

Construction of a diabody (small recombinant bispecific antibody) using a refolding system

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Diabodies are the recombinant bispecific antibodies (BsAbs), constructed from heterogeneous single-chain antibodies. Usually, diabodies have been prepared from bacterial periplasmic fraction using a co-expression vector (i.e. genes encoding two chains were tandemly located under the same promoter). Some diabodies, however, cannot be expressed as a soluble material owing to inclusion body formation, which limits the utilization of diabodies in various fields. Here we report an improved method for the construction of diabodies using a refolding system. As a model, a bispecific diabody binding to adenocarcinoma-associated antigen MUC1 and to CD3 on T cells was studied. One chain consisted of a VH specific for MUC1 linked to a VL specific for CD3 with a short polypeptide linker (GGGGS). The second was composed of a VL specific for MUC1 linked to a VH specific for CD3. The two hetero scFvs were independently obtained from intracellular insoluble fractions of *Escherichia coli*, purified, mixed stoichiometrically (at an equivalent molar ratio of 1:1) and refolded. The refolded two hetero scFv has a hetero-dimeric structure, with complete specificity for both target cells [i.e. MUC1 positive cells and CD3 positive lymphokine-activated killer cells with a T cell phenotype (T-LAK)]. Evaluation of the *in vitro* efficacy of T-LAK with the diabody by growth inhibition assay of cancer cells demonstrated maximum growth inhibition of cancer cells to reach ~98% at an effector:target ratio (E:T ratio) of 10, almost identical with that with anti-MUC1×anti-CD3 chemically synthesized BsAbs (c-BsAbs). This is the first report of the construction of a diabody using a refolding system.

Keywords: bispecific antibody/diabody/diabody-T-LAK cell/MUC1/refolding system

Abbreviations: BsAb, bispecific antibody; scFv, single-chain Fv antibody; T-LAK, lymphokine activated killer with T cell phenotype; c-BsAb, chemically synthesized BsAb; BDC, bile duct carcinoma; SCID, severe combined immunodeficient; SEA, *Staphylococcus enterotoxin A*; HAMA, human anti-mouse antibody; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; BS, bacterial supernatant; PP, periplasmic; ICS, intracellular soluble; ICIS, intracellular insoluble.

Introduction

Recent advances in genetic engineering have made it possible to prepare fragments of immunoglobulins consisting of variable domains linking with polypeptide linkers [single-chain antibodies (scFvs) (Bird *et al.*, 1988)]. With regard to recombinant BsAb, diabodies or dimeric antibody fragments consisting of two non-covalently associated single-chain antibodies have attracted particular attention. In order to form two binding sites, the variable domains of two antibodies (A and B) are arranged as VH_A-VL_B (VH domain of antibody A and VL domain of antibody B) on one chain and VH_B-VL_A (VH domain of antibody B and VL domain of antibody A) on the other (Holliger *et al.*, 1993). Diabodies are one of the smallest recombinant BsAbs and the distance between the two antigen-binding sites is only 6.5 nm on average, which is less than half the distance in IgG (Perisic *et al.*, 1994). This compact size would be expected to contribute to rapid pharmacokinetics, low immunogenicity and high tumor penetration (Wu *et al.*, 1996).

Usually, diabodies have been prepared from bacterial periplasmic fraction (Holliger *et al.*, 1993, 1999; Helfrich *et al.*, 1998; Kipriyanov *et al.*, 1998). Some diabodies, however, cannot be expressed as a soluble material and therefore the utilization of diabodies in various fields is limited. Here we report a method for the construction of diabodies using a refolding system. As a model, construction of a diabody reactive with both the adenocarcinoma-associated antigen MUC1 and the CD3 molecule on T cells (Mx3 diabody) was studied. The target antigen in this work, MUC1, is overexpressed in various epithelial carcinomas, such as those arising in the pancreas, stomach, colon and ovarian and bile ducts (Ban *et al.*, 1989; Ioannides *et al.*, 1993; Yamashita *et al.*, 1993). In our previous study of human bile duct carcinoma (BDC)-grafted SCID mice, specific targeting therapy consisting of i.v. administration of T-LAK cells sensitized with c-BsAbs, i.e., anti-MUC1×anti-CD3 and anti-MUC1×anti-CD28, demonstrated remarkable inhibition of tumor growth (Katayose *et al.*, 1996). Preparation of the chemically synthesized BsAb (c-BsAb), however, is time consuming and laborious with low yield and therefore an alternative method was sought.

