 60 minutes at 25°C. Plates were washed thrice and incubated with 100 μL soluble Ep-CAM protein conjugated with biotin (Micromet, Munich, Germany) at a final concentration of 2 μg/mL for 60 minutes at 25°C. Plates were washed thrice followed by incubation for 60 minutes at 25°C with 100 μL streptavidin conjugated with alkaline phosphatase (DAKO) at a final concentration of 0.5 μg/mL. Plates were washed thrice, incubated for 60 minutes with 180 μL substrate (1 mg/mL p-NPP dissolved in 0.2 mol/L Tris buffer; Sigma) at 25°C and the absorbance (405 nm) was read on Power WaveX select (Bio-Tek Instruments, Bad Friedrichshall, Germany). The bscEp-CAMxCD3 concentration in different test samples was calculated referring to a bscEp-CAMxCD3 standard calibration curve.

Pharmacokinetic calculations of bscEp-CAMxCD3 were done by the pharmacokinetic software package WinNonlin Professional 4.1 (Pharsight Corporation, Mountain View, CA, 2003). Parameters were determined by noncompartmental analysis. The noncompartmental analysis was based on model 201 (i.e., bolus injection). The terminal elimination half-life t1/2 was calculated using a log-linear regression of the concentration data including the last four sampling time points with a measurable concentration of bscEp-CAMxCD3.

### Results

**In vitro activity of bscEp-CAMxCD3 against human colon carcinoma cells.** The in vitro efficacy of bscEp-CAMxCD3 dimer was tested in a FACS-based cytotoxicity assay using CD3-positive cell-enriched human PBMCs of healthy donors as effector cells and Ep-CAM–positive SW480 human colon carcinoma cells as target cells. Effector cells were mixed with target cells at an effector-to-target ratio of 5:1 and incubated with 5-fold serial dilutions (0.02 pg/mL–200 ng/mL) of bscEp-CAMxCD3 for 2, 8, 16, and 24 hours. The activity of bscEp-CAMxCD3 was dose-dependent and strongly increased over a 24-hour time period (Fig. 1A). After 2-hour incubation in the presence of bscEp-CAMxCD3, no significant tumor cell lysis was detected. Tumor cell lysis was first detectable after 8-hour incubation but remained low with 8% lysis above background. Following 16 hours of incubation, maximal specific lysis reached 50% and half-maximal lysis was reached at a bscEp-CAMxCD3 concentration of 0.7 ng/mL (6.3 pmol/L). Twenty-four-hour incubation resulted in 80% specific lysis at 8 ng/mL bscEp-CAMxCD3 with an EC50 value of 0.2 ng/mL (1.8 pmol/L). The exquisite specificity of bscEp-CAMxCD3 was investigated with CHO cell lines stably expressing or not expressing human Ep-CAM. Whereas virtually no cell lysis was observed in the presence of T cells and bscEp-CAMxCD3 with Ep-CAM–negative CHO cells (Fig. 1B), Ep-CAM–positive CHO cells were efficiently subjected to redirected lysis. The experiment also shows that rodent antigen-positive cells can be eliminated by human T cells redirected through bscEp-CAMxCD3.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Dose response and kinetics of bscEp-CAMxCD3–mediated cell lysis of SW480 human colon carcinoma and Ep-CAM–positive/negative CHO cells. A, 1 × 10^5 CD3- enriched PBMCs of healthy donors were incubated with 2 × 10^5 SW480 colon carcinoma cells (effector-to-target ratio 5:1) in the presence of 5-fold serial dilutions of bscEp-CAMxCD3 for 2, 8, 16, and 24 hours. B, 2 × 10^5 CHO cells or 2 × 10^5 CHO cells expressing Ep-CAM were tested for specific lysis at 8 ng/mL bscEp-CAMxCD3. Bars, SD of triplicate measurements.
Pharmacokinetic properties of bscEp-CAMxCD3. Twenty C57BL/6 mice were i.v. injected with 100 µg bscEp-CAMxCD3 and groups of five mice each bled at different time points. BscEp-CAMxCD3 serum concentrations were quantified by a bscEp-CAMxCD3-specific ELISA and a serum concentration versus time profile generated. Peak bscEp-CAMxCD3 serum concentrations in C57BL/6 mice were detected 2 minutes after bolus i.v. injection. BscEp-CAMxCD3 concentrations reached the lower limit of quantification of 1 µg/mL within 24 hours. The bscEp-CAMxCD3 serum concentration versus time curve exhibited a biexponential curve progression with an early distribution phase between 0 and 2 hours and a terminal elimination phase. Elimination rate constants were determined and resulted in a distribution half-life ($t_{1/2A}$) of 0.59 ± 0.17 hours and a terminal elimination half-life ($t_{1/2B}$) of 5.3 ± 0.4 hours. Bolus injection of 100 µg bscEp-CAMxCD3 resulted in a mean maximum serum concentrations ($C_{max}$) of 77.6 ± 8.3 µg/mL and an associated exposure (AUClast) of 206.3 ± 3.6 hour × µg/mL. The clearance was determined with 0.47 ± 0.01 mL/h and the volume of distribution with 3.54 ± 0.25 mL.

