Carcinoembryonic Antigen (CEA)-specific T-Cell Activation in Colon Carcinoma Induced by Anti-CD3×Anti-CEA Bispecific Diabodies and B7×Anti-CEA Bispecific Fusion Proteins

Philipp Holliger, Oliver Manzke, Mary Span, Robert Hawkins, Bernd Fleischmann, Liu Qinghua, Jürgen Wolf, Volker Diehl, Olivier Cochet, Greg Heribert Bohlen

ABSTRACT

Two bispecific recombinant molecules, an anti-CD3×anti-carci-noembryogenic antigen (CEA) diabody and a B7×anti-CEA fusion protein, were tested for their capacity to specifically activate T cells in the presence of CEA-expressing colon carcinoma cells. T-cell activation by the anti-CD3×anti-CEA diabody required close contact to CEA-positive cells and resulted in diabody-mediated cytotoxicity against the target cells. Additionally, CD28-mediated costimulation in combination with anti-CD3×anti-CEA diabodies induced activation of autologous T cells in CEA-positive primary colon carcinoma specimens, as determined by flow cytometry. The high specificity of the bispecific diabody approach could be further enhanced by the use of B7×anti-CEA fusion proteins because the costimulatory CD28-signaling to the T cells strictly depended on the expression of CEA on the target cells.

We demonstrate that displaying engagement sites for the T-cell antigens CD3 and CD28 on the surface of colon carcinoma cells is a suitable way to activate and retarget T cells in a highly tumor-specific manner. For clinical purposes, B7×anti-tumor-associated antigen (TAA) fusion proteins, which are equally effective but more specific compared with anti-CD28 monoclonal anti-bodies, thus may improve the tumor specificity of anti-CD3×anti-TAA bispecific antibodies. Furthermore, B7-negative tumors can be converted into B7-positive tumors by B7×anti-TAA fusion proteins without the need for B7 gene transfer to the malignant cells.

INTRODUCTION

Tumor cells that are not destroyed by cytotoxic therapy regimens, such as chemotherapy or radiation, are assumed to be the cause for treatment failure and tumor relapse (1). The remaining tumor cells, denoted as minimal residual disease, resemble a target for immunotherapeutic strategies. In solid tumors, several TAAs are that are expressed on the malignant cells have been identified thus far. Among these, the CEA is expressed on the majority of adenocarcinomas of the large intestine and can be used as a model antigen for immunotherapeutic targeting of solid tumors.

Cellular immunotherapy of malignant diseases intends to redirect autologous effector cells toward the tumor. The recruitment of T lymphocytes is of particular interest due to their key role in antigen-specific cellular immune responses and their capacity to develop immunological memory. Although T-cell infiltrates appear to be present in many tumors (2, 3), they usually fail to attack the malignant cells, and the reasons for the lack of immunosurveillance of malignant diseases still remain to be clarified. There are several scenarios of the possible interaction between malignant cells and the immune system that may lead to the manifestation of a tumor. Active immune escape mechanisms of the tumor cells (4), ongoing but inappropriate antitumoral immune responses (5), as well as immune “ignorance” of the malignant cells (6) have been reported. However, there is strong evidence, that antitumoral activity can be induced or restored by the activation of tumor-specific T cells (2, 6).

The use of anti-CD3×anti-tumor bispecific antibodies is an attractive and highly specific approach in antitumoral immunotherapy. These antibodies can be used not only to redirect preactivated cytotoxic T cells toward the tumor (7–9), but, moreover, are able to stimulate resting or even anergic T cells if sufficient costimulatory signaling (for example, via the CD28-B7 pathway) is provided (10, 11). Artificial signaling via the CD3 antigen mimicks the physiological antigen-specific activation of T lymphocytes by MHC-bound antigen. On the other hand, CD28-costimulation by monoclonal antibodies with signal transduction activity can replace B7 as the physiological ligand for CD28. The simultaneous use of CD3 and CD28 monoclonal antibodies may, thus, substitute for the T-cell stimulatory capacity of professional antigen-presenting cells (12). However, to avoid a systemic T-cell activation that may result in undesirable clinical side effects, the CD3/CD28 signaling has to be localized strictly to the tumor site.