The method reported here can be summarized as follows: mixing stoichiometrically two hetero scFvs that expressed in *Escherichia coli* separately and purified from the intracellular insoluble fraction, followed by guanidine phase-removing dialysis and addition of L-arginine and oxidized glutathione to the refolding solution in the early folding stage. The diabody constructed could be recovered with high yield and sufficient activity for both target antigens. In addition, this Mx3 diabody was able to retarget T-LAK cells to MUC1-positive cancer cells with efficiency and specificity for MUC1 antigen in growth inhibition assays of cancer cells. The method reported here would have potential in widening the scope of diabody utilization in various fields.

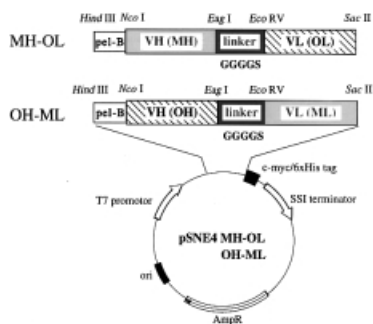


Fig. 1. Schematic representation of two kinds of hetero scFv expression vectors. VH and VL region genes of MUSE11 designated as MH and ML and VH and VL region genes of OKT3 as OH and OL, respectively. AmpR denotes the ampicillin resistance gene; c-myc, a sequence encoding an epitope recognized by 9E10 mAb; ori, origin of DNA replication; 6× His, a sequence encoding six C-terminal histidine residues; pelB, signal peptide sequence of bacterial pectate lyase.

Materials and methods

Monoclonal antibodies (mAbs) and hybridoma cell lines

For the construction of the diabody, MUSE11 and OKT3 hybridoma cell lines were used as the sources of V region genes. The MUSE11 mAb (produced by Dr Hinoda, Sapporo Medical University, Sapporo, Japan) (Hinoda *et al.*, 1993) was a mouse IgG₁ directed to epithelial mucin MUC1 antigen. OKT3 mAb was a mouse IgG_{2a} directed to the human CD3ε-chain (Van Wauwe *et al.*, 1980; Salmeron *et al.*, 1991).

Cloning and construction of two kinds of hetero scFv expression vectors, MH-OL and OH-ML

Primers for the preparation of VH and VL genes of MUSE11 and OKT3 mAb were designed according to previous reports (Hinoda *et al.*, 1993; Arakawa *et al.*, 1996; Asano *et al.*, 2000). Total RNA was isolated from hybridoma cell lines using Dynabeads oligo(dT)₂₅ (Dyna, Oslo, Norway) and first strand cDNA was prepared using a first strand cDNA synthesis kit (Life Sciences, St. Petersburg, FL). VH and VL regions were amplified separately by PCR amplification. For their assembly, VH and VL domains of HyHEL-10, a mAb raised against hen egg white lysozyme which contains the pSNE4 vector (Tsumoto *et al.*, 1997) with a GlyGlyGlyGlySer short linker, were replaced by those of MUSE11 or OKT3. VH and VL region genes of MUSE11 were designated MH and ML and VH and VL region genes of OKT3 as OH and OL, respectively. One hetero scFv were replaced with OH and ML and the other with MH and OL. Thus the hetero scFv genes, OH-ML and MH-OL, were constructed as shown in Figure 1.

In order to enhance the stability of the VH region of OKT3, the cysteine residue in position 100A of the VH domain was replaced with serine (Kipriyanov *et al.*, 1997) by Kunkel's method (Kunkel, 1985).

Expression of OH-ML and MH-OL

The *E. coli* strain BL21 (DE3) transformed with pSNE4 MH-OL or OH-ML was grown at 28°C in 2× YT broth supplemented with 100 µg/ml ampicillin to the early stationary phase. In order to induce hetero scFv production, isopropyl-1-thio-β-D-galactopyranoside (IPTG) (1 mM) was added to the culture and the cells were grown overnight at 28°C. From 200 ml of culture, four fractions, namely bacterial supernatant (BS), periplasmic (PP), intracellular soluble (ICS) and intracellular insoluble (ICIS) fractions, were obtained. The BS fraction was obtained from the culture medium by centrifugation (2000 g,

35 min). The cell pellet was resuspended in 10 ml of 20 mM Tris-HCl (pH 7.5), 0.5 M sucrose, 0.1 mM EDTA, kept at room temperature for 5 min and 40 ml of cold water were added in order to induce osmotic shock. It was kept on ice for 30 min and the cell suspension was then centrifuged at 2000 g for 35 min at 4°C. The PP fraction was obtained from the supernatant. The remaining cells were resuspended in 10 ml of PBS, ultrasonicated at 200 W for 15 min and centrifuged at 4500 g for 20 min. The ICS fraction was recovered from the supernatant. The ICIS fraction was obtained by solubilizing the sonicated cell pellet with 10 ml of 6 M GuHCl-PBS and kept overnight at 4°C.

