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SEA-scFv as a Bifunctional Antibody: Construction of a Bacterial Expression System and Its Functional Analysis

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A SEA-antibody single chain Fv (SEA-scFv) fusion protein was produced by bacterial expression system in this study. SEA-scFv has both staphylococcal enterotoxin A (SEA) effects and antibody activity directed at the epithelial mucin core protein MUC1, a cancer associated antigen. It was expressed mostly in the cytoplasm as an insoluble form. The gene product was solubilized by guanidine hydrochloride, refolded by conventional dilution method, and purified using metal-chelating chromatography. The resulting SEA-scFv fusion protein preparation was found to react with MUC1 and MHC class II antigens and had the ability to enhance cytotoxicity of lymphokine activated killer cells with a T cell phenotype against a human bile duct carcinoma cell line, TFK-1, expressing MUC1. This genetically engineered SEA-scFv fusion protein promises to be an important reagent for cancer immunotherapy.

The prognosis of patients with BDC is poor because of the difficulty of curative resection even with the recent advance in surgical technologies (1). As an approach to improve this situation, immunotherapy has attracted great attention. Previous studies of human BDC-grafted SCID mice, with specific targeting therapy, consisting of i.v. administration of T-LAK cells sensitized with two kinds of bispecific antibodies, demonstrated remarkable inhibition of tumor growth (2). However complete cures could not be obtained. To develop another strategy that could be used together with this specific targeting therapy, we have concentrated our attention on superantigens (SAgs). SAgs, named by White et al. 1989 (3), are the most potent known activators of human T lymphocytes. They bind MHC class II molecules and activate T cells expressing a certain Vβ type of TCR (4). To use this potential for adoptive immunotherapy, SEA and MUSE11 mAb directed to the epithelial mucin antigen MUC1, widely expressed in adenocarcinomas, were conjugated chemically. SEA binds T-LAK cells through MHC class II molecules and thereby activates them. MUSE11 mAb, on the other hand, increases the ability of T-LAK cells to target MUC1 after introduction of an appropriate ligand. We therefore speculated that SEA-conjugated MUSE11 mAb (SEA-MUSE11) might enhance the specific cytotoxic activity of T-LAK cells against MUC1-positive tumors. Really marked suppression of tumor growth was observed in BDC-grafted SCID mice administered T-LAK cells preincubated with SEA-MUSE11 antibodies (5).

As a next step, we constructed a MUSE11 scFv antibody by gene engineering methods in order to overcome disadvantages with IgG mAb. Since scFvs have been shown to have better tumor penetration and clearance (6, 7), they represent potentially useful molecules for targeted delivery of drugs, toxins or radionuclides to tumors. Then, recombinant SEA (reSEA)

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Abbreviations used: SEA, staphylococcal enterotoxin A; scFv, single-chain Fv; SAg, superantigen; Fv, antibody variable region fragment; Gu-HCl, guanidine hydrochloride; MHC, major histocompatibility complex; T-LAK, lymphokine activated killer cells with T cell phenotype; BDC, bile duct carcinoma cell; SCID, severe combined immunodeficiency; IL-2, interleukin 2; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodesyl sulfate; FITC, fluorescein isothiocyanate; SEA-MUSE11, SEA-conjugated MUSE11 antibody; reSEA, recombinant SEA.

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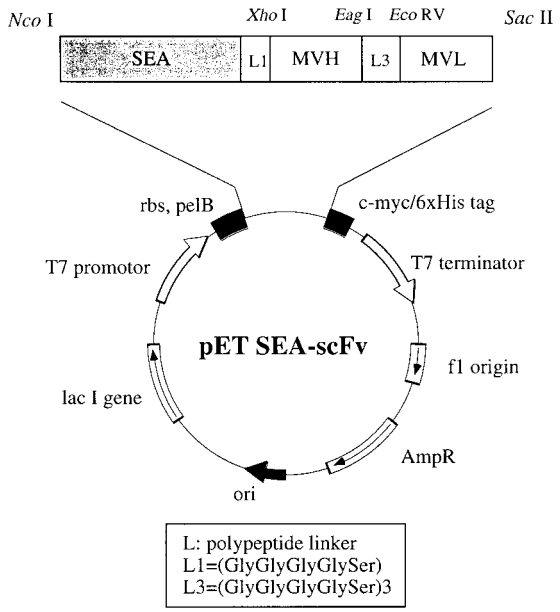

FIG. 1. Schematic representation of the SEA-scFv expression vector. AmpR denotes the ampicillin resistance gene; c-myc, a sequence encoding an epitope recognized by 9E10 mAb; ori, origin of DNA replication; 6 x His, a sequence encoding six C-terminal histidine residues; pelB, signal peptide sequence of bacterial pectate lyase; rbs, a sequence a encoding ribosome binding site for starting translation.

was produced by gene engineering. Furthermore, SEA-MUSE11 scFv fusion protein has been constructed by genetically engineering methods, which should bind to both MUC1-positive cancer cells and T-LAK cells, with the aim of clinical application for BDC or other MUC1-positive carcinomas.

