Humanization of the Bispecific Epidermal Growth Factor Receptor × CD3 Diabody and Its Efficacy as a Potential Clinical Reagent

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Abstract

Purpose: Bispecific antibodies (BsAb) have been exploited as both cancer immunodiagnostics and cancer therapeutics and show promise in clinical trials of cancer imaging and therapy. For development of BsAbs as clinical reagents, we have focused on construction of small recombinant BsAbs, called bispecific diabodies. Here, we constructed and characterized a humanized bispecific diabody.

Experimental Design: We have reported significant antitumor activity of an anti-epidermal growth factor receptor (EGFR) × anti-CD3 bispecific diabody (Ex3) in *in vitro* cytotoxicity assays and *in vivo*. We humanized the Ex3 diabody (hEx3) by grafting the complementarity-determining region and compared its biological properties with those of Ex3. We also tested its physiologic stability and ability to alter survival in xenografted mice.

Results: The final yield of hEx3 was 10 times that of Ex3, and refolded hEx3 and Ex3 showed identical binding profiles in EGFR-positive cell lines and EGFR-transfected Chinese hamster ovary cells. hEx3 showed dose-dependent cytotoxicity to EGFR-positive cell lines, which could be specifically inhibited by parental monoclonal antibody IgGs against EGFR or CD3 antigens. The heterodimeric structure was retained in PBS for 6 months, and growth inhibition was maintained after incubation under physiologic conditions. Coadministration of hEx3 with T-LAK cells and interleukin-2 prolonged the survival of nude mice with human colon carcinoma.

Conclusions: The humanized diabody hEx3 is an attractive molecule for cancer therapy and may provide important insights into the development of EGFR-based cancer-targeting reagents.

The epidermal growth factor receptor (EGFR) is overexpressed in a wide range of human malignancies, and its expression level correlates with a poor clinical outcome in patients with any of several cancers. The proposal 20 years ago that EGFR was an attractive target molecule for cancer therapy led to the development of several classes of anti-EGFR agents, including monoclonal antibodies (mAb) and receptor tyrosine kinase inhibitors (1–3). One of these agents, cetuximab, is a chimeric mAb that binds to EGFR, blocks ligand binding, and thus prevents receptor activation and downstream signaling (4). Cetuximab was effective in phase II trials, and cotreatment with cetuximab and irinotecan gave better results than cetuximab alone (4–7).

Bispecific antibodies (BsAb) have drawn considerable attention owing to their unique affinity for two different antigens. Most are designed to redirect T cells toward non–MHC-restricted tumor cells by cross-linking tumor cell surface antigens and the CD3-TCR complex on T cells. CTLs, the most potent killer cells of the immune system, cannot be engaged by monoclonal antibodies because T lymphocytes lack Fcγ receptors. The efficacy of monoclonal antibodies for cancer treatment is still limited, leaving great potential for further improvements for example by BsAbs (8, 9). Although BsAbs have therapeutic potential, the classic preparation methods hybrid hybridoma and chemical conjugation are time consuming and laborious (10).

Recent advances in recombinant DNA technologies have made it feasible to generate smaller BsAbs, called diabodies, consisting of only two VH and two VL domains from two different antibodies (11–13). Diabodies are the smallest BsAbs available, and the distance between the two antigen-binding sites is less than half of IgG (14). This compactness contributes not only to low immunogenicity and high tumor penetration but also rapid clearance from the circulation (15). Although large-scale preparation of diabodies with bacterial expression systems is possible because of their small size, the yield is typically a few mg/mL from *Escherichia coli* or mammalian cells, and the former system requires protein refolding to obtain functional proteins (16–18).

Recently, we successfully prepared functional diabodies from the bacterial intracellular insoluble fraction using an *in vitro* refolding system that may allow for industrial-scale diabody production (19–21). We previously constructed an

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anti-EGFR/anti-CD3 bispecific diabody, termed Ex3, which specifically targeted both LAK with T-cell phenotype (T-LAK) and resting peripheral blood mononuclear cells against EGFR-positive cell lines. Furthermore, coadministration of the Ex3 diabody with T-LAK cells in bile duct carcinoma-xenografted severe combined immunodeficient mice resulted in pronounced inhibition of tumor growth (22).

Here, we describe the construction of a humanized Ex3 (hEx3) by complementarity-determining region (CDR) grafting. Refolded hEx3 showed identical biological activity to that of Ex3, including significant inhibition of *in vitro* tumor growth. hEx3 was stable under physiologic conditions, and coadministration with T-LAK prolonged the survival of nude mice xenografted with human colon carcinoma. To the best of our knowledge, this is the first report of the *in vivo* antitumor effects of a fully humanized diabody prepared by refolding and therefore provides important insights for constructing EGFR-targeted therapeutic antibodies.