SDS-PAGE and Western blotting

Aliquots of 1 ml of culture supernatant served for analysis. The total proteins in each fraction precipitated with 6% trichloroacetic acid (TCA) and 0.083% deoxycholate were subjected to SDS-PAGE as reported previously (Laemmli, 1970), under reducing conditions, and proteins were stained with Coomassie Brilliant Blue R-250. Next, proteins in the gel were blotted to nitrocellulose membranes (Amersham, Little Chalfont, UK) and treated with blocking buffer consisting of PBS, 0.05% Tween 20 and 4% skim milk (Difco, Detroit, MI) at room temperature for 1 h. Thereafter, they were incubated with peroxidase-conjugated anti-His tag mAb (Santa Cruz Biotechnology, Santa Cruz, CA) followed by signal enhancement using the ECL detection system (Amersham).

Purification and refolding of diabody

From the ICIS fraction that contained the majority of OH-ML and MH-OL, purification was carried out separately. After solubilization with 6 M GuHCl-PBS, the proteins were applied to a 2 ml TALON metal affinity resin column (Clontech, Palo Alto, CA), followed by extensive washing with PBS, 6 M GuHCl and 1 mM imidazole. Thereafter, OH-ML and MH-OL were eluted with three buffer solutions made of PBS, 6 M GuHCl and imidazole. The concentration of imidazole in the elution buffer was sequentially increased from 1 to 10 and to 500 mM. OH-ML and MH-OL eluted from the metal affinity resin were diluted with 6 M GuHCl-PBS to a concentration of 0.1 mg/ml (4 µM) prior to refolding.

The dialysis method was adopted for stoichiometric association of two chains. Optimization of refolding conditions using dialysis in principle followed Tsumoto *et al.* (1998). Two chains were mixed stoichiometrically and refolded by guanidine-removal dialysis with GuHCl-PBS with addition of L-arginine (Arg) (Tsumoto *et al.*, 1998). In brief, the denaturant of the mixture of OH-ML and MH-OL (20 ml) was removed by guanidine-removal dialysis with GuHCl-PBS (1000 ml, pH 7.9, adjusted with NaOH). The dialysis buffers (pH 7.9) used for refolding were as follows: (1) PBS containing 0.4 M Arg; (2) PBS containing 0.4 M Arg and 200 mM oxidizing reagent (glutathione, oxidized form; Sigma); (3) PBS containing sequentially decreased GuHCl (3, 2, 1, 0.5 and 0 M) with 0.4 M of Arg added during the final dialysis stage (i.e. 0.5 and 0 M GuHCl); (4) PBS containing sequentially decreased GuHCl (3, 2, 1, 0.5 and 0 M) and oxidizing reagent (glutathione, oxidized form; Sigma) was introduced in 1 and 0.5 M GuHCl-PBS dialysis buffer (pH 7.9) and 0.4 M of Arg was added during the final dialysis stage (i.e. 0.5 and 0 M GuHCl). The refolding solution was finally dialyzed against PBS (pH 7.9) overnight, centrifuged at 4500 g for 20 min at 4°C and the supernatant was filtered with a 0.22 µm ultrafiltration membrane (Millipore, Tokyo, Japan). The ratio of the soluble

proteins remaining in the supernatant to the denatured ones subjected to refolding was determined spectrophotometrically, i.e. division of OD₂₈₀ after refolding by OD₂₈₀ before refolding.

Gel-filtration chromatography

Gel-filtration chromatography was performed using a Superdex 75 column (Amersham-Pharmacia) (1.0×100 cm) pre-equalized with PBS, pH 7.9. Molecular masses were calibrated with calibration proteins for the gel chromatography combitekh (Boehringer Mannheim, Germany). A 1 ml volume of sample (0.2 mg/ml) was loaded at a flow-rate of 0.5 ml/min and 2.0 ml fractions were collected.

Cell lines

The human BDC cell line (TFK-1) (Saijyo *et al.*, 1995), reactive with MUSE11 mAb, was used as a target and a human hepatocellular carcinoma cell line (HT-17) as a control. MUC1 overexpressed CHO cells were prepared as described previously (Asano *et al.*, 2000). These cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (designated the culture medium below).

Preparation and stimulation of T-LAK cells

T-LAK cells were prepared from peripheral blood mononuclear cells by stimulation with solid-phase OKT3 mAb (10 µg/ml) and IL-2 (100 IU/ml), as reported previously (Katayose *et al.*, 1996).