MATERIALS AND METHODS

DNA sequence. Primers for preparation of the scFv (single chain Fv) gene for the MUSE11 monoclonal antibody were designed from the previous data (8). A MUSE11 hybridoma cell line producing mouse IgG1 antibody directed to MUC1 antigen (9, 10) was used as the source of V region genes (8, 11, 12). Total RNA was isolated from the hybridoma cells using Dyna beads oligo (dT)25 (Dynal A. S, Oslo, Norway) and first strand cDNA was prepared using a first strand cDNA synthesis kit (Life Sciences, St. Petersburg, Florida). VH and VL regions were amplified separately by PCR amplification using an Ig prime kit (Novagen, Madison, WI). For assembly, VH and VL domains of HyHEL10, an antibody to hen egg white lysozyme, contained in the pSNE4 vector (13), were replaced respectively by those from the MUSE11 hybridoma. For amplification of SEA gene by PCR, primers were designed according to the data of Betley and Mekalonos (14). Staphylococcus aureus strain 129 producing SEA was used as the source of the SEA gene. Oligonucleotide pair primers for the construction of SEA-scFv fusion protein gene were designed as follows: up (5′-ATTCCATGGCTAGCGAGAAAAGCGAAGAAA-3′) and down (5′-ATCTCGAGTGAACCTCCACCTCCACTTGTATATA-AATA-3′) for the SEA gene, adding a short linker peptide to C-terminal, and up (5′-ATCTCGAGGCCGATATCCAGCTGCAGG-AGT-3′) and down (5′-AGCCGCGGCTTTCAGCTCCAGCTTGGTCC-3′) for the MUSE11 scFv gene, with the pET-20b plasmid vector employed for construction of the fusion protein (Fig. 1).

MUSE11 scFv antibody and recombinant SEA (reSEA) protein. Construction of MUSE11 scFv and reSEA is to be reported elsewhere (Sakurai, manuscript in preparation). Briefly, VH and VL regions were amplified separately. For assembly, VH and VL domains of HyHEL10 contained in the pSNE4 vector (13), were replaced respectively. For construction of reSEA, the SEA gene was modified by introducing flanking *Nco* I and *Sac* II sites by PCR. Direct expression vector pUT-SEA was constructed by inserting the modified SEA gene in the vector pUT17 (15). The *E. coli* strain BL21 (DE3) was used for the transformation of the expression vectors of pSNE4 MUSE11 scFV and pUT-SEA.

Construction of the SEA-scFv fusion protein expression vector. A plasmid for production of SEA-scFv fusion protein was constructed as shown in Fig. 1. PCR modification was carried out to the SEA gene by adding a short linker peptide (GGGGS) and a *Xho* I restriction site to the 3′-terminal site. A *Xho* I site was also added to the 5′-terminal and an *Eag* I site to the 3′-terminal site of the VH gene. VH and VL were linked by a L3 linker (GGGGS)3 and a *Sac* II restriction site was introduced into the 3′-terminal of the VL gene. Then, the SEA-scFv fusion protein gene was inserted into the pET 20b vector (Novagen, Madison, WI).

Production of the fusion protein. The *E. coli* strain BL21 (DE3) pLysS transformed with pET SEA-scFv was grown at 37°C in 2x YT broth, supplemented with 100 μg/ml ampicillin, to the early stationary phase. In order to induce fusion protein production, isopropyl-1-thio-β-D-galactopyranoside (IPTG) (1mM) was added to the culture and the cells were grown overnight at 25°C. From 200 ml 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 culture medium supernatant by centrifugation (2000 x g, 30 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, and kept at room temperature for 5 min, then 40 ml of cold water was added in order to give an osmotic shock. After being kept on ice for 30 min, the cell suspension was centrifuged at 2000 x g for 30 min at 4°C to obtain the PP fraction. The remaining cells were resuspended in 10 ml of TE buffer consisting of 0.1M Tris-HCl (pH 8.0) and 0.1mM EDTA, ultrasonicated at 200 W for 15 min and centrifuged at 4500 x g for 30 min to recover the ICS fraction in the supernatant. The ICIS fraction was obtained by solubilizing the sonicated cell pellet with 10 ml of 6 M Gu-HCl, 0.1 M Tris-HCl (pH 8.0), 0.2 M NaCl by stirring overnight at 4°C. These four fractions were kept at 4°C until examination of activity.

SDS-PAGE and Western blotting. One ml of culture supernatant served for analysis. The total proteins in each fraction precipitated with 6% trichloroacetic acid (TCA) and 0.083% deoxycholate were applied for SDS-PAGE as reported previously (16), under reducing conditions, and proteins were stained with coomassie brilliant blue. Next, elecrophoresed proteins were blotted to nitrocellulose membranes (Amersham, Little Chalfont, Buckinghamshire, England). The membranes were treated with blocking buffer consisting of PBS, 0.05% Tween 20 and 4% skim milk (Difco, Detroit, Michigan) at room temperature for 1 h, then incubated with either 9E10 antibody directed to the c-myc peptide tag (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-SEA rabbit polyclonal antibody (S-7656, Sigma St. Louis, MO). Thereafter, they were incubated with peroxidase conjugated anti-mouse IgG (Amersham, Buckinghamshire, England) or goat anti-rabbit IgG (Amersham), followed by signal enhancement using the ECL detection system (Amersham).

Purification of fusion protein. From the ICIS fraction which contained the majority of fusion protein, purification was carried out. After solubilization with 6 M Gu-HCl, the proteins were applied to a 1 ml TALON metal affinity resin column (CLONTECH, Palo Alto, CA), followed by extensive washing with 20 mM Tris-HCl (pH 8.0), 6 M Gu-HCl, 1 mM imidazole and 0.2 M NaCl. Thereafter, fusion protein was eluted with four buffer solutions made of 20 mM Tris-HCl (pH 8.0), 6 M Gu-HCl, 0.2 M NaCl, and imidazole. The concen-

tration of imidazole in the elution buffer was sequentially increased from 10, 50, 100, to 500 mM.

Refolding of fusion protein. Fusion protein eluted from TALON metal affinity resin column was refolded by phased-guanidine removing dialysis with 20 mM Tris-HCl (pH 8.0), 0.2 M NaCl, and Gu-HCl. The concentration of Gu-HCl in the dialysis buffer was sequentially decreased (3, 2, 1, 0.5, and 0 M). Thereafter, fusion protein was dialysed against PBS containing 0.1 M NP-40 (N-0896, Sigma). Precipitates developing during dialysis were removed by centrifugation at 5500 x g for 20 min and the supernatant used as a fusion protein sample for the tests.

Cell lines. The human BDC cell line (TFK-1), reactive with MUSE11 mAb, was used as a target and a human hepatocellular carcinoma cell line (HT-17) as a control (17). Burkitt's lymphoma cell line (Raji) expressing MHC class II was used for flow cytometry. 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 as culture medium below).

T-LAK cells. T-LAK cells as effector cells were prepared from peripheral blood mononuclear cells by stimulation with solid phase OKT-3 mAb and IL-2 (100 IU/ml), as reported previously (5).

Cytotoxicity assay. Cytotoxicity was determined with a MTS assay kit (CellTiter 96TM AQUEOUS, Promega Co. Madison, WI) as reported elsewhere (5). Briefly, ten thousand 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., Cambridge, MA). They were cultured overnight to allow adherence to the plate, and after removing the culture medium, 50 μl aliquots of various concentrations of fusion protein in culture medium and 50 μl of effector cell suspension were added to each well. After culture for 48 h, the wells were washed with PBS three times. This was followed by addition of MTS/phenazine methosulfate solution. Plates were read on a microplate reader (Bio-Rad model 3550) at 490 nm after incubation for 1-3 h at 37°C and the % cytotoxicity calculated as reported previously (2).

Flow cytometric analysis. In order to determine binding ability of scFv, recombinant SEA and SEA-scFv fusion protein to cells, flow cytometry was carried out. In case of MUSE11 scFv binding, TFK-1 cells (5 x 105) were incubated with 50 μl of MUSE11 scFv (200 μg/ml) first, then, mouse anti-c-myc 9E10 (Santa Cruz Biotechnology). Finally, FITC-conjugated goat anti-mouse IgG was used. For binding of reSEA, Raji cells (MHC class II positive, 5 x 105) were incubated with 50 μl of reSEA. They were then incubated with rabbit anti-SEA (Sigma), and finally stained with FITC-conjugated goat anti-rabbit IgG(H+L, 0833, Immunotech Inc., Westbrook, ME). For SEA-scFv binding test, test cells (TFK-1 or T-LAK cells) (5 x 105) were first incubated with 50 μl of SEA-scFv fusion protein (40 μg/ml). After washing with PBS plus 0.1% NaN3, they were exposed to rabbit anti-SEA (Sigma) for 30 min and then FITC conjugated goat anti-rabbit IgG (Immunotech Inc.,). Stained cells were analyzed by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA).

RESULTS

Fusion protein production and purification. SDS-PAGE (12.5% gel) analysis of proteins obtained from E. coli cells harboring pET SEA-scFv at different steps of purification is shown in Fig. 2A. On the lanes 1 and 2, various proteins of different sizes were found. On the lane 3 (ICIS fraction), about 60 kDa protein was dominant. In flow through (lane 4) and wash fractions (lanes 5 and 6) from TALON chromatography, proteins of different sizes were seen. However, single band of 60 kDa protein was clearly detected on the lane 7, where proteins eluted from TALON chromatography were applied.

Western blotting analysis of the fusion proteins in the four fractions obtained from *E. coli* cells harboring pET SEA-scFv is shown in Fig. 2B. Proteins detected by anti-c-myc mAb and by anti-SEA antibody were shown in the left and right panels, respectively. Strong band of 60 kDa was detected on the lanes 1-5 (left panel) and lanes 1-4 (right panel). Among them, the strongest band of 60 kDa protein was seen on the lanes 4 (ICIS fraction) in the left and right panels, although other fractions contained smaller amounts of SEA-scFv fusion protein. Since SEA-scFv fusion protein has both c-myc tag and SEA epitopes, 60 kDa protein found in Figs. 2 and 3 was identified as SEA-scFv fusion protein.

