**MUC1-specific Targeting Immunotherapy with Bispecific Antibodies: Inhibition of Xenografted Human Bile Duct Carcinoma Growth**

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**ABSTRACT**

For the purpose of establishing a new adaptive immunotherapy for bile duct carcinoma (BDC), we synthesized two bispecific antibodies (BsAbs), MUC1 × CD3 BsAb constructed with MUSE11 (anti-MUC1 tumor antigen) and OKT-3 (anti-CD3), and MUC1 × CD28 BsAb constructed with MUSE11 and 15E8 (anti-CD28) antibodies. These two BsAbs reacted well with both MUC1-positive target tumor cells and effector lymphokine-activated killer (LAK) cells. Investigation of in vitro cytotoxicity [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay] revealed that the MUC1 × CD3 BsAb could antigen-specifically enhance the cytotoxicity of LAK cells. Addition of the two BsAbs (MUC1 × CD3 BsAb plus MUC1 × CD28 BsAb) in vitro resulted in a 60% cytotoxicity, similar to that obtained with BsAb (MUC1 × CD3) alone. Interleukin 12-induced LAK cells demonstrated far greater cytotoxicity (50%) than their interleukin 2-induced counterparts (LAK cells), and this was also enhanced by the BsAbs. When 2 × 10² LAK cells sensitized with both kinds of BsAbs were administered four times i.v. to BDC-grafted severe combined immunodeficient mice (tumor size 5 mm in diameter), inhibition of tumor growth was observed. Thus, BsAb-LAK therapy for control of BDC warrants clinical trials.

**INTRODUCTION**

With the much-improved surgical techniques of hepatic lobectomy, the resection rate for BDC has increased. However, the long-term survival of patients undergoing such surgery remains far from satisfactory, because curative resection is very difficult and remnant cancer cells frequently give rise to advanced lesions and death. This is largely due to the fact that the BDC generally spreads extensively along the intrabiliary bile ducts, often also demonstrating a multifocal origin from areas of epithelial dysplasia. The unique progression of BDC, with discontinuous invasion through lymphogenous or perineural spaces, is also known to contribute to the poor prognosis (1). After surgery for BDC, we have tried multimodal therapy, including i.v. and intra-arterial chemotherapy, and extracorporal and intracanalicular irradiation. These treatments have certainly prolonged survival as compared to surgical resection alone, but recurrence from remnant BDC still occurs in spite of aggressive therapy. The clinical status of this problem at the present time requires that we develop a new adjuvant therapy capable of eliminating remnant BDC.

In the field of immunotherapy, application of BsAbs with dual specificities, one for tumor antigens and another for effector cells, is a promising approach. BsAbs have been shown to redirect and trigger effector cells, and various investigators have demonstrated that BsAb, when administered with effector cells like LAK cells, can enhance specific cytotoxicity and exert strong effects in vivo (2–5). These results stimulated us to produce BsAbs reactive with BDC. When considering BsAb therapy for BDC, selection of a target antigen on the tumors is important. As a target molecule, MUC1 (a mucin core protein) was selected for BsAb in the present study because of the specificity and broad overexpression by neoplasms arising from the bile ducts. Successful adoptive immunotherapy with two kinds of BsAbs in the Hodgkin cell-grafted SCID mice (5) also triggered our examination of simultaneous administration of double BsAbs in the BDC-grafted SCID mouse model. Here, we report the results of in vitro and in vivo immunotherapy using BsAbs.

**MATