Efficient Construction of a Diabody Using a Refolding System: Anti-Carcinoembryonic Antigen Recombinant Antibody Fragment

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Recombinant fragments of the variable region of antibodies are useful in many experimental and clinical applications. However, it can be difficult to obtain these materials in soluble form after their expression in bacteria. Here, we report an efficient procedure for preparing several variable-domain fragments (Fv), single-chain Fv (scFv), and a diabody (the smallest functional bispecific antibody) of anti-carcinoembryonic antigen (CEA) antibody by overexpression in *Escherichia coli* in inclusion bodies, using a refolding system to obtain renatured proteins. Two types of refolded Fv were prepared: (i) Heavy and light chains of the immunoglobulin variable regions (VH and VL, respectively) were coexpressed with a dicistronic expression vector (designated Fv_{co}); (ii) VH and VL were expressed separately, mixed stoichiometrically, and refolded (designated Fv_{mix}). All samples refolded with high efficiency; Fv_{co}, Fv_{mix}, scFv, and the bispecific diabody bound to several CEA-positive cell lines, exactly as did soluble Fv fragments secreted by *E. coli* (Fv_{sol}) and the parent IgG. The refolded fragments inhibited binding of the parent IgG to CEA-positive cell lines, indicating that their epitope is identical to that of IgG. The bispecific diabody, which combined variable-region fragments of anti-CEA antibody with variable-region fragments of anti-CD3 antibody, was also prepared using the refolding system. This refolded diabody could bind to lymphokine-activated killer cells. In addition, its cytotoxicity toward human bile duct carcinoma TFK-1 and other several other CEA-positive cell lines was concentration-dependent. Taken together, our results suggest that a refolding procedure can be used to prepare various functional antibody fragments (Fv, scFv, and diabody).

Key words: adoptive immunotherapy, bispecific diabody, CEA, inclusion body.

Recombinant antibody fragments, such as variable-domain fragments (Fv) and single-chain antibodies (scFv), have several potential advantages compared with intact monoclonal antibodies (*1–3*). Their smaller molecular size enables them to be prepared using a bacterial expression system, instead of requiring time-consuming, laborious hybridoma technology. The compact size of the fragments facilitates their use in diagnosis and therapy, because it contributes to rapid tumor targeting and blood clearance, more uniform distribution in tumors, and lower potential for eliciting an immune response (*4–7*). Overexpression of recombinant antibodies in *Escherichia coli*, however, often leads to the formation of insoluble inclusion bodies in the cytoplasmic or periplasmic space (*8*). We previously reported a system for preparing functional soluble scFvs from these insoluble aggregates by an *in-vitro* refolding system, and the same protocol could be used to refold heterodimeric proteins, *i.e.*, Fv and bispecific diabody (*9–11*). The applicability of the refolding procedure to other antibody molecules is intriguing for researchers in various fields.

In this study, we assessed the usefulness of the refolding procedure for preparing anti-carcinoembryonic antigen (CEA) antibody, T84.66. First, we constructed a coexpression vector for anti-CEA Fv, produced the anti-CEA Fv in *E. coli*, and compared refolded Fv (Fv_{co}) obtained from the intracellular insoluble fraction (ICIS) with soluble Fv (Fv_{sol}) obtained from the culture supernatant. Second, we constructed separate single expression vectors for heavy and light chains of immunoglobulin variable regions (VH and VL), expressed them in *E. coli*, purified the gene products

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Abbreviations: Ab, antibody; CEA, carcinoembryonic antigen; Cx3, antibody with bispecificity for CEA and CD3; E:T, effector:target ratio; FITC, fluorescein isothiocyanate; Fv, antibody variable-domain fragment; Gu-HCl, guanidinium hydrochloride; ICIS, intracellular insoluble; IPTG, isopropyl-1-thio-β-D-galactopyranoside; L-Arg, L-arginine; mAb, monoclonal antibody; MTS, 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MW, molecular weight; Mx3, antibody with bispecificity for MUC1 and CD3; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; scFv, single-chain Fv fragment; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; T-LAK, lymphokine-activated killer with T cell phenotype; VH, variable region of immunoglobulin heavy chain; VL, variable region of immunoglobulin light chain; β-ME, 2-mercaptoethanol.

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from the ICIS, mixed them in a 1:1 molar ratio under denaturing conditions, and then refolded them to form Fv_{mix}.

