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Induction of Human T Lymphocyte Cytotoxicity and Inhibition of Tumor Growth by Tumor-Specific Diabody-Based Molecules Secreted from Gene-Modified Bystander Cells

Belen Blanco,* Phillip Holliger,† Richard G. Vile,‡ and Luis Alvarez-Vallina²*

Infiltrating T cells are found in many malignancies, but they appear to be mostly anergic and do not attack the tumor, presumably because of the absence of activation and/or costimulatory signals. We describe a strategy for cellular antitumor immunotherapy by the in situ production of soluble bifunctional Ab-based molecules that activate and retarget T cells to the tumor. We genetically modified cells to simultaneously secrete two bifunctional molecules, a bispecific diabody directed against the carcinoembryonic Ag (CEA) and the CD3ε chain of the TCR (αCEA × αCD3), and a fusion protein comprising the extracellular portion of B7-1 fused to a bivalent anti-CEA diabody (B7-αCEA). Together, αCEA × αCD3 and B7-αCEA proved potent at inducing the activation, proliferation, and survival of primary human T cells. When producer cells were cocultured with primary T cells and CEA⁺ cancer cells, αCEA × αCD3 and B7-αCEA acted in combination to activate and retarget T cell cytotoxicity and completely abrogate tumor growth in the coculture. Furthermore, the introduction of just a few such producer cells at the tumor site efficiently inhibited the growth of established human colon carcinoma xenografts. Despite a cumbersome generation process, the use of autologous gene-modified producer cells opens the way for a new diabody-based gene therapy strategy of cancer.


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 Ag-specific immune responses and their capacity for developing immunological memory. However, T cell-based cancer immunotherapies present significant obstacles. To date, the majority of the T cell-defined tumor Ags identified are unmutated differentiation Ags that are recognized as self, and there is little or no evidence that these Ags can function as tumor rejection Ags (1, 2). Furthermore, different epitopes from the same Ag may be hidden from the immune system, depending on proteolytic processing, differential binding to allelic MHC variants, and the TCR repertoire of the individual (3). In general, tumor cells themselves provide poor targets for immunological responses. In many cases, surface expression of MHC molecules is down-regulated or lost altogether, and changes in the peptide transport or proteosome functions (4, 5) provide for poor peptide display on the MHC. Furthermore, there is an insufficient expression, or absence, of costimulatory molecules (6) and secretion of mediators that depress cellular immunity (7).

An effective strategy for cancer immunotherapy, therefore, has to overcome the above obstacles and efficiently activate and retarget the body’s T cells to attack the tumor. Among the most promising strategies are those that combine the high specificity of Ab molecules with the efficient trafficking properties and effector functions of immune cells (8). These Ag-selective cell therapies (ASCT)³ are designed to convert therapeutically important native Ags expressed on the cell surface (tumor-associated Ags) into recruitment points of effector functions and to promote MHC-independent activation of mature effector T cells. These therapies include the use of bispecific Abs (BsAb) (9) and genetic manipulation of the recognition specificity of T cells by grafting the recognition specificity of an Ab onto the signaling components of the TCR/CD3 complex to create a chimeric immune receptor (CIR) (10, 11).

Although their effectiveness in cancer therapy has been extensively proved in different in vitro and in vivo models (8, 9), both ASCT approaches present limitations. The therapeutic potential of exogenously administered BsAbs can be limited by their short half-life and poor accessibility to tumor sites (12, 13). Moreover, systemic administration of BsAbs can also lead to serious side-effects and toxicity (14, 15) due to the acute release of cytokines. A major drawback of CIR-based therapies is that tumor-specific signals are restricted to gene-modified cells. Further complications include the potentially large antigenic diversity in target cell populations and, in many tumors, the high serum levels of soluble tumor Ag (16).

