J. Biochem. **135**, 555–565 (2004)
DOI: 10.1093/jb/mvh065

A New Recombinant Single Chain Trispecific Antibody Recruits T Lymphocytes to Kill CEA (Carcinoma Embryonic Antigen) Positive Tumor Cells *In Vitro* Efficiently

Xiang-Bin Wang[1], Bao-Feng Zhao[2], Qi Zhao[1], Jin-Hua Piao[1], Jing Liu[1], Qing Lin[1] and Hua-Liang Huang[*,1]

[1]*The Institute of Genetics and Developmental Biology, Chinese Academy of Science, 100101, Beijing, China; and* [2]*Lanzhou University, School of Life Science, 73000, Lanzhou, China*

Received February 1, 2004; accepted March 3, 2004

Anti-tumor associated antigen (TAA)·CD3·CD28 trispecific antibody(TsAb) is able to provide two signals for fully and continuously activation of T lymphocytes and recruit them around tumor cells, presenting an attractive concept in tumor immunotherapy. Here, a new single chain trispecific antibody (scTsAb), named CEA-scTsAb, was constructed by fusion of anti-CEA (Carcinoma Embryonic Antigen) single chain antibody (scFv), anti-CD3 scFv and anti-CD28 VH, spaced by polypeptide interlinkers taken from the fragment of constant region (FC) of human IgG and human serum albumin (HSA). It was expressed in *Escherichia coli* at low temperature (30°C) with up to 50% of the antibody being present in soluble form. After one step of DEAE anion chromatography, the soluble product was sufficiently pure for further *in vitro* activity assays. First, it was proved that CEA-scTsAb could recognize three antigens (CEA, CD28 and Jurkat cell membrane antigen) specifically and could distinguish antigen positive cells from antigen negative cells *in vitro*. Then fresh PBMC (peripheral blood mononuclear cells), without being pre-treated by co-stimulatory reagents, such as IL-2 or CD28 mAb, were used as effector cells to test their ability to mediate tumor specific cytolysis of CEA-positive tumor cells, SW1116. It was found by photomicrography that T lymphocytes were attracted to SW1116 cells in the presence of CEA-scTsAb, which resulted in effective cytolysis of tumor cells. As shown by MTT assay, the efficacy of tumor specific cytolysis mediated by CEA-scTsAb related to both the quantity and activation of PBMC. At an effector cells/target cells ratio (E/T) of 5, it was proved by dual-color FACS with propidium iodide (PI) and FITC-annexin V that both necrosis and apoptosis of tumor cells were causes of tumor specific cytolysis. In summary, a new single chain trispecific (CEA × CD3 × CD28) antibody was constructed and characterized carefully in this paper and was found to possess functions: (i) to activate T lymphocytes independently of additional co-stimulatory signal, (ii) to attract activated T lymphocytes around CEA-positive tumor cells, (iii) to attack CEA-positive tumor cells with recruited T lymphocytes. Because it recognizes a widely distributed tumor antigen (CEA), with moderate molecular weight (about 75 kDa) and a simple production procedure, and is able to mediate a high level of tumor specific cytolysis without any additional co-stimulating reagents, CEA-scTsAb is very promising for the task of immunotherapy in future.

Key words: CD3, CD28, CEA, single chain trispecific antibody.

It is generally accepted that activation of T cells needs two distinct signals. The first signal depends on the interaction of CD3/TCR complex on T cells with MHC/antigen peptide complex on APC (antigen presenting cells), while the second is produced by ligation of co-stimulatory molecules (such as CD28, CD40) on T cells with their ligands on APC (*1*, *2*). Dual signals are required to activate T lymphocytes fully and continuously, while simply providing the first signal tends to activate T cells transiently and then induce AICD (activation induced cell death) (*3*, *4*), or result in immunological tolerance (*4*). Recognized as allogenous cells, tumor cells could be attacked by CTL (Cytotoxic T Lymphocytes) *in vivo*. However, tumor cells evade the surveillance of the immune system for many reasons, such as weak antigenicity, lack of co-stimulatory ligands (B7-1/B7-2) and so on (*5*, *6*).

To circumvent the problem of antigen recognition, a T-cell-based immunotherapy model with bispecific antibodies (BsAb) against tumor associated antigen (TAA) and CD3 has been designed, and has been validated by many *in vitro* or *in vivo* (pre-clinical and clinical) tests (*7–9*) . Most such antibodies generally function much better in the presence of a co-stimulatory reagent, such as anti-CD28 mAb (*10–12*). It has also been shown that a combi-

[*]To whom correspondence should be addressed at. Group 102, the Institute of Genetics and Developmental Biology, Chinese Academy of Science, Beijing, 100101, China. Fax: +86-10-8072-6906, E-mail: hlhuang@genetics.ac.cn

natorial approach using two kinds of BsAb (TAA × CD3 and TAA × CD28) was more effective than using them separately (*13–15*). The role of co-stimulation in immunotherapy with BsAb or other T-cell-based immune strategies was judged not only to facilitate T-cell activation but also to prevent T-cell depletion by apoptosis, which is the result of T-cell AICD (*16*). A recombinant single chain BsAb (CD19 × CD3) was recently reported to mediate specific cytolysis of malignant B cells independent of any co-stimulatory signals (*17–20*). However as the fate of T cells activated by this BsAb was not clarified in these reports, it is necessary to show further whether T-cell AICD could be circumvented or was caused by this antibody.

To provide two signals for T-cell activation in a single molecule or a molecular polymer, TAA × CD3 × CD28 trispecific antibody (TsAb) was considered potentially superior to BsAb in tumor immunotherapy. With the development of antibody engineering, methods for constructing TsAb have also matured. The first generation of TsAb was constructed by chemical coupling of three different Fab fragments [$(Fab)_3$ TsAb (*21–23*)] or two $F(ab)_2$ fragments [$(F(ab)_2)_2$ TsAb (*24*)]. Then the development of recombinant DNA technique brought us two multimeric types. scFvs associate with each other with shortened interlinkers located between VH and VL. It was found that scFvs with an interlinker of 3–12 residues dimerized to form a diabody, while those with an interlinker length of less than 3 residues trimerized into a triabody. In this way, TsAb could in principle be trimerized from three different scFv in principle (*25–27*). The other type of multimeric trispecific antibody, Fab-$(scFv)_2$, was constructed with Fab chains as an efficient heterodimerization scaffold (*28–31*). Several disadvantages are associated with the above TsAbs, including laborious production procedures, high molecular weight and potential instability *in vivo*.

Since the first construction of TsAb in 1991(*22*, *23*), two opposite viewpoints have arisen concerning its therapeutic application *in vivo*. The negative viewpoint centers on to non-target dependent T-cell activation mediated by $[F(ab)_2]_2$ TsAb (*22*). However, it was suggested that this effect could be eliminated in practice by using recombinant TsAbs rather than chemically coupled TsAbs.

To circumvent these disadvantages, a new scTsAb was constructed here by fusion of three antibody fragments (anti-CEA scFv, anti-CD3 scFv and reshaped anti-CD28 VH) in a single molecule, spaced with FC interlinker (from the fragment of constant region 2 (CH2) of human IgG1) and HSA interlinker (from human serum albumin (HSA)) in tandem. These two interlinkers have been used in constructing anti-ovarian carcinoma × CD3 scBsAb previously without any negative effect on dual binding specificity, while remarkably long half-life *in vivo* was detected for HSA-interlinker scBsAb (*32–34*). Reshaped anti-CD28 VH was reshaped from VH of anti-CD28 mAb (9.3) by molecular directed evolution (*35*, *36*), and was included in this scTsAb to provide enough co-stimulating signal for T-cell activation in the presence of target tumor cells, while reducing its molecular weight.

MATERIALS AND METHODS

Plasmids, Strains and Cells—Plasmid vector pTMF (*37*) was constructed by us previously based on pET-28a+ (Novagen) (*38*, *39*). Anti-CEA scFv was constructed by overlapping PCR (not published) according to the published amino acid sequence of mouse anti-human CEA mAb (*40*). Anti-CD3 scFv was constructed in our laboratory (not published) with genes (VH, VL) amplified from hybridoma cells (*41*) (UCTH1). Anti-CD28 VH was reshaped from VH of anti-CD28 mAb (9.3 strain) with the strategy of molecular directed evolution (*35*, *36*).

E. coli strain *TOP10* was used for maintaining plasmids and cloning throughout the experiments. *E.coli* strain *BL21 (DE3) star* (Novagen) was used for expression of CEA-scTsAb.