Finally, Cx3 diabody, a heterodimeric protein that targets CEA and CD3, was also prepared by our refolding method. Diabodies have usually been prepared from the bacterial periplasmic fraction. However, bacteria cannot produce some diabodies in a soluble form; thus it is difficult to obtain enough for therapeutic purposes. We previously reported that the refolding system enabled us to prepare functional diabody molecules even in these cases (*10*). Here, we describe the utilization of an *in vitro* refolding system for preparing a uniform, heterodimeric, bispecific diabody directed against the tumor-associated antigen CEA.

MATERIALS AND METHODS

Amplification of Anti-CEA Ab Variable-Region Genes—Mouse B cell hybridoma T84.66, which secretes a monoclonal antibody (mouse IgG1, κ chain) that reacts with CEA, was obtained from our Cell Resource Center and used as the source of genes for the antibody variable region. The genes were amplified by PCR using a synthesized primer based on the known sequences of VH and VL (*12*), and using synthesized first-strand cDNA from T84.66 hybridoma as the templete.

Construction of Expression Vectors of Recombinant Antibody Fragment (Fig. 1)—The VH and VL regions of T84.66 were designated TH and TL, respectively. Expression vectors for the TH and TL chains were each constructed as follows: amplified fragments were digested with *Nco*I and *Sac*II and cloned into pSNE4 MHL-G3, which is a previously constructed T7-promoter-based expression vector for the scFv of epithelial mucin MUC1 antigen-specific MUSE-11 monoclonal antibody (*9*). The expression vector for T84.66 scFv was constructed by sequentially replacing MH of the MHL in pSNE4 MHL-G3 with TH by digestion with *Nco*I and *Eag*I, and ML of the MHL in pSNE4 MHL-G3 with TL by digestion with *Eco*RV and *Sac*II.

The vector for coexpressing both TH and TL was constructed by replacing the MUSE11 VH and VL chains in the pSNE4 MHML vector, as described (*9*).

The THOL, which is one component of the Cx3 diabody, was constructed by replacing the VH portion of the pSNE4 MHOL, which is one of the expression vectors for the Mx3 diabody, with Gly-Gly-Gly-Gly-Ser as a short linker between the VH and VL domains (*10*). OHTL, the other component of the Cx3 diabody, was similarly constructed by replacing the VL portion of the pSNE4 OHML with the same pentapeptide linker.

Expression and Purification of Antibody Fragments—Antibody fragments were expressed and purified as described previously (*9, 13*). In brief, cultures of *E. coli* strain BL21 (DE3) transformed with each expression vector were incubated at 37°C in LB broth. When the optical density at 600 nm reached 0.8, 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to the culture to induce protein production, and the cells were further grown overnight. Cells were separated from the culture by centrifugation (2,000 ×*g*, 35 min) and resuspended in 10 ml of phosphate-buffered saline (PBS), ultrasonicated at 150 W for 15 min, and centrifuged at 4,500 ×*g* for 20 min.

The separated intracellular insoluble (ICIS) fraction was solubilized overnight at 4°C in 10 ml of 6 M Gu-HCl/PBS. Solubilized proteins were purified on a TALON Metal Affinity Resin column (Clontech, Palo Alto, CA).

To obtain soluble anti-CEA Fv (Fv_{sol}), culture supernatants were salted out with ammonium sulfate at 80% saturation, and precipitates were collected by centrifugation at 4,500 ×*g* for 20 min. These precipitates were dissolved in PBS overnight, dialyzed three times against PBS, and purified or a TALON Metal Affinity Resin column. Gel filtration on a Superdex 75 pg column (Amersham, Little Chalfont, Buckinghamshire, UK) was carried out to obtain the 1:1 heterodimeric protein complex .