Flow cytometric analysis

Cells (5×10⁵) were first incubated on ice with 100 µl of diabody (100 µg/ml) for 30 min as the first Ab. After washing with PBS–0.1% NaN₃, they were exposed to 9E10 anti-c-myc mAb (Santa Cruz Biotechnology) as the second and then FITC-conjugated anti-mouse IgG as the third Ab for 30 min on ice. The stained cells were analyzed by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA).

Absorption test

For the absorption test, 200 µl of diabody (100 µg/ml) were incubated with 5×10⁶ TFK-1 or T-LAK cells on ice for 30 min. After centrifugation, 200 µl of supernatant were collected and half of each was added to TFK-1 and T-LAK cells (5×10⁵ cells) for detection of residual antibody activity. After incubation on ice for 30 min, the cells were exposed to the second (9E10 mAb) and the third Ab (FITC-conjugated anti-mouse IgG). Flow cytometric analysis was performed as described in the previous section.

Growth inhibition assay of cancer cells

Growth inhibition assays of cancer cells were performed with an MTS assay kit (CellTiter 96TM aqueous non-radioactive cell proliferation assay; Promega, Madison, WI) as described previously (Shinoda *et al.*, 1998; Sakurai *et al.*, 1999). Briefly, 10 000 target cells in 100 µl of culture medium were distributed to 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 plate. After removing the culture medium, 100 µl of T-LAK cells preincubated with various concentrations of diabody at 4°C for 30 min were distributed into each well. After culture for 48 h at 37°C, each well was washed with PBS three times. This was followed by addition of MTS–phenazine methosulfate solution (Promega) diluted with culture medium. The plates were read on a microplate reader (Bio-Rad Model 3550) at 490 nm after incubation for 1 h at 37°C. The percentage growth inhibition of cancer cells was calculated

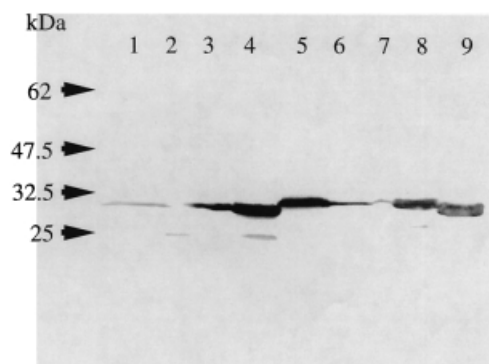


Fig. 2. Western blotting analysis of MH-OL and OH-ML. Molecular size markers (kDa) are shown on the left. Experimental procedures were as described in Materials and methods. Lanes 1–4 show four fractions for MH-OL and lanes 5–8 show those for OH-ML. A 1 ml volume of culture supernatant served for analysis. Lanes: 1 and 5, proteins in the BS fraction; 2 and 6, proteins in the PP fraction; 3 and 7, proteins in the ICS fraction; 4 and 8, proteins in the ICIS fraction; 9, HyHEL-10 scFv (scFv reactive with hen egg white lysozyme, positive control for anti-His tag mAb).

as follows: percentage growth inhibition of cancer cells = $[1 - (A_{490} \text{ of experiment} - A_{490} \text{ of background}) / (A_{490} \text{ of control} - A_{490} \text{ of background})] \times 100$.

Results

Expression of the Mx3 diabody in *E. coli*

The MH-OL and OH-ML hetero scFvs were separately produced using *E. coli* strain BL21(DE3) harboring the plasmid pSNE4 MH-OL or pSNE4 OH-ML. To estimate the amount of expressed hetero-scFvs in the four fractions, Western blotting analysis was performed (Figure 2). Most MH-OL and OH-ML and especially MH-OL, was found in the intracellular fraction and only small amounts of gene products were secreted into the periplasmic and bacterial culture supernatant fractions. Considerable amounts of OH-ML could be obtained by ammonium sulfate precipitation from PP or BS. On the other hand, preparation of MH-OL-soluble proteins was difficult from PP or BS (data not shown).

Purification and refolding of diabody

To obtain diabody from insoluble fractions of OH-ML and MH-OL, we optimized the refolding system as reported by Tsumoto *et al.* (1998). First, immobilized metal affinity chromatography under denatured conditions was carried out to purify OH-ML and MH-OL. The proteins eluted from the affinity column were diluted with 6 M GuHCl–PBS to 0.1 mg/ml (4 µM). The diluted OH-ML and MH-OL were mixed stoichiometrically before refolding, followed by dialysis against the buffer. Without Arg, significant aggregation of proteins (~50% of total proteins) was observed in the final dialysis step, indicating that the appropriate addition of Arg is critical for avoiding aggregation. More than 0.2 M Arg was effective (data not shown). Sequential removal of GuHCl from the refolding solution increased the recovery of the proteins and introduction of an oxidizing reagent (glutathione, oxidized form) in 1 and 0.5 M GuHCl–PBS dialysis buffer (pH 7.9) minimizes the aggregation of the proteins. Little aggregation of proteins was observed in the course of refolding and more than 95% of the proteins were recovered in the supernatant (the soluble fraction) under optimized refolding conditions.