Establishment of a human colon carcinoma xenograft model in nonobese diabetes/severe combined immunodeficiency mice. To evaluate the in vivo efficacy of bscEp-CAMxCD3, the human SW480 cell line was used to establish a xenograft model in NOD/SCID mice. With a s.c. inoculum of 1 × 10⁶ tumor cells, all injected NOD/SCID mice developed rapidly growing tumors of similar growth kinetics (data not shown). Inoculation of 1 × 10⁶ SW480 cells also resulted in 100% tumor take but growth kinetics were slower and interanimal variability was higher. Injection of 1 × 10⁵ SW480 cells did not result in the formation of palpable tumors. An inoculum of 5 × 10⁶ SW480 cells was, therefore, selected for in vivo experiments to assure 100% tumor take, to keep tumor variability low, and to induce an intermediate kinetic of SW480 tumor growth.

Because bscEp-CAMxCD3 is specific for both human CD3 and human Ep-CAM, human-derived effector cells had to be coincubated with the tumor cells expressing human Ep-CAM. To this end, unstimulated human PBMC from healthy donors were used and premixed with Ep-CAM–positive SW480 tumor cells followed by s.c. injection of cell mixes into NOD/SCID mice. To mimic the situation within human tumors more closely, a low effector-to-target ratio of 1:1 or 1:2 (5 × 10⁶ or 2.5 × 10⁶ PBMC plus 5 × 10⁶ SW480 cells) was used. PBMC donor samples were analyzed by flow cytometry to determine the effector-to-target ratio with respect to CD3-, CD4-, and CD8-positive T effector cells. All PBMC samples used had similar percentages of T-cell subpopulations (i.e., 46.5 ± 6.8% CD3-positive cells and 31.7% ± 4.9% CD3/CD4 and 14.1% ± 2.6% CD3/CD8 double-positive cells). The effector-to-target ratios, as are relevant for bscEp-CAMxCD3 activity, were therefore smaller than 1:1; it was ~1:2 for total CD3-, 1:3 for CD4-, and 1:7 for CD8-positive T cells.

BscEp-CAMxCD3 dose finding in the SW480 human xenograft model. Based on the in vitro efficacy and the pharmacokinetic analysis of bscEp-CAMxCD3, a daily i.v. dosing scheme was selected for the dose finding study. Four animals per treatment group were i.v. treated with 0.01, 0.1, 1, and 10 µg bscEp-CAMxCD3 per injection starting 1 hour after inoculation of the PBMC/SW480 cell mixture. BscEp-CAMxCD3 treatment was repeated once daily for 4 consecutive days and outgrowth of solid s.c. SW480 tumors determined by caliper measurements (Fig. 2). A clear dose response for the antitumor activity of bscEp-CAMxCD3 was observed. Following treatment with the lowest dose level of 5 × 0.01 µg bscEp-CAMxCD3, four of four animals developed SW480 tumors and there was no reduction in tumor growth observed compared with the SW480 only and the SW480 + PBMC control group. Five consecutive doses of 0.1, 1, and 10 µg bscEp-CAMxCD3 per injection induced complete growth inhibition of SW480 tumors in four of four mice until day 60 after SW480 tumor cell inoculation. At the end of the study on day 82, all mice of the 10 and 1 µg dose group were still free of detectable tumor and only one animal in the 0.1 µg dose group had recurrence of a SW480 tumor.