Refolding of Antibody Fragments Using Step-Wise Dialysis—To obtain functional antibody fragments from the ICIS fraction, a step-wise dialysis method was applied to allow the fragments to refold. Purified antibody fragments were diluted to 7.5 μM with 6 M Gu-HCl/PBS ("denaturing buffer"), or the heterodimeric proteins were mixed in a 1:1 ratio so that the concentration of each fragment type was 3.75 μM, and the overall fragment concentration was 7.5 μM. A step-wise dialysis method was used as previously reported (*8*). In brief, 10 ml of each (denatured) recombinant antibody (7.5 μM protein concentration) was reduced with 375 μM β-ME, and then the guanidine was gradually removed by dialyzing the protein against decreasing concentrations of Gu-HCl in PBS (500 ml, 4°C, 12 h). The concentration of Gu-HCl in the dialysis buffer was lowered sequentially (3, 2, 1, 0.5, and 0 M). An oxidizing reagent (glutathione, oxidized form, Sigma) and 0.4 M of L-arginine were included in the 1- and 0.5-M Gu-HCl/PBS dialysis buffers. The solution containing the refolded proteins was centrifuged at 4,500 ×*g* for 20 min. Fv_{co} was subjected to an additional purification step, gel filtration on a Superdex 75 pg column (Amersham-Pharmacia), to obtain uniformly heterodimeric proteins.

SDS-PAGE and Western Blotting—Aliquots (500 μl) of culture supernatant were used for analysis of the antibody fragments. The total proteins in each fraction, precipitated with 6% trichloroacetic acid (TCA) and 0.083% deoxycholate, were subjected to dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions as previously reported (*11*). The gels were stained with Coomassie Brilliant Blue R-250 (*14*). Then the proteins in the gel were blotted onto nitrocellulose membranes (Amersham) and incubated with peroxidase-conjugated anti–His tag monoclonal antibody (Invitrogen), followed by signal enhancement using the ECL Detection System (Amersham Bioscience).

Preparation and Stimulation of Effector Cells—For the induction of T-LAK cells, PBMC were isolated by density-gradient centrifugation from a healthy volunteer and cultured for 48 h in culture medium supplemented with 100 IU ml^{-1} recombinant human interleukin-2, which was kindly supplied by Shionogi Pharmaceutical (Osaka), at a cell density of 1×10^6 ml^{-1} in a culture flask (A/S Nunc, Roskilde, Denmark) coated with OKT3 monoclonal antibody (10 μg ml^{-1}). The T-LAK cells were then transferred to another flask and expanded in culture medium containing 100 IU ml^{-1} of interleukin-2 for 2–3 weeks (*15*).

Flow Cytometric Analysis—All test cells (5×10^5) were first incubated with 100 μl of recombinant antibody fragments (about 100 μg ml^{-1}) as the first antibody for 30 min on ice. After washing with PBS containing 0.1% NaN_3, the cells were exposed to 9E10 anti–c-myc monoclonal antibody

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(Santa Cruz Biotechnology, CA) as the second antibody, and then to FITC-conjugated anti–mouse IgG as the third antibody for 30 min on ice. The stained cells were analyzed by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA).

Blockage of T84.66 IgG and OKT3 IgG Binding to CEA-Positive Cell Lines and T-LAK by Recombinant Antibody Fragments—The cells for the inhibition test were incubated with 200 μl of purified recombinant antibodies (200 μl ml^{-1}) on ice for 30 min. Next, 10 μl of parent mouse IgG (100 μl ml^{-1}) was added, and the incubation was continued for 30 min to allow competition between recombinant and parent antibodies for binding. The cells were washed three times with PBS containing 0.1% NaN_3 and stained with FITC-conjugated goat anti–mouse IgG to detect parent IgG binding. The stained cells were analyzed by flow cytometry (FACS Calibur).

In Vitro Growth Inhibition Assay—*In vitro* growth inhibition of various cell lines was assayed with an MTS assay kit (CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI). Target cells, 5,000 cells in 100 μl of culture medium, were distributed into each well of a half-area (A/2) 96-well flat-bottomed plate (Costar, Cambridge, MA). They were cultured overnight to allow adhesion to the bottoms of the wells. After removing the culture medium by aspiration, 100 μl of T-LAK cells plus various concentrations of recombinant antibodies were added to each well. After culture for 48 h at 37°C, each well