Determination of molecular weight of diabody

Determination of the molecular weight of the diabody by gel-filtration chromatography revealed a major peak at a retention

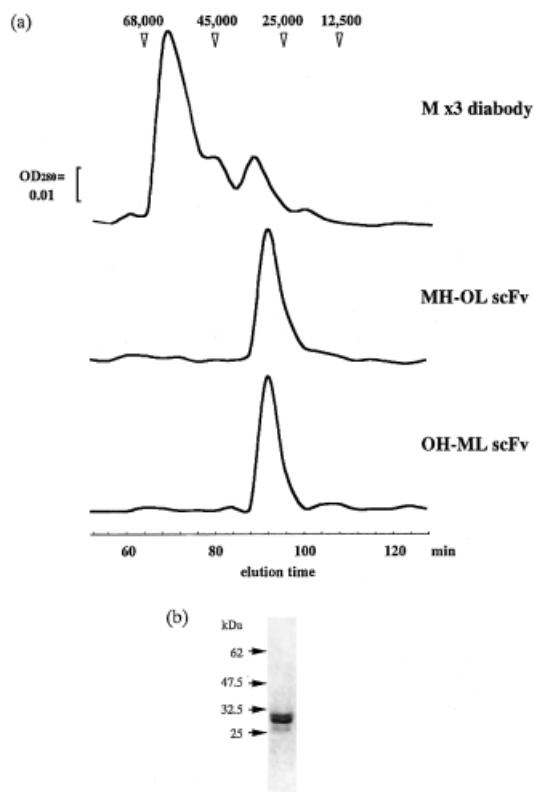


Fig. 3. Gel filtration of refolded Mx3 diabody and each chain. (a) 1 ml of refolded proteins adjusted to 0.2mg/ml was loaded at a flow-rate of 0.5 ml/min. Under this condition, the retention time of the standards is shown under the horizontal line. (b) SDS-PAGE of the dimer fraction after gel filtration. Molecular size markers (kDa) are shown on the left. The upper band is the OH-ML (MW 29 800) and the lower band the MH-OL (MW 29 300).

time between 45 and 68 kDa compared with calibration proteins (Figure 3). SDS-PAGE of a sample taken from this peak showed equal quantities of the two bands (Figure 3). Under reducing conditions, separation into two hetero scFvs occurred and two bands, MH-OL (MW 29 300) and OH-ML (MW 29 800) could be seen, indicating that this peak contained MH-OL and OH-ML. These results strongly suggest that refolded hetero antibody fragments associate with each other stoichiometrically, forming a diabody. On the other hand, refolded OH-ML scFv or MH-OL scFv formed monomer, not dimer, when refolded alone (Figure 3). In addition, formation of hetero-dimer was not observed when each chain refolded separately was mixed (data not shown). These results indicate that the stoichiometric mixing of each chain before refolding is critical for construction of a diabody. Final yields of Mx3 diabody were about 6 mg from each 1 l of culture of OH-ML and MH-OL.

Flow cytometric analysis

Binding of refolded diabody to cell lines was examined by flow cytometry (Figures 4 and 5). Strong reactivities were observed with MUC1-positive TFK-1 cells (Fig. 4) and MUC1 overexpressed CHO cells (Fig. 5), the specificity of which was almost identical with that of the parental IgG (MUSE11). It was observed that the diabody did not react with MUC1-negative HT17 cells (data not shown). It also reacted well with T-LAK cells (CD3 positive) (Figure 4), with a specificity slightly weaker than that of the parental IgG (OKT3). The

