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Functional Fv fragment of an antibody specific for CD28: Fv-mediated co-stimulation of T cells

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Abstract The most predominant co-stimulation pathway, which is critical for T cell activation and proliferation, is the CD28-B7 pathway. The anti-CD28 monoclonal antibody (mAb) also provides a co-stimulatory signal to T cells. In order to construct a functional Fv fragment (complex of VH and VL domains) of anti-CD28 antibody using a bacterial expression system, cDNA encoding the variable regions of immunoglobulin from 15E8 hybridoma cells was cloned and expressed in *Escherichia coli*. The Fv fragment was obtained as a soluble protein from the periplasmic fraction and showed a binding pattern similar to parental IgG. The Fv fragment induced proliferation of peripheral blood mononuclear cells in the presence of anti-CD3 or anti-CD2 mAb and enhanced anti-tumor activity of anti-MUC1 × anti-CD3 bispecific antibody when tested with lymphokine-activated killer cells with T cell phenotype. Thus, the anti-CD28 Fv fragment will be promising not only for the study of co-stimulation, but also for cancer immunotherapy.

Key words: CD28-B7; Co-stimulation; Fv; Bacterial expression; Immunotherapy

1. Introduction

Activation of T cells initially occurs by the engagement of the T cell receptor (TCR)/CD3 with an antigen/major histocompatibility complex complex. However, this first signal alone cannot induce complete T cell activation, and the secondary signaling pathways are important for T cell activation and prevention of T cell anergy. The most universally characterized co-stimulatory molecule is CD28, whose ligands are the B7-1 (CD80) and B7-2 (CD86) molecules on the antigen presenting cells [1–3]. B7-CD28 co-stimulation in the presence of TCR/CD3 signal results in the promotion of cell cycle progression, an increase in cytokine production [4–6], up-regulation of interleukin (IL)-2 receptors [7–9] and T cell proliferation [3,10]. This co-stimulatory signal is also delivered by interaction between CD28 and anti-CD28 monoclonal antibody (mAb). Administration of anti-CD28 antibody in vivo resulted in the induction of CD40 ligand expression on T cells and also subsequent expansion of B cells [11]. Thus, CD28 signaling has a great influence on the systemic immune system, and protein engineering of anti-CD28 antibody would be an important step for further characterization of the CD28-B7 co-stimulatory pathway and anti-CD28 antibody-mediated biotherapy.

Recent protein engineering technology has made the development possible of recombinant antibodies suitable for diagnostics, therapy and industrial applications such as protein purification or catalysis. Because of its smaller molecular size, utilization of variable fragments of antibodies, i.e. Fv (complex of VH and VL domains) or single chain Fv [12], would be attractive. The small molecular size enables antibody fragments to exhibit unique characteristics, including application as immunodiagnostic and immunotherapeutic reagents, due to better penetration into solid tumor tissues [13], lower immunogenicity [14] and improved pharmacokinetics. Furthermore, these recombinant antibody fragments are easily produced in large quantities and at low-cost by an *Escherichia coli* expression system, and functional investigation can be performed by site-directed mutagenesis of the Fv fragment. Here, we report the first successful construction of a bacterial expression system for anti-CD28 Fv fragment and describe the fragment's functional characteristics.

2. Materials and methods

2.1. Oligodeoxyribonucleotide primers

Primers for mouse V-gene amplification by polymerase chain reaction (PCR) were obtained from the Ig prime kit (Novagen). Other primers used in this study were synthesized on an Applied Biosystem model 391 DNA synthesizer and purified by polyacrylamide gel electrophoresis (PAGE) under denatured conditions.

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Abbreviations: TCR, T cell receptor; mAb, monoclonal antibody; VH, heavy chain variable domains; VL, light chain variable domains; PBMC, peripheral blood mononuclear cell; MUC1, mucin core protein 1; bsAb, bispecific antibody; IL, interleukin; T-LAK, lymphokine-activated killer with T cell phenotype; Fv, complex of VH and VL domains; PCR, polymerase chain reaction; BS, bacterial supernatant; PP, periplasmic; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; BrdU, bromodeoxyuridine; MTS, (3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazorium, inner salt

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2.2. mAbs and hybridoma cell lines

The anti-CD28 hybridoma cell line 15E8 (mouse IgG1) [15] was kindly provided by Dr. R. van Lier (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands). Two mAbs, TS2/18.1.1 (anti-CD2, mouse IgG1) and OKT3 (anti-CD3, mouse IgG2a), were obtained from the respective hybridoma cells. The MUSE11 mAb, a mouse IgG1 directed at epithelial mucin core protein 1 (MUC1) antigen [16], was used for preparation of bispecific antibody (bsAb).