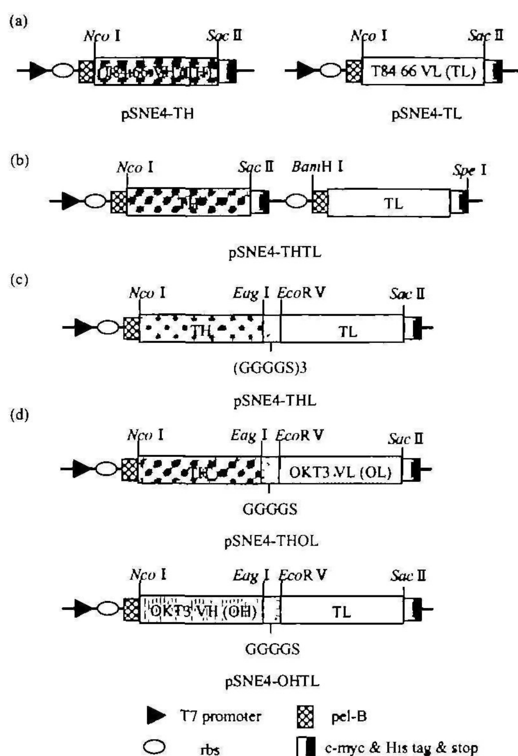

Fig. 1. **Schematic showing the construction of expression vectors.** (a) The single expression vector for the VH and VL domains of antibody T84.66 are designated TH and TL, respectively. (b) Coexpression vector for TH and TL. (c) T84.66 single-chain Fv (scFv) expression vector. (d) Two different scFv expression vectors for Cx3 diabody, THOL and OHTL. The positions of important restriction sites used in constructing these vectors are shown. *rbs*, ribosome binding region; *pel-B*, signal peptide sequence of bacterial pectate lyase; His tag, sequence encoding six C-terminal histidine residues; c-myc, sequence encoding an epitope recognized by 9E10 monoclonal antibody.

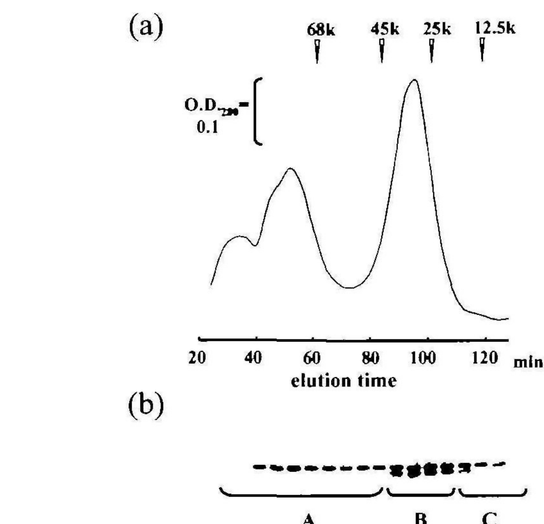

Fig. 3. **Gel filtration of Fv_{sol} after IMAC purification.** (a) A sample (1 ml) of proteins eluted from a metal-affinity chromatography column was loaded onto a Superdex at a flow rate of 0.5 ml min^{-1}. The retention times of the standards are shown above the horizontal line. (b) SDS-PAGE of each fraction; the A fraction range appears to be a multimer of VH that is expressed in excess; the B range is the uniform heterodimer fraction, *i.e.*, Fv; the C range appears to be a monomer of VH and VL.

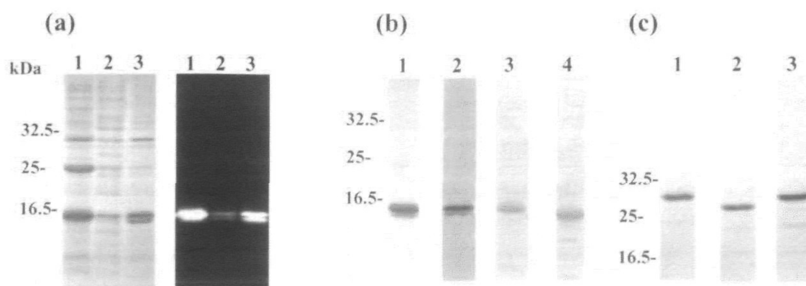

Fig. 2. **Expression and purification of recombinant anti-CEA antibody fragments.** (a) SDS-PAGE (left) under reducing conditions and Western blot (right) stained with the anti-His tag monoclonal antibody of each fraction of *E. coli* BL21 (DE3) cells expressing anti-CEA antibody fragments. Lanes: 1, proteins in the bacterial culture supernatant; 2, proteins in the intracellular soluble fraction; 3, proteins in the intracellular insoluble fraction (inclusion bodies). (b) SDS-PAGE under reducing conditions of Fv fragments obtained by immobilized metal affinity chromatography (IMAC) under denaturing conditions (except for Fv_{sol}). Lanes: 1, Fv_{sol}; 2, Fv_{co}; 3, anti-CEA VH; 4, anti-CEA VL. (c) SDS-PAGE under reducing conditions of scFv and diabody fragments purified using IMAC under denaturing conditions. 1, single-chain Fv; 2, THOL; 3, OHTL.