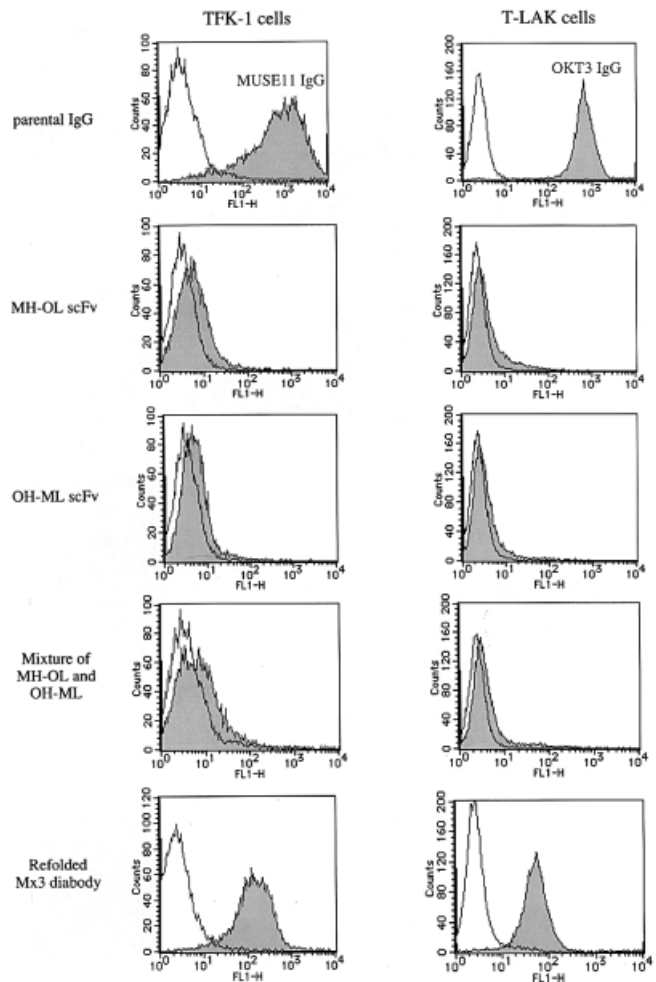


Fig. 4. Flow cytometry profiles of Mx3 diabody tested for TFK-1 and T-LAK cells. Open histograms, cells incubated with PBS as negative control; filled histograms, cells incubated with parental IgGs, MH-OL, OH-ML, mixture of MH-OL with OH-ML after refolding each chain and refolded Mx3 diabody. When these (10 μ g/ml) were used as the first Abs, mouse anti-c-myc 9E10 Ab was the second Ab and finally stained with FITC-conjugated goat anti-mouse IgG.

results for reactivity are in line with the affinity of scFv or diabody usually being lower than that of parental IgG (Kipriyanov *et al.*, 1997), owing to there being one binding site per molecule (Figure 4). However, the diabody retains sufficient MUC1 specificity for targeting, judging from the results of growth inhibition assays with MUC1-positive and -negative cell lines (Figures 6 and 7).

It was found that MH-OL alone, OH-ML alone and a mixture of each chain after refolding did not react with either TFK-1 or T-LAK cells (Figure 4), indicating that refolded diabody showed bispecificity for the targets.

Absorption test

In order to confirm the bivalency of the diabody, i.e. whether every molecule refolded has two binding sites, an absorption test was performed. If the refolded diabody does not have two binding sites, some molecules specific for one target antigen cannot react with another target. After reaction with either 5×10^6 TFK-1 or T-LAK cells at 4°C, the supernatant did not contain any diabody (Table I), indicating that one molecule reacted with both TFK-1 and T-LAK cells. This result suggests that each molecule has both binding sites of two antibody molecules.

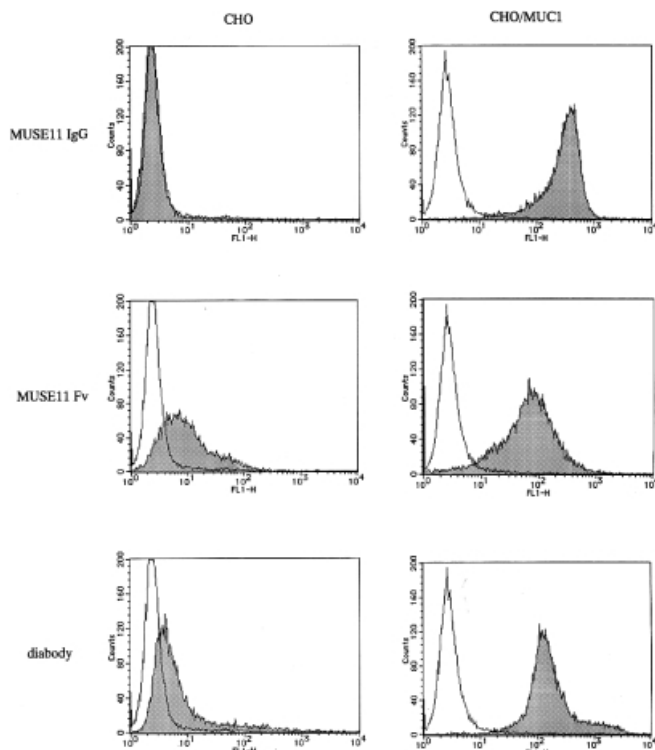


Fig. 5. Flow cytometry profiles of Mx3 diabody tested for CHO and MUC1-overexpressed CHO cells. Open histograms, cells incubated with PBS as negative control; filled histograms, cells incubated with MUSE11 IgG, Fv and refolded Mx3 diabody. When these (10 μ g/ml) were used as the first Abs, mouse anti-c-myc 9E10 Ab was the second Ab and finally stained with FITC-conjugated goat anti-mouse IgG.