Both strategies have to overcome the consequences of the dual nature of the T cell activation process. It is generally accepted that T cell activation requires two distinct signals (17). The first signal depends on ligation of the TCR/CD3 complex. The second, or costimulatory, signal can be provided by cell surface molecules that complement TCR/CD3-mediated events. Among these, the

³ Abbreviations used in this paper: ASCT, Ag-selective cell therapy; BsAb, bispecific Ab; CEA, carcinoembryonic Ag; CIR, chimeric immune receptor; dAb, diabody; DCM, DMEM complete medium; IRES, internal ribosomal entry site; RCM, RPMI complete medium; scFv, single-chain variable fragment; SN, supernatant.
interaction between members of the B7 family (B7-1, CD80; B7-2, CD86) on APCs with CD28 on T cells has been shown to play a key role in initiating and maintaining T cell responses (18). Co-stimulation appears to be required not only for T cell activation, but also to prevent deletion of activated T cells by rendering them apoptosis resistant (19). Recently, it has been shown that CD28 triggering is required for efficient elimination of tumor cells in immunotherapy with BsAbs (20, 21) and other T cell-based immune strategies (22). Therefore, an effective ASCT strategy must target both pathways and provide tumor-specific activation signals to the entire pool of T cell effectors (transrecruitment and multi-effector activation) present at the tumor site.

Here we describe a novel strategy for promoting a tumor site-restricted T cell activation and induction of cytolyis through the in situ production of bifunctional Ab molecules by gene-modified cells. We chose two Ab molecules based on the diabody (dAb) format (23), comprising two Ab variable fragment heads of the same or different specificities, arranged back-to-back (24). The first was a bispecific dAb (α-carcinomembryonic Ag (αCEA) × αCD3) directed against the ε-chain of the TCR/CD3 complex and against CEA, a cell surface protein expressed in 80% of colon cancers and a range of other malignancies (25). The second was a fusion protein (B7-dIII) comprising the two extracellular domains of human B7.1 joined to a bivalent anti-CEA dAb. We generated vectors that allow the secretion of both molecules from the same cell, and we demonstrated that the secreted dAb-based molecules provide both functional and tumor-specific signals 1 and 2 for the activation of primary human T cells. We also show that coexpression of these molecules by producer cells provides an effective means to redirect effector functions in primary T lymphocytes and target T cell cytotoxicity, specifically against CEA-expressing tumor cells. These results open the way for a new dAb-based gene therapy strategy of human cancer.

Materials and Methods

Antibodies

The mAb used included: OKT3 (Ortho Biotech, Raritan, NJ) specific for human CD3ε, DAL-1 (SeroTec, Oxford, U.K.) specific for human CD80, 85A12 (SeroTec) specific for human CD66e, and YTH 913.12 (SeroTec) specific for human CD28. Anti-Myc (9E10) mAb was obtained from Sigma-Aldrich (St. Louis, MO). The polyclonal Abs used included an HRP-coupled goat anti-human CD28. Anti-Myc (9E10) mAb was obtained from Sigma-Aldrich, and a range of other malignancies (25). The second was a fusion protein (B7-dIII) comprising the two extracellular domains of human B7.1 joined to a bivalent anti-CEA dAb. We generated vectors that allow the secretion of both molecules from the same cell, and we demonstrated that the secreted dAb-based molecules provide both functional and tumor-specific signals 1 and 2 for the activation of primary human T cells. We also show that coexpression of these molecules by producer cells provides an effective means to redirect effector functions in primary T lymphocytes and target T cell cytotoxicity, specifically against CEA-expressing tumor cells. These results open the way for a new dAb-based gene therapy strategy of human cancer.

Cells and culture conditions

293T, HT-29, and HCT-116 cells were grown in DMEM supplemented with 10% FCS (Invitrogen Life Technologies, Carlsbad, CA), referred to as DMEM complete medium (DCM). Jurkat (clone E6-1), HUT-78, K562, and HeLa cells were maintained in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS, referred as to RPMI complete medium (RCM). All these cell lines were obtained from American Type Culture Collection (Manassas, VA). HeLa-CEA cells (26) were grown in RCM supplemented with 750 μg/ml G418 (Invitrogen Life Technologies). MKN45 cells (27) were cultured in RCM.