2.3. cDNA synthesis, PCR amplification of variable region genes of 15E8 hybridoma

Total RNA was isolated from hybridoma 15E8 cells using Dynabeads oligo(dT) (Dynal) and the first strand cDNA synthesis was performed using a first strand cDNA synthesis kit (Life Sciences). PCR amplification of the heavy and light chain variable domain (VH and VL) families was performed separately using an Ig prime kit (Novagen). The amplified V-genes were cloned into TA vector (Invitrogen), followed by DNA sequencing using the Auto Read Sequencing kit (Pharmacia) according to the recommendations of the manufacturer.

2.4. Construction of 15E8 Fv co-expression vector

The cloned VH or VL genes were modified by introducing flanking *Nco*I and *Sac*II sites by PCR and inserted into pSNE4 vector [17], which is a derivative of pGEM-2 (Promega). Thus, pSNE4-15E8 VH (Fig. 1A) and pSNE4-15E8 VL (Fig. 1B) were constructed first. Next, the gene fragment from rbs to the stop codon, including the 15E8 VL gene in pSNE4-15E8 VL, was modified by introducing flanking *Bam*HI and *Spe*I sites by PCR and inserted into pSNE4-15E8 VH. The gene was tandemly arranged with rbs-pelB-15E8 VH-rbs-pelB-15E8 VL, designated as pSNE4-15E8 Fv (Fig. 1C). This vector has two kinds of C-terminal peptide tags: the c-myc peptide tag for detection by 9E10 mAb (Santa Cruz Biotechnology) and the 6×histidine tag for purification by metal-immobilized affinity chromatography.

2.5. Expression of 15E8 Fv

For production of 15E8 Fv, the *E. coli* strain BL21 (DE3) transformed with pSNE4-15E8 Fv was grown to the early stationary phase at 28°C in 2×YT broth supplemented with 100 μg/ml ampicillin. To induce the expression of 15E8 Fv, 1 mM isopropyl-1-thio-β-D-galactopyranoside was added and the cells were grown overnight. The bacterial supernatant (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 and 0.1 mM EDTA, kept at room temperature for 5 min and then 40 ml of cold water was added in order to induce osmotic shock. The suspension was kept on ice for 30 min and then centrifuged at 2000×*g* for 35 min at 4°C. The periplasmic (PP) fraction was obtained from the supernatant.

2.6. Sodium dodecyl sulfate (SDS)–PAGE and Western blotting

The proteins in each fraction were precipitated with 6% trichloroacetic acid and 0.083% deoxycholate and then applied to SDS–PAGE under reducing conditions as reported previously [18]. The proteins were stained with Coomassie brilliant blue R-250. Next, proteins in the gel were blotted to nitrocellulose membranes (Amersham-Pharmacia). Thereafter, the blots were incubated with peroxidase-conjugated anti-His tag mAb (Santa Cruz Biotechnology) followed by signal enhancement using the ECL detection system (Amersham-Pharmacia).

2.7. Purification of soluble 15E8 Fv

Soluble fractions (BS and PP) were salted out with ammonium sulfate at 70% saturation, and precipitates were collected by centrifugation at 4500×*g* for 20 min. The precipitates were dissolved in phosphate-buffered saline (PBS) (pH 7.9) overnight and dialyzed with PBS (pH 7.9) for three times. They were purified with a TALON metal affinity resin column (Clontech) and eluted with three buffer solutions made of sequentially increasing concentrations of imidazole (1, 10 to 500 mM)/PBS. Minor impurities were removed by gel filtration on a Superdex 75 pg column (Amersham-Pharmacia) (1.0×100 cm), pre-equilibrated with PBS, pH 7.4.

2.8. Preparation of lymphokine-activated killer cells with T cell phenotype (T-LAK)

T-LAK cells were prepared as reported previously [19]. Briefly, peripheral blood mononuclear cells (PBMCs) from healthy volunteers were isolated by density gradient centrifugation and cultured for 48 h in culture medium (RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin) and 100 IU/ml IL-2 (Shionogi Pharmaceutical Co. Ltd.) in a culture flask precoated with OKT3 mAb (10 μg/ml). The proliferating cells were then transferred to another flask and allowed to expand for 2 weeks in the culture medium containing 100 IU/ml IL-2.

2.9. Flow cytometry

T-LAK cells were incubated with purified 15E8 Fv (100 μl) on ice for 30 min. Cells were washed with 0.1% NaN_3/PBS three times, and then incubated with mouse anti-c-myc antibody (9E10) as the secondary antibody. Cells were stained with FITC-conjugated goat anti-mouse IgG (Cappel Lab.) as the tertiary antibody. The stained cells were analyzed by FACS Calibur (Becton Dickinson). For positive control, 15E8 IgG was used as the first antibody and FITC-conjugated goat anti-mouse antibody as the second antibody.

2.10. Blockage of 15E8 IgG binding to T-LAK cells by 15E8 Fv

T-LAK cells were incubated with 50 μl of purified 15E8 Fv (0.28 mg/ml) on ice for 30 min. Next, 10 μl of 15E8 IgG (0.1 mg/ml) was added and incubated for 30 min to compete with 15E8 Fv binding. The cells were washed with 0.1% NaN_3/PBS three times and stained with FITC-conjugated goat anti-mouse IgG to detect 15E8 IgG binding. The stained cells were analyzed by FACS Calibur.

2.11. Proliferation assay of PBMCs

Proliferation of mitogen-activated human PBMCs was performed using a Cell Proliferation enzyme-linked immunosorbent assay (ELISA) system (Amersham Life Science Ltd.). Briefly, 1×10^5 freshly isolated PBMCs suspended in 0.2 ml culture medium were distributed into each well of a 96-well flat-bottomed plate (Sumitomo Bakelite Ltd.) in the presence of various concentrations of 15E8 Fv or 15E8 IgG together with anti-CD3 or anti-CD2 mAb. After incubation for 48 h at 37°C, bromodeoxyuridine (BrdU) labeling reagent was added and the cells were incubated for an additional 24 h. The detection procedure was according to the manufacturer's assay protocol for suspension cells. The optical density was measured using a plate reader (Bio-Rad model 3550) at 450 nm.

2.12. Expression of CD25 on antibody-activated PBMCs

Freshly isolated 2×10^5 PBMCs suspended in 0.2 ml culture medium were distributed into each well of a 96-well flat-bottomed plate in the presence of OKT3 mAb or 15E8 Fv or their combination. After 72 and 96 h, cells were harvested, washed with 0.1% NaN_3/PBS and incubated with FITC-conjugated anti-IL-2R (CD25) mAb (Becton Dickinson). After 30 min incubation on ice, the cells were washed twice and analyzed for CD25 expression by FACS Calibur.

2.13. Cancer cell line

Human bile duct carcinoma cell line, TFK-1 [20], which is positive for adenocarcinoma-associated antigen MUC1 [19], was used as the target for growth inhibition assay.

2.14. Growth inhibition assay of cancer cells

Effectiveness of bsAb (anti-MUC1×anti-CD3 bsAb) in specific targeting immunotherapy of cancer and production of the bsAb was described in a previous report [19]. Growth inhibition assays of cancer cells were performed using a MTS ((3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazorium, inner salt) assay kit (Promega Co.). Briefly, 5000 target cells in 100 μl culture medium were distributed to each well of a half-area (A/2) 96-well flat-bottomed plate (Costar Corp.) and cultured overnight. After removing the culture medium, 100 μl of T-LAK cells, preincubated with various concentrations of bsAb (anti-MUC1×anti-CD3) [19] and 15E8 Fv for 30 min, was distributed into each well. After culture for 48 h at 37°C, each well was washed with PBS three times. This was followed by the addition of MTS/phenazine methosulfate solution (Promega Co.) diluted with culture medium. The plates were read on a microplate reader at 490 nm after incubation for 1 h at 37°C. The growth inhibition of cancer cells was calculated as follows: % growth inhibition of cancer cells = $[1-(A_{490}$ of experiment $-A_{490}$ of background$)/(A_{490}$ of control $-A_{490}$ of background$)]\times100$.