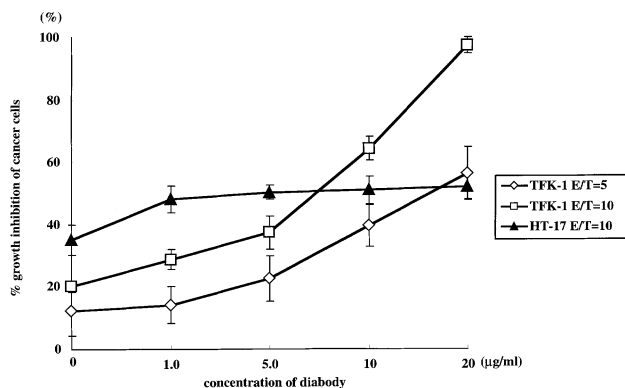


Fig. 6. Growth inhibition assay using T-LAK cells preincubated with various concentrations of diabody Mx3. After preincubation of T-LAK cells with diabody Mx3 at 4°C for 30 min, the mixture was added to TFK-1 (\diamond , results with an E:T ratio of 5:1; \square , results with an E:T ratio of 10:1) or HT-17 (\blacktriangle , results with an E:T ratio of 10:1) target cells as effector cells and co-cultured for 48 h at 37°C. The percentage growth inhibition of cancer cells was determined by 48 h MTS assay. Data are from triplicate determinations.

Growth inhibition of cancer cells with T-LAK cells preincubated with diabody attachment of T-LAK cells mediated by Mx3 diabody

In order for this diabody to inhibit the growth of cancer cells (TFK-1) with T-LAK cells, various concentrations of Mx3 diabody preincubated with T-LAK cells were mixed with target TFK-1 cells. Depending on the concentration of diabody, the percentage growth inhibition of cancer cells increased and reached about 98% with an E:T ratio of 10:1 when the target

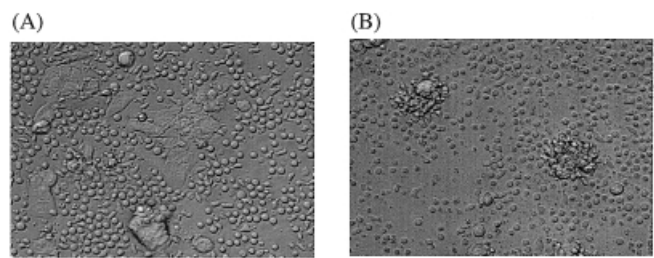


Fig. 7. Phase-contrast micrographs of cells in the growth inhibition assay of TFK-1 cells. TFK-1 cells were co-cultured for 6 h at 37°C with T-LAK cells, which had been preincubated with (A) or without (B) Mx3 diabody (20 μ g/ml, E:T = 10:1).

Table I. Absorption test of Mx3 diabody^a

First Ab	Test cells	
	TFK-1	T-LAK
Negative control	6.15% (1)	1.03% (1)
Diabody	94.73% (51.5)	98.45% (22.2)
Supernatant after absorption with TFK-1	5.41% (0.98)	0.59% (0.94)
Supernatant after absorption with T-LAK	6.12% (1.04)	0.63% (0.93)

^aFigures in the table are percentages of positively stained cells. Those in parentheses are relative fluorescence intensities, calculated as the ratios of mean fluorescence intensities of test cells to negative controls. Data are from triplicate determinations.

cells were TFK-1 (Figure 6). With T-LAK cells alone as a control, 20% growth inhibition was obtained. To determine whether growth inhibition of cancer cells by diabody is due to MUC-1, HT-17 cells (MUC1 negative) (Katayose *et al.*, 1996) were used as target cells for this growth inhibition assay (Figure 6). Mx3 diabody enhanced growth inhibition dose-dependently against MUC1-positive TFK-1 cells, but not against MUC1-negative HT-17 cells.

