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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 CD3ζ 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. The Journal of Immunology, 2003, 171: 1070–1077.

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 transporters 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 restimulate T cells. 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 (BsAbs) (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 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...

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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 cytolysis 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 (a-carcinomembryonic Ag (oCEA) × αCD3) directed against the ϵ-chain of the TCR/CD3 complex and against CEA, a cell surface protein expressed in 80–90% of colon carcinomas and a range of other malignancies (25). The second was a fusion protein (B7-1/CEA) 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 91312 (Serotec) specific for human CD28. Anti-Myc (9E10) mAb was obtained from Sigma-Aldrich, an FITC-conjugated goat anti-mouse IgG ( Sigma-Aldrich), an FITC-conjugated goat anti-rat IgG (Serotec). For direct staining, the following FITC- or PE-conjugated mAbs (Beckman-Coulter, Miami, FL) were used: 39C1.5 (anti-human CD2), UCHT-1 (anti-human CD3ε), SFC112T4D11 (anti-human CD4), SFC112T4D11 (anti-human CD4), B9.11 (anti-human CD8), RMO54 (anti-human CD14), B9.11 (anti-human CD8), RMO54 (anti-human CD14), SFC112T4D11 (anti-human CD4), B9.11 (anti-human CD8), RMO54 (anti-human CD14), B9.11 (anti-human CD8), RMO54 (anti-human CD14), SFC112T4D11 (anti-human CD4), B9.11 (anti-human CD8), RMO54 (anti-human CD14), B9.11 (anti-human CD8), RMO54 (anti-human CD14), SFC112T4D11 (anti-human CD4), B9.11 (anti-human CD8), RMO54 (anti-human CD14), B9.11 (anti-human CD8), RMO54 (anti-human CD14), SFC112T4D11 (anti-human CD4), B9.11 (anti-human CD8), RMO54 (anti-human CD14), B9.11 (anti-human CD8), RMO54 (anti-human CD14), SFC112T4D11 (anti-human CD4), B9.11 (anti-human CD8), RMO54 (anti-human CD14), B9.11 (anti-human CD8), RMO54 (anti-human CD14), SFC112T4D11 (anti-human CD4), B9.11 (anti-human CD8), RMO54 (anti-human CD14), B9.11 (anti-human CD8), RMO54 (anti-human CD4, H299 (anti-human CD20), and N901 (anti-human CD56).

Cells and culture conditions

293T, HT-29, and HCT-116 cells were grown in DMEM supplemented with 10% FCS (In vitrogen Life Technologies, Carlsbad, CA), referred to as DMEM complete medium (DMC). Jurkat (clone E6-1), HUT-78, K562, and HeLa cells were maintained in RPMI 1640 (In vitrogen Life Technologies) supplemented with 10% FCS, referred to as 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 (In vitrogen Life Technologies). MKN45 cells (27) were cultured in RCM.

Construction of vectors

The two V72 MFE-VE-OKT3 and V72 OKT3-VeMFE chains were amplified from plasmid pUC119 oCEA/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 backbone of plasmid pVOM.oNIP (28) to obtain plasmids pBB1 (containing VHMF-VE-OkT3) 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 pdAb1 and pdAb2, respectively. The HindIII/NotI fragment derived from the pdAb1 was introduced into the HindIII/NotI site of pdAb2, resulting in pdAb1bis. Plasmid pHRES-Bgeo (provided by A. Smith; Medical Research Council Laboratory of Molecular Biology) was digested with PstI to remove an XhoI site and was religated to form pHRES.new, in which an oligonucleotide (oligonucleotides 11 and 12) carrying two stop codon was introduced between NotI and XhoI. In this plasmid we cloned the fragment NotI/XhoI from pBB2 to obtain pHRES-dAb2. pdAb3 was constructed introducing the fragment NotI/NotI from pHRES-dAb2 in pdAb1bis. Several digestions were made to check that the fragment had been inserted in the correct direction. To construct plasmids pH32 (−His6-Myc tag) and pLV32.tag (+His6-Myc tag), the 1520-bp HindIII/NotI fragment derived from the plasmid pUC19.B7-1/MFE-232 (29) was cloned in pREP9 (In vitrogen Life Technologies) or pCEP4.6/Hmyc (28), respectively.

Cell transfections

293T cells were transfected with plasmids pdAb1, pdAb2, pdAb3, pR32, and pLV32.tag using Lipofectamine (In vitrogen Life Technologies). Supernatant was recollected at 48 h and analyzed for oCEA×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, pdAb1-, and pdAb3-transfected 293T cells (293T.dAb-1 and 293T.dAb) were selected in DCM with 150 μg/ml hygromycin B (In vitrogen Life Technologies); pdAb2- and pR32-transfected 293T cells (293T.dAb-2 and 293T.B7) were selected in DCM with 2 mg/ml G418 (In vitrogen Life Technologies). To generate double transfectants, 293T.dAb-1 and 293T.dAb were transfected with plasmid pdAb2 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 pdAb3 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 dAb and B7-1 fusion protein secretion by ELISA (29).