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was washed with PBS three times to remove effector cells and dead target cells. Then 95 μl of culture medium and 5 μl of a fresh mixture of MTS/phenazine methosulfate solution (Promega) were added to each well. The plates were incubated for 1 h at 37°C and then read on a microplate reader (Bio-Rad model 3550) at 490 nm. Growth inhibition of target cells was calculated as follows: percent growth inhibition of target cells=[1–(A_{490} of experiment–A_{490} of background)/(A_{490} of control–A_{490} of background)]×100 (*16*).

RESULTS

Expression and Purification of Recombinant Anti-CEA Antibody Fragments—The following expression vectors for recombinant anti-CEA antibody fragments were constructed (Fig. 1): coexpression of Fv (genes encoding VH and VL are arranged dicistronically, Fig. 1b), single expression of Fv (VH and VL chains were expressed separately as inclusion bodies, Fig. 1a), scFv (Fig. 1c), and the THOL and OHTL components of the Cx3 diabody (Fig. 1d).

These antibody fragments were shown to be more than

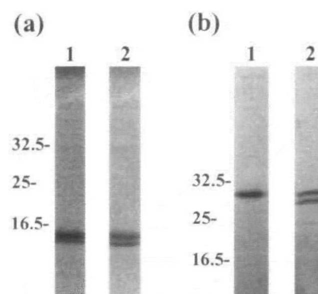

Fig. 4. **SDS-PAGE analysis of refolded recombinant antibodies.** SDS-PAGE under reducing conditions of each refolded anti-CEA antibody fragment. (a) Fv fragment. Lanes: 1, Fv_{co}; 2, Fv_{mix}. (b) Lanes: 1, scFv; 2, Cx3 diabody.