Jurkat (human acute lymphoblastic leukemia cell line) (*42–44*), SW1116 (human colorectal carcinoma cell line) (*45*), BCL (human liver cancer cell line) (*46*), MCF-7 (human breast cancer cell) (*47*), A549 (human lung carcinoma cell line) (*48*), EJ (human bladder cancer cell line) (*49*), SKOV3 (human ovarian cancer cell line) (*50*), CNE-2 (human low differentiated nasophoryngeal carcinoma cell line) (*51*), TJ905 (human glioblastoma cell line) (*52*) were stored in our laboratory. All of these cells except SW1116 were cultured in RPMI-1640 medium (Hyclone) containing 10% FBS (fetal bovine serum) (Hyclone) at 37°C in a 5% CO_2 incubator. SW1116 was cultured in L15 medium (Hyclone) containing 10% FBS at 37°C in a 5% CO_2 incubator.

Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll density centrifugation from enriched preparations of lymphocytes obtained from local blood banks. Erythrocytes were removed from PBMC by erythrocyte lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 100 μM EDTA) and thrombocytes were removed *via* the supernatant obtained after centrifugation of PBMC at 100 ×*g* for 10 min. PBMC were cultured in L15 medium containing 10% FBS at 37°C in a 5% CO_2 incubator.

Antibodies—Anti-cmyc tag mAb (9E10) and HRP conjugate of polyclonal goat anti-mouse IgG were purchased from Santa Cruz Biotechnology. FITC conjugate of goat anti-mouse IgG was purchased from BD Company.

Construction of CEA-scTsAb—The backbone DNA fragment (Fig. 1A) containing a series of restriction sites and coding sequences for two interlinkers was prepared previously by overlapping PCR (unpublished) with 12 oligonucleotides, p1–p12 (Fig. 2). FC interlinker (human IgG1 CH2, 297–322, 5′NSTYRVVSVLTVLHQDWLNGKEYKCK3′) and HSA interlinker (from D3 domain of human serum albumin, 5′FQNALLVRYTKKVPQVSTPTLVEVS3′) had been previously used as interlinkers for BsAb (ovarian carcinoma × CD3) without affecting its two independent binding specificities (*33*). Cut with *Nco*I/*Bam*HI, the fragment was ligated into pTMF to produce pTRI (Fig. 1B). The DNA fragment encoding anti-CEA scFv was cut out from anti-CEA scFv/pTMF with *Xho*I/*Eco*RI and ligated into pTRI to produce CEA/pTRI. The DNA fragment encoding anti-CD3 scFv was amplified from anti-CD3 scFv/pTMF with two primers, CD3-up and CD3-down (Fig. 2) and endowed with two new restriction sites *Sca*I/*Sal*I at two ends. The product of PCR was cut with *Sca*I/*Sal*I and ligated into CEA/pTRI to produce

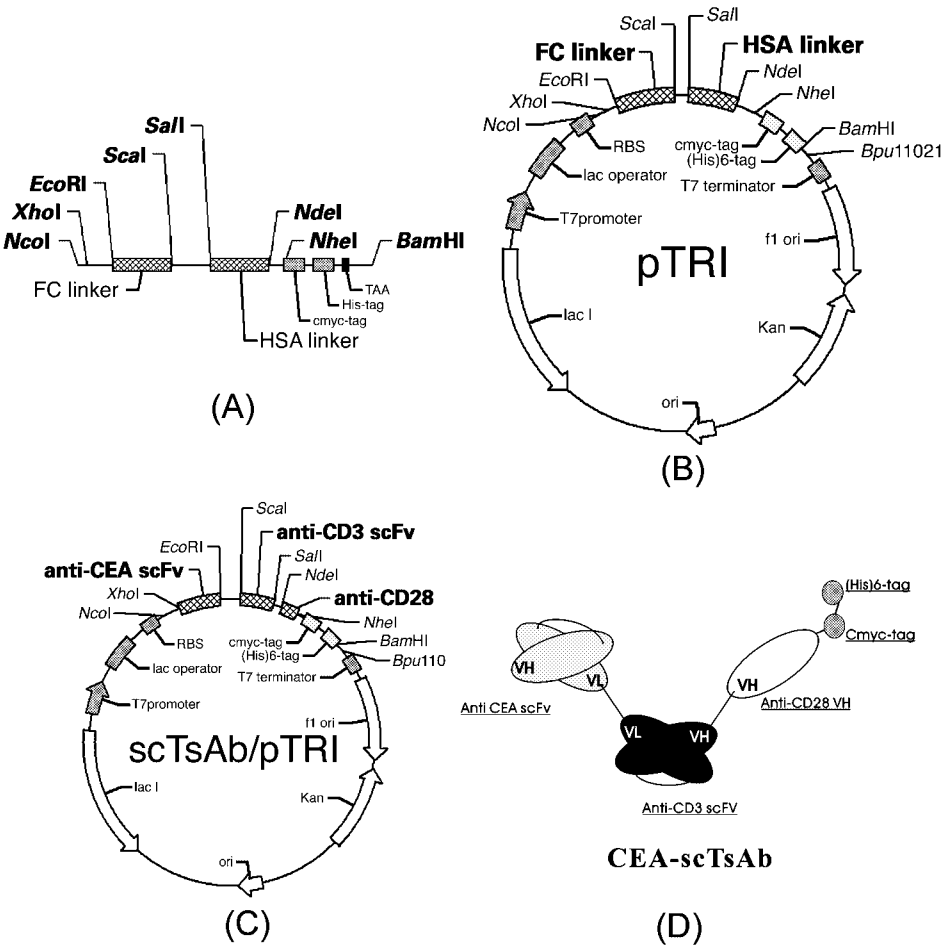

Fig. 1. **Construction of CEA-scTsAb.** (A) Parent vector pTMF, (B) The backbone DNA fragment inserted in pTRI, (C) Schematic map of DNA coding CEA-scTsAb inserted in pTRI/ CEA-scTsAb, (D) Imagined structure of CEA-scTsAb.

CEA/CD3/pTRI. Using a similar method, the DNA fragment encoding ant-CD28 VH was endowed with two new restriction sites *Nde*I/*Nhe*I by amplifying from anti-CD28 VH/pTMF with two primers, CD28-up and CD28-down (Fig. 2) and ligated into CEA/CD3/pTRI to produce the final plasmid: CEA-scTsAb/pTRI. Thus a new single chain trispecific antibody was constructed and named CEA-scTsAb.

All restriction enzymes, T4 DNA ligase, Pfu polymerase and dNTP were purchased from Promega. The Plasmid Isolation Kit and Agarose Gel DNA Purification Kit were products of Takara Biotech. (Dalian).

Expression Protocol—A single clone was picked up from a LB-K plate [10 g/liter tryptone(GIBCO), 5 g/liter yeast extract(GIBCO), 5 g/liter NaCl, 15 g/liter agrose and 100 μg/ml kanamycin, pH 7.5 [spread with BL21(DE3) star transformed with CEA-scTsAb/pTRI and cultured in 5 ml of LB-K medium (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl and 50 μg/ml kanamycin, pH 7.5) at 37°C overnight. This was then diluted 1/100 into 250 ml LB-K medium and cultured at 37°C till the A_{600} of the culture reached 0.4–0.6. After adding IPTG (isopropyl-1-thio-β-D-galactoside) [Takara Biotech. (Dalian)] to a final concentration of 0.2 mmol/liter, CEA-scTsAb was induced at 30°C for 4 h. The cells were harvested by centrifugation at 12,000 rpm for 10 min. The pellet was re-suspended in 50 ml equilibrium buffer (20 mmol/ml NaCl, 50 mmol/ml Tris-HCl, pH 8.0) and then lysed by sonication. After centrifuging the suspension at 12,000 rpm for 20 min, the supernatant containing soluble CEA-scTsAb and the pellets containing insoluble CEA-scTsAb were both stored at –80°C. By use of a

Name	Sequence (from 5' end to 3' end)
P1	*NcoI* *XhoI* TAT *ACCATGGGT* *CTCGAG*
P2	*XhoI* *EcoRI* TATACCATGGGT *CTCGAG* ATGTACCCGCGCGGTAACACTAGT *GAATTC* AACAGCACGTA
P3	AGCCAGTCCTGGTGCAGTACGGTGAGGACGCTTACAACCCGGTACGTGCTGTTGAATTC
P4	*ScaI* CTGCACCAGGACTGGCTGAATGGCAAGGAATACAAATGCAAG *AGTACT* TCTAGAATGTA
P5	*SalI* *ScaI* CGAACCAGCAGCGCATTCTGGAA *GTCGAC* GTTACCGCGCGGGTACATTCTAGA *AGTACT*
P6	AATGCGCTGCTGGTTCGTTACACCAAGAAAGTACCCCAAGTGTCAACTCCAACTCCTGT
P7	*NdeI* GCGGTACCGTTACCGCGCGGGTACAT *CATATG* TGAGACCTCTACAGGAGTTGGAGTTGA
P8	CGCGGTAAC GGTACC GCGCTGGAAGTTGACGAAACCTACGTTCCGAAAGAATTTAACGC
P9	*NheI* TC *GCTAGC* CCCATCCGCGGGATGTCAGCGTGGAAGGTGAAGGTTTCCGCGTTAAATTCTTTCGG
P10	*NheI* ATCGAGCTCATGTACCCGCGCGGTAAC *GCTAGC* GAACAAAAACTCATCTCAGAAGAGGA
P11	TATTGCTCGTGATGGTGATGATGTGCGGCCCCATTCAGATCCTCTTCTGAGATGAG
P12	*BamHI* CTCGAC *GGATCC* TTATTGCTCGTGATGGTG
CD3-up	*ScaI* AG *AGTACT* GAGGTGAAGCTGGTGGAGTCT
CD3-down	*SalI* AA *GTCGAC* AGCGCGCTTCAGTTCCAGTTT
CD28-up	*NdeI* CA *CATATG* CAGGTACAGCTACAGGAA
CD28-down	*NheI* AT *GCTAGC* GGAAGATACGGTTACCAGGG