To specifically target colon carcinoma cells via the TAA CEA, we engineered a bispecific diabody recognizing the e-chain of the CD3-TCR complex, as well as CEA. To provide tumor-specific CD28 costimulation, a second, bivalent CEA-specific diabody was produced and fused with two extracellular domains of the human B7.1 protein. The diabody format was chosen, as for clinical purposes genetically engineered antibody fragments are considered to be advantageous over murine immunoglobulins due to their rapid penetration into tumor tissue, fast clearance of unbound diabody from the circulation, and minimal immunogenicity (13, 14). Diabodies consist of four antibody V-domains and are formed by two cross-paired scFvs. They are produced by bacterial fermentation and have been proved to be effective in retargeting preactivated CTLs to tumor cells (15).

We show that anti-CD3×anti-CEA bispecific diabodies, together with B7×anti-CEA bispecific fusion proteins, can be used to provide CEA-expressing tumor cells with binding specificities for both CD3 and CD28 antigens. This resulted in the activation of T cells and the redirection of cytotoxicity toward CEA-expressing tumor cells. Furthermore, autologous tumor-infiltrating lymphocytes in primary patient tumor specimens could be activated by anti-CD3×anti-CEA bispecific diabodies together with CD28 costimulation, pointing toward a potential clinical application of these immunotherapeutic agents.

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4 The abbreviations used are: TAA, tumor-associated antigen; CEA, carcinoembryogenic antigen; scFv, single-chain variable fragment; IMAC, immobilized metal chelate affinity chromatography; PBMC, peripheral blood mononuclear cell.

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Table 1 Primers

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**Materials and Methods**

Construction and Isolation of Anti-CD3×Anti-CEA Diabodies and B7×Anti-CEA Fusion Proteins. TG1 was used for the propagation of plasmids and the expression of antibody fragments. A five-linker anti-CD3 diabody (O5) was constructed, as described (15), by amplification of VH OKT3 with primers 1 and 2 (Table 1) and Vk OKT3 with primers 3 and 4. The VH digested with Sfi/I/SacI and the Vk digested with NotI/SacI were simultaneously ligated into Sfi/I-NotI-digested pUC119His6myc. An anti-CEA scFv MFE-23 (K. Chester) was converted to a 5-linker bivalent diabody (M5), as described above. An internal BstEI site in M5 Vk was removed by site-directed mutagenesis (Amersham, Braunschweig, Germany) using oligo 6 to create M5B. The two antibody specificities M5 and OKT3 were then combined to the bispecific diabody MO5 as follows: VH OKT3 was amplified with primers 2 and 7 and digested with Ascl/SacI. Vk OKT3 was obtained by PCR amplification of O5 diabody with primers 1 and 8, digested with Ascl, and then partially digested with BstEI. A vector fragment of diabody M5B was cut with BstEl/SacI and the VH and the VK fragments of OKT3 were simultaneously digested. Human B7.1 was combined with M5B to the bispecific diabody fusion protein B7M as follows: B7 was amplified with primers 9 and 10 and M5B diabody was amplified with primers 1 and 11. The B7 fragment was cut with NcoI/XhoI, the M5B fragment was cut with XhoI/NotI, and the two fragments were ligated simultaneously into pUC119His6myc cut with NcoI/NotI. Soluble diabody was expressed as described (16). After induction, cells were transferred to 22°C for expression. The diabodies were purified from concentrated culture supernatant by single-step IMAC (17).

ELISA was performed using recombinant CEA (CalBiochem, Bad Soden, Germany) as antigen coated at a concentration of 2 μg/ml PBS at 4°C overnight and blocking the plate with 3% BSA/PBS for 2 h at room temperature. B7M fusion protein binding to CEA was detected with biotinylated anti-B7.1 IgM (Harlan Seralab, Loughborough, United Kingdom) and horseradish peroxidase conjugated Streptavidin (Amersham). The binding of the bispecific diabody M05 to CEA was detected using the monoclonal mouse antibody 9E10) directed against c-myc (18). The binding to CD3 and CD28 was assessed by indirect immunofluorescence staining of Jurkat T cells (CD3/CD28 double positive). As second-step reagents, anti-c-myc antibodies and FITC-labeled goat antimouse IgG antibodies (SBA, Birmingham, AL) were used. The Jurkat cells were then analyzed on a flow cytometer (Becton Dickinson, Heidelberg, Germany).