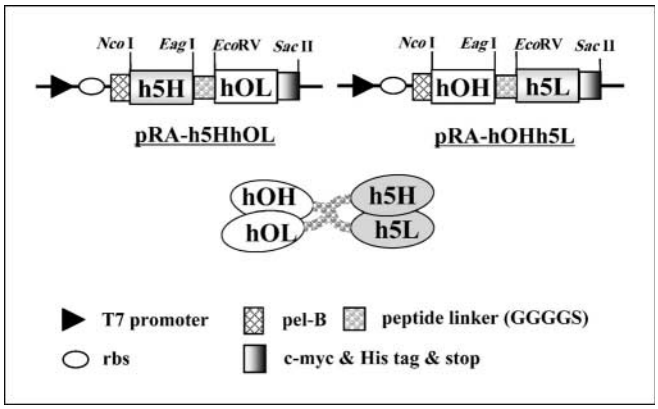

Fig. 1. Schematics of two scFv expression vectors and hEx3 diabody: h5HhOL and hOHh5L. Positions of important restriction enzyme sites used in construction of these vectors. rbs, ribosome-binding region; pel-B, a signal peptide sequence of bacterial pectate lyase; His tag, a sequence encoding six COOH-terminal histidine residues; c-*myc*, a sequence encoding an epitope recognized by the 9E10 monoclonal antibody.

Materials and Methods

Cloning of anti-EGFR variable region genes. The mouse hybridoma cell line 528, which produces an anti-human EGFR antibody (IgG2a), was used as the source of variable region genes (23, 24). The *VH* and *VL* genes of the hybridoma cells were cloned with the synthesized primers and PCR methods reported previously (25). The expression vector of 528 single-chain Fv (scFv) was constructed from these cloned genes, and 528 scFv was prepared for sequence verification because myeloma cell lines frequently produce aberrant sequences (26).

Humanization of 528 Fv and construction of fully humanized diabody expression vector. The anti-EGFR Fv was humanized by CDR-grafting methods based on previous reports (27–29). The sequence of Xu-12 VH (DNA Data Bank of Japan; http://getentry.ddbj.nig.ac.jp/getstart-j.html; accession no. AF062257; ref. 30) was chosen for the template of 528 VH, and the sequence of BR55-2 VL (accession no. A25561) was chosen for the template of 528 VL by homology searches with human antibodies using the BLAST sequence program. *VH* and *VL* sequences containing the CDR sequences were designed by substituting the 528 CDRs with the chosen sequences, and then constructed by PCR overlap methods with synthesized primers optimized for *E. coli*. To characterize the activity of humanized 528 Fv, we also constructed the scFv as described above. All of the functions of the 528 scFv were retained; thus, we used these sequences for construction of fully humanized bispecific diabody for clinical use. The genes for the humanized anti-CD3 antibody OKT3 Fv were similarly constructed with whole synthesized and *E. coli* codon-optimized genes (31–33). The VH and VL regions of humanized 528 Fv are designated h5H and h5L, and those of humanized OKT3 Fv, used a previously published humanized sequence, are designated hOH and hOL, respectively (34). The two hetero-scFvs of humanized anti-EGFR × anti-CD3 bispecific diabody (designated hEx3) are designated h5HhOL and hOHh5L, and each corresponding gene (*h5HhOL* and *hOHh5L*) was inserted into the pRA vector, which is a previously constructed T7 promoter–based expression vector (Fig. 1; ref. 35).

Preparation of the diabody molecule. Preparation of the diabodies from an *E. coli* intracellular insoluble fraction by refolding has been described previously (19, 21, 36). In brief, vector-transformed cultures of *E. coli* strain BL21 (DE3) were incubated at 37°C in Luria-Bertani broth. When the absorbance at 600 nm reached 0.8, 1 mmol/L isopropyl-1-thio-β-D-galactopyranoside 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 minutes), resuspended in 10 mL of PBS, ultrasonicated at 150 W for 15 minutes, and centrifuged at 4,500 × *g* for 20 minutes. The separated intracellular insoluble fraction was solubilized overnight at 4°C in 10 mL of 6 mol/L guanidinium hydrochloride/PBS (Gu-HCl/PBS). Solubilized proteins were purified through a TALON Metal Affinity Resin column (Clontech, Palo Alto, CA).