Construction of vectors

The two V72 MFE-VeOKT3 and V72 OKT3-VeMFE chains were amplified from plasmid pUC119 αCEA/αCD3 (27) with primers pairs 1 and 2 or 3 and 4, respectively (Table I). These PCR products were reamplified with primers pairs 5 and 6 or 7 and 8. The ClaI/NotI-digested PCR fragments were ligated into the ClaI/NotI-digested backbone of plasmid pVOM.eNIP (28) to obtain plasmids pBB1 (containing VHMF-E-VeOKT3) and pBB2 (containing VHOKT3-VeMFE). The sequence was verified using primers 9 and 10. The HindIII/NotI fragments from plasmids pBB1 and pBB2 were cloned into the episomal expression EBV-based vectors pREP9.6/Hmyc or pCEP4.6/Hmyc (28) to obtain plasmids pAda1 and pAda2, respectively. The HindIII/NotI fragment derived from the pAda1 was introduced into the HindIII/NotI site of pAda2, resulting in pAda1bis. Plasmid pRES-Bgeo (provided by A. Smith; Medical Research Council Laboratory of Molecular Biology) was digested with PstI to remove an XhoI site and was ligated to form pRESnew, in which an oligonucleotide (oligonucleotides 11 and 12) carrying two stop codons was introduced between NotI and XbaI. In this plasmid we cloned the fragment NolI/XhoI from pBB2 to obtain pRES- dAda2. pAda3 was constructed introducing the fragment NotI/NolI from pRES-dAda2 in pAda1bis. Several digestions were made to check that the fragment had been inserted in the correct direction. To construct plasmids pR32 (−His6-Myc tag) and pLAV32.tag (+His6-Myc tag), the 1520-bp HindIII/NotI fragment derived from the plasmid pUC19.B7-I/MFE-23- yck (29) was cloned in pREP9 (Invitrogen Life Technologies) or pCEP4.6/Hmyc (28), respectively.

Cell transfections

293T cells were transfected with plasmids pAda1, pAda2, pAda3, pR32, and pLAV32.tag using Lipofectamine (Invitrogen Life Technologies). Supernatant was recollected at 48 h and analyzed for αCEA×αCD3 dAb and/or B7-αCEA fusion expression by ELISA (29) and SDS-PAGE and Western blotting using anti-Myc mAb. To generate stable cell lines, pAda1- and pAda3-transfected 293T cells (293T.dAda-1 and 293T.dAda) were selected in DCM with 150 μg/ml hygromycin B (Invitrogen Life Technologies); pAda2- and pR32-transfected 293T cells (293T.dAda-2 and 293T.dAda) were selected in DCM with 2 mg/ml G418 (Invitrogen Life Technologies). To generate double transfectants, 293T.dAda-1 and 293T.dAda were transfected with plasmid pAda2 or pR32 respectively, and selected in DCM with 150 μg/ml hygromycin B and 2 mg/ml G418. Jurkat, HUT-78, and K562 cells were transfected with pAda3 plasmid using Superfect (Qiagen, Hilden, Germany) and were selected in RCM supplemented with 400 or 250 μg/ml hygromycin B. Supernatants from stable transfected cell populations were analyzed for dAda and B7-1 fusion protein secretion by ELISA (29).

Table I. Oligonucleotide sequencesa

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
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<tbody>
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<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>ATTGTGATCGAAGCTTG</td>
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<tr>
<td>3</td>
<td>CAGCTGTGATGCAAAT</td>
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</tr>
<tr>
<td>7</td>
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</tr>
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<td>8</td>
<td>GAGTCATCTCGCGCGCCCGCTTTATTTCCACGTTGCC</td>
</tr>
<tr>
<td>9</td>
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</tr>
<tr>
<td>10</td>
<td>AAACACAGATGGCTGCG</td>
</tr>
<tr>
<td>11</td>
<td>GGCGCATAATAAT</td>
</tr>
<tr>
<td>12</td>
<td>CTAGATTATTAGTGC</td>
</tr>
</tbody>
</table>

a Sequences of the various primers applied for the construction of the vectors, and subsequent verification of vector sequences.
Expression and purification of the recombinant dAb-based molecules

TG1 was used for propagation of the plasmids pUC119 MFE23/OKT3 and pUC119.B7-1/MFE-23 and the expression of Ab fragments. Soluble dAb-based molecules were expressed as previously described (23) and were purified from concentrated culture supernatant by single-step IMAC.