3. Results

3.1. Cloning of variable domains of 15E8 anti-CD28 antibody

VH and VL genes of 15E8 hybridoma cells were amplified by PCR using an Ig prime kit. Amplified V-genes were cloned into TA vector and sequenced. Nucleotide and deduced amino acid sequences of VH and VL clones are shown in Fig. 2. The 15 amino acid sequences from N-termini of each chain deduced from cloned DNA sequences were identical to the sequences analyzed from the parental IgG (data not shown). By comparing the amino acid sequences with the Kabat's database (sixth edition) of immunoglobulin sequences, obtained from the World Wide Web, the VL domain of 15E8 was identified as a member of the mouse immunoglobulin kappa chain subgroup V, and the VH domain as a member of the heavy chain subgroup IIIc.

3.2. Expression and purification of 15E8 Fv

15E8 Fv was produced using *E. coli* strain BL21 (DE3) harboring the plasmid pSNE4-15E8 Fv (Fig. 1C). We confirmed the expression of 15E8 Fv using SDS–PAGE (Fig. 3A) followed by Western blotting analysis using anti-His tag mAb (Fig. 3B). Both 15E8 VH and 15E8 VL were secreted into the PP and bacterial culture supernatant fractions. Under reducing conditions, Fv separated into VH and VL, and two bands could be detected corresponding to the calculated molecular weight of 16 500 Da for 15E8 VH and 15 200 Da for 15E8 VL. The soluble Fv was purified by immobilized metal affinity chromatography, followed by gel filtration. Under gel filtration, the purified Fv fragment has a molecular weight of ca. 25 kDa (Fig. 4), indicating that the Fv fragment is a hetero-dimer composed of a stoichiometric association of VH with VL. Final yields of 15E8 Fv were about 4 mg from 1 l culture.

3.3. Reactivity of 15E8 Fv for T-LAK cells

The reactivity of the 15E8 Fv was examined by flow cytometry. Purified soluble 15E8 Fv reacted with CD28-positive T-LAK cells, showing a similar positive pattern to that of parental 15E8 IgG (Fig. 5A).

In order to determine that this 15E8 Fv has the same antibody specificity as parental IgG, a blocking test was performed and reactivity was examined by flow cytometry. In brief, T-LAK cells were incubated with 15E8 Fv as the first competitor. Next, 15E8 IgG was added, and the binding of

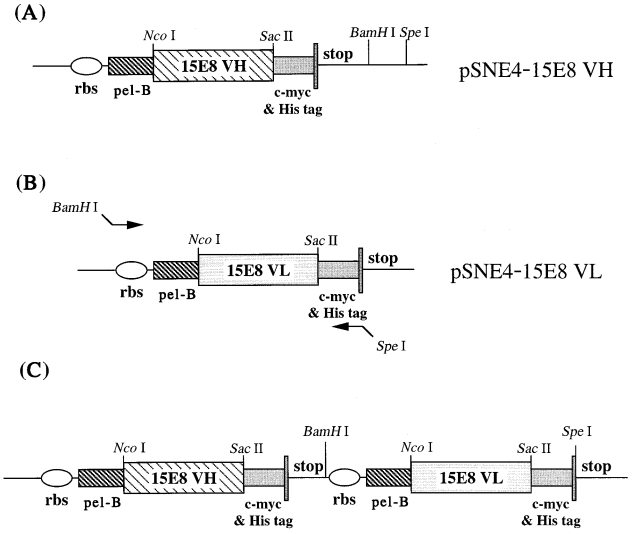

Fig. 1. Schematic construction of 15E8 Fv expression vectors. (A) 15E8 VH expression vector; (B) 15E8 VL expression vector; (C) 15E8 Fv expression vector. The positions of primers and important restriction sites used for constructing 15E8 Fv expression vectors are shown. c-myc, a sequence encoding an epitope recognized by the mAb 9E10; 6×His, a sequence encoding six C-terminal histidine residues; rbs, a sequence encoding the ribosome binding region.