To obtain functional diabody fragments from the intracellular insoluble fraction, we used a stepwise dialysis method to allow the fragments to refold. Purified hetero-scFvs (h5HhOL and hOHh5L) were diluted to 7.5 μmol/L with 6 mol/L Gu-HCl/PBS, and then these were mixed in a 1:1 ratio. This denatured scFv mixture (10 mL, with 3.75 μmol/L diabody) was reduced with 375 μmol/L 2-mercaptoethanol, and then guanidine was gradually removed by dialyzing the protein against decreasing concentrations of Gu-HCl in PBS (500 mL, 4°C, 12 hours). The concentration of Gu-HCl in the dialysis buffer was lowered sequentially (3, 2, 1, 0.5, and 0 mol/L). An oxidizing reagent (glutathione, oxidized form, Sigma, St. Louis, MO) and 0.4 mol/L of L-arginine were included in the 1 mol/L Gu-HCl/PBS and 0.5 mol/L Gu-HCl/PBS dialysis buffers. The solution containing the refolded proteins was centrifuged at 4,500 × *g* for 20 minutes to remove insoluble material. Then, concentrated sample was filtered through a 0.22-μm ultrafiltration membrane (Millipore, Tokyo, Japan) and stored in PBS at 4°C.

Preparation of T-LAK cells. For the induction of T-LAK cells, peripheral blood mononuclear cells were isolated by density-gradient centrifugation of serum from a healthy volunteer and cultured for 48 hours in medium supplemented with 100 IU mL^{-1} recombinant human interleukin-2, kindly supplied by Shionogi Pharmaceutical Co. (Osaka, Japan), at a cell density of 1×10^6 mL^{-1} in a culture flask (A/S Nunc, Roskilde, Denmark) precoated with OKT3 mAb (10 μg mL^{-1}). The proliferated cells were then transferred to another flask and expanded in culture medium containing 100 IU mL^{-1} of interleukin-2 for 2 to 3 weeks, as reported previously (37).

Flow cytometric analyses. Test cells (1×10^6) were first incubated on ice with 10 μg (final concentration, 1 μmol/L) of recombinant antibody for 30 minutes. After washing with PBS plus 0.1% NaN_3, they were exposed to FITC-conjugated (FITC conjugated) 9E10 anti-c-*myc* mAb (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes on ice. The stained cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA).

Blocking tests were also done with flow cytometry: EGFR/Chinese hamster ovary (CHO) or T-LAK cells (1×10^6) were incubated on ice with or without 4 μg (final concentration, 0.13 μmol/L) competing parent-mAb 528 IgG or OKT3 IgG, respectively. After washing with PBS plus 0.1% NaN_3, they were incubated with 2.5 μg (final concentration,

0.25 μmol/L) hEx3 diabody for 30 minutes on ice, further washed, and incubated on ice with the FITC-conjugated 9E10 anti-c-*myc* mAb. Flow cytometry was then done as described (20, 22).

In vitro ***growth inhibition assay.*** *In vitro* growth inhibition of various cell lines was assayed with a 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt assay kit (CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI). The target cells (5,000 in 100 μL of culture medium) were plated on 96-well, half-well area (*A*/2), flat-bottomed plates (Costar, Cambridge, MA). Cells were cultured overnight to allow well adhesion. After removal of the culture medium by aspiration, 100 μL of T-LAK cells (effector cells) plus various concentrations of recombinant antibodies were added to each well, giving a final target/effector cell ratio of 5 or 10. After culture for 48 hours at 37°C, each well was washed with PBS thrice to remove effector cells and dead target cells, and 95 μL of culture medium plus 5 μL of a fresh mixture of 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt/phenazine methosulfate solution (Promega) were added to each well. The plates were incubated for 1 hour at 37°C and then read on a microplate reader (Bio-Rad model 3550) at 490 nm. The growth inhibition of target cells was calculated as follows: percentage growth inhibition of target cells = [1 − (A_{490} of experiment − A_{490} of background) / (A_{490} of control − A_{490} of background)] × 100 (37, 38).

A blocking test using parental mAb IgGs (528 or OKT3) or nonspecific IgGs (MUSE11, anti-MUC1 mAb or OKT8, anti-CD8 mAb) was also done: after removal of the overnight culture medium of target cells, 100 μL of T-LAK cells plus 1 pmol/mL hEx3 diabody and various concentrations of parental IgGs were added to each well. After culture for 48 hours at 37°C, detection with 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt solution was done as above.

Gel filtration chromatography. Gel filtration analysis with a Hiload Superdex 200-pg column (16/300; Amersham Biosciences, Piscataway, NJ) was used to evaluate the long-term stability of hEx3 dimers. The column was equilibrated with 50 mmol/L Tris-HCl (pH 8) containing 200 mmol/L NaCl, and then 2 mL of purified protein was applied to the column at a flow rate of 0.5 mL/min. The hEx3 peak corresponding to the dimer molecular weight was collected and then reanalyzed under the same conditions after 2 weeks or 6 months.

Stability test under physiologic conditions. To examine *in vitro* stability, hEx3 diabody was preincubated at 37°C for 1 or 24 hours in human plasma. Growth inhibition was then compared with untreated hEx3 by the 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt assay. The control condition was T-LAK cells and human plasma diluted with medium.