Flow cytometry. Reactivity of MUSE11 scFv to TFK-1 cells was examined by flow cytometry (Fig. 3A). MUSE11 scFv reacted positively with TFK-1 cells, though reactivity was weak compared with parental MUSE11 mAb. reSEA produced in our laboratory also reacted positively with Raji cells through class II molecule (Fig. 3B).

Reactivity of SEA-scFv fusion protein was examined by flow cytometry. When SEA-scFv (40 μg/ml) was added to TFK-1 cells, it reacted positively with TFK-1 cells (Fig. 3C), however, it did not react with HT-17 carcinoma cells (data not shown). When T-LAK cells were used for test cells, it also reacted positively (Fig. 3D), indicating our SEA-scFv has bispecificity similar to SEA-MUSE11 antibody.

Fusion protein-mediated T-LAK cell cytotoxicity. Various concentrations of SEA-scFv fusion protein or SEA-MUSE11 were added to TFK-1 target and T-LAK effector cells in the MTS assay. In Fig. 4A, both SEA-scFv and SEA-MUSE11 were adjusted to the same molar concentration, since the molecular weight of SEA-MUSE11 antibody (180 kDa) was completely different from SEA-scFv (60 kDa). The concentration in Fig. 4A indicated that of SEA-scFv fusion protein. The cytotoxicity of T-LAK cells was about 20%, and this was much enhanced to about 70% by addition of the SEA-scFv fusion protein dose-dependently. Similarly SEA-MUSE11 enhanced T-LAK cell cytotoxicity (Fig. 4A).

As a control study, HT-17 cells (MUC1 negative hepatocellular cell line) were used for target of SEA-scFv mediated T-LAK cell cytotoxicity (Fig. 4B). SEA-scFv enhanced slightly cytotoxicity to HT-17 cells, but with lesser degree compared to TFK-1 cells.

In the absence of T-LAK cells, various concentrations of SEA-scFv fusion protein were added to TFK-1 cells in order to examine toxicity of SEA-scFv fusion protein. Cytotoxicity was almost negligible even at the concentration of 1 μg/ml (data not shown).

Specificity of fusion protein cytotoxicity. To verify the specificity of the cytotoxicity in this study, blocking tests were performed. When anti-SEA antibody was added to the SEA-scFv mediated T-LAK cell cytotoxic-