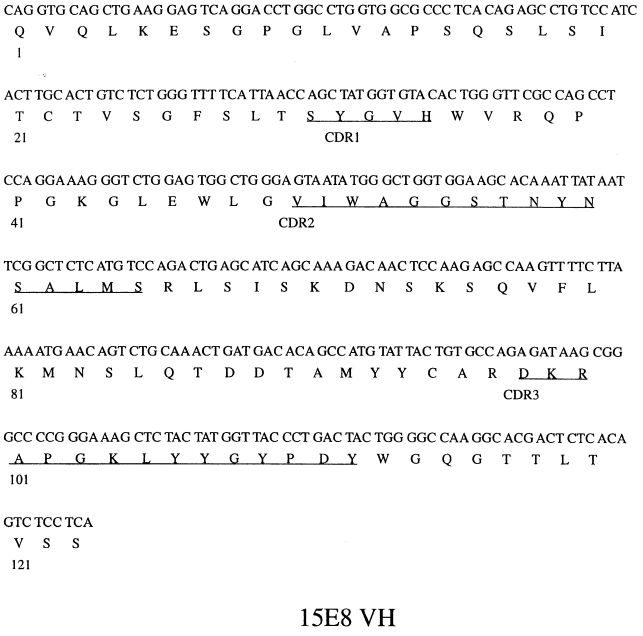

Fig. 2. Nucleotide sequences and deduced amino acid sequences of the VH and VL regions of 15E8 hybridoma. Complementarity determining regions 1, 2 and 3 are underlined.

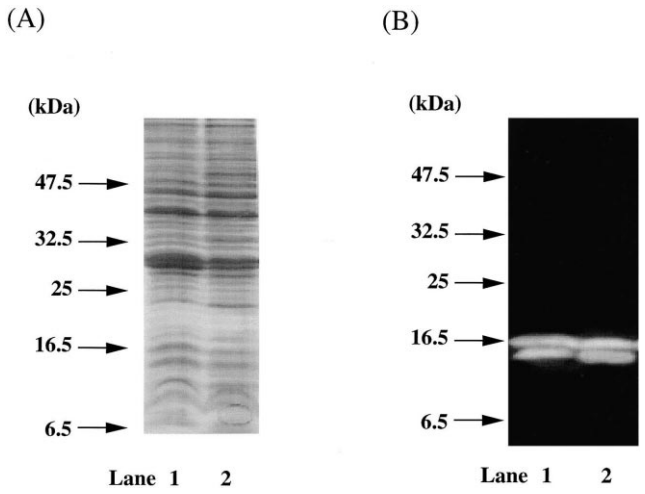

Fig. 3. (A) SDS–PAGE and (B) Western blot of each fraction of *E. coli* BL21 (DE3) cells expressed 15E8 Fv fragment. Lanes: 1, culture supernatant of Fv; 2, PP fraction of Fv. The positions of molecular size markers are shown on the left.

15E8 IgG was detected by FITC-conjugated anti-mouse IgG. As shown in Fig. 5B, reactivity of 15E8 IgG was apparently blocked by 15E8 Fv, indicating that 15E8 Fv has the same antibody specificity as 15E8 IgG.

3.4. Proliferation of PBMCs stimulated by various mAbs

To assess the co-stimulatory function of the 15E8 Fv fragment, two types of proliferation assays for PBMCs were performed. The first used a combination of anti-CD3 mAb OKT3 and 15E8 Fv, and the second used a combination of anti-CD2 mAb TS2/18.1.1 and 15E8 Fv. In the presence of OKT3 mAb (0.5 μg/ml), proliferation of PBMCs was enhanced dose-dependently by 15E8 Fv. This activity of 15E8 Fv was almost equal to parental 15E8 IgG (Fig. 6A).