Diabody-mediated tumor inhibition in nude mice. Female 5-week-old nude mice were primed i.p. with rabbit anti-asialo GM1 (Wako Pure Chemical Industries Ltd., Osaka, Japan) to deplete natural killer cells. Three days later, mice were given an i.p. injection of 5×10^6 CoLo TC (human colon carcinoma). Starting 4 days later, the mice were given a daily i.p. injection of 2×10^7 T-LAK cells plus 500 IU interleukin-2, with or without 5 μg diabody/day/mouse, for four consecutive days and were monitored weekly for survival.

Results

Cloning and humanization of the variable region of 528 anti-EGFR antibody. Cloned genes encoding VH and VL of 528 anti-EGFR antibody were used to construct a scFv to verify the sequence, because myeloma cell lines often produce aberrant mRNAs. This scFv specifically bound to EGFR in flow cytometry and recognized the same epitope as the parent IgG in a blocking assay (data not shown); we, therefore, humanized this gene. The CDR-grafting methods for humanization of 528 Fv are described in Materials and Methods.

Construction of* E. coli *expression system and preparation of hEx3. The two scFvs of hEx3 (h5HhOL and hOHh5L) were prepared using an *E. coli* expression system. Results of SDS-PAGE and Western blotting showed that each gene product primarily existed in intracellular fractions (Fig. 2). All of the hEx3 genes were synthesized to optimized *E. coli* codons. The refolding efficiency was >50%, which is relatively high for a bispecific diabody. Refolded hEx3 could be prepared with high purity (>95%) and with stoichiometric association of hetero-scFvs (Fig. 2). The final yield of hEx3 was ~10 times that of Ex3.

Binding properties of hEx3 in flow cytometry. Binding of hEx3 to the targeted antigens was confirmed by flow cytometry. Strong reactivity was observed with EGFR-positive TFK-1 (human bile duct carcinoma) and CD3-positive T-LAK, with identical affinity to that of the parental IgGs (528 or OKT3) and Ex3 (data not shown). hEx3 also bound to other EGFR-positive cell lines OCUCh-LM1 (human bile duct carcinoma), HuCC-T1 (human bile duct carcinoma), and EGFR/CHO (CHO cells transfected with EGFR) but not to the EGFR-negative cell line MCF7 (human breast adenocarcinoma) or normal CHO (Fig. 3A). Parental mAbs significantly and competitively inhibited the binding of hEx3 to T-LAK and EGFR/CHO, suggesting that they recognize the same epitope (Fig. 3B).

Growth inhibition of cancer cells with T-LAK cells. The effect of hEx3 on cancer cell growth was evaluated using T-LAK as an effector: low doses of hEx3 (0.1-1.0 pmol/mL, or ~60 ng/mL) dose-dependently inhibited cell growth of the TFK-1, OCUCh-LM1, and HuCC-T1 cell lines (Fig. 4A). Additionally, hEx3 showed the same or slightly higher cytotoxicity than Ex3, despite a 40-fold lower affinity for EGFR (K_a values of the mouse and humanized 528 Fv to EGFR, as estimated from isothermal titration calorimetry, are 8.17×10^8 and 1.89×10^7, respectively). K_a values against to CD3 of the diabody consisted of mouse OKT3 is 4.5×10^7 for reference, and it is reported that mouse and humanized OKT3 show almost same property (34, 39). hEx3 did not enhance T-LAK cytotoxicity against MCF7 or non–EGFR-transfected CHO cells (Fig. 4B). Furthermore, these effects were completely blocked by the

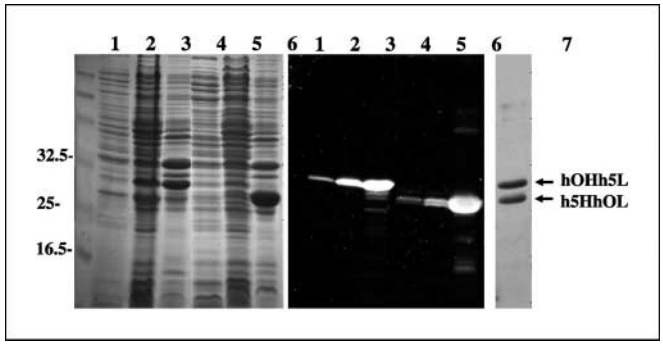

Fig. 2. SDS-PAGE (*left* and *right*) under reducing conditions and Western blot (*middle*) stained with anti-His tag monoclonal antibody of *E. coli* BL21 (DE3) cells expressing h5HhOL (*lanes 1-3*) and hOHh5L (*lanes 4-6*). Molecular size markers (in kDa) are on the left. Lanes 1 and 4, proteins in the bacterial culture supernatant; lanes 2 and 5, proteins in the intracellular soluble fraction; lanes 3 and 6, proteins in the intracellular insoluble fraction; lane 7, refolded hEx3 diabody.