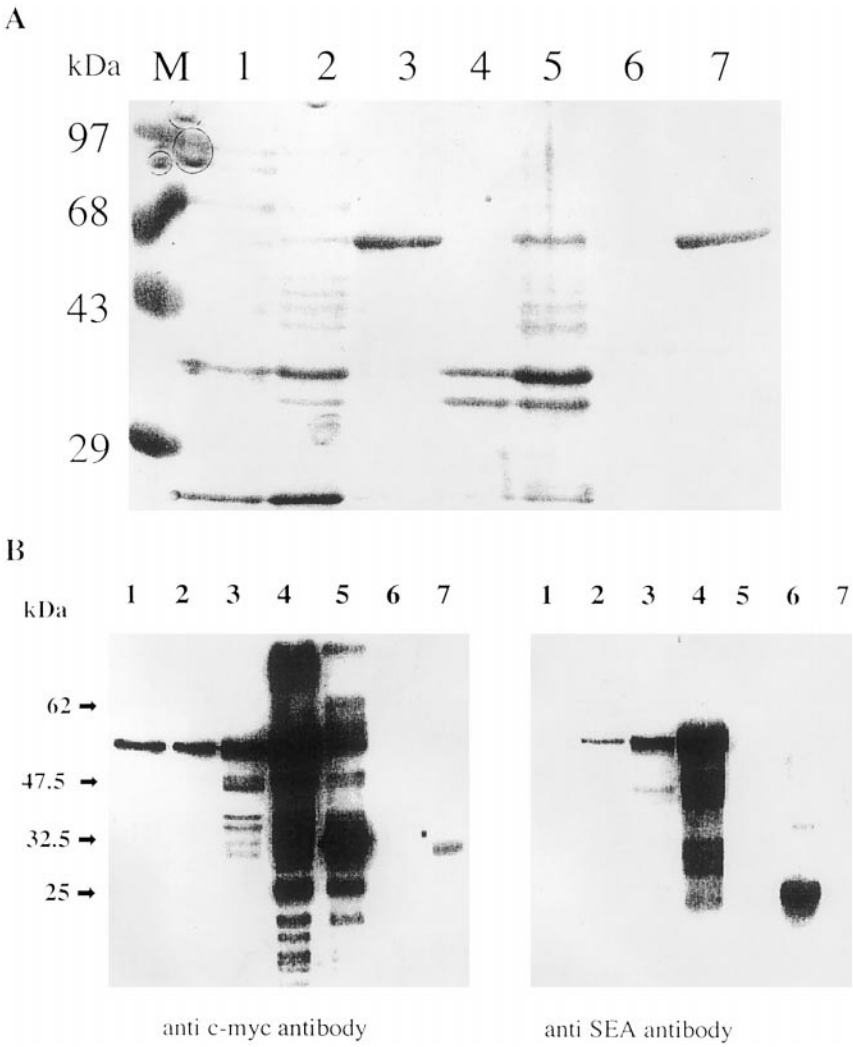

FIG. 2. (A) SDS-PAGE (12.5% gel) analysis of proteins at different steps of fusion protein purification. Molecular size markers (values in kDa) are shown on the left. Lane 1 shows *E. coli* culture medium (BS fraction); Lane 2, PP and ICS fractions; Lane 3, ICIS fraction; Lane 4, TALON chromatography flow through solution; Lanes 5 and 6, TALON chromatography wash solution; Lane 7, TALON chromatography elution fraction. Proteins were stained with coomassie brilliant blue. (B) Western blotting analysis of SEA-scFv fusion protein in the four fractions. Molecular size markers (kDa) are shown on the left. The proteins produced in the *E. coli* cultures were electrophoresed (SDS-PAGE), blotted to nitrocellulose membrane, and incubated with blocking buffer at room temperature for 1 h. One membrane (left) was incubated with anti-c-myc 9E10 mAb, and then peroxidase conjugated goat anti-mouse IgG. Finally, peroxidase signals were enhanced with the ECL detection system. Another membrane (right) was incubated with rabbit anti-SEA antibody, and then peroxidase conjugated goat anti-rabbit IgG. Finally peroxidase signals were enhanced by ECL detection system. Lanes 1, proteins in the BS fraction; Lanes 2, proteins in the PP fraction; Lanes 3, proteins in the ICS fraction; Lanes 4, proteins in the ICIS fraction; Lanes 5, MUSE11 scFv antibody (positive control for 9E10 mAb), Lanes 6, SEA (Toxin Technology, Inc., Sarasota, FL); Lanes 7, HyHEL10 scFv (scFv reactive with hen egg white lysozyme, positive control for 9E10 mAb).

ity, significant reduction of cytotoxicity was observed, dose dependently (Fig. 5A). However, slight reduction of cytotoxicity was noted when MUSE11 anti-MUC1 antibody was added to the SEA-scFv mediated T-LAK cell cytotoxicity (Fig. 5B).

DISCUSSION

Bacterial SAgs are most potent activators of human T cells (14). They bind MHC class II and certain Vβ of TCR and activate them unlike the case with conventional antigen recognition (18-22). Activated T cells proliferate and produce cytokines, e.g., tumor necrosis factor α, INFγ, IL-1, IL-2 and IL-6 (23-27), consequently, rapidly binding to MHC class II positive tumor cells in the presence of SAgs and lysing them. To apply this activity to various tumor cells, regardless of expression of MHC class II, SAg-conjugated antibodies against tumor associated antigens have been developed (28-33). Previously, we reported effective adoptive