Fig. 6B shows the results of the combination of anti-CD2 mAb with 15E8 Fv. When 15E8 Fv alone was added to PBMCs, enhancement of proliferation was very marginal. In contrast to this, remarkable proliferation of PBMCs was observed using a combination of 15E8 Fv and anti-CD2 mAb

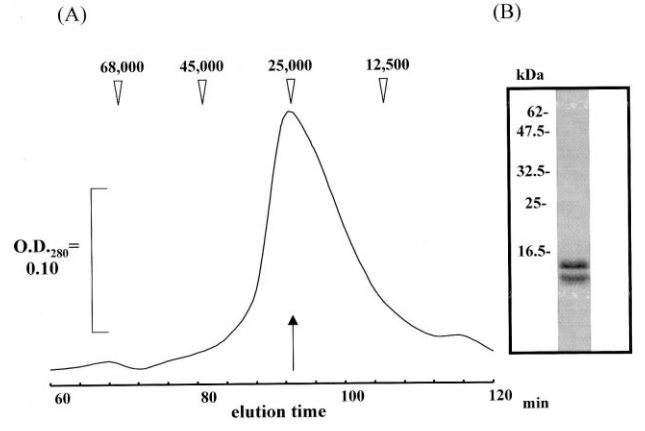

Fig. 4. Gel filtration of 15E8 Fv. (A) 1 ml of proteins eluted from immobilized metal affinity chromatography was loaded at a flow rate of 0.5 ml/min. Under this condition, the retention time of the standards is shown under the horizontal line. (B) SDS–PAGE of the eluted fraction. The fraction indicated in (A) by the arrow has been analyzed. Molecular size markers (kDa) are shown on the left.

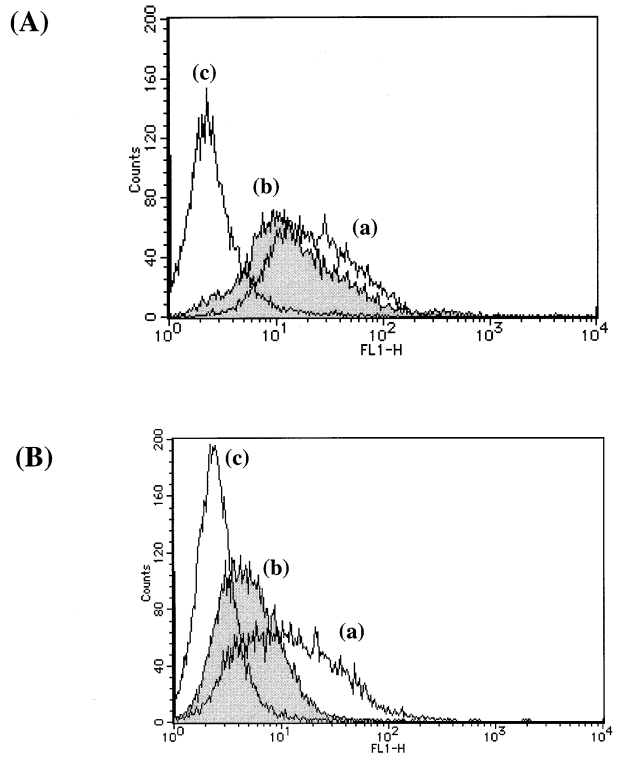

Fig. 5. Reactivity of 15E8 Fv to T-LAK cells was examined by flow cytometry. (A) (a) T-LAK cells were incubated with 15E8 IgG (primary antibody), following FITC-conjugated anti-mouse IgG (secondary antibody). (b) T-LAK cells were first incubated with 15E8 Fv first, then anti-c-myc (9E10) mAb (secondary antibody). Finally, cells were incubated with FITC-conjugated anti-mouse IgG as the tertiary antibody. (c) PBS was used instead of the primary antibody (15E8 Fv) as negative control. (B) Competition between 15E8 Fv and IgG was examined by blocking test. The reactivity of 15E8 IgG to T-LAK cells was examined by flow cytometry. (a) T-LAK cells were incubated with 15E8 IgG (as positive control), followed by FITC-conjugated anti-mouse IgG. (b) T-LAK cells were incubated with 15E8 Fv (primary antibody). Next, cells were incubated with 15E8 IgG. Finally FITC-conjugated anti-mouse IgG was added to examine competition between 15E8 Fv and IgG. (c) T-LAK cells were incubated with 15E8 Fv, followed by incubation with FITC-conjugated anti-mouse IgG (negative control).

(1.0 μg/ml), indicating that 15E8 Fv fragment has a co-stimulatory function.