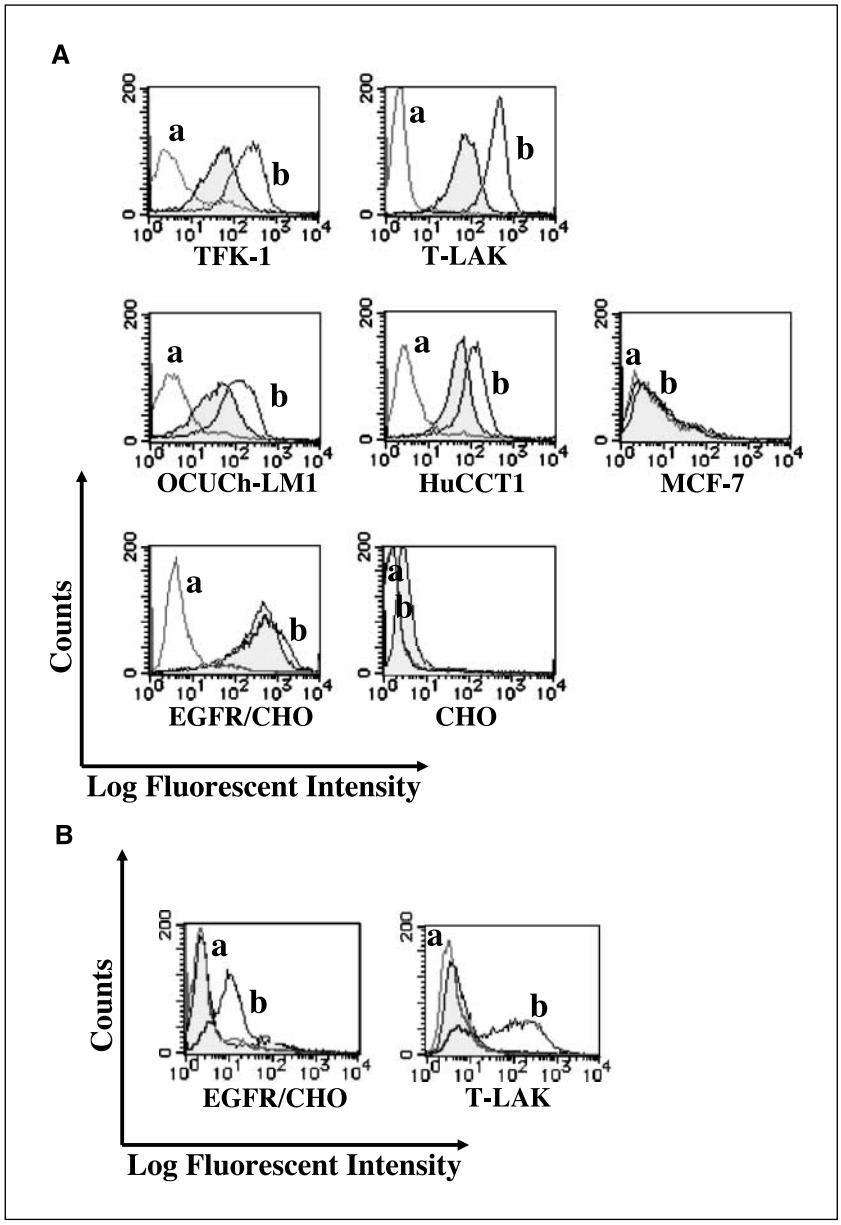

Fig. 3. *A,* reactivity of hEx3 with T-LAK cells and several cell lines. Cells incubated with PBS as a negative control (*a*) and with either OKT3 parental IgG for T-LAK cells or 528 IgG for the others followed by staining with FITC-conjugated anti-mouse IgG, as a positive control (*b*). Shaded areas, cells incubated with hEx3, then stained with FITC-conjugated anti-c-myc 9E10 antibody. *B,* blocking test by competing parental IgGs. Cells incubated with PBS as a negative control (*a*) and with hEx3 as a positive control (*b*). Shaded areas, cells pretreated with parental IgG (OKT3 for T-LAK and 528 for EGFR/CHO) first and reacted with hEx3 and then stained with FITC-conjugated anti-c-*myc* 9E10 antibody.

parental IgGs or 528 or OKT3 antibodies (Fig. 4C). These results indicate that cytotoxicity was induced in an EGFR- and CD3-specific manner, and that cross-linking of target and effector cells is more important than T-cell activation by the anti-CD3 agonist antibody or growth inhibition by the anti-EGFR antagonist antibody. We also confirmed the dual-specificity of hEx3 by absorption test according to previous report (data not shown; ref. 22).