Specificity of the antitumor activity of bscEp-CAMxCD3. A dose level of 1 µg, which was shown to be highly efficacious in the dose-finding experiment, was selected to investigate the specificity of bscEp-CAMxCD3–induced inhibition of SW480 tumor growth (Fig. 3). Two cohorts of six NOD/SCID mice were inoculated s.c. with 5 × 10⁶ SW480 cells in the absence of human PBMC, whereas the remaining cohorts were s.c. injected with mixtures of 5 × 10⁶ SW480 cells and 2.5 × 10⁶ unstimulated human PBMC of healthy donors. One cohort thereof additionally received 2.5 × 10⁶ PBMC by the i.v. route to mimic the presence of peripheral T cells. Treatment with PBS vehicle control, bispecific antibody control, and bscEp-CAMxCD3 was started 1 hour after SW480 tumor cell inoculation and continued on days 1, 2, 3, and 4 thereafter.

Treatment with bscEp-CAMxCD3 in the absence of human PBMC did not induce any antitumor effect compared with the PBS vehicle control group, indicating that the antitumor effect was strictly dependent on the presence of human effector cells. Treatment with the control vehicle PBS and a bispecific control antibody in the presence of human effector cells also did not induce antitumor effects, demonstrating that neither human effector cells as such nor treatment with a bispecific control antibody, having binding activity for CD3 but not for Ep-CAM, could elicit an antitumor effect. In contrast, treatment with bscEp-CAMxCD3, in the presence of human effector, induced a complete suppression of tumor growth. There was no difference whether human effector cells were exclusively present at the tumor site or also present in circulation after their i.v. injection 1 hour later, demonstrating that peripheral effector cells did not impair the efficacy of bscEp-CAMxCD3 in situ.
Treatement of established SW480 tumors by bscEp-CAMxCD3 at different time points. We next explored the therapeutic effect of bscEp-CAMxCD3 on palpable s.c. growing SW480 tumors. Six animals per group were either treated with PBS control buffer or bscEp-CAMxCD3 1 hour after inoculation of the PBMC/SW480 mixture. In the other groups, start of treatment was delayed to days 4, 8, and 12 after inoculation, respectively, when s.c. growing tumors had reached volumes between 50 and 200 mm³. A dose of 1 μg bscEp-CAMxCD3 on 5 consecutive days was selected because this treatment schedule had been shown to strongly inhibit SW480 tumor outgrowth (see Figs. 2 and 3).

In the early treatment group, bscEp-CAMxCD3 induced complete inhibition of SW480 tumor growth in six of six animals (Fig. 4A). BscEp-CAMxCD3 treatment at days 4 to 8 reduced the size of established SW480 tumors to the limit of detection in six of six animals until day 18 (Fig. 4B). At day 25, two of six mice had recurring SW480 tumors. At the end of the study on day 42, three of six animals had their tumors eradicated. Statistical analysis of the mean tumor volume of the control group versus the bscEp-CAMxCD3 treatment group showed highly significant differences until the end of the study (P < 0.0005). Treatment of established tumors between days 8 to 12 strongly inhibited tumor growth in six of six animals (Fig. 4C). At day 25, tumors were still absent in two of six animals. By the end of the study on day 42, one of six animals was still tumor free and mean tumor volume of the bscEp-CAMxCD3 treatment group was significantly lower between day 12 and the end of the study than that of the control group (P < 0.03). BscEp-CAMxCD3 treatment between days 12 to 16 showed a growth inhibitory trend in four of six animals (Fig. 4D). At the end of the study, six of six animals developed SW480 tumors and mean tumor volume was not significantly lower than in the control group.

Eradiation of established SW480 tumors. Conditions were sought that would lead to an eradication of established tumors during the entire observation period. I.v. treatment with bscEp-CAMxCD3 was started when SW480 tumors had reached a medium size of 100 mm³ (Fig. 5). Cohorts of six animals were injected with PBS control buffer or 1 and 10 μg bscEp-CAMxCD3 for 5 consecutive days, respectively. Delayed treatment with 5 × 1 μg between days 8 and 12 induced eradication of established tumors in five of six animals (Fig. 5A). In one animal, the established SW480 tumor started to shrink until day 18 but thereafter started to regrow. Delayed treatment from days 8 to 12 with a 10 μg dose of bscEp-CAMxCD3 led to eradication of established SW480 tumors in six of six animals with no recurrence seen until the end of the study on day 63 (Fig. 5B).