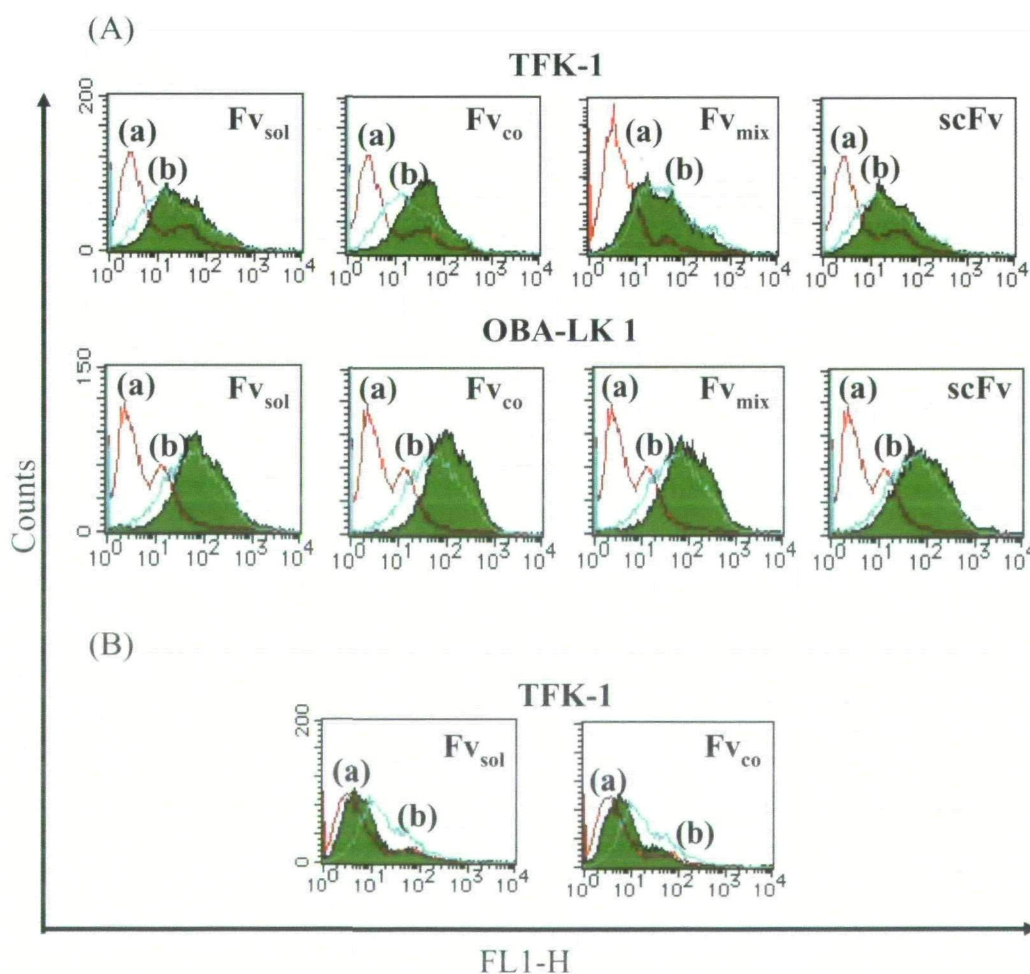

Fig. 5. **Flow-cytometry analysis of anti-CEA antibody fragments.** (A) Reactivity of Fv_{sol}, Fv_{co}, Fv_{mix}, and scFv to TFK-1 (top panel) and OBA-LK1 (bottom panel). Lines: (a) cells incubated with PBS as a negative control and (b) parent T84.66 IgG, followed by staining with FITC-conjugated anti-mouse IgG, as a positive control; solid areas: cells incubated with the indicated anti-CEA antibody fragment. Cells were incubated with these Fv fragments, then mouse anti–c-myc 9E10 antibody was added, followed by staining with FITC-conjugated anti-mouse IgG. (B) Competition between Fv fragments and IgG. Lines: (a) TFK-1 cells were incubated with Fv, followed by incubation with FITC-conjugated anti-mouse IgG (negative control) and (b) TFK-1 cells were incubated with T84.66 IgG, followed by FITC-conjugated anti-mouse IgG (positive control); solid areas: TFK-1 cells were incubated with Fv. Next, cells were incubated with T84.66 IgG. Finally FITC-conjugated anti-mouse IgG was added to determine competition between Fv and IgG.

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95% pure, and the molecular weights estimated from SDS-PAGE and Western blotting using anti-His tag antibody corresponded well with the theoretical calculated molecular weight of each protein (Fig. 2). Soluble Fv_{sol} was indeed obtained from the culture supernatant; however, the amount of VH in the Fv_{sol} exceeded that of VL. To obtain Fv_{sol} containing stoichiometrically equal amounts of VH and VL, the Fv_{sol} was additionally purified by size-exclusion chromatography (Fig. 3). The yields of recombinant antibodies were, for Fv_{sol}, approximately 1 mg per liter of culture supernatant; and for the other recombinant antibodies (obtained from the inclusion bodies), over 10 mg per liter.

Refolding of Recombinant Antibodies—To obtain soluble, functional recombinant antibodies from ICIS fractions, we used the refolding system described in "MATERIALS AND METHODS." First, each purified protein was dissolved in denaturing buffer. Step-wise dialysis was used to gradually remove the denaturant, allowing the proteins to refold. The refolding efficiencies of the Fv_{co}, Fv_{mix}, and scFv fragments and of the Cx3 diabody were 93, 96, 87, and 64%, respectively. Proper refolding was supported by the ability of the two domains of Fv_{mix} and of the diabody to associate into 1:1 molar complexes; however, one domain was in excess in Fv_{co} (Fig. 4). Therefore, Fv_{co}, like Fv_{sol}, required further purification by gel filtration to obtain a uniform heterodimeric molecule after the refolding step (Figs. 3 and 4).

Binding of Anti-CEA Fv and scFv Studied by Flow Cytometry—The binding activity of each anti-CEA antibody fragment was assessed by flow cytometry using several CEA-positive cell lines. The refolded antibody fragments, and also the parent anti-CEA antibody T84.66, could bind to TFK-1 (human bile duct carcinoma), OBA-LK1 (human lung carcinoma), CEA-transfected CHO cells, and other cell lines (some of the data are shown in Fig. 5). The results were almost identical for the Fv fragment prepared from the soluble fraction. Moreover, all recombinant antibodies inhibited the binding of the parent T84.66 IgG to both TFK-1 (Fig. 5) and OBA-LK-1 (data not shown) cells, indicating that these fragments recognized the same epitope.