Long-term and physiologic stability testing. The long-term stability of hEx3 was examined by re-chromatography using a gel filtration column. Two major elution peaks were found in the gel filtration chromatograph of hEx3 after refolding (Fig. 5A, *a*). The first large peak corresponds to the molecular weight of the hEx3 dimer, and the second small one corresponds to a monomer fraction consisting mainly of hOH5L, as confirmed by SDS-PAGE (data not shown). Storage for 2 weeks (Fig. 5A, *b*) or 6 months (Fig. 5A, *c*) did not induce conversion to the monomer or other protein degradation. Furthermore, hEx3 retained a heterodimeric structure after one freeze (at −20°C)/thaw (4°C) cycle in PBS (data not shown). Physiologic stability is also a critical factor for potential therapeutic reagents. hEx3 almost fully retained its cytotoxic activity after incubation with human plasma at 37°C (Fig. 5B), indicating that hEx3 has sufficient stability for use *in vivo*.

Tumor inhibition in nude mouse by hEx3. To determine the *in vivo* antitumor activity of hEx3, mice were inoculated with CoLo TC (human colon carcinoma) cells and then treated for 4 days with T-LAK plus interleukin-2 in the presence or absence of 5 μg of hEx3. hEx3 and Ex3 both prolonged survival, despite the transient, short-term treatment and low doses of recombinant antibody (Fig. 6). Ex3 and hEx3 treatment also clearly improved the physical condition of tumor-inoculated mice. Although Ex3- and hEx3-treated mice died within 19 and 29

weeks, respectively, weekly i.p. treatments could potentially extend these survival times. We also confirmed that the residual level of endotoxin derived from *E. coli* was suitable for *in vivo* use with the PYROGENT Gel Clot Assay (Cambrex, East Rutherford, NJ; data not shown).

Discussion

Recombinant BsAbs offer several advantages over classic BsAbs prepared by chemical cross-linking or fusion of two hybridoma clones. One BsAb format, the diabody, possesses