IL-2 release assay

Jurkat cells (5 × 10⁴/well) were stimulated in duplicate under various conditions in round-bottom, 96-well microtiter plates with mitomycin C (Kyowa Hakko Kogyo, Tokyo, Japan)-inactivated target (HeLa; HeLa-CEA, HT-29, HCT-116, or MKN45) cells at a 1:1 E:T cell ratio. Where indicated, irradiated effector cells were cultured in wells precoated with anti-CD3 mAb (10 μg/ml) either alone or with soluble rat anti-CD28 mAb (2.5 μg/ml) and goat anti-rat IgG (20 μg/ml) (30). The plates were incubated at 37°C in 5% CO₂/95% air. After 20 h, supernatants (SNs) were harvested and analyzed for IL-2 secretion using a commercially available ELISA (Diaclone, Besançon, France).

T cell proliferation assays

To produce effector cells for the functional studies, PBMCs were isolated from the buffy coat fraction of normal donor peripheral blood by density gradient centrifugation and subsequently passed through a nylon-wool syringe. The enriched T cell preparation contained >85% T cells (CD3⁺), <5% B cells (CD20⁺), and ~10% other cells as determined by flow cytometric analysis (data not shown). Enriched primary T cells (5 × 10⁵ well) were stimulated in triplicate under various conditions in 96-well microtiter plates with mitomycin C-inactivated target (HeLa, HeLa-CEA, or MKN45) cells at different E:T cell ratios. Where indicated, concentrated filtered cell-free SN from 48-h cultures of either untransfected (293T) or stable transfected (293T.dAb or 293T.dAb and 293T.B7) cells was added. As controls, effector cells were cultured in wells precoated with anti-CD3 mAb (10 μg/ml) either alone or with soluble rat anti-CD28 mAb (2.5 μg/ml) and goat anti-rat IgG (20 μg/ml) (30). The plates were incubated at 37°C in 5% CO₂/95% air. After 20 h, supernatants (SNs) were harvested and analyzed for IL-2 secretion by a liquid scintillation counter (Wallac Oy, Turku, Finland).

Flow cytometry

The expression of CD66e on HeLa, HeLa-CEA, HT-29, HCT-116, and MKN45 and the binding of the secreted oCEA × αCD3 dAb to CD3⁺ cells were studied as previously described (27, 29). Enriched primary T cells were treated with appropriate dilutions of FITC- and PE-conjugated mAbs. The samples were analyzed with an EPICS XL (Coulter Electronic, Hialeah, FL).

Cytotoxicity assays

For induction of cytotoxicity, enriched primary T cells (2 × 10⁵/well) were stimulated in duplicate under various conditions in 24-well plates with irradiated target (HeLa or HeLa-CEA) cells at a 10:1 E:T cell ratio in AIM-V medium (Life Technologies, Gaithersburg, MD). Where indicated, concentrated filtered cell-free SN from 72-h cultures of either untransfected (293T) or stable transfected (293T.dAb or 293T.dAb.B7) cells was added. The plates were incubated at 37°C in 5% CO₂/95% air. After 5 days T cells were harvested and incubated (10³/well) in 96-well plates with 10⁷ fluorochrome PKH67-labeled target (HeLa or HeLa-CEA) that had been pre-incubated (1 h, 4°C) with 100 μl of concentrated filtered cell-free SN from 72-h culture of 293T.dAb cells. The uptake of propidium iodide (5 μg/ml) by avital PKH67⁺ cells was determined by flow cytometry after 4 h at 37°C. The percentage of avital HeLa or HeLa-CEA cells was calculated as the 100-fold ratio of (experimental uptake − spontaneous uptake) / (maximal cell number − spontaneous uptake) (21). For cytotoxic studies in Transwell systems, a polyethylene terephthalate filter insert (6.5 mm diameter) with 0.4-μm pores (Falcon, BD Biosciences, Bedford, MA) was used. Pre-established monolayers of target cells (HeLa, HeLa-CEA, or MKN45) were coincubated with primary T cells (2 × 10⁵) in the lower compartment, and 293T cells (2 × 10⁴), either unmodified (293T) or stably transfected (293T.dAb, or 293T.dAb.B7), were added to the upper compartment. After 96 h the Transwell insert was removed, and the nonadherent cells were removed by washing with PBS. Adherent cells were fixed in 1% glutaraldehyde in PBS, stained with 0.1% crystal violet, and examined under the microscope.