Colon carcinoma xenografting in nude mice and tumor imaging with C7 (Amersham)-labeled diabodies was performed, as described (19).

**Cytotoxicity Assay.** PBMCs of healthy donors were isolated by Ficoll density centrifugation (Pharmacia Biotech, Freiburg, Germany) and subsequently stimulated with trispecific anti-CD3×anti-CD2×anti-CD28 (20 ng/ml) antibodies (M. Glennie, Tenovus Laboratory, General Hospital, Southampton, United Kingdom). After 5 days, the cells were harvested and positively enriched (CD8) by magnetic-activated cell sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). The labeling of target cells with lanthanides and measurement of specific cytolyis in time-resolved fluorometry has been described elsewhere (20). Specific lysis was determined after 4 h in a time-resolved fluorometer (DeLilfa, Wallac, Turku, Finland).

Calcium Imaging Analysis. CEA-positive tumor cells (11576) were grown as a confluent layer on glass coverslips. Jurkat T cells (1 × 10^6/ml) were loaded with the cell impermeant dye fura-2AM (0.5 μM; Molecular Probes, Leiden, the Netherlands) for 10 min at 37°C in RPMI 1640 (Life Technologies, Inc., Eggenstein, Germany). The cells were washed and incubated with the bispecific diabody MO5 (5 μg/ml in RPMI 1640). The glass coverslips were superfused for 10 min with Ringer solution (140 mM NaCl, 4.5 mM KCl, 2 mM MgCl_2, 2 mM CaCl_2, 10 mM Hepes, and 5.5 mM glucose (pH 7.35)) in a temperature-controlled chamber (room temperature or 35°C), then the fura-2AM-loaded Jurkat T cells were dropped into the recording chamber. T cells in contact with tumor cells and unbound T cells were selected for monitoring the intracellular calcium concentration.

Monochromatic excitation light (340 nm, 380 nm) was coupled to an inverted microscope (135 M; Zeiss, Jena, Germany) through a quartz light guide. The fluorescence images of the Jurkat cells were acquired (exposure time of 50–100 ms at 0.6 Hz) through a 470-nm interference filter using an intensified charge-coupled device camera (Theetha, München, Germany) connected to the microscope. Paired images (340/380 nm) were background subtracted, and the ratio images were displayed. The concentration of intracellular-free calcium ([Ca^{2+}]_i) was calculated using a curser-defined areas using the equation of Grynkiewicz et al. (21). R_{\text{max}} was obtained in the presence of ionomycin and 10 mM Ca^{2+} and R_{\text{min}} with excess EGTA (R_{\text{max}} 5.0, R_{\text{min}}, 0.4, F380/F340 11.0).

**Autologous T-Cell Stimulation in Primary Colon Carcinoma Specimens.** Specimens of tumor tissue from the large intestine were obtained during surgery and immediately placed into 20 ml of complete medium [RPMI 1640, 10% fetal bovine serum, 2.5% glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 25 mM NaHCO_3, 1 mM sodium pyruvate, and 50 μg/ml gentamicin].

**CEA-SPECIFIC T-CELL ACTIVATION IN COLON CARCINOMA**

![Fig. 1. Schematic illustration of diabodies (A) and fusion proteins (B). Construction of the bispecific diabody MF23B/OKT3/5 (MO5) and the bispecific diabody fusion protein B7.1-MFE23B/5 (B7M). Both constructs are under control of the lac promoter (P) with lacZ ribosome binding site (rbs) and the pbEl leader sequence (L1) for secretion to the periplasm. The COOH-terminal His6myc tag appended was for immunodetection and IMAC purification.](image-url)
Table 2: Binding specificities of anti-CD3 × anti-CEA diabodies and B7 × anti-CEA fusion proteins

<table>
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<td>B7 × anti-CEA</td>
<td>+</td>
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<td>ND(^{b})</td>
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\(^{a}\) Colon carcinoma xenografts in nude mice.
\(^{b}\) ND, not determined.