Efficacy of bscEp-CAMxCD3 against xenografts from human ovarian cancer metastases. Human tumors can be substantially different with respect to cell composition, growth rate, and tumor cell heterogeneity from those established in animal models by implantation of homogenous tumor cell lines. We, therefore, also investigated the in vivo efficacy of bscEp-CAMxCD3 in a NOD/SCID xenograft model where metastatic tumor tissue derived surgically from the peritoneal cavity of ovarian cancer patients was implanted. Of note, in this xenograft model, no additional immune effector cells were used such that the sole source of T effector cells were tumor infiltrated lymphocytes (TIL) and residual blood lymphocytes of the primary ovarian tumor samples.

Primary ovarian tumor samples from patients were cut into equal pieces of ~100 mm³ and s.c. implanted into NOD/SCID mice. From a total of six patient samples, tumor pieces were partially accepted in immunodeficient mice in four cases and showed either stable size or growth during the observation period of 70 days. The primary ovarian tumor samples from these four patients (patients 1–4) were each implanted as 12 equally sized pieces into two groups of six NOD/SCID mice each. One group of six NOD/SCID mice was i.v. treated with PBS control buffer at the indicated time points, whereas the other group was treated with 5 μg bscEp-CAMxCD3 per i.v. injection. Four additional pieces of each tumor were characterized for intersample and intrasample heterogeneity with respect to the percentage of tumor cells by morphologic criteria, and for the relative number of Ep-CAM-, CD3-, CD4-, and CD8-positive cells by immunohistochemical staining with respective antibodies. H&E staining of tumor specimen revealed a tumor cell percentage of 70% to 95%, 50% to 90%, 10% to 50%, and 10% for samples 1 to 4, respectively. The number of Ep-CAM, expressing cells was closely correlated with the number of tumor cells. Analysis of the effector cell populations resulted in 0.83 ± 1.2% CD3-positive and 0.38 ± 0.42% CD8-positive cells for sample 1. Sample 2 showed 0.83 ± 0.45% CD3-positive and 0.2 ± 0.23% CD8-positive cells; sample 3 had 6.8 ± 5% CD3-positive, 1.6 ± 1.8% CD8-positive and 4.1 ± 2.8% CD8-negative cells. Sample 4 contained 5.8 ± 7.4% CD3-positive and 0.8% CD8-negative cells. In tumor samples 1, 2, and 4, no CD4-positive cells could be detected.

Tumor pieces from the four patient samples grew slowly in NOD/SCID mice. With samples 3 and 4, tumor take was poorest and even in PBS control group only one of six and two of six animals, respectively, had measurable xenografts at the end of the study (i.e., 70 days after implantation). In the bscEp-CAMxCD3–treated cohort with samples 3 and 4, mean tumor volume during the study period was lower compared with the PBS control group, and at the end of the study all bscEp-CAMxCD3–treated animals were free of detectable tumor (data not shown). With patient samples 1 and 2, the rate of tumor take was higher and tumor growth was more consistent (Fig. 6). In both experiments, five of six
animals in the PBS control group were tumor positive at the end of the study whereas only three of six mice had detectable tumors in the bscEp-CAMxCD3–treated group. With patient sample 1, mean tumor volume in bscEp-CAMxCD3 treated mice was significantly lower between days 24 and 31 compared with the PBS-treated animals (P < 0.05; Fig. 6A). With patient sample 2, a therapeutic effect was most pronounced resulting in a significant difference in mean tumor volume of the control group versus the bscEp-CAMxCD3–treated group (P < 0.05) between days 29 until end of the study on day 70 (Fig. 6B).