TABLE I. **Reactivity of T84.66 IgG and recombinant antibodies.**

Cell lines	T84.66 IgG	Fv_{mix}	Cx3 diabody
TFK-1 (bile duct)	++	++	++
OBA-LK 1 (lung)	++	++	++
MCF-7 (breast)	+	+	+
OCUCh-LM1 (bile duct)	++	+	+
WiDr-TC (colon)	+	+	+
HuCCT1 (bile duct)	–	–	–
CEA transfected CHO	+	+	+

*Figure in the table are relative fluorescence intensities compared with negative control.

Flow-Cytometry Analysis of Cx3 Diabody—The binding of the refolded heterodimeric Cx3 diabody to several cancer cell lines (Fig. 6 and Table I) and to lymphokine-activated T-cells (T-LAK cells) (Fig. 6) was examined by flow cytometry. Strong reactivities for cell lines were observed, similar to the results for the parent antibody and recombinant Fvs described above. The reactivity of the diabody with T-LAK cells was weaker than that of OKT3 IgG. The intrinsic affinity of scFv and diabody for target antigens is identical to that of the parent IgG (the apparent affinity is decreased due to the bivalency of the IgG molecules). The lower affinity of the diabody, relative to OKT3 IgG, for T-LAK was reported previously (*17, 18*). The ability of Cx3 to inhibit the binding of the parent IgG, as well as the bifunctionality of the Cx3 diabody (*i.e.*, the binding of each binding site in the bispecific antibody), were confirmed by an absorption test (data not shown) (*10*).

Inhibition of Cancer Cell Growth by T-LAK Cells—The effects of the refolded Cx3 diabody on cancer cells were evaluated in a growth-inhibition assay using several cancer cell lines (TFK-1, OBA-LK1, MCF7 [human breast adenocarcinoma], and WiDr-TC [human colon carcinoma]) as targets and T-LAK cells as the effector. The percent inhibition of cancer cell growth by Cx3 increased at E:T ratios of 5:1

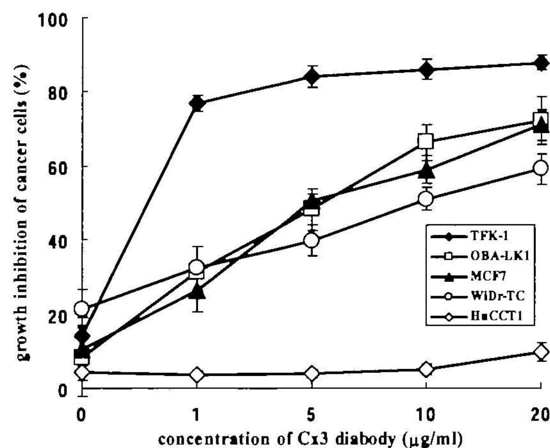

Fig. 7. **Growth inhibition assay of five cancer cell lines.** Percent growth inhibition was determined by a 48-h MTS assay, in which Cx3 diabody and T-LAK cells (effectors) were added to these targets: TFK-1 (◆), OBA-LK1 (□), MCF7 (▲), WiDr-TC (○), and HuCCT1 (◇) (targets), at an E:T ratio of 5:1. Data are mean values from a minimum of three determinations.

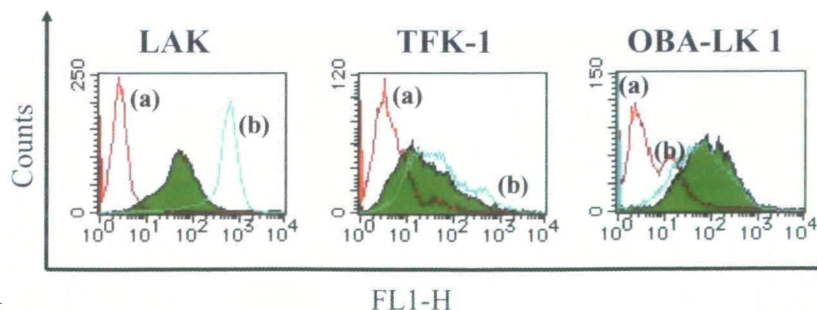

Fig. 6. **Flow-cytometry analysis of Cx3 diabody.** Reactivity of Cx3 diabody to T-LAK (left) and two cancer cell lines, TFK-1 (middle) and OBA-LK1 (right). Lines: (a) cells incubated with PBS as a negative control and (b) T84.66 IgG (OKT3 IgG to T-LAK) as a positive control; solid areas: cells incubated with Cx3 diabody. Cells were incubated with Cx3 diabody, then mouse anti–c-myc 9E10 antibody was added, followed by staining with FITC-conjugated anti-mouse IgG.