3.5. Induction of activation marker (CD25) on PBMCs by anti-CD3 mAb and 15E8 Fv

CD25 expression on PBMCs cultured in the presence of different combinations of anti-CD3 mAb and 15E8 Fv was tested by flow cytometry (Fig. 7). Each mAb or Fv was used at a concentration of 0.25 μg/ml. OKT3 alone induced considerable CD25 expression, which increased until 72 h, but declined at 96 h. Induction of CD25 expression by 15E8 IgG or Fv alone was negligible. However, the combination of OKT3 and 15E8 Fv (or 15E8 IgG) enhanced CD25 expression remarkably, maintaining it at a high level at 96 h.

3.6. Enhancement of anti-tumor activity of anti-MUC1×anti-CD3 bsAb by 15E8 Fv

In our previous study, we reported the usefulness of bsAb in specific targeting immunotherapy of cancer [19]. Therefore, the co-stimulatory function of 15E8 Fv for cancer immuno-

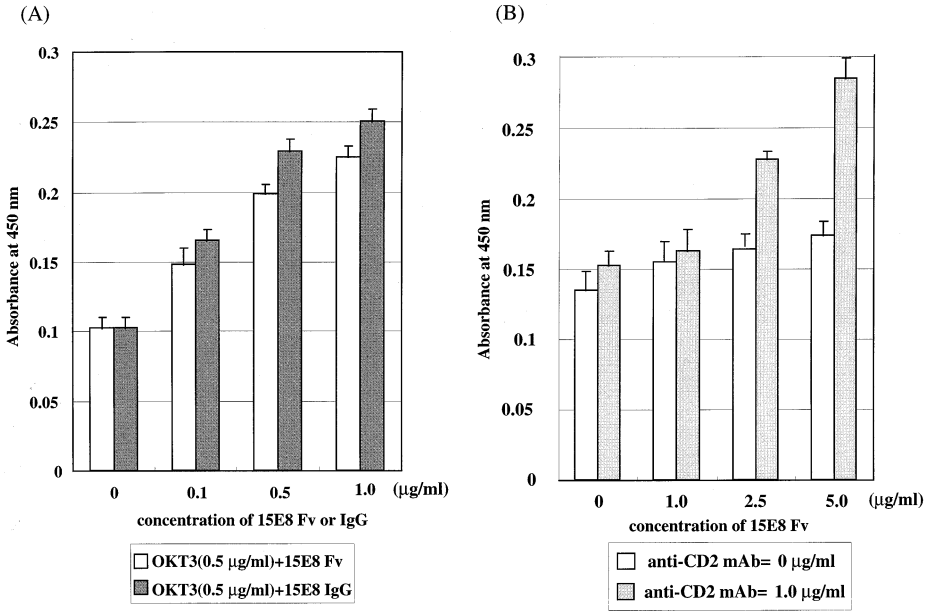

Fig. 6. PBMC proliferation assay (72 h BrdU incorporation test). Freshly isolated PBMCs were incubated for 72 h with anti-CD3 mAb (OKT3), anti-CD2 mAb (TS2/18.1.1), 15E8 Fv, 15E8 IgG or a combination of these antibodies. BrdU was then added to the culture. Incorporation of BrdU was measured for 72 h by Cell Proliferation ELISA system. (A) Combination of OKT3 mAb with 15E8 Fv. (B) Combination of anti-CD2 mAb with 15E8 Fv. Data are mean values of triplicate determinations.

therapy was examined by MTS growth inhibition assay of cancer cells. MUC1-positive cell line TFK-1 cells were used as the target and T-LAK cells as the effector. At an effector:-target ratio of 5:1, the effect of bsAb (anti-MUC1 × anti-CD3) was enhanced dose-dependently by the addition of 15E8 Fv (Fig. 8).

4. Discussion

The T cell-specific receptor CD28 is an important regulator of the immune system. CD28 strongly enhances T cell functions, which are essential for an effective antigen-specific response. The primary antigen signal alone cannot induce complete T cell activation, and the second co-stimulation pathway, interaction between B7 on the antigen presenting cells [21,22] and CD28, is important for T cell activation and proliferation. Since this co-stimulatory signal is also delivered by treatment with anti-CD28 antibody, development of mass production methods of anti-CD28 antibody by gene engineering is desirable. Toward this end, we tried to construct the Fv fragment of anti-CD28 antibody (15E8 [15]).