TFK-1 and T-LAK cells were observed during the growth inhibition assay using a phase-contrast microscope (Figure 7). When diabody and T-LAK cells were added to TFK-1 cells and co-cultured, they started to surround the target cells after about 2 h. TFK-1 cells surrounded and attacked by diabody-mediated T-LAK cells began to detach from the bottom of the wells in the course of the cultivation. This phenomenon was not observed in the absence of the Mx3 diabody.

These results indicate that the diabody bridges the T-LAK cells with cancer cells (TFK-1), leading to growth inhibition of the cancer cells.

Discussion

In previous studies, diabodies were obtained from the periplasmic fraction or bacterial supernatant as soluble proteins using co-expression vector. Although a co-expression vector of Mx3 diabody has also been constructed, diabody could not be obtained from the bacterial supernatant (data not shown). This is because almost all of the hetero scFv, especially MH-OL, expressed in *E. coli* accumulates in the intracellular insoluble fractions (Figure 2), requiring the refolding system.

Usually, a dilution method is used for refolding insoluble proteins (Rudolph and Lilie, 1996; Misawa and Kumagai, 1999). However, refolding at low protein concentration did not lead to stable hetero-dimer (i.e. diabody) formation (data not shown), although each chain was refolded and concentrations of

the polypeptide above the micromolar level were required for efficient dimer formation. Therefore, in this work, the dialysis method was used for construction of the diabody by refolding.

Using the refolding system reported here, we succeeded in obtaining a bispecific diabody. The critical points for an increase in the refolding efficiency are as follows: (1) appropriate addition of Arg, >0.2 M Arg being effective (data not shown); (2) sequential removal of GuHCl from the refolding solution; and (3) introduction of oxidizing reagent in 1 and 0.5 M GuHCl–PBS dialysis buffer. Aggregation of proteins could be almost completely avoided under these conditions.

Gel filtration of this refolded diabody demonstrated a peak at about 60 kDa (Figure 3) and SDS–PAGE of a sample taken from this peak showed the same amounts of MH-OL and OH-ML under reducing conditions (Figure 3). This peak did not contain the homodimer of OH-ML scFv or MH-OL scFv, because OH-ML scFv alone or MH-OL scFv alone never formed a dimer when refolded alone (Figure 3). These results suggest that MH-OL and OH-ML in the diabody are non-covalently associated. It was found that MH-OL alone, OH-ML alone and a mixture of each chain after refolding did not form a dimer, suggesting that the non-covalent hetero-dimer can be formed when each chain is mixed before refolding.

The refolded diabody showed bispecificity for the targets from flow cytometric analyses (Figures 4 and 5) and bivalency of the diabody was demonstrated by an absorption test (Table I) and growth inhibition assay of cancer cells (Figures 6 and 7). It was found that MH-OL alone, OH-ML alone and a mixture of each chain after refolding did not react with either TFK-1 or T-LAK cells (Figure 4), indicating that a bifunctional heterodimer can be formed under the conditions reported here.

In the field of immunotherapy, bispecific antibodies (BsAbs) have been used for redirecting T cells toward tumor cells by cross-linking the cell-surface antigen on tumor cells with the CD3–TCR complex on killer T cells. However, sufficient BsAb production by chemical conjugation (c-BsAb) or the quadroma method (Bohlen *et al.*, 1993) is very time-consuming and laborious so an alternative method for BsAb production was desirable. Furthermore, since BsAbs have inevitable problems with human anti-mouse antibody (HAMA) reactions (Mountain and Adair, 1992), downsizing and/or humanizing of BsAbs are necessary (von Mehren and Weiner, 1996). Thanks to the development of recombinant DNA technology, however, various recombinant BsAbs based on scFv are now possible such as diabody (Holliger *et al.*, 1993), bispecific (scFv)₂ (Gruber *et al.*, 1994) and (scFv)₂ conjugated by leucine zipper (de Kruif and Logtenberg, 1996). The advantages of diabodies as compact agents (Perisic *et al.*, 1994) with rapid pharmacokinetics and good penetration to tumor tissues (Wu *et al.*, 1996) underlie the numerous examples of biologically functional bispecific diabodies potentially suitable for T-cell retargeted tumor therapy of human carcinomas or lymphomas in the literature (Holliger *et al.*, 1996, 1999; Zhu *et al.*, 1996; Helfrich *et al.*, 1998; Kipriyanov *et al.*, 1998; Arndt *et al.*, 1999). The refolding system reported here would broaden the scope of recombinant antibody fragments.

In conclusion, we have documented the successful construction of a recombinant bispecific diabody reactive with the MUC1 molecule and CD3, employing a refolding system. The Mx3 diabody-mediated T-LAK cells showed growth inhibition for TFK-1. The system reported here would broaden the scope of recombinant antibody fragments in various fields.

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