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(Fig. 7) and 10:1 (data not shown) in a dose-dependent manner (although the degree of inhibition differed among cell lines due to their differing sensitivities to T-LAK cells). The antigen specificity of the Cx3 diabody was confirmed by its inability to inhibit the growth of a CEA-negative cell line, HuCCT1 (human bile duct carcinoma).

CEA is expressed on most adenocarcinomas and has been used as a model antigen for immunotherapeutic targeting of solid tumors. Our constructed diabody also bound to some cancer cell lines (Table I) and inhibited their growth (Fig. 7), indicating the applicability of the Cx3 diabody to cancer immunotherapy.

DISCUSSION

CEA is a cancer antigen that has been extensively investigated as a means of targeting malignant tumors. Anti-CEA monoclonal antibodies have been used for therapeutic purposes, *e.g.*, in diagnosis, tumor targeting, and imaging of remaining tumor cells in minimal residual disease (*19–22*). Recombinant anti-CEA fragments (Fv, scFv, scFv dimer, minibody, and diabody), because of their smaller molecular size, should offer advantages over intact monoclonal antibodies, such as rapid tumor targeting, rapid blood clearance, more uniform tumor distribution, and a lower potential of eliciting a human immune response (*5, 23, 24*).

Recent advances in genetic engineering have made it more feasible to prepare these fragments in several expression systems (*25, 26*). These antibody fragments are usually prepared from soluble fractions, *e.g.*, the culture supernatant or periplasmic fraction of *E. coli*, the supernatant of transfected mammalian-cell cultures, or extracts of plant leaves. The prepared proteins are functional, but require complicated purification procedures, which tend to decrease the yield of recombinant proteins, usually to less than 1 mg $liter^{-1}$ (*27*).

Here we describe an *in vitro* refolding system for preparing Fv, scFv, and diabody, which target the tumor-associated carcinoembryonic antigen CEA. Refolding enabled us to obtain functional, soluble recombinant antibodies, including heterodimeric molecules, from insoluble ICIS aggregates. Since the level of expressed proteins is higher in the ICIS than in the culture supernatant, preparing antibody fragments from ICIS increases the yield, enables the use of more convenient purification procedures, and yields products in high purity (*28, 29*).

In this study, we showed that the Fv, whose VH and VL chains were coexpressed and refolded (Fv_{co}), had almost the same binding activity as Fv prepared from the soluble fraction (Fv_{sol}). In both Fv_{co} and Fv_{sol}, however, the quantity of the VH domain was larger than that of the VL domain, and gel filtration thus had to be used (after refolding, for Fv_{co}) to obtain uniform Fv.

Fv_{mix}, which was obtained by mixing VH and VL in equal concentrations under denaturing conditions and then refolding, and the smallest bispecific antibody (diabody), which was refolded under the same conditions as Fv_{mix}, formed uniform 1:1 dimers and did not need additional purification methods. These refolded heterodimeric proteins (including the diabody) were also functional. Recently, the failure of diabody obtained from ICIS to refold was reported. This failure was attributed to incorrect dimerization of the proteins, possibly due to a lack of domain swapping in the periplasmic fraction (*30*). Our present results, however, indicate that functional diabody can be prepared by refolding under carefully controlled conditions. Although efforts to establish the best refolding procedures for diabodies should be continued, some diabodies can already be prepared by refolding, and thus, the molecular mechanisms involved in stable diabody formation can be investigated. The phage-display system (*31–33*) might be useful for preparing stable diabody molecules.

In conclusion, the refolding procedure developed here is useful for preparing anti-CEA recombinant antibody fragments, including heterodimeric antibody fragments. The procedure also enabled us to produce a bispecific diabody, one that was reactive with CEA on several cancer cell lines and with the CD3 molecule on T-LAK cells. The Cx3 diabody inhibited the growth of many cancer cell lines, which indicates that these refolded proteins may eventually be useful as clinical reagents. Consequently, the refolding procedure reported here should broaden the range of applications of bispecific diabodies in various fields.

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