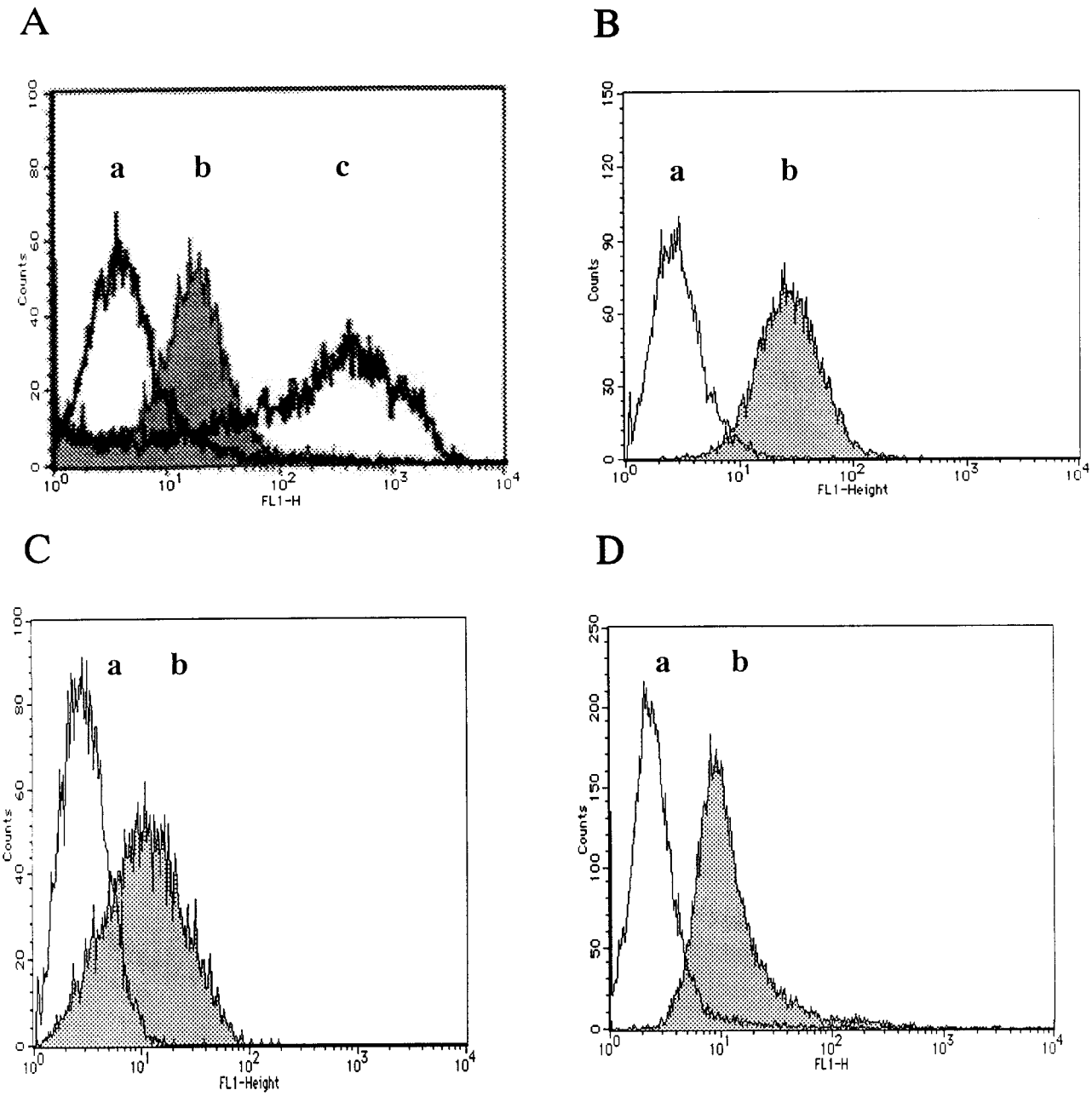

FIG. 3. Flow cytomety analysis. (A) TFK-1 cells were incubated with MUSE11 IgG mAb (c), then incubated with FITC-conjugated goat anti-mouse IgG. For determination of MUSE11 scFv binding to MUC1, TFK-1 cells were incubated with PBS only (a) or MUSE11 scFv (200 μg/ml) (b) first, then mouse anti-c-myc 9E10. Finally, FITC-conjugated goat anti-mouse IgG was employed. (B) For determination of SEA activity, Raji cells (MHC class II positive) were incubated with PBS only (a) or re SEA (b). They were then incubated with rabbit anti-SEA, and finally stained with FITC-conjugated goat anti-rabbit IgG. (C) For determination of SEA-scFv fusion protein antibody binding to MUC1, TFK-1 cells were incubated with PBS (a) or SEA-scFv fusion protein (40 μg/ml) (b) first, then rabbit anti-SEA. Finally, FITC-conjugated goat anti-rabbit IgG was employed. (D) For determination of SEA activity of the fusion protein, T-LAK cells (MHC class II positive) were incubated with PBS (negative control) (a) or SEA-scFv fusion protein (40 μg/ml) (b). They were then incubated with rabbit anti-SEA, and finally stained with FITC-conjugated goat anti-rabbit IgG.

immunotherapy by T-LAK cells retargeted with SAg-conjugated antibody to MUC1 in xenografted SCID mice with SEA-MUSE11 antibody (5). Remarkable tumor growth inhibition was observed in BDC-grafted SCID mice administered 2 x 107 T-LAK cells preincubated with 2 μg SEA-MUSE11 antibody together with IL-2 i.v. for 4 consecutive days, when tumor size was 5 mm in diameter. For application of this therapy to clinical trials, chemical construction of SEA-conjugated antibody is not practical, because large amounts of