1640 with 2 mM Glutamal-I, 5% FCS, 25 μg/ml gentamicin (Life Technologies, Inc.), and 10 μg/ml ciprofloxacin (Bayer, Leverkusen, Germany). The carcinoma tissue was minced with a scalpel, turned into a single cell suspension using a homogenizer, and aggregates were removed with a 400-μm cell strainer (Becton Dickinson). The expression of CEA on the tumor cells was determined by staining with 50 μl of anti-CEA antibody A5B7 (10 μg/ml; Ref. 22) and indirect immunofluorescence staining with 50 μl of FITC-coupled goat antimouse IgG [20 μg/ml (SBA)], followed by analysis on a flow cytometer (Becton Dickinson). PBMCs were obtained from 20 ml of the patient’s venous blood by Ficoll (Pharmacia) density centrifugation and were resuspended in complete medium.

PBMCs (2.5 × 10⁶) were then coincubated with the autologous colon carcinoma cells (5 × 10⁵) in 2000 μl of complete medium containing the indicated diabodies, control antibodies, and anti-CD28 antibodies at a final concentration of 1 μg/ml. The stimulation assays were performed for 5 days in 24-well plates (Greiner, Frickenhausen, Germany) at 37°C and 7% CO₂ in a humidified atmosphere. After the incubation period, cell culture supernatants were harvested and stored at -20°C for further IFN-γ analysis by ELISA (Laboserv, Giessen, Germany). The cells were resuspended in PBS, stained with fluorochrome-conjugated antibodies [anti-CD45, anti-CD14, anti-CD3, anti-CD19, anti-CD16, anti-CD4, anti-CD8, anti-CD25, anti-HLA-DR (Becton Dickinson), and anti-HLA-ABC (DAKO, Glostrup, Denmark)] or anti-CEA antibody A5B7 and FITC-conjugated goat antimouse IgG (SBA), and analyzed by flow cytometry.

Costimulation of T Lymphocytes with B7 × Anti-CEA Fusion Proteins. CD4-positive T-helper lymphocytes from healthy donors were isolated from the PBMC fraction by depletion of cells that express CD8, CD19, CD16, and CD14, using magnetic-activated cell sorting (Miltenyi). The remaining CD4-positive T cells were incubated for 10 days with 20 ng/ml anti-CD2 × anti-CD3 × anti-CD28 trispecific antibodies in complete medium, followed by an additional 5-day incubation without stimulation. After depletion of dead cells by Ficoll density centrifugation, 1 × 10⁷ T cells were coincubated with 2.5 × 10⁵ cells of the CEA-positive carcinoma cell line L1576 or the CEA-negative B cell line LAZ509 (each of them fixed in 0.05% glutaraldehyde) in 150 μl of complete medium in a 96-well flat-bottomed plate (Greiner). Antibodies and diabodies were added either directly to the stimulation cultures, or the tumor cells were preincubated with the antibodies and then washed before the T cells were added. The concentration of IFN-γ in the culture supernatant was assessed by ELISA (Laboserv).

RESULTS

Production of Bispecific Proteins and Antigen-binding Analysis. The genetically engineered constructs, the anti-CD3 × anti-CEA bispecific diabody and the B7 × anti-CEA fusion protein, are shown schematically in Fig. 1. Both proteins were secreted from Escherichia coli, harvested directly from the culture supernatant, and purified by IMAC. IMAC yielded an average of 1 mg/liter culture for the anti-CD3 × anti-CEA bispecific diabody and 0.2 mg/liter for the B7 × anti-CEA fusion protein, which is in accordance with reports on other diabodies or antibody fragments (23, 24). The antigen-binding properties of the two constructs are summarized in Table 2. Both the bispecific diabody anti-CD3 × anti-CEA and the dimeric fusion protein B7 × anti-CEA bound to CEA, as determined by ELISA and by in vivo localization studies in human colon carcinoma xenografts in nude mice (data not shown). Flow cytometric analyses revealed binding of...
both protein constructs to the human T cell line Jurkat, which expresses the CD3-antigen for anti-CD3×anti-CEA diabody binding and CD28 as a ligand for B7.