Animal studies

Animal studies were reviewed and approved by the animal care and use committee of Mayo Clinic and are in accordance with the guidelines of the Department of Health and Human Services. We used human HCT-116 cells in these studies as a low CEA-expressing cancer cell line. HCT-116 cells (2 × 10⁶/animal) and irradiated (25 GY) transfectected (293T) or stably transfected dAb-producer (293T.dAb or 293T.dAb.B7) cells (1.5 × 10⁷/animal) were s.c. implanted into the dorsal space of 5-wk-old female athymic nude mice (The Jackson Laboratory, Bar Harbor, ME). On day 8, when tumors were palpable, the animals received intratumoral injections of human PBLs (1 × 10⁶) that were preactivated in vitro with plastic immobilized anti-CD3 mAb (1 μg/ml), soluble anti-CD28 mAb (1 μg/ml), and low dose IL-2 (50 U/ml). Tumor volumes were determined at various time points; the formula: width² × length ÷ 0.52 for approximating the volume of a spheroid was applied.

Results

Design and expression of a bispecific αCEA×αCD3 two-chain dAb

Starting from the variable genes of the mAbs MFE23 (directed against human CEA) (31) and OKT3 (which recognizes the human CD3e chain) (32), we designed constructs for the expression of a five-amino acid linker, bispecific, two-chain dAb in eukaryotic cells. DAb chain 1 (VHMFE23-VoOKT3) and DAb chain 2 (VHOKT3-VoMFE23) (27) were cloned into mammalian expression vectors (pdAb1 and pdAb2, respectively) containing the human oncostatin M leader sequence (Fig. 1A). PdAb1 is a bicistronic vector containing the IRES sequence of the encephalomyocarditis virus, preceded by the dAb1 chain and followed by the dAb2 chain (Fig. 1B). Transfection of human 293T cells with pdAb1 plasmids or cotransfection with both pdAb1 and pdAb2
plasmids resulted in the secretion of functional dAb. The secreted αCEA × αCD3 dAb showed high stability under physiological conditions (data not shown), bound specifically to CEA as determined by ELISA (Fig. 2A), and bound specifically to the surface of CD3+ cells as determined by flow cytometry (data not shown). No binding to CD3− cell lines was observed (data not shown). Western blot analysis, under reducing conditions, of culture medium from 293T cells transfected with plasmid pdAb3 (Fig. 2B) demonstrated that the migration pattern of the secreted dAb was consistent with the predicted m.w. Functional dAb was not detected in the cell culture supernatant of 293T cells transfected with either pdAb1 or pdAb2 alone (Fig. 2A).

We next investigated whether our bicistronic vector allowed the efficient secretion of αCEAαCD3 dAb by human hemopoietic cells.