Table I. Oligonucleotide sequences

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<td>GCCGATATAAAAT</td>
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<tr>
<td>12</td>
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*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/MFE-23 and the expression of Ab fragments. Soluble dAb-based molecules were expressed as previously described (23) and were purified from concentrated cell culture supernatant by single-step IMAC.

IL-2 release assay
Jurkat cells (5 \times 10^4/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, purified recombinant oCEA × αCD3 dAb at 1 μg/ml or concentrated filtered cell-free supernatant from 48-h cultures of either untransfected (293T) or stable transfected (293T.dAb or 293T.dAb.B7 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% CO_2/95% 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 \times 10^5 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.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% CO_2/95% air. After 20 h, supernatants (SNs) were harvested and analyzed for IL-2 secretion using a commercially available ELISA (Diaclone, Besançon, France).

Flow cytometry
The expression of CD66e on HeLa, HeLa-CEA, HT-29, HCT-116, and MKN45 cells 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 Electronics, Hialeah, FL).

Cytotoxicity assays
For induction of cytotoxicity, enriched primary T cells (2 \times 10^5/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% CO_2/95% air. After 5 days T cells were harvested and incubated (10^4/well) in 96-well plates with 10^4 fluorescent 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) to (maximal cell number – spontaneous uptake) (21). For cytostatic 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 \times 10^5) in the lower compartment, and 293T cells (2 \times 10^5), 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 \times 10^9/animal) and irradiated (25 Gy) untransfected (293T) or stably transfected dAb-producer (293T.dAb or 293T.dAb.B7) cells (1.5 \times 10^9/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 \times 10^6) 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: width^2 × 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 (VHMF23-VαOKT3) and DAb chain 2 (VHOKT3-VαMFE23) (27) were cloned into mammalian expression vectors (pDAb1 and pDAb2, respectively) containing the human oncostatin M leader sequence (Fig. 1A). pDAb3 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 pDAb3 plasmids or cotransfection with both pDAb1 and pDAb2

![FIGURE 1. Genetic structure of dAb-based constructs. A, Monocistronic vectors containing either the proximal (pDAb1) or the distal (pDAb2) chain of the bispecific (αCEA × αCD3) dAb (MFE/OKT3). B, Bicistronic vector containing both dAb chains and the encephalomyocarditis virus IRES sequence. The direction of transcription is indicated by the arrows. The Hist6-Myc tag appended is for immunodetection.](http://www.jimmunol.org/)
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 × 10⁶ 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.
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 μg/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) tumor cell line; HeLa-CEA, an intermediate CEA producer; or HCT-116, a low CEA producer/

FIGURE 3. A, Diagrammatic representation of the B7.1-αCEA dAb (MFE) fusion gene construct (29). The direction of transcription is indicated by the arrows. The His6-Myc tag appended is for immunodetection. B, Production of αCEA × αCD3 dAb and/or B7-αCEA fusion protein by human cells. The functionality of secreted dAb-based molecules was demonstrated by ELISA against plastic immobilized human CEA (■) and BSA (□). Bound αCEA × αCD3 dAb was detected with an anti-Myc mAb, and bound B7-αCEA was detected with an anti-CD80 mAb (DAL-1). C, Western blot analysis of B7-αCEA fusion protein secreted into the cell culture supernatant either by untransfected (3) or single transfected (pLAV32.tag) human 293T cells (2). Lane 1, B7-αCEA fusion purified from bacterial culture supernatants, D, Assessment of the effectiveness of secreted B7-αCEA in providing costimulation from cellular Ag to T cells activated by the secreted αCEA × αCD3 dAb. Approximately 5 × 104 Jurkat cells were stimulated (E:T cell ratio = 1:1) with inactivated CEA-negative (HeLa) or CEA-positive (MKN45 or HeLa-CEA) target cells in the presence of cell-free supernatant (10 μl) from cultures of 293T cells stably transfected with pDAβ3 plasmid (293T.dAb) and different amounts of cell-free supernatant (0–30 μl) from cultures of 293T cells stably transfected with pR32 plasmid (293T.B7).
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+/H11001 amounts (10%, v/v) of cell-free supernatant from gene-modified producer cells were added to cocultures of Jurkat cells with CEA+/H11001 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 transfectants (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+ tumor cell lines (Fig. 4). In a standard [3H]thymidine proliferation assay, the human-secreted αCEA×αCD3 and B7-αCEA) to supply effective and tumor-specific signals 1 and 2.

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 cytolyis 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, which 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

![FIGURE 4.](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org) Induction of T cell proliferation by human-secreted αCEA × αCD3 and B7-αCEA molecules. A standard [3H]thymidine proliferation assay was performed with human unstimulated primary T cells cultured (E:T cell ratio = 1:1) with inactivated CEA-negative HeLa (A) or CEA-positive MKN45 (B) target cells. Where indicated, concentrated filtered cell-free SN from cultures of either untransfected (293T) or stably transfected (293T.dAb or 293T.dAb.B7) cells was added. As controls, effector and target cells were cultured alone.
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 \(\alpha\text{CEA} \text{ Ab} (42)\). 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...