**T-Cell Activation and Redistribution of T-Cellular Cytotoxicity.**

After the binding of the anti-CD3×anti-CEA diabody to both of its designated antigens was confirmed, we assessed whether this bispecific molecule was capable to activate T cells and to redirect cytotoxic T cells toward CEA-positive target cells.

T-cell activation induced by the anti-CD3×anti-CEA diabody was analyzed at the single-cell level in Jurkat T cells by monitoring the concentration of free cytosolic calcium (Fig. 2A). Only T cells in contact with CEA-expressing tumor cells showed an increased intracellular calcium concentration after treatment with the diabody. If incubated with monospecific anti-CD3 diabodies, or with control diabodies (anti-CD3×anti-nitrophenyl), or without preincubation with diabodies, T cells did not show any changes in [Ca^{2+}], on contact with tumor cells (data not shown). The cytosolic calcium concentration showed regular rhythmic oscillations within the first minute after contact with CEA-positive tumor cells (Fig. 2B), which lasted from several seconds up to 15 min. The oscillations and the sustained rise of [Ca^{2+}], depended on a transmembrane Ca^{2+} influx, Cd^{2+} (1 msi) and isomolar replacement of extracellular Na^{+} by K^{+} blocked the oscillations (Fig. 2C) and resulted in a decrease in [Ca^{2+}].

The bispecific diabody was found to induce cytolysis of CEA-expressing target cell lines (H498 and L1576) by CTLs in a dose-dependent manner (Fig. 3). Specific cytolyis was observed at concentrations of the bispecific diabody from 10 µg/ml to 10 ng/ml, whereas neither parental bivalent diabodies nor irrelevant bispecific diabodies (anti-CD3×anti-nitrophenyl) induced cytolysis of the CEA-positive target cells. Additionally, CEA-negative target cell lines REH (pre-B-cell) or HL60 (myelomonocytic cell line) were not killed on incubation with cytotoxic T cells and anti-CD3×anti-CEA bispecific diabodies (data not shown).

**Activation of Autologous T Cells in Colon Carcinoma.**

To investigate the T-cell stimulatory capacity of the bispecific diabodies in an experimental setting with MHC-identity between target and responder cells, we performed stimulation assays with primary colon carcinoma specimens, as determined by flow cytometric phenotyping of the stimulation cultures (Fig. 4, B-D), the expression of T cell activation markers as CD25 (Fig. 4E), and the production of human IFN-γ (data not shown). These effects were not detectable in the CEA-negative tumor specimen, indicating the CEA specificity of the T-cell activation induced by the anti-CD3×anti-CEA bispecific diabody in combination with costimulation via the CD28-molecule.

**T-Cell Costimulation with B7×anti-CEA Fusion Proteins.**

To confirm the CEA specificity of the bispecific diabody approach and, more importantly, to restrict not only the CD3-activating moiety but also the CD28 costimulation to the tumor cells, we introduced a second bispecific molecule, the B7×anti-CEA fusion protein. Additional experiments were performed with the defined CEA-positive carcinoma cell line L1576 and the CEA-negative B-lymphoma cell line LAZ509 to assess the costimulatory capacity, as well as the CEA specificity of B7×anti-CEA fusion proteins.

In the presence of L1576 colon carcinoma cells and anti-CD3×anti-CEA bispecific diabodies, CD4-positive T cells from healthy donors secreted comparable amounts of IFN-γ after costimulation by both anti-CD28 antibodies and B7×anti-CEA fusion proteins (Fig. 5A). However, if the colon carcinoma cells were preincubated with the diabodies and fusion proteins and then washed before the addition of the T cells, the costimulatory capacity of anti-CD28 antibodies was diminished. Furthermore, decreased IFN-γ production of the T cells was observed if CEA-negative target cells (Fig. 5B) were preincubated with either bivalent anti-CD28 antibodies or B7×anti-CEA fusion proteins. These findings demonstrate that the costimulatory signaling provided by B7×anti-CEA fusion proteins depended on the expression of CEA on the target cell surface, whereas anti-CD28 antibodies were removed from the stimulation cultures by washing of the tumor cells.