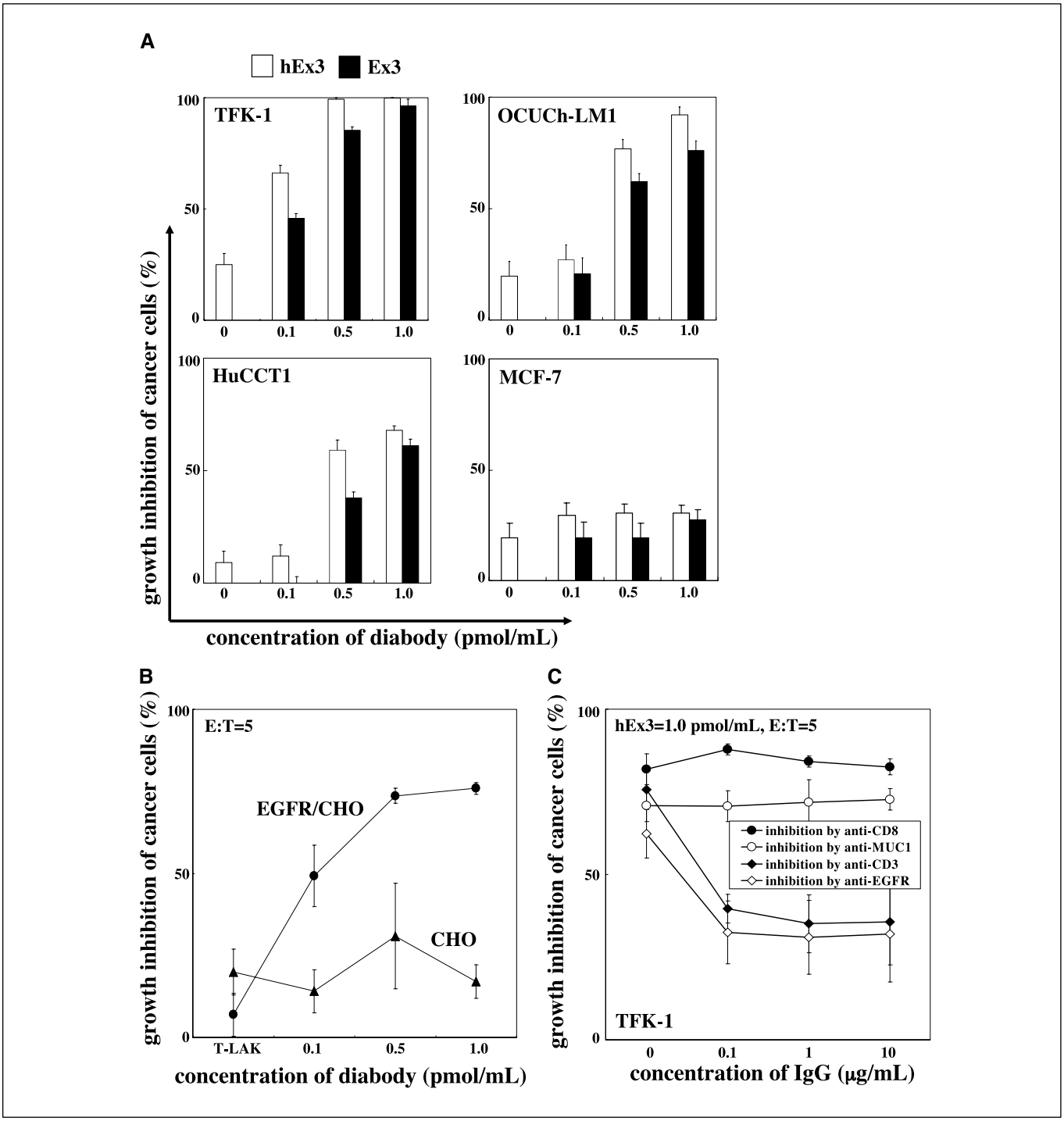

Fig. 4. *A,* percentage of growth inhibition was determined by a 48-hour 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt assay, in which Ex3, hEx3 diabody, and T-LAK cells (effectors) were added to the EGFR-positive cell lines, TFK-1, OCUCh-LM1, and HuCCT1 or the EGFR-negative line MCF-7. *B,* antigen-specific growth inhibition of hEx3 was determined in normal and EGFR-transfected CHO cells. *C,* inhibition of hEx3 activity by parental or nonspecific mAb IgGs. TFK-1 cells were cocultured with T-LAK cells in the presence of hEx3 (1.0 pmol/mL) in combination with parental IgG. The growth inhibition assay was done at an effector/target (*E:T*) ratio of 5:1. Points, mean from at least triplicate determinations.

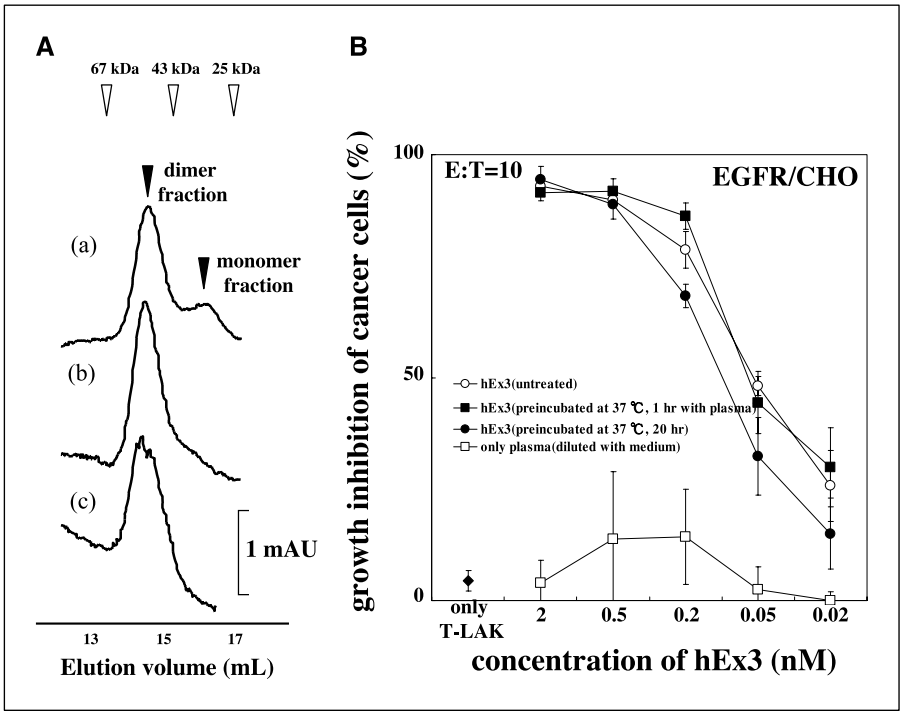

Fig. 5. *A,* gel filtration was done on a Hiload Superdex 200-pg column (16/300). *a,* chromatograph of hEx3 immediately after refolding. The dimer fraction was collected and subjected to re-chromatography under the same conditions after 2 weeks (*b*) or 6 months (*c*). *B, in vitro* stability of hEx3 was examined by growth inhibition assay for EGFR/CHO at an effector/target (*E:T*) ratio of 10:1. hEx3 was preincubated at 37°C for 20 hours (●) or at 37°C for 1 hour with human plasma (■), and the remaining activity was compared with untreated hEx3 (○). T-LAK cells only (♦) and human plasma diluted with medium (□) were used as controls.

advantages, such as humanization, enhancement of tumor penetration, reduction of immunogenicity due to the small molecular size compared with classic BsAbs, and simpler production at high yields using bacterial expression systems (8, 9).