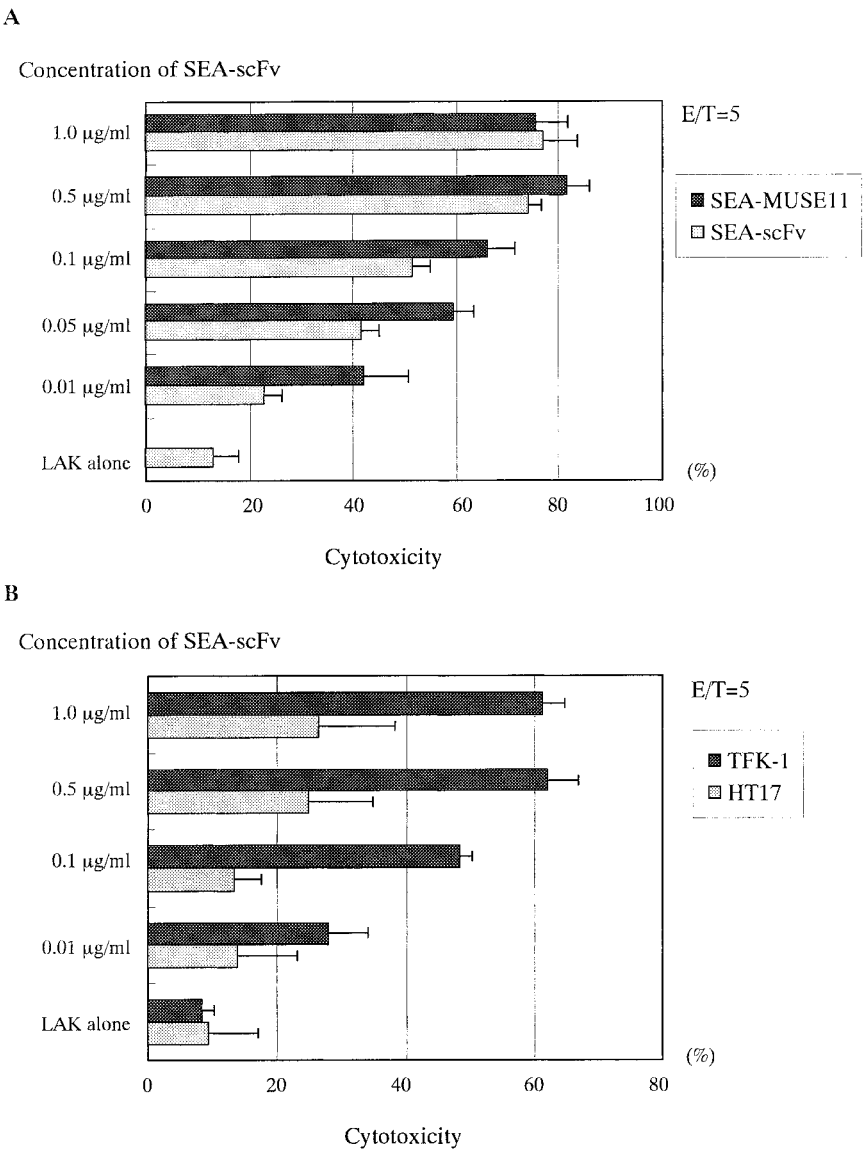

FIG. 4. (A) Cytotoxicity of T-LAK cells against TFK-1 cells with various concentrations of chemically conjugated SEA-MUSE11 or SEA-scFv fusion protein. Each protein was added to the culture as described in the text. Both SEA-scFv and SEA-MUSE11 were adjusted to the same molar concentration in this figure, in order to avoid the effects of the size difference between SEA-scFv (60 kDa) and SEA-MUSE11 (180 kDa). Namely, when 0.1 μg/ml of SEA-scFv was used, 0.3 μg/ml SEA-MUSE11 was used to adjust the molar concentration. The cytotoxicity was determined by 48 h MTS assay at an E:T ratio of 5:1. Columns show results (means; bars, SD) of at least triplicate determinations. (B) Cytotoxicity of T-LAK cells against TFK-1 cells or HT-17 cells with various concentrations of SEA-scFv fusion protein. The fusion protein was added to the culture as described in the text, and the cytotoxicity was determined by 48 h MTS assay at an E:T ratio of 5:1. Columns show results (means; bars, SD) of at least triplicate determinations.

fusion protein are required for patients. Chemical conjugation production of SEA-MUSE11 antibodies is too time-consuming and laborious. This is the reason why we focused production of SEA-scFv fusion protein by a gene engineering method in this study. The cost of SEA-scFv could thereby be reduced as compared with that of SEA-MUSE11 antibody, and this approach was also time-saving.

In addition, this scFv fusion protein has other merits compared with SEA-conjugated antibody produced by chemical conjugation. First, scFv lacks the IgG Fc portion which is the main antigen causing induction of human anti-mouse antibodies (HAMA) when administered to patients (34-37). Second, this scFv would be expected penetrate to tissues better than SEA-conjugated IgG antibodies since its size is much smaller (6, 7). Third, genetic modification of SEA-scFv is relatively easy by site-directed mutagenesis (38, 39), and we have already produced gene-modified fusion proteins. For example, we have generated mutated