Fig. 2. **Oligonuleotides used for constructing CEA-scTsAb.**

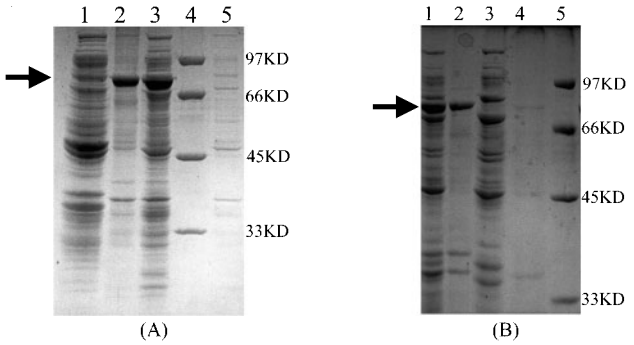

Fig. 3. **Analysis of the expression (A) and purification (B) of CEA-scTsAb.** (A) 12% SDS-PAGE under reducing conditions. Lanes 1 and 5, supernatant and pellet of empty vector (pTRI); lanes 2 and 3, pellet and supernatant of CEA-scTsAb/pTRI, lane 4, protein molecular weight standard (Shanghai Biochemical Institute), (B) Lane1, supernatant of CEA-scTsAb/pTRI; lane 2, flow-through from the column of DEAE chromatography; lane 3, fraction eluted by 1 mol/liter NaCl; lane 4, fraction eluted by 1 mol/liter NaOH; lane 5, protein molecular weight standard (Shanghai Biochemical Institute).

standard protocol (*53*), SDS-PAGE under reducing condition and Western blotting were used to analyze the distribution of CEA-scTsAb between soluble and insoluble fractions. With mouse anti-cmyc-tag mAb (9E10) as primary antibody and HRP conjugate of goat anti-mouse IgG as secondary antibody, the result of the Western Blotting was visualized with DAB (3,3′-diamionbenzidene) (Sigma) as substrate.

DEAE Anion Chromatography—First, a 16 mm × 20 cm column was packed with 20 ml of DEAE Sepharose Fast Flow (Amersham Biosciences). After equilibrating the column with 5 volumes of equilibrium buffer (as above), the supernatant containing soluble expressed CEA-scTsAb was loaded at a rate of 1 ml/min. The flow-through fraction was collected, which contained purified CEA-scTsAb. Most host proteins were eluted from the column by 2 volumes of elution buffer (500 mM NaCl, 50 mM Tris-HCl, pH 8.0). Finally, the column was washed with 2 volumes of washing buffer (1mol/liter NaCl). By use of a standard protocol (*53*), three fractions (flow through fraction, eluted fraction and washed fraction) and non-purified supernatant were analyzed by SDS-PAGE under reducing conditions. After dialyzing against PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4 and 0.24 g KH2PO4, 1 liter of volume, pH 7.4) overnight at 4°C, purified CEA-scTsAb was quantified by the Bradford method according to Ref. (*54*) and stored at –80°C separately for further activity assay.

ELISA—Purified CEA-scTsAb was used in an ELISA to test its binding specificity to three antigens (CD28-FC chimera (R&D company), CEA (Fitzgerald, Germany), and Jurkat cell membrane antigen). To prepare Jurkat cell membrane antigen, 5×10^6 Jurkat cells were collected by centrifugation at 1,000 rpm for 10 min and suspended in 0.5 ml of PBS. The suspension was sonicated and centrifuged at 1,000 rpm for 10 min. The protein concentration of the supernatant containing Jurkat cell membrane antigen was quantified by the Bradford method (*55*). Then 10 mg/ml of Jurkat cell membrane antigen and 1 mg/ml of the other two antigens (CD28-FC chimera and CEA) were coated in a 96-well ELISA plate in coating buffer (1.36 g Na_2CO_3, 7.35 g $NaHCO_3$, 1 liter of volume, pH 9.2). With mouse anti-cmyc-tag mAb (9E10) as primary antibody, HRP conjugate of goat anti-mouse IgG as secondary antibody and OPD (*o*-phenylenediamine, Sigma) as visualizing substrate, the absorbance of triplicate samples was measured at 490 nm.

FACS Assay of Binding to Antigen Positive Cells—Binding to both CD3/CD28-positive cells (PBMC, Jurkat) and eight kinds of tumor cells (SW1116, SKOV3, BCL, MCF-7, EJ, CNE-2, A549, TJ905) was analyzed with direct FACS. Purified CEA-scTsAb was labeled with fluorescein isothiocyanate (FITC) by the Clark method (*56*). Briefly, CEA-scTsAb was concentrated to 5 mg/ml and dialyzed overnight against 25 mM $NaCO_3$–$NaHCO_3$ buffer, pH 9.6. FITC was then dissolved in the same buffer to a final concentration of 0.1 mg/ml. After incubation at 4°C for 20 h, the solution was dialyzed against PBS to remove unbound FITC.

Samples of 5×10^5 cells were collected separately by centrifuging at 1,000 rpm for 10 min. These cells were then resuspended in 500 μl of PBS and re-centrifuged at 1,000 rpm for 10 min. The pellets were gently dispensed in 100 μl of PBS containing CEA-scTsAb and the suspension incubated at 4°C for 30 min. The binding of CEA-scTsAb to these two kinds of cells was analyzed by flow cytometry (FACS Calibur, Becton Dickinson, Mountain View, CA). Ten thousand cells were collected per test and the results were analyzed with Cellquest software (Becton Dickinson, Mountain View, CA). While viewing a FSC vs SSC dot plot of unstained cells, T lymphocytes from PBMC were gated for further analysis.

Morphological Analysis—After mixing PBMC (effector cells) with SW1116 cells (target cells) in L15 medium (10% FBS) at an E/T of 5, and adding purified CEA-scTsAb at a concentration of 1 μg/ml, the mixture was incubated at 37°C for 30 h in 5% CO_2 incubator. Then morphological changes of tumor cells and PBMC were observed under a 40× object lens with an OLYMPUS IMT-2 inverted microscope, and recorded by photomicrography.

MTT Assay of Tumor Specific Cytolysis and T-Cell Proliferation—After mixing SW1116 cells (target cell) with PBMC (effector cell) in L15 medium containing 10% FBS at a certain E/T, purified CEA-scTsAb was added to mediate specific cytolysis by recruiting activated PBMC. After incubating the mixture at 37°C for 48 h in a 5% CO_2 incubator, PBMC were removed by discarding the medium and washing with an equal volume of PBS (200 μl/well) once. Then the viability of adherent tumor cells was analyzed by the MTT method (MTT from Sigma) in quadruplicate. Tumor specific cytolysis was calculated with the following formula:

Percent of tumor specific cytolysis (%) = $[A_{600}\ (E/T) - A_{600}\ (E/T/A)]/[A_{600}\ (E/T) - A_{600}\ (M)] \times 100\%$; A_{600} (E/T): absorbance of the wells without adding CEA-scTsAb; A_{600} (E/T/A): absorbance of the wells added with CEA-scTsAb; A_{600} (M): absorbance of the wells with added L15 medium only without any cells or antibodies.