To date, however, there is no example of a clinical trial with recombinant BsAbs and only a few examples of the *in vivo* effects using tumor-xenografted mice (9, 16, 40, 41). Two main reasons for this limited success are the relatively low yield (only a few mg/mL) and instability during long-term storage or under physiologic conditions (9). Physiologic instability results from the dissociation of noncovalently linked dimers into monomers. Coexpression with molecular chaperones to improve solubility and efforts to stabilize dimer formation through mutations or artificial linkers have not led to dramatic improvements (42, 43). Additionally, administration with costimulatory molecules, such as the B7 family or anti-CD28 agonistic mAb, was required to successfully treat tumor-xenografted mice with a bispecific diabody (44).

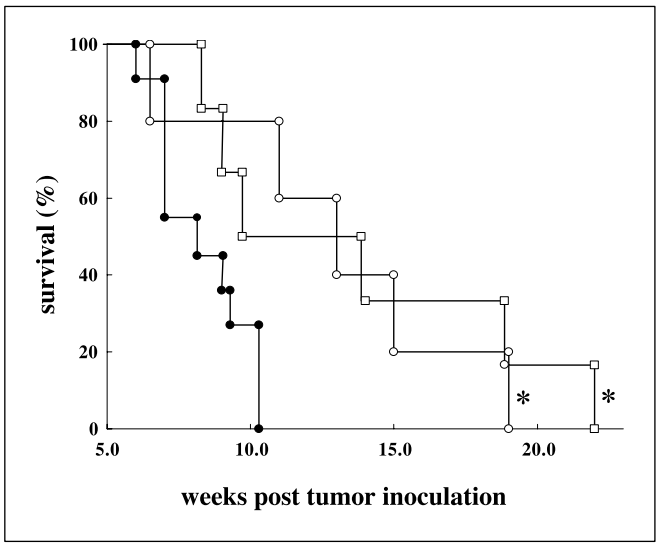

Fig. 6. Effects of Ex3 and hEx3 on the growth of xenografted human colon carcinoma in nude mice. Each mouse was given 5×10^6 CoLoTC cells i.p. and was treated 96 hours later with i.p. injection of 2×10^7 T-LAK cells plus 500 IU IL-2 (●, $n = 11$), with 5 μg of Ex3 (○, $n = 5$) or 5 μg of hEx3 (□, $n = 6$) for four consecutive days. Statistical analysis using the log-rank test showed that survival is significantly prolonged in mice coinjected with Ex3 and hEx3 compared with mice injected with only T-LAK (*, $P < 0.01$), and differences were not observed in between injected with Ex3 and hEx3.

Recently, we have reported the preparation of a functional diabody targeting EGFR and CD3, termed Ex3, from *E.coli* intracellular insoluble using an *in vitro* refolding system. Ex3 was stable and retained binding reactivity after incubation at 37°C for 48 hours. Furthermore, administration of Ex3 and T-LAK cells, without other costimulatory factors, to tumor-xenografted mice resulted in pronounced inhibition of tumor growth, with complete tumor disappearance in half of the mice (22).

In this report, we humanized the Ex3 to attempt to lower the immunogenicity for clinical use. The humanization was done by the CDR-grafting method, with whole synthesized and *E. coli* codon-optimized genes to improve yield. hEx3 had identical biological properties to those of Ex3 (i.e., antigen-specific binding activity and growth inhibition), despite a 40-fold lower binding affinity. The hEx3 dimer fraction slightly increased after humanization (data not shown), and the fractionated dimer showed long-term stability. Incubation under physiologic conditions did not change hEx3 function. The weak interaction of the Fv region of OKT3, the opposite portion of Ex3, has already been reported (45); therefore, the high stability of hEx3 putatively results from the strong interaction between the VH and VL of the 528 cell line.

It is very difficult to estimate the percentage of diabody retaining full function in refolded solution, especially when

it contains monomers, dimers, and other forms, such as multimers. However, it is at least expected that dimer fraction of refolded diabody has almost full function because it showed identical growth inhibition effect to that of soluble diabody prepared using mammalian expression system (data not shown). Therefore, comparable functional diabody using the refolding system may allow for industrial-scale diabody production.

Ex3 or hEx3 coadministration with T-LAK cells improved survival and the physical condition of xenografted mice despite transient administration, a relatively low dose (total 20 μg), and no other costimulatory factors. To the best of our knowledge, this report is the first to show *in vivo* antitumor activity of a fully humanized diabody prepared by refolding.

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