Discussion

This study shows an extraordinary efficacy of bscEp-CAMxCD3 to induce at low effector-to-target ratios a cytotoxic activity of previously unstimulated T cells against human tumor cells. BscEp-CAMxCD3 was active in preventing tumor outgrowth and in eradicating in immunodeficient mice established tumors derived from both a colon carcinoma cell line and human metastatic tissues from ovarian cancer patients. With bscEp-CAMxCD3, it was, for the first time, possible to analyze the effect of a member of the BiTE class on established tumors because of the rapid outgrowth of SW480 cell–derived tumors and the use of primary human tumor tissue. The high efficacy of bscEp-CAMxCD3 at low doses and at low effector-to-target ratios is remarkable. The observation that repeated i.v. doses of 1 or 10 μg bispecific antibody not only reduced SW480 tumor volume to undetectable levels but also prevented recurrence of tumor for >2 months suggests that tumor cell elimination by BiTE-activated T cells was complete. This therapeutic effect was very specific. It did not occur with human T cells and vehicle alone nor in the presence of very low doses of bscEp-CAMxCD3. Likewise, it could not be induced by a BiTE molecule with specificity for CD3 but lack of binding to SW480 tumor cells. This is consistent with a previous study testing the CD19-specific BiTE bscCD19xCD3 against CD19-positive tumors (12). There, it was shown that bscEp-CAMxCD3 was not effective in preventing outgrowth of Ep-CAM–negative tumors. Lastly, BscEp-CAMxCD3 was not active in the absence of human T cells and efficacy was not affected if extra human T cells were present in the circulation of mice. In the current xenograft model, a possible effect of peripheral T cells on the efficacy could only be addressed in a limited fashion. Therefore, a new animal model is currently being developed that is using a murine CD3-/Ep-CAM–bispecific antibody construct recruiting murine T cells in immunocompetent mice.

The high in vivo potency of bscEp-CAMxCD3 is comparable with that of bscCD19xCD3, a single-chain bispecific antibody present in a monomeric form, which has shown high potency in preventing outgrowth of s.c. CD19-positive human B lymphoma in NOD/SCID mice (12). Previously, bscCD19xCD3 could not be tested for its activity against solid tumors because the long delay in s.c. B lymphoma outgrowth surpassed the limited life span of human T effector cells in NOD/SCID mice.

To evaluate the effect of bscEp-CAMxCD3 on established tumors, we had to establish a tumor model with fast outgrowth resulting in palpable tumors within 1 week after cell inoculation. The reduced efficacy seen with delayed treatments of bscEp-CAMxCD3...
beyond 8 days post inoculation may have several reasons. One is the limited half-life of human T cells within NOD/SCID mice. Another one is the higher tumor volume that will increase the workload for cytotoxic T cells as well as decrease tumor penetration for the bispecific antibody construct.

BscEp-CAMxCD3 and other BiTE molecules harboring a human-specific anti-CD3 single-chain antibody are strictly dependent in their activity on human T cells. No antitumor activity is possible in the absence of human T cells (11) and no cross-reactivity with T cells from other species with the exception of chimpanzee was observed. In the case of the human colon carcinoma xenograft model, T cells were supplied in the form of freshly isolated, unstimulated human PBMC. The activation status of such T cells was documented earlier (12). In the present experiments, the relevant fraction of cytotoxic T cells with the CD8 phenotype were present at a low effector-to-target ratio of only 1:7 in the mixtures with tumor cells. This effector-to-target ratio may still be overestimated given that a significant proportion of CD8-positive T cells are still naïve and have no cytotoxic granules yet.

In the case of metastatic ovarian cancer tissue, human T cells could only have originated from the tumor itself. As shown in a vast body of literature (22–25) and has been shown in the present study by immunohistochemical analysis, only a few percent of cells in tumors are CD3/CD8 double-positive cytotoxic T cells. These could have either rested next to tumor cells as TILs or were present in blood of tumor vasculature. Our efficacy data suggest that these few T cells can be activated by i.v. administered bscEpCAMxCD3 against Ep-CAM–positive tumor cells, resulting in a significant reduction of tumor size. TILs were frequently described as being anergic and occasionally specific for tumor-associated peptide antigens (26, 27). Our data suggest that such tumor-specific anergic T cells may become reactivated by BiTEs and mount a tumor-specific T-cell response in addition to the BiTE-activated response. Moreover, BiTEs were shown to fully activate T cells leading to proliferation, cytokine production, and expression of cell adhesion molecules (12). In particular, secretion of tumor necrosis factor, IFNγ, and chemokines by activated T cells may add to a therapeutic effect through concerted immune cell attraction and stimulation.