**FIGURE 2.** Characterization of dAb molecules produced by human cells. A, Secretion of functional αCD3 × αCEA dAb into the cell culture supernatant by human embryonic kidney 293T cells transfected with pdAb1, pdAb2, or pdAb3 plasmid or cotransfected with both pdAb1 and pdAb2 plasmids. The functionality of secreted dAb was demonstrated by ELISA against plastic immobilized human CEA (■) and BSA (□). Bound dAb was detected with an anti-Myc mAb. B, Western blot analysis of αCEA × αCD3 dAb secreted into the cell culture supernatant either by untransfected (1) or transfected (pdAb3) human 293T cells (2). Lane 3, Recombinant dAb (rdAb) purified from bacterial culture supernatants. M, molecular mass standards (kilodaltons). The blot was developed with an anti-Myc mAb. C, Secretion of functional dAb into the cell culture supernatant of 293T, Jurkat T, or K562 cells stably transfected with pCEP4 or pdAb3 plasmid. D, Assessment of the capability of secreted αCD3 × αCEA dAb to act as an efficient Ag-specific activator molecule for T cells in the presence of CEA-expressing tumor cells. Approximately 5 × 10⁴ Jurkat cells were stimulated (E:T cell ratio = 1:1) with inactivated CEA-negative HeLa (□) or CEA-positive MKN45 (■) target cells in the presence of medium, rdAb, or cell-free supernatant from cultures of either untransfected 293T cells or stably transfected cells with plasmid pdAb3 (293T.dAb). The level of IL-2 secreted was compared with those obtained after stimulation with anti-CD3ε mAb or with anti-CD3ε mAb and anti-CD28 mAb.
With this aim, CD3\(^+\) (Jurkat) and CD3\(^-\) (K562) cell lines were stably transfected with pdAb3 plasmid. K562 cells are multipotential blasts that spontaneously differentiate into progenitors of the erythrocytic, granulocytic, and monocytic series (33). Transfected K562 cells secreted functional dAb, while no dAb was detected in the supernatant of Jurkat cell transfectants (Fig. 2C). Similar results were observed in other human T cell line (HUT-78; data not shown). The endoplasmic reticulum is the natural site of Ab assembly, being the residence of molecular chaperones that assist in the correct folding of Ig molecules (34). Presumably, in CD3\(^+\) cell lines the αCEA × αCD3 dAb interacted with newly synthesized CD3 e-chains in the endoplasmic reticulum, and the complex was degraded (35, 36). Transfected Jurkat cells showed a normal pattern of expression of surface TCR/CD3 (data not shown).

T cell activation by human-produced αCEA × αCD3 dAb

To assess whether the human secreted dAb was capable of acting as an efficient activator molecule for T cells in the presence of CEA-expressing tumor cells, we performed different cocultures of Jurkat cells with either CEA\(^-\) or CEA\(^+\) tumor cell lines. After a 20-h incubation period, cell-free supernatants were collected to measure IL-2 secretion by ELISA (Fig. 2D). When cocultured with CEA\(^-\) (HeLa) cells in either the presence or the absence of bacterial or mammalian-produced αCEA × αCD3 dAb, Jurkat cells did not secrete significant levels of IL-2. However, when Jurkat cells were cocultured with CEA\(^+\) cells in the presence of cell-free supernatant from cultures of 293T.dAb cells, a significant level of IL-2 was produced (Fig. 2D and data not shown). The level of IL-2 was higher than that observed when tumor-specific cross-linking was conducted by the recombinant αCEA × αCD3 dAb (1 \(\mu\)g/ml). Under these conditions, Jurkat IL-2 secretion was similar to that observed after stimulation with plastic-immobilized anti-CD3ε mAb, but it was far from reaching the level obtained by optimal stimulation with anti-CD3ε plus anti-CD28 mAbs (Fig. 2D).

We have previously shown that B7-αCEA bispecific fusion proteins (Fig. 3A) can be secreted by gene-modified human cells (T and non-T cell lines) and that the secreted B7-αCEA fusion (Fig. 3B) bound specifically to the surface of CEA-expressing cancer cells, triggering potent costimulation of T cells when combined with approaches targeting the TCR pathway (29). Western blot analysis under reducing conditions demonstrated that the migration patterns of both bacterial and mammalian-produced B7-αCEA dAb fusions were similar (Fig. 3C). To assess whether the human secreted αCEA × αCD3 dAb and the B7-αCEA dAb fusion protein were capable of acting synergistically to produce maximal levels of IL-2, we performed different cocultures of Jurkat cells with either CEA\(^-\) (HeLa) or CEA\(^+\) (MKN45, a high CEA producer) expressing tumor cell line; HeLa-CEA, an intermediate CEA producer; expressing tumor cell line; HT-29, a low CEA producer; or HCT-116, a low CEA producer/
expressing tumor cell line) tumor cell lines (Fig. 3D and data not shown). As expected, the addition of cell-free supernatant from cultures of 293T.B7 cells (stably transfected with a B7-αCEA fusion gene-coding vector; Fig. 3A) resulted in a pronounced and dose-dependent enhancement of IL-2 secretion (Fig. 3D). The addition of B7-αCEA preserved cell viability (data not shown), indicating that it could mimic CD28-mediated T cell activation and survival (29). This effect was not observed when cell-free supernatant from untransfected 293T cells was used (not shown).