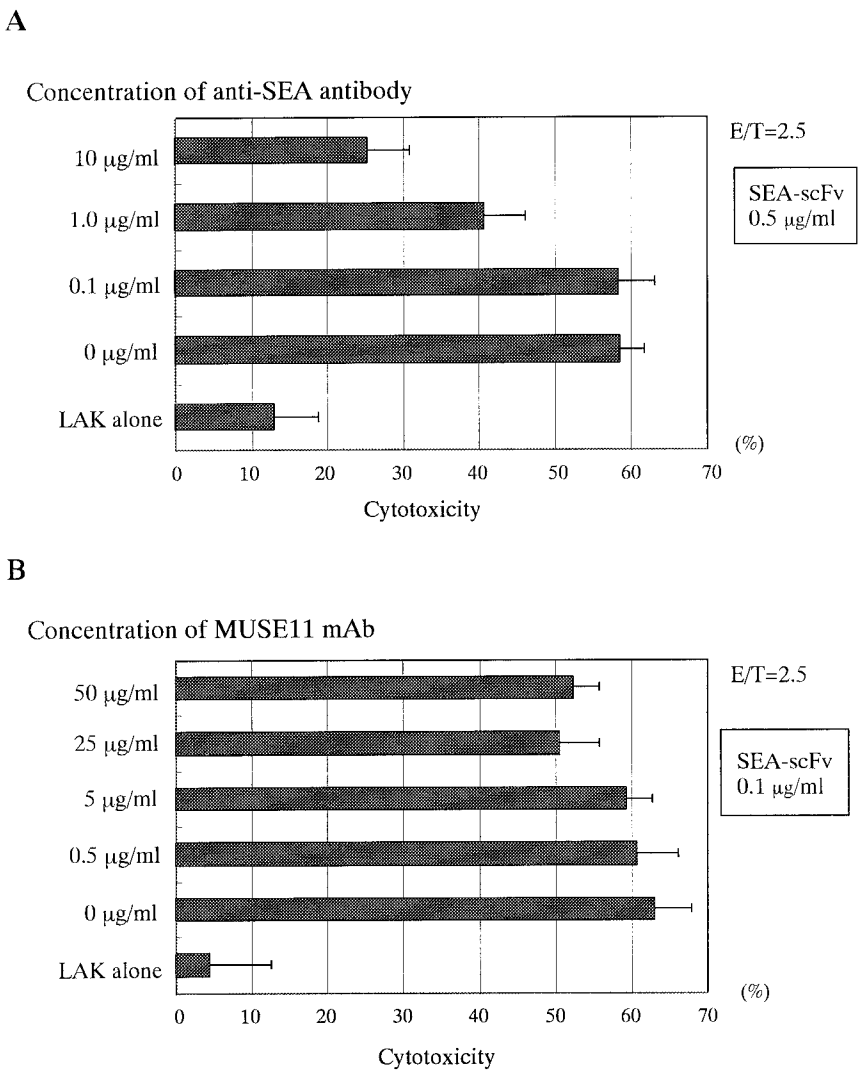

FIG. 5. (A) Blocking tests with various concentrations of anti-SEA antibody. T-LAK cell cytotoxicity against TFK-1 cells by T-LAK cells was determined by 48 h MTS assay in the presence of SEA-scFv fusion protein (0.5 μg/ml) at an E:T ratio of 2.5:1, with various concentrations of added anti-SEA antibody. Columns show results (means; bars, SD) of at least triplicate determinations. (B) Blocking tests with various concentrations of MUSE11 mAb. T-LAK cell cytotoxicity against TFK-1 cells was determined by 48 h MTS assay in the presence of SEA-scFv fusion protein (0.1 μg/ml) at an E:T ratio of 2.5:1, with various concentrations of added MUSE11 mAb. Columns show results (means; bars, SD) of at least triplicate determinations.

SEA by changing Asp at 227 of the original SEA (wild type) to Ala, in order to reduce nonspecific binding of wild SEA to class II positive cells. This mutated SEA has potential for T cell activation, but reduced reactivity with MHC class II, and this might cause lower side effects in comparison with wild SEA when administered to patients (33, 40). The specificity and function of this mutated SEA-scFv, are now under examination.

In the Figs. 3A and 3B, much higher concentration of MUSE11 scFv and SEA-scFv were required to obtain positive results in the flow cytometry compared with the parental mAbs, though they reacted with target cells positively. This may be because the affinity of scFv is usually lower than that of parental mAb (41).

The cytotoxicity of T-LAK cells was about 20%, and this was much enhanced to about 70% by addition of the SEA-scFv fusion protein dose-dependently in the same way as the chemically conjugated SEA-MUSE11 (Fig. 4A). However, cytotoxicity enhancement by SEA-scFv fusion protein against the HT-17 cells (MUC1 negative) was much lower (Fig. 4B), indicating the SEA-scFv has specificity to MUC1 positive cells. In the absence of T-LAK cells, SEA-scFv fusion protein did not show any cytotoxicity to TFK-1 cells. Therefore, it could be concluded that enhanced cytotoxicity by SEA-scFv fusion protein depends on the specificity of MUSE11 mAb and T cell activation by SEA.

In order to confirm the specificity, blocking tests using anti-SEA or MUSE11 mAb were performed. When anti-SEA antibodies were added to cytotoxicity tests, apparent inhibition of cytotoxicity was observed (Fig. 5A). Slight reduction of cytotoxicity was noted when MUSE11 anti-MUC1 antibody was added in Figure 5B. This may be because the antibody activity of the SEA-scFv fusion protein had lower affinity against MUC1 antigens than MUSE11 IgG. This is correlated with observation in the flow cytometry (Figs. 3A and 3B), where both MUSE11 scFv and SEA-scFv had lower reactivity than parental MUSE11 mAb.

In conclusion, we have succeeded in constructing an SEA-scFv fusion protein in a bacterial expression system and this promises to be a good reagent for cancer immunotherapy.

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