**DISCUSSION**

In industrialized countries, colon carcinoma represents one of the major neoplasms and has a high mortality (25). Particularly in advanced stages, a definite cure cannot be achieved by surgery alone due to the persistence of malignant cells. Thus, alternative therapeutic approaches toward minimal residual disease are desirable, and immunotherapeutic strategies are currently under investigation.

The clinical value of a humoral immunotherapeutic targeting of colon carcinoma micrometastasis by monoclonal antibodies was recently demonstrated (26). A different immunotherapeutic approach
Fig. 4. T-cell stimulation in primary colon carcinoma. Colon carcinoma tissue specimens were analyzed for CEA expression by flow cytometry (A). PBMCs obtained from the patients were incubated for 5 days with medium (B) or anti-CD3×anti-CEA bispecific diabodies and monospecific anti-CD28 antibodies (C). After 5 days, the cells were harvested and analyzed by flow cytometry for differences in the numbers of CD4+ and CD8+ T cells, and the T cell:tumor cell ratio was calculated from the flow cytometric data (D). The expression of CD25 (α chain of interleukin-2 receptor) was analyzed on both CD4+ and CD8+ T cells after the antibody stimulation and is related to the incubation with medium (E).
toward tumors is the redirection of cellular effector systems such as T cells, natural killer cells, or FcR-positive cells (granulocytes, macrophages) by bispecific antibodies, a treatment modality that has been examined in preclinical and clinical studies (10, 27–31). However, the progress of these immunotherapeutic agents into clinical applications has been slow, mainly due to the low yields of clinical grade bispecific agents, as well as the development of human immune responses because bispecific antibodies usually were produced by modifications of complete murine immunoglobulins.

Among the different formats of recombinant bispecific molecules, diabodies resemble antibody fragments consisting of four antibody V-domains and are formed by two cross-paired scFvs, resulting in a molecular weight of $\sim M_r 55,000$ (16). Because diabodies lack the Fc fragment, human immune responses against the diabodies should be neglectable, which is advantageous over hybrid-hybridoma-derived bispecific antibodies with regard to clinical applications, if repeated administrations are required. Diabodies are secreted in functional form from E. coli bacteria, with yields of up to 1 mg/liter, and can be isolated by a single purification step. Their small size facilitates penetration into tumor tissues (13), and the lack of the Fc portion avoids the Fc receptor-mediated bystander killing (27).

We describe the functional properties of an anti-CD3×anti-CEA diabody that was constructed to redirect autologous T cells toward CEA-expressing tumor cells. As shown by imaging of intracellular calcium concentration in T cells, the bispecific diabody was able to activate T cells through engagement of the CD3-TCR protein complex. The increases in calcium were only observed if T cells were cross-linked to CEA-positive tumor cells and, consequently, the bispecific anti-CD3×anti-CEA diabody was effective in redirecting human T cells to specifically lyse CEA-positive colon carcinoma cells in vitro.

As T-cell-based antitumoral immunotherapies have to consider the sufficient activation of the effector cells, systemic interleukin-2 administration (9), or infusion of ex vivo generated lymphokine-activated killer cells should be performed (7, 8). However, the systemic preactivation of T cells has to take unwanted clinical effects into account that can be induced by the unspecifically activated effector cells. On the contrary, costimulation via the CD28/B7 pathway (32) has the potential to avoid these obstacles if the costimulatory signaling can be restricted to tumor-specific T cells or to T cells in the tumor cell environment.