Significantly, maximal levels of IL-2 were observed when small amounts (10%, v/v) of cell-free supernatant from gene-modified producer cells were added to cocultures of Jurkat cells with CEA-positive MKN45 cells (Fig. 3D). These results suggest that a small number of producer cells are able to secrete sufficiently high levels of dAb-based molecules (αCEA × αCD3 and B7-αCEA) to supply effective and tumor-specific signals 1 and 2.

**Simultaneous secretion of bispecific αCEA×αCD3 dAb and B7-1 αCEA dAb fusion**

Next we investigated whether the same cell population was able to produce both the bispecific dAb and the B7 dAb fusion protein. 293T.B7 cells were transfected with pdAb3 plasmid. Double transfecants (293T.dAb.B7) secreted both proteins in a functionally active form (Fig. 3B and data not shown).

**Activation of primary T cells by secreted dAb-based molecules**

To assess the effectiveness of secreted dAb-based proteins in providing both activation and costimulatory signals from cellular Ag to primary T cells, unstimulated PBLs from healthy donors were cocultured with CEA-positive MKN45 cells (data not shown). In a standard 3H-thymidine proliferation assay, the human-secreted αCEA×αCD3 dAb induced proliferation of unstimulated primary T cells only in the presence of CEA-expressing cancer cells (Fig. 4B). In the presence of CEA− cells, the secreted dAb exerted almost no proliferative stimulus (Fig. 4A). The addition of supernatant from double-transfected 293T.dAb.B7 cells resulted in a strong increase in T cell proliferation compared with cells incubated in the presence of supernatant from single-transfected 293T.dAb cells (Fig. 4B).

**Induction of cytotoxic activity by secreted dAb-based molecules**

The ability of secreted dAb molecules to induce tumor cell lysis by redirecting T cell-mediated cytotoxicity was investigated using two different in vitro tests. First, the secreted αCEA × αCD3 dAb was found to induce cytolysis of CEA− target cell lines in a conventional assay, but only when using primary T cells previously stimulated with CEA+ cells in the presence of both αCEA × αCD3 dAb and B7-αCEA (Fig. 5A). Prestimulation with only the αCEA × αCD3 dAb was not effective. No cytotoxic activity was observed using the CEA− HeLa cell line as target cell (Fig. 5A). To approximate in vivo conditions and to investigate the ability of locally produced dAb molecules to induce tumor cell lysis by unstimulated PBLs, we used Transwell cell culture dishes. In this system CEA− (HeLa) or CEA+ (HeLa-CEA or MKN45) target cells and freshly isolated primary T cells were cocultivated with either untransfected (293T) or stably transfected dAb producer (293T.dAb or 293T.dAb.B7) cells (Fig. 5B). At a target:effector:producer ratio of 1:1:1, T cells activated with both αCEA × αCD3 and B7-αCEA exhibited stronger cytotoxicity to CEA− cell lines than T cells that were activated only with αCEA × αCD3 (Fig. 5C and data not shown). No cytotoxic activity was achieved after cocultivation with untransfected 293T cells or when nonexpressing CEA cell lines were used as targets (Fig. 5C). The omission of T cells produced no cytotoxicity (data not shown).

**Antitumor effect of locally produced dAb-based molecules**

To determine the in vivo antitumor activity of locally produced dAb-based molecules, we established a xenotransplant model of the human colon carcinoma cell line HCT-116, that expresses very low levels of CEA on the cell surface (data not shown). Cohorts of four mice were injected in the s.c. dorsal space with a mixture (1/1) of HCT-116 tumor cells and irradiated 293T cells (untransfected or stably transfected dAb producer). Significantly, gene-modified 293T cells secreted active dAb-based molecules (αCEA × αCD3 or αCEA × αCD3 and B7-αCEA) at detectable levels for >2 wk
post-radiation (data not shown). When tumors became palpable, animals were treated with a single intratumoral injection of pre-activated human T cell effectors. Sustained expression of the dAb effector molecules at the tumor site significantly \((p < 0.05)\) delayed the growth rate of established tumors (Fig. 6). Unmodified 293T cells had no effect on tumor growth.