15E8 Fv and parental IgG showed an identical binding pattern (Fig. 5A) and the blocking test confirmed that both recognized the same epitope of CD28 (Fig. 5B). 15E8 Fv enhanced proliferation of PBMCs in the presence of anti-CD3 mAb, as did its parental IgG (Fig. 6A). Similarly, 15E8 Fv also enhanced anti-CD2 activity as demonstrated by proliferation of PBMCs (Fig. 6B). The co-stimulatory activity of anti-CD28 mAb [23] was successfully imparted to 15E8 Fv. The function of 15E8 Fv was substantiated by the enhanced IL-2 receptor (CD25) expression, wherein that 15E8 Fv plus anti-CD3 mAb induced lasting and enhanced expres-

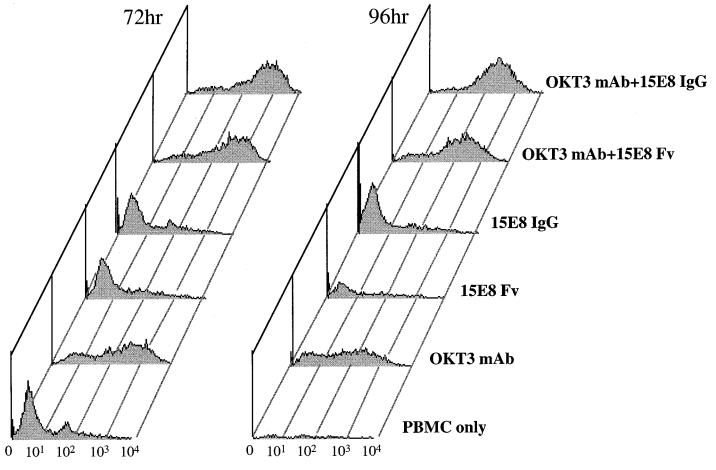

Fig. 7. Expression of CD25 (IL-2 receptor) on cultured PBMCs. Freshly isolated PBMCs (2×10^5/well) were cultured in the presence of OKT3 mAb, 15E8 Fv, 15E8 IgG or their combination. After 72 and 96 h, cells were harvested and stained with FITC-conjugated anti-CD25 mAb. CD25 expression of the cells was analyzed by flow cytometry.

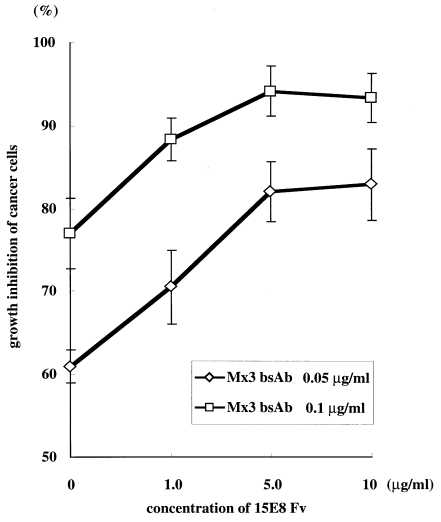

Fig. 8. 15E8 Fv enhances anti-tumor activity of bsAb. TFK-1 cells (target) and T-LAK cells (effector) were cultured in the presence of bsAb (anti-MUC1 × anti-CD3) (0.05 or 0.1 μg/ml), with or without 15E8 Fv for 48 h. Each well was washed three times, and then MTS/phenazine methosulfate solution was added. After 60 min, the microplate was read on a microplate reader at 490 nm. E:T ratio = 5:1. Data are mean values of triplicate determinations.

sion of IL-2 receptor (Fig. 7). Moreover, the effect of this functional co-stimulation by 15E8 Fv was reflected in the anti-tumor activity mediated by bsAb (Fig. 8). These results clearly indicate that the Fv fragment exhibits significant co-stimulatory activity, nearly equal to the parental 15E8 IgG. Therefore, this 15E8 Fv can be applied in further studies, such as the analysis of antibody-mediated co-stimulation by site-directed mutagenesis of variable domains, or the construction of recombinant bsAb, diabodies [24–26].

In conclusion, we have succeeded in constructing a bacterial expression system for the anti-CD28 Fv fragment, which has the same specificity and almost identical functional characteristics as its parental anti-CD28 antibody. The Fv fragment will be promising not only for study of co-stimulation, but for cancer immunotherapy.

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