Stimulation assays with primary colon carcinoma isolates revealed that the activation of autologous T lymphocytes induced by bispecific anti-CD3×anti-CEA diabodies is dependent on the expression of

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**Fig. 5. CEA-specific costimulation of T cells.**

CD4-positive T-helper lymphocytes of healthy donors ($1 \times 10^5$) were cocultured with $2.5 \times 10^4$ glutaraldehyde-fixed cells of the CEA-positive carcinoma cell line L1576 (A) or the CEA-negative B cell line LAZ509 (B) and the indicated antibodies (1 $\mu$g/ml). Prewashing of the targets was performed by incubation of the tumor cells with both indicated antibodies, followed by a washing step before the T cells were added. The concentration of human IFN-γ was measured in the culture supernatant by ELISA and is related to the incubation of T cells and tumor cells with medium (stimulation index = 1). The incubation of T cells with each of the antibody combinations, but without tumor cells, served as control and did not result in IFN-γ production.
CEA on the colon carcinoma cells. In these assays, the costimulatory signal required for the optimal activation of resting T cells was provided by monospecific bivalent anti-CD28 antibodies. To restrict not only the CD3-activating moiety, but also the costimulatory signaling to the CEA-positive tumor cells, we produced a second bispecific molecule (a B7×anti-CEA fusion protein) to display engagement sites for the T-cell antigen CD28 on the surface of colon carcinoma cells. Our data clearly demonstrate that the signaling of the B7×anti-CEA fusion proteins required the presence of CEA-positive target cells. In contrast, neither the presence of CEA-negative cells nor the use of bivalent anti-CD28 antibodies did induce a sufficient T-cell activation. This experimental setting reflects the in vivo situation in which bispecific molecules with a TAA-binding moiety enrich at the tumor cell surface, whereas unbound antibodies are removed from the circulation. Therefore, this strategy for activation and retargeting of resting T cells is highly tumor-specific because the optimal T-cell stimulation will only be induced in the presence of cells expressing the TAA. Thus, we conclude that the tumor specificity of anti-CD3×anti-CEA bispecific antibodies can be improved by the use of B7×anti-TAA fusion proteins as costimulatory agents, being equally effective but more specific compared with anti-CD28 monoclonal antibodies. Additionally, in contrast to the use of anti-CD28 antibodies (33), B7×anti-TAA fusion proteins can interact with both CD28 and CTLA4, thus providing a more physiological coactivation.

Although our strategy was designed to both activate and to target T cells, the display of B7 on the tumor cell surface may also amplify weak antitumor responses without the need for application of anti-CD3×anti-CEA bispecific antibodies as “artificial” T-cell activators. In animal models, it has been shown that the immunogenicity of B7-negative tumors can be enhanced by ex vivo transfection of B7 into the malignant cells (34). However, because the tumors have to be exploited for genetic modifications, this approach not only inevitably destroys the tumor architecture but also results in the removal of tumor-infiltrating lymphocytes, which are considered to be partially tumor-specific. In this regard, the use B7×anti-CEA fusion proteins may be a more “physiological” way of B7-transfer to the malignant cells that depends only on the availability of antibodies against a suitable TAA (35).

We have shown that anti-CD3×anti-CEA bispecific diabodies and B7×anti-CEA bispecific fusion proteins can be used for the redirection of T cells toward CEA-expressing tumors. The simultaneous display of ligands for both of the T-cell antigens CD3 and CD28 on the tumor cell surface induces activation of anergic T cells, and the cytotoxic activity of which can then be redirected toward the malignant cells. Importantly, this approach is highly dependent on the expression of CEA as the TAA on the malignant cells. In this study, CEA served as a model antigen as one of the well-characterized TAAs expressed on solid tumors. However, “universal” anti-CD3×anti-hapten bispecific diabodies5 and B7×anti-hapten fusion proteins can be used in combination with haptenized tumor-ligands such as antibodies, antibody fragments, hormones, or cytokines for high-specific targeting of a wide variety of human malignancies. Compared with bispecific antibodies generated by the “classical” technologies, these recombinant bispecific molecules may have a greater ability to assert themselves as immunotherapeutic agents. However, clinical testing is necessary to judge on the clinical value of this bispecific antibody approach.

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