**Discussion**

We have demonstrated proof-of-principle for a novel genetic strategy of T cell activation by paracrine secretion of tumor-specific, dAb-based molecules targeting both TCR and CD28 pathways. Bispecific \(\alpha\text{CEA} \times \alpha\text{CD3}\) two-chain dAb molecules were properly assembled in vivo and secreted in a functionally active form by gene-modified human CD3-negative cells. Although various bispecific Ab fragments (tandem single-chain variable fragment (scFv) and single-chain diabodies) have already been shown to be expressed and secreted by eukaryotic cells (37, 38), our results demonstrate for the first time that human cells can be engineered to simultaneously produce a bispecific, two-chain dAb and a chimeric B7 dAb fusion protein. The use of diabodies would have some advantages over the use of other formats of bispecific Abs, including the lack of an Fc portion, thus avoiding the killing of FcR-positive bystander cells (39) and their small size for better penetration of tumors (40). The anti-CEA Ab used in this study has shown excellent tumor localization in colon carcinoma patients in an scFv format (41) and in colon-carcinoma xenografts in nude mice, both as a bispecific dAb \(\alpha\text{CEA} \times \alpha\text{CD3}\) (data not shown) as well as a bivalent \(\alpha\text{CEA} \text{dAb}\) (42). Tumor-specific dAb-based molecules were secreted at high levels and were able to efficiently activate un-stimulated human peripheral blood T cells to proliferate and eliminate CEA-expressing tumor cells. Notably, locally produced dAbs showed significant cytotoxic activity in vivo against established tumors and only required the infusion of small numbers of functional T cells.

We see several advantages over current BsAb- or CIR-based approaches in our strategy. These include 1) the provision to tumor-associated Ag-expressing tumor cells, with binding specificities for both CD3 and CD28 receptors, from a single type of

**FIGURE 5.** Specific target cell lysis by human primary T cells redirected with dAb-based molecules. A. For induction of cytotoxicity, primary T cells were stimulated (E:T cell ratio = 10:1) with irradiated CEA-negative HeLa (■) or CEA-positive HeLa-CEA (▲) target cells in the presence of cell-free supernatant from cultures of either untransfected (293T) or stably transfected (293T.dAb or 293T.dAb.B7) cells. After 5 days T cells were harvested and incubated (E:T cell ratio = 100:1) with fluorochrome PKH67-labeled target (HeLa or HeLa-CEA) cells that had been preincubated (1 h, 4°C) with \(\alpha\text{CEA} \times \alpha\text{CD3}\) dAb containing cell-free culture supernatant. After a 4-h incubation period, the percentages of avital HeLa and HeLa-CEA cells were determined. B and C. Specific target cell lysis by human primary T cells redirected with dAb-based molecules locally secreted from gene-modified cells. B. Scheme of the Transwell cell culture chamber used. In the lower chamber (L), monolayers of inactivated CEA-negative (HeLa) or CEA-positive (HeLa-CEA) target cells were incubated. In the upper chamber (U), unmodified (293T) or stably transfected (293T.dAb or 293T.dAb.B7) 293T cells were added. C. After 96 h, adherent cells (lower chamber) were fixed, stained with crystal violet, and photographed.

**FIGURE 6.** Inhibition of in vivo tumor growth by locally produced dAb-based molecules. Groups of four athymic nude mice were s.c. injected with a mixture of \(2 \times 10^6\) human colon carcinoma HCT-116 cells and \(2 \times 10^6\) irradiated 293T cells, either untransfected (293T) or stably transfected (293T.dAb or 293T.dAb.B7). When tumors became palpable, animals received a single intratumoral injection of preactivated human T cells. The mean tumor volume and SE are shown for each time. The statistical significance of differences between groups was computed using Student’s \(t\) test (\(*, p < 0.05\)).

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