SW1116 cells were adjusted to 10^6/ml in L15 medium containing 10% FBS and 25 μg/ml mitomycin C (Sigma), and incubated at 37°C for 20 min in a 5% CO_2 incubator to arrest proliferation. Mitomycin C was removed by

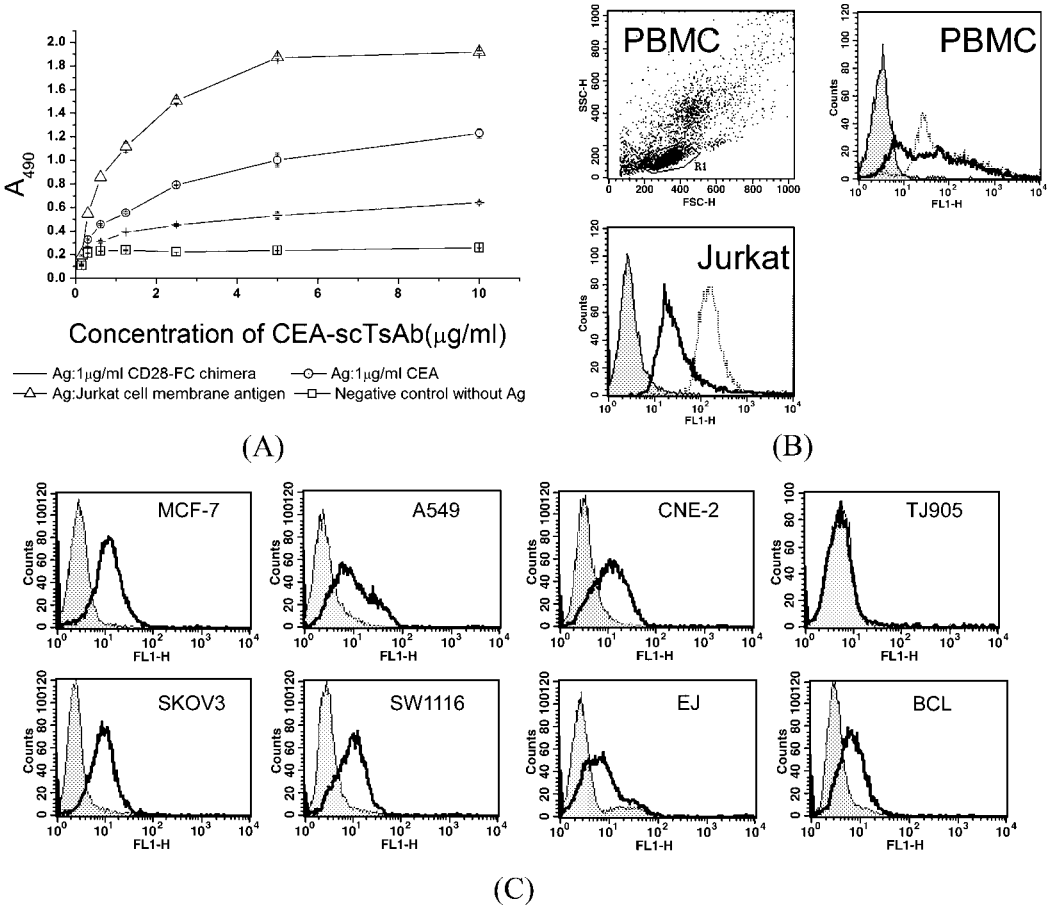

Fig. 4. **Analysis of the binding activity of CEA-scTsAb.** (A) ELISA assay of the binding specificity to three antigens (CEA, CD28-FC chimera and Jurkat cell membrane antigen) with anti-cmyc tag mAb(9E10) as primary antibody. (B) Direct FACS assay of the specific binding to CD3/CD28-positive cells. Note that only T lymphocytes in gate R1 were analyzed. In the overlaid maps, the shadowed histogram represents the control without any FITC conjugate of CEA-scTsAb, while the hollow ones with the thick line or the broken line were the samples with 1 μg/ml or 10 μg/ml FITC conjugate of CEA-scTsAb respectively. (C) Direct FACS assay of the specific binding of CEA-scTsAb to CEA-positive tumor cells. In the overlaid maps, the shadowed histogram represents the control without FITC conjugate of CEA-scTsAb, while those with the thick line were the samples with 1 μg/ml FITC conjugate of CEA-scTsAb.

washing with 1ml PBS three times. After mixing treated SW1116 cells with PBMC at a certain E/T, purified CEA-scTsAb was added to activate PBMC. After incubating the mixture at 37°C for 48 h in a 5% CO_2 incubator, the mixture was centrifuged at 1,000 rpm for 20 min. The pellet was re-suspended in an equal volume of PBS and centrifuged at 1,000 rpm for 20 min again. Then the viability of PBMC and tumor cells was analyzed in quadruplicate with the MTT assay. The stimulating index of CEA-scTsAb to PBMC (SI) was calculated with the formula:

SI = [A_{600} (E/T/A)/A_{600} (E/T)]; A_{600} (E/T): absorbance of the wells without added CEA-scTsAb; CEA-scTsAb; A_{600} (E/T/A): absorbance of the wells with added CEA-scTsAb.

FACS Assay of Tumor Specific Cytolysis—After mixing SW1116 cells (target cell) with PBMC (effector cell) in L15 medium (10% FBS) at an E/T of 5, and adding purified CEA-scTsAb, the causes for tumor specific cytolysis were analysed by dual-color FACS (*57*) (PI/FITC-annexin V, BD company). Specifically, after incubation at 37°C for a certain time in a 5% CO_2 incubator, the cell mixture was washed with PBS once and then co-incubated with PI and FITC-conjugated annexin V (BD company) in binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4) at 4°C for 20 min. After four fold dilution with binding buffer, the triplicate samples were then analyzed by flow cytometry (FACS Calibur, BD company) using Cellquest software (BD company). While viewing the FL1 *vs.* FL2 dot plot of the gated cells, FL2-%FL1 compensation (20%) and FL1-%FL2 compensation (0.8%) were adjusted to ensure that no events for single positive tubes [annexin V-FITC (+) or PI (+) only] were recorded in the negative quadrants. Tumor specific cytolysis was then calculated with the following formula:

Percent of Tumor Specific Cytolysis (%) =[VC – VA]/VC × 100%; VC: Percent of Control Viable Cells; VA: Percent of Viable Cells with Added CEA-scTsAb.

RESULTS

Construction of CEA-scTsAb—The backbone DNA fragment shown in Fig. 1A was prepared by overlapping PCR (unpublished) with 12 oligonucleotides, p1–p12 (Fig. 2) and inserted into parent vector pTMF (*37*), to produce

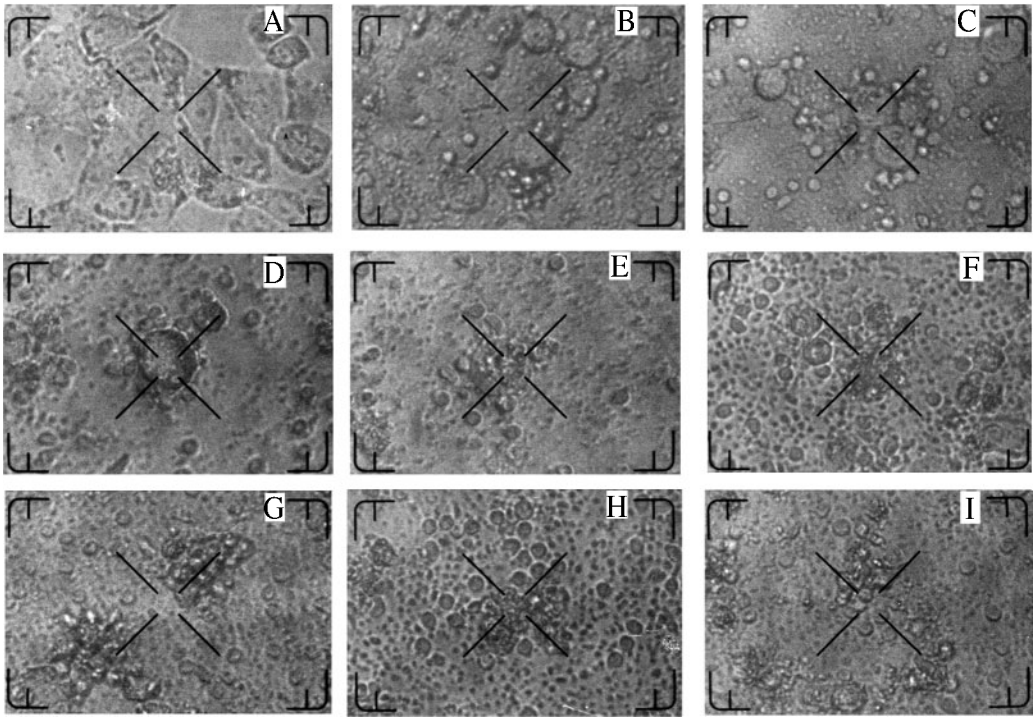

Fig. 5. **Photomicrography of cytolysis of SW1116 cells mediated by CEA-scTsAb.** (A) SW1116 cells alone, (B) SW1116 cells mixed with PBMC at an E/T of 5 without adding CEA-scTsAb; (C–I) SW1116 cells mixed with PBMC at an E/T of 5 and CEA-scTsAb (1 μg/ml)

pTRI (Fig. 1B). CEA-scTsAb was constructed by inserting three DNA fragments encoding three different antibody fragments (anti-CEA scFv, anti-CD3 scFv and anti-CD28 VH) into pTRI one by one. The schematic map of scTsAb/pTRI and imagined structure of scTsAb are displayed in Fig. 1, C and D, separately. It should be emphasized that the variable domains of anti-CEA scFv and anti-CD3 scFv in CEA-scTsAb were in a different order SSfrom each other, which ensured the correct dimerization of VH with VL from the same scFv (*58*).

Soluble Expression in Cytoplasm and Purification by DEAE Anion Chromatography—As shown in Fig. 3A, the molecular weight of CEA-scTsAb was about 76 kDa and the percentage of soluble expression reached 50%. With DEAE anion chromatography, CEA-scTsAb from the soluble fraction extracted by sonication was collected from the flow-through fraction with a purity of about 70%, while most of the host proteins were in the eluted fraction, and only a small amount of residual CEA-scTsAb was found in the washed fraction (shown in Fig. 3B). Thus CEA-scTsAb was purified primarily without changing the solution condition throughout the process of purification.

Characterization of Binding Activity—As shown in Fig. 4A, CEA-scTsAb bound to three antigens (including CEA, CD28-FC chimera and Jurkat cell membrane antigen)

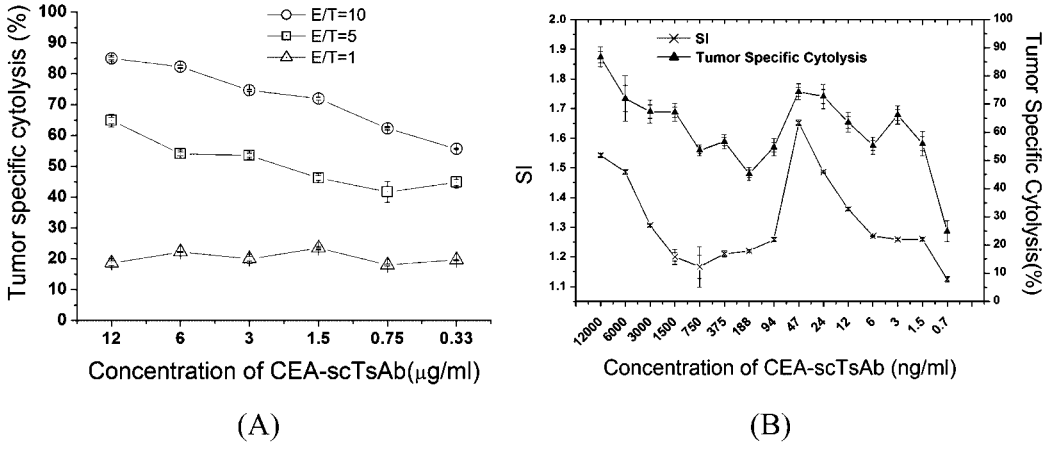

Fig. 6. **MTT assay of tumor-specific cytolysis.** (A) Effect of decreasing E/T on the efficacy of tumor-specific cytolysis, (B) Correlation between the proliferation of PBMC and tumor-specific cytolysis at an E/T ratio of 5.

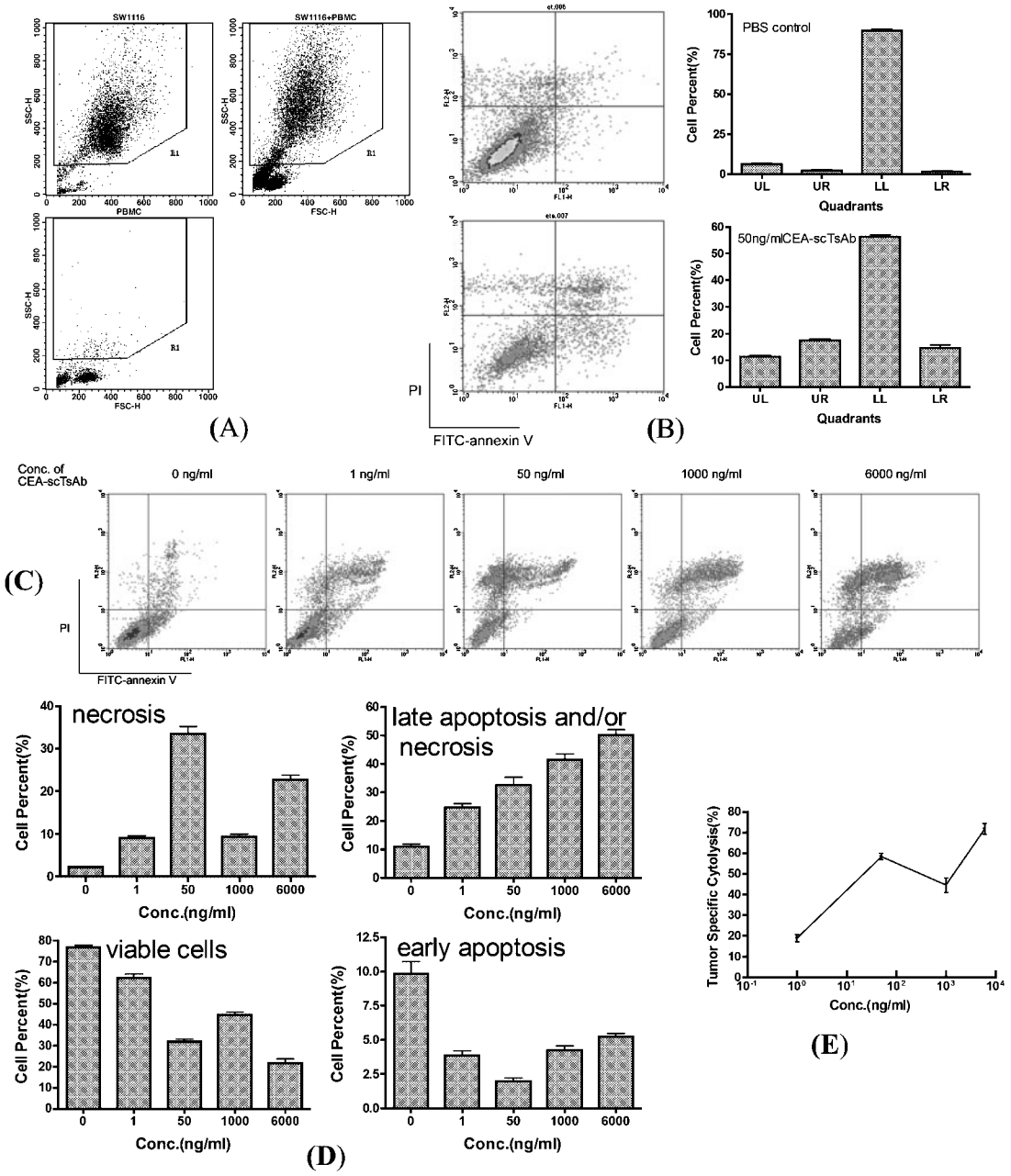

Fig. 7. **Dual-color FACS (PI/FITC-annexin V) assay of tumor-specific cytolysis.** (A) The tumor cells were gated in R1. (B) FL1 *vs.* FL2 dot plot of the gated cells at 50 ng/ml of CEA-scTsAb after co-incubation for 10 h. Compensation: FL2-FL1% = 20%; FL1-FL2% = 0.8%. 10,000 cells were acquired per test. Quadrant statistics: necrosis cells in upper left (UL), necrosis and/or late apoptosis cells in upper right (UR), viable cells in lower left (LL) and early apoptosis cells in lower right (LR). The results of triplicate samples were calculated statistically. (C) FL1 vs FL2 dot plot of the gated cells at different concentration of CEA-scTsAb (0, 1, 50, 1,000, 6,000 ng/ml) after co-incubation for 50 h. Compensation: FL2-FL1% = 20%; FL1-FL2% = 0.8%. 30,000 cells were acquired per test. (D) The results of triplicate samples were calculated statistically. (E) Tumor-specific cytolysis calculated from the percentage of viable cells in (D).

independently and specifically. As CD3 and CD28 are both expressed on Jurkat cells (*44*), CEA-scTsAb bound to the Jurkat cell membrane antigen most strongly. On the other hand, with anti-CD28 derived from a reshaped single domain antibody fragment (VH) (*35*), it bound to CD28-FC chimera most weakly. Thus it was deduced that the two interlinkers (Fc interlinker and HSA interlinker) in CEA-scTsAb did not affect the independent antigen-binding ability of three antibody fragments (anti-CEA scFv, anti-CD3 scFv and anti-CD28 VH).

The ability of CEA-scTsAb to recognize antigen-expressing cells (CEA, or CD3 or/and CD28 expressing cells) was evaluated by direct FACS. As shown in Fig. 4B, CEA-scTsAb recognized both T lymphocytes (gated PBMC) and Jurkat cells specifically. As shown in Fig. 4C, seven tumor cell lines (MCF-7, SKOV3, A549, SW1116, CNE-2, EJ, BCL) with different tissue origins were binding-positive, while only TJ905 was binding-negative. With dual binding ability to both CEA-positive tumor cells and CD3/CD28-positive T cells, CEA-scTsAb was

endowed with the dual function of activating T cells and recruiting them around CEA-expressing tumor cells.

Morphological Analysis—Morphological changes of co-incubated target cells (SW1116), effector cells (fresh PBMC) and CEA-scTsAb were observed with an inverted microscope. Both single-cultured SW1116 cells and those co-incubated with PBMC without any CEA-scTsAb were regarded as negative controls (shown in Fig. 5, A and B). It was found that T lymphocytes gathered around target cells (Fig. 4, C and D) obviously at first in the presence of CEA-scTsAb, which then caused the target cells to break up (Fig. 5, E–I). This result proved that CEA-scTsAb could activate and recruit T lymphocytes to attack CEA-positive tumor cells.

MTT Assay of Tumor Specific Cytolysis—Tested quantitatively by MTT assay, the percent of tumor specific cytolysis was found be improved by increasing E/T (Fig. 6A), which demonstrated that the efficacy of tumor specific cytolysis changed proportionally with the quantity of effector cells.

At a fixed E/T of 5, the proliferation of effector cells induced by CEA-scTsAb was tested by MTT assay. In order to find the correlation between the proliferation of effector cells and tumor specific cytolysis, the percent of tumor specific cytolysis was tested as a control under the same conditions. As shown in Fig. 6B, with the decrease in CEA-scTsAb concentration, the proliferation of PBMC and tumor specific cytolysis decreased simultaneously. In the first phase (12 μg/ml–750 ng/ml) and the third phase (50 ng/ml–0), it changed with the concentration of CEA-scTsAb almost proportionally. In the second phase (750 ng/ml–50 ng/ml), it changed in inverse proportion. Two peak values of tumor specific cytolysis between 12 μg/ml and zero were designated as about 85% and 75% separately. These results indicated that it was the activated PBMC that killed tumor target cells specifically. The more PBMC were activated, the more target cells were killed. In future, experiments will be necessary to find the real causes for the synchronously "phasic" changes between the proliferation of effector cells and tumor specific cytolysis. In any case, these results demonstrated that a high level of tumor specific cytolysis was induced at low E/T without any pre-treatment of effector cells.

FACS Analysis of Tumor Specific Cytolysis—As shown in Fig. 7A, tumor cells were gated in R1 for further analysis, while viewing a FSC vs SSC dot plot of unstained cells, in which only a few cells from PBMC were present. As debris from both tumor cells and PBMC were too small to be included, only intact cells were tested. As shown in Fig. 7B, about 50% of tumor cells were induced to death after 10 h in the presence of 50 ng/ml CEA-scTsAb, among which cell death in three quadrants cells accounted for about one third. Thus it can be concluded that tumor cells died of both necrosis and apoptosis in this T-cell-based cytolysis model with CEA-scTsAb.

Further, triplicate samples containing different concentrations of CEA-scTsAb (0, 1, 50, 1,000, 6,000 ng/ml) were analyzed under the same conditions, the representative results of which are shown in Fig. 7C. Except for the samples containing 50 ng/ml CEA-scTsAb, most cell death appeared in the UR quadrant, which included late apoptosis and/or a part of necrosis, while early apoptosis in the LR quadrant and necrosis in the UL each quadrant accounted for only a small part. At 50 ng/ml, more cells appeared in the UL quadrant as necrosis, while early apoptosis in the LR quadrant decreased slightly. The data of quadrants of triplicate samples are displayed in Fig. 7D. It was found that the percentage in the UR quadrant increased step by step synchronously with the concentration of CEA-scTsAb, with that in the LR at all concentrations was under 10%. The percentage of viable cells in the LL quadrant decreased step-by-step, except for that at 50 ng/ml. Also, at that concentration, an outburst of necrosis occurred unexpectedly, like that at the top concentration. Tumor-specific cytolysis was calculated from the percentage of viable cells and is shown in Fig. 7E, which displays "phasic" changes in accordance with the results of MTT assay. Tumor specific cytolysis induced by CEA-scTsAb at 50 ng/ml was much higher than that at 1,000 ng/ml, and lower than that at 6,000 ng/ml. Additionally, the value of tumor-specific cytolysis here was a little lower than that of the MTT assay, as cell debris of tumor cells was too small to be contained in gate R1.

DISCUSSION

It is well established that a single injection of a trispecific antibody (TAA × CD3 × CD28) *in vivo* will be more effective in mediating tumor specific cytolysis than using either of two BsAbs (TAA × CD3 and TAA × CD28) alone. In this paper, a new single-chain trispecific antibody (CEA × CD3 × CD28) named CEA-scTsAb was constructed by fusion of anti-CEA scFv, anti-CD3 scFv and anti-CD28 VH, which were spaced by FC interlinker and HSA interlinker in tandem. It was expressed in the cytoplasm of *E. coli* in soluble form at low temperature (30°C) and purified by collecting the flow-through fraction from DEAE anion chromatography with a purity of about 70%. Circumventing the laborious steps of denaturation and renaturation, the expression and purification procedure was simple enough to provide enough purified product for further activity assay.

Using the purified products, the binding specificity of CEA-scTsAb to three kinds of antigen (CEA, CD28-FC chimera and Jurkat cell membrane antigen) was affirmed by ELISA. As the Jurkat cell expresses both CD3 and CD28 on its surface, the specific binding to CD3 needs to be confirmed with further experiments, for example anti-CD3 mAb competent-ELISA. Its ability in recognizing both CEA-expressing cells and CD3/CD28-expressing cells was tested. Thus it was concluded that with FC interlinker and HSA interlinker located in the intervals between the three antibody segments, three antigen-binding specificities were kept independent. Also, CEA-scTsAb had the ability to activate T cells by providing dual stimulating signals for them and recruiting them around tumor cells. The morphological changes of tumor cells co-incubated with fresh PBMC and CEA-scTsAb showed the actual effect of CEA-scTsAb in recruiting T lymphocytes to kill target tumor cells. As tested by MTT assay, a high level of tumor specific cytolysis was induced at a low E/T of 5 without any pre-treatment of effector cells.

Two anti-ovarian carcinoma × CD3 scBsAbs were previously constructed using FC interlinker or HSA inter-

linker (*33*). A cytotoxicity assay showed that pre-treatment of effector cells with co-stimulatory reagents, such as IL-2 and CD28 mAb, was prerequisite for strong tumor specific cytolysis (*32*). Here, the presence of anti-CD28 VH in CEA-scTsAb made external co-stimulating reagents unnecessary.

Based on all these results, CEA-scTsAb was found to have three functions: (i) To activate T lymphocytes without any foreign co-stimulatory signals, (ii) To attract activated T lymphocytes to CEA-positive tumor cells, (iii) to attack CEA-positive tumor cells with recruited T lymphocytes.

Surprisingly and interestingly, it was found that with increasing CEA-scTsAb concentration, both T-cell proliferation and tumor specific cytolysis decreased with two peaks between 0 and 12 μg/ml. Cytolysis tested by dual-color FACS led to a similar conclusion, which is discussed below. So within different ranges of concentration, CEA-scTsAb acted on T cells in different ways. A hypothesis is proposed here to explain this phenomenon. At a fixed E/T, CEA-scTsAb built a bridge between target tumor cells and T cells. At first, T cells were activated by CEA-scTsAb absorbed on target tumor cells at low concentration. Then excess CEA-scTsAb acted on T cells in a target-independent way without cross-linking on target cells, which may compete with cell-bound CEA-scTsAb to provide a weaker signal for T-cell activation. Finally, increasing antibody concentration led to the accumulation of redundant CEA-scTsAb on T cells, which helped to circumvent the inhibitive threshold even without being cross-linked. Therefore, adopting a lower optimized concentration of CEA-scTsAb *in vivo* may help to reduce the risk of non-target-dependent cytolysis.

Cells can die in either of two ways: necrosis and apoptosis. Membrane damage caused by physical or chemical injury leads to necrosis; apoptosis is distinguished from necrosis by needing regulation, requiring new gene expression, and inducing morphologic changes in the nucleus, DNA laddering and membrane blebbing. The changes in membrane composition leading to extracellular exposure of phosphatidylserine (PS) residues occur early in the apoptotic cycle (*59*). Exposed PS residues strongly bind annexin V, a natural ligand, in a calcium-dependent manner (*60*). As necrotic cells also expose PS residues and lose membrane function simultaneously soon after cell injury, a DNA-binding dye, propidium iodide (PI), was generally used in tandem with FITC-conjugated annexin V to analyze the causes of tumor-specific cytolysis (*57*). According to the principle of this method, the quadrants of dots represent four fates of target cells. Among them, three quadrants have been designated definitely: viable cells in PI(–)/annexinV(–), early apoptosis in PI(–)/annexinV(+), and necrosis in PI(+)/annexinV(–). The dual positive quadrant of PI(+)/annexinV(+) was deduced to be late apoptosis and/or necrosis. Accordingly, we tested target cells after co-incubation for 10 h and observed early apoptosis and necrosis definitely. Thus both necrosis and apoptosis were causes of tumor specific cytolysis in this T-cell-based immunotherapy model with CEA-scTsAb. After extending incubating time to 50 h, cell death accumulated in the dual-positive quadrant, while only a small amount of early apoptosis was detected. Here, similarly to the result from MTT assay, tumor-specific cytolysis calculated from the percent of viable cells displaying "phasic" changes due to the outburst of necrosis in two peaks.

There are two major pathways involved in CTL-induced cell death of tumor cells (*61*, *62*). (i) Perforin and granule exocytosis cytotoxicity. Perforin is released from granules of activated CTL and causes damage to target cell membranes. Various granzymes, and possibly other granule constituents that co-secret with perforin, can enter the target cells to induce apoptosis. (ii) Fas/Fas ligand (FasL)-interaction-induced cytotoxicity. The expression of FasL is enhanced on activated CTL cells, which interacts with its receptor, Fas, on tumor cells to induce apoptosis. In this T-cell-based immunotherapy model with CEA-scTsAb, tumor cells were induced to both necrosis and apoptosis. Thus, it may be suggested that necrosis of target cells is caused by perforin-made pores on the membrane, and apoptosis is induced by either the granzyme pathway or FasL/Fas ligation.

CEA-scTsAb, which recognizes widely distributed tumor antigen (CEA), has a moderate molecular weight (about 75 kDa) and a simple production procedure, and is able to mediate a high level of tumor-specific cytolysis without any additional co-stimulating reagents such as IL-2 or anti-CD28 mAb, unlike most presently available BsAbs, provides us a promising prospect for tumor immunotherapy. Further experiments need to be done to test its superiority to BsAb and other designed advantageous features, such as non-target-cell-dependent activation of T cells, avoidance of activation-induced cell death of T cells, and so on.

This work was supported by Grant 2001AA215381 from the National High Technology Research and Development Program of China.

REFERENCES

1. Baxter, A.G. and Hodgkin, P.D. (2002) Activation rules: the two-signal theories of immune activation. *Nat. Rev. Immunol.* **2**, 439–446
2. Bernard, A., Lamy, and Alberti, I. (2002) The two-signal model of T-cell activation after 30 years. *Transplantation* **73**, S31–35
3. Tang, Q., Smith, J.A., Szot, G.L., Zhou, P., Alegre, M.L., Henriksen, K.J., Thompson, C.B., and Bluestone, J.A. (2003) CD28/B7 regulation of anti-CD3-mediated immunosuppression in vivo. *J. Immunol.* **170**, 1510–1516
4. Van Parijs, L., Ibraghimov, A., and Abbas, A.K. (1996) The roles of costimulation and Fas in T cell apoptosis and peripheral tolerance. *Immunity* **4**, 321–328
5. Salih, H.R., and Nussler, V. (2001) Commentary: Immune escape versus tumor tolerance: how do tumors evade immune surveillance? *Eur. J. Med. Res.* **6**, 323–332
6. Foss, F.M. (2002) Immunologic mechanisms of antitumor activity. *Semin. Oncol.* **29**, 5–11
7. Withoff, S., Helfrich, W., de Leij, L.F., and Molema, G. (2001) Bi-specific antibody therapy for the treatment of cancer. *Curr. Opin. Mol. Ther.* **3**, 53–62
8. Kudo, T., Suzuki, M., Katayose, Y., Shinoda, M., Sakurai, N., Kodama, H., Ichiyama, M., Takemura, S., Yoshida, H., Saeki, H., Saijyo, S., Takahashi, J., Tominaga, T., and Matsuno, S. (1999) Specific targeting immunotherapy of cancer with bispecific antibodies. *Tohoku J. Exp. Med.* **188**, 275–288
9. Bauer, S., Renner, C., Juwana, J.P., Held, G., Ohnesorge, S., Gerlach, K., and Pfreundschuh, M. (1999) Immunotherapy of human tumors with T-cell-activating bispecific antibodies:

stimulation of cytotoxic pathways in vivo. *Cancer Res.* **59**, 1961–1965
10. Manzke, O., Tesch, H., Lorenzen, J., Diehl, V., and Bohlen, H. (2001) Locoregional treatment of low-grade B-cell lymphoma with CD3xCD19 bispecific antibodies and CD28 costimulation. II. Assessment of cellular immune responses. *Int. J. Cancer* **91**, 516–522
11. Manzke, O., Tesch, H., Borchmann, P., Wolf, J., Lackner, K., Gossmann, A., Diehl, V., and Bohlen, H. (2001) Locoregional treatment of low-grade B-cell lymphoma with CD3×CD19 bispecific antibodies and CD28 costimulation. I. Clinical phase I evaluation. *Int. J. Cancer* **91**, 508–515
12. Cochlovius, B., Kipriyanov, S.M., Stassar, M.J., Schuhmacher, J., Benner, A., Moldenhauer, G., and Little, M. (2000) Cure of Burkitt's lymphoma in severe combined immunodeficiency mice by T cells, tetravalent CD3 (CD19 tandem diabody, and CD28 costimulation. *Cancer Res.* **60**, 4336–4341
13. Kodama, H., Suzuki, M., Katayose, Y., Shinoda, M., Sakurai, N., Takemura, S., Yoshida, H., Saeki, H., Asano, R., Ichiyama, M., Imai, K., Hinoda, Y., Matsuno, S., and Kudo, T. (2002) Specific and effective targeting cancer immunotherapy with a combination of three bispecific antibodies. *Immunol. Lett.* **81**, 99–106
14. Holliger, P., Manzke, O., Span, M., Hawkins, R., Fleischmann, B., Qinghua, L., Wolf, J., Diehl, V., Cochet, O., Winter, G., and Bohlen, H. (1999) 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. *Cancer Res.* **59**, 2909–2916
15. Jung, G., Brandl, M., Eisner, W., Fraunberger, P., Reifenberger, G., Schlegel, U., Wiestler, O.D., Reulen, H.J., and Wilmanns, W. (2001) Local immunotherapy of glioma patients with a combination of 2 bispecific antibody fragments and resting autologous lymphocytes: evidence for in situ t-cell activation and therapeutic efficacy. *Int. J. Cancer* **91**, 225–230
16. Daniel, P.T., Kroidl, A., Kopp, J., Sturm, I., Moldenhauer, G., Dorken, B., and Pezzutto, A. (1998) Immunotherapy of B-cell lymphoma with CD3 × 19 bispecific antibodies: costimulation via CD28 prevents ″veto″ apoptosis of antibody-targeted cytotoxic T cells. *Blood* **92**, 4750–4757
17. Loffler, A., Gruen, M., Wuchter, C., Schriever, F., Kufer, P., Dreier, T., Hanakam, F., Baeuerle, P.A., Bommert, K., Karawajew, L., Dorken, B., and Bargou, R.C. (2003) Efficient elimination of chronic lymphocytic leukaemia B cells by autologous T cells with a bispecific anti-CD19/anti-CD3 single-chain antibody construct. *Leukemia* **17**, 900–909
18. Dreier, T., Baeuerle, P.A., Fichtner, I., Grun, M., Schlereth, B., Lorenczewski, G., Kufer, P., Lutterbuse, R., Riethmuller, G., Gjorstrup, P., and Bargou, R.C. (2003) T cell costimulus-independent and very efficacious inhibition of tumor growth in mice bearing subcutaneous or leukemic human B cell lymphoma xenografts by a CD19-/CD3-bispecific single-chain antibody construct. *J. Immunol.* **170**, 4397–4402
19. Dreier, T., Lorenczewski, G., Brandl, C., Hoffmann, P., Syring, U., Hanakam, F., Kufer, P., Riethmuller, G., Bargou, R., and Baeuerle, P.A. (2002) Extremely potent, rapid and costimulation-independent cytotoxic T-cell response against lymphoma cells catalyzed by a single-chain bispecific antibody. *Int. J. Cancer* **100**, 690–697
20. Loffler, A., Kufer, P., Lutterbuse, R., Zettl, F., Daniel, P.T., Schwenkenbecher, J.M., Riethmuller, G., Dorken, B., and Bargou, R.C. (2000) A recombinant bispecific single-chain antibody, CD19 × CD3, induces rapid and high lymphoma-directed cytotoxicity by unstimulated T lymphocytes. *Blood* **95**, 2098–2103
21. Wong, W.M., Vakis, S.A., Ayre, K.R., Ellwood, C.N., Howell, W.M., Tutt, A.L., Cawley, M.I., and Smith, J.L. (2000) Rheumatoid arthritis T cells produce Th1 cytokines in response to stimulation with a novel trispecific antibody directed against CD2, CD3, and CD28. *Scand J. Rheumatol.* **29**, 282–287
22. Jung, G., Freimann, U., Von Marschall, Z., Reisfeld, R.A., and Wilmanns, W. (1991) Target cell-induced T cell activation with bi- and trispecific antibody fragments. *Eur. J. Immunol.* **21**, 2431–2435
23. Tutt, A., Stevenson, G.T., and Glennie, M.J. (1991) Trispecific F(ab′)3 derivatives that use cooperative signaling via the TCR/CD3 complex and CD2 to activate and redirect resting cytotoxic T cells. *J. Immunol.* **147**, 60–69
24. French, R.R. (1998) Production of bispecific and trispecific F(ab)2 and F(ab)3 antibody derivatives. *Methods Mol. Biol.* **80**, 121–134
25. Kortt, A.A., Dolezal, O., Power, B.E., and Hudson, P.J. (2001) Dimeric and trimeric antibodies: high avidity scFvs for cancer targeting. *Biomol. Eng.* **18**, 95–108
26. Dolezal, O., Pearce, L.A., Lawrence, L.J., McCoy, A.J., Hudson, P.J., and Kortt, A.A. (2000) ScFv multimers of the anti-neuraminidase antibody NC10: shortening of the linker in single-chain Fv fragment assembled in V(L) to V(H) orientation drives the formation of dimers, trimers, tetramers and higher molecular mass multimers. *Protein Eng.* **13**, 565–574
27. Atwell, J.L., Breheney, K.A., Lawrence, L.J., McCoy, A.J., Kortt, A.A., and Hudson, P.J. (1999) scFv multimers of the anti-neuraminidase antibody NC10: length of the linker between VH and VL domains dictates precisely the transition between diabodies and triabodies. *Protein Eng.* **12**, 597–604
28. Willems, A., Leoen, J., Schoonooghe, S., Grooten, J., and Mertens, N. (2003) Optimizing expression and purification from cell culture medium of trispecific recombinant antibody derivatives. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **786**, 161–176
29. Schoonjans, R., Willems, A., Schoonooghe, S., Leoen, J., Grooten, J., and Mertens, N. (2001) A new model for intermediate molecular weight recombinant bispecific and trispecific antibodies by efficient heterodimerization of single chain variable domains through fusion to a Fab-chain. *Biomol. Eng.* **17**, 193–202
30. Schoonjans, R., Willems, A., Schoonooghe, S., Fiers, W., Grooten, J., and Mertens, N. (2000) Fab chains as an efficient heterodimerization scaffold for the production of recombinant bispecific and trispecific antibody derivatives. *J. Immunol.* **165**, 7050–7057
31. Somasundaram, C., Sundarapandiyan, K., Keler, T., Deo, Y.M., and Graziano, R.F. (1999) Development of a trispecific antibody conjugate that directs two distinct tumor-associated antigens to CD64 on myeloid effector cells. *Hum. Antibodies* **9**, 47–54
32. Min Fang, R.Z., Zhi Yang, Zhong Zhang, Hua Li, Xue-Tao Zhang, Qing Lin, and Hua-Liang Huang, . (2004) Characterization of an anti-human ovarian carcinoma_antihuman CD3 bispecific single-chain antibody with an albumin-original interlinker. *Gynecol. Oncol.* in press
33. Min Fang, X.J., Zhi Yang, Cong-Xiao Yu, Cheng-Chang Yin, Hua Li, Rui Zhao, Zhang-Zhang, Qin-Lin, Hua-Liang Huang. (2003) Effect of inter-linker on the activaty of single chain bispecific antibody. *Chines Sci. Bull.* **48**, 1912–1918
34. Fang M, Z.R., Li H, Jiang X, Yin C, Lin Q, Huang H. (2002) Construction and expression of an anti-human ovarian carcinoma-anti-human CD3 bispecific single-chain antibody and its refolding studies. *High Technol. Lett.* **12**, 47–50
35. Cheng, J., Wang, X., Zhang, Z., and Huang, H. (2002) Construction and expression of a reshaped VH domain against human CD28 molecules. *Prep. Biochem. Biotechnol.* **32**, 239–251
36. Cheng, J.L., Wang, X.B., Zhang, Z., Liu, J., Yao, X.S., and Huang, H.L. (2002) A method for constructing reshaping single-domain antibody. *Yi Chuan Xue Bao* **29**, 189–195
37. Zhang, Z., Li, Z.H., Wang, F., Fang, M., Yin, C.C., Zhou, Z.Y., Lin, Q., and Huang, H.L. (2002) Overexpression of DsbC and DsbG markedly improves soluble and functional expression of single-chain Fv antibodies in Escherichia coli. *Protein Exp. Purif.* **26**, 218–228
38. Rosenberg, A.H., Lade, B.N., Chui, D.S., Lin, S.W., Dunn, J.J., and Studier, F.W. (1987) Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* **56**, 125–135

39. Studier, F.W., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**, 60–89
40. Koga, H., Kanda, H., Nakashima, M., Watanabe, Y., Endo, K., and Watanabe, T. (1990) Mouse-human chimeric monoclonal antibody to carcinoembryonic antigen (CEA): in vitro and in vivo activities. *Hybridoma* **9**, 43–56
41. Borst, J., Prendiville, M.A., and Terhorst, C. (1983) The T3 complex on human thymus-derived lymphocytes contains two different subunits of 20 kDa. *Eur. J. Immunol.* **13**, 576–580
42. Schneider, U., Schwenk, H.U., and Bornkamm, G. (1977) Characterization of EBV-genome negative ″null″ and ″T″ cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. *Int. J. Cancer* **19**, 621–626
43. Weiss, A., and Stobo, J.D. (1984) Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. *J. Exp. Med.* **160**, 1284–1299
44. Weiss, A., Manger, B., and Imboden, J. (1986) Synergy between the T3/antigen receptor complex and Tp44 in the activation of human T cells. *J. Immunol.* **137**, 819–825
45. Leibovitz, A., Stinson, J.C., McCombs, W.B., 3rd, McCoy, C.E., Mazur, K.C., and Mabry, N.D. (1976) Classification of human colorectal adenocarcinoma cell lines. *Cancer Res.* **36**, 4562–4569
46. Ganjei, P., Nadji, M., Albores-Saavedra, J., and Morales, A.R. (1988) Histologic markers in primary and metastatic tumors of the liver. *Cancer* **62**, 1994–1998
47. Kammerer, U., Thanner, F., Kapp, M., Dietl, J., and Sutterlin, M. (2003) Expression of tumor markers on breast and ovarian cancer cell lines. *Anticancer Res.* **23**, 1051–1055
48. Dorvillius, M., Garambois, V., Pourquier, D., Gutowski, M., Rouanet, P., Mani, J.C., Pugniere, M., Hynes, N.E., and Pelegrin, A. (2002) Targeting of human breast cancer by a bispecific antibody directed against two tumour-associated antigens: ErbB-2 and carcinoembryonic antigen. *Tumour Biol.* **23**, 337–347
49. Tomita, Y., Arakawa, F., Hirose, Y., Liao, S., Khare, P.D., Kuroki, M., Yamamoto, T., and Ariyoshi, A. (2000) Carcinoma-associated antigens MK-1 and CEA in urological cancers. *Anticancer Res.* **20**, 793–797
50. Osaki, T., Tanio, Y., Tachibana, I., Hosoe, S., Kumagai, T., Kawase, I., Oikawa, S., and Kishimoto, T. (1994) Gene therapy for carcinoembryonic antigen-producing human lung cancer cells by cell type-specific expression of herpes simplex virus thymidine kinase gene. *Cancer Res.* **54**, 5258–5261
51. Kuo, W.R., Tsai, S.M., Jong, S.B., and Juan, K.H. (1996) Significance of tumour markers in nasopharyngeal carcinoma. *J. Otolaryngol.* **25**, 32–36
52. Xia, Z., Pu, P., and Huang, Q. (2001) [Effect of transfected Cx43 gene on the gap junction intercellular communication and the human glioma cells proliferation]. *Zhonghua Zhong Liu Za Zhi* **23**, 465–468
53. Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning: a Laboratory Manual*, 3rd ed. 3 vols., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
54. Ausubel, F.M. (1999) *Short protocols in Molecular Biology: a Compendium of Methods from Current Protocols in Molecular Biology*, 4th ed., Wiley, New York
55. Darbre, A. (1986) *Practical Protein Chemistry: a Handbook*. analytical methods., Wiley, Chichester [Sussex]; New York
56. Clark, W.R. (1991) *The Experimental Foundations of Modern Immunology,* 4th ed., Wiley, New York ; Chichester
57. Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods* **184**, 39–51
58. Kipriyanov, S.M., Moldenhauer, G., Braunagel, M., Reusch, U., Cochlovius, B., Le Gall, F., Kouprianova, O.A., Von der Lieth, C.W., and Little, M. (2003) Effect of domain order on the activity of bacterially produced bispecific single-chain Fv antibodies. *J. Mol. Biol.* **330**, 99–111
59. Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M., and Green, D.R. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* **182**, 1545–1556
60. Koopman, G., Reutelingsperger, C.P., Kuijten, G.A., Keehnen, R.M., Pals, S.T., and van Oers, M.H. (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* **84**, 1415–1420
61. Lowin, B., Hahne, M., Mattmann, C., and Tschopp, J. (1994) Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature* **370**, 650–652
62. Kagi, D., Vignaux, F., Ledermann, B., Burki, K., Depraetere, V., Nagata, S., Hengartner, H., and Golstein, P. (1994) Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* **265**, 528–530