Cancer cells in an established tumor show high heterogeneity at various levels. They can differ with respect to target antigen expression, acquired immune escape mechanisms, growth behavior, and accessibility to drug and T cells. Likewise, the local frequency and state of activity of cytotoxic T cells can be very different in tumors. These variables of primary and metastatic human tumor tissue may reduce response rates to T cell–recruiting bispecific antibodies at all conceivable levels. By studying

**Figure 5.** Effect of bscEp-CAMxCD3 on established SW480 tumors in NOD/SCID mice. Cohorts of six NOD/SCID mice were inoculated s.c. with 5 \( \times 10^6 \) SW480 in the absence (PBS control) or presence of 5 \( \times 10^6 \) unstimulated human PBMC of healthy donors (effector-to-target ratio 1:1). Animals were either treated with PBS control vehicle or bscEp-CAMxCD3 via the tail vein injection on days 8, 9, 10, 11, and 12. A, 1 µg bscEp-CAMxCD3 was used per injection. B, 10 µg bscEp-CAMxCD3 were used per injection. Mean tumor growth curve is shown for mice treated with PBS control vehicle only (\( \bullet \)). For bscEp-CAMxCD3–treated animals, individual tumor growth curves are shown (\( \Delta \)). Points, means; bars, SD. Arrows, bscEp-CAMxCD3 treatment time points.

**Figure 6.** Effect of bscEp-CAMxCD3 on the growth of primary human ovarian tumor xenografts in NOD/SCID mice. Primary patient tumor samples derived from peritoneal metastasis of ovarian cancer origin were cut into pieces of ~100 mm\(^3\) and single pieces s.c. implanted into the right flank of NOD/SCID mice. Cohorts of six animals were treated with PBS vehicle control (\( \bullet \)) or 5 µg bscEp-CAMxCD3 (\( \circ \)) via tail vein injection on days 6 to 10, 20 to 24, and 34 to 38 (A); or on days 3 to 7 and 17 to 21 (B). Points, mean values of tumor growth curves (\( n = 6 \)); bars, SD. Arrows, bscEp-CAMxCD3 treatment time points. Asterisks, readings that were statistically significant (\( P < 0.05 \)) from readings of the PBS group.
metastatic ovarian cancer tissue in immunodeficient mice, we attempted to take the heterogeneity of human tumor tissue into consideration. With all four patient xenografts that were accepted, we have seen that bsEp-CAMxCD3 caused a reduction in tumor size or even loss of the xenograft, whereas vehicle controls had significantly less rejections. These effects suggest that i.v. administered bspecific antibody reached tumor-resident T cells and that reactivated T cells were present in sufficient number to diminish tumor cell numbers. The current data are limited for a number of reasons. For future experiments, larger patient sample numbers are required to estimate an in vivo response rate and even longer observation periods are desirable to study the durability of responses. Likewise, larger sample numbers from one tumor are also desirable to allow inclusion of more control conditions, such as parental antibodies and BiTEs of different target specificity. Lastly, authentic tumor material from humans presents with a high degree of heterogeneity with respect to growth characteristics in immunodeficient mice. Our in vivo data are corroborated by an ex vivo study with metastatic peritoneal ovarian cancer samples (18). In this study, resected tumor samples were dissociated and resulting cell mixtures incubated with bsEp-CAMxCD3. After 24 hours, the number of Ep-CAM/CA125-positive tumor cells was determined by cell sorting and the percentage of cell tumor lysis was determined. The response rate to bsEp-CAMxCD3 was 80% (17 of 21 ovarian cancer patient samples). Again, the only source of human T cells was the tumor tissue itself.

In metastatic ovarian cancer, a high proportion of tumor cells is Ep-CAM positive (18, 19) and considerable overexpression of Ep-CAM on ovarian cancer tissue relative to normal ovarian epithelium has been reported (21). Late-stage ovarian cancer is, therefore, an interesting target indication for treatment with Ep-CAM–specific immunotherapeutics. An Ep-CAM–specific antibody of the triamo class is currently being developed as a palliative treatment of metastatic ovarian cancer. It has shown promising clinical results in the reduction of malignant ascites (28). Triomas are different from BiTEs in that this class of recombinant antibodies activates antigen-presenting cells in addition to T cells leading to a broader, but perhaps less controllable, immune response with the potential of a vaccination effect (29). BiTEs are considerably smaller than triomas, which might be beneficial with respect to tumor penetration and activation of TILs, and exclusively activate T cells in the presence of tumor target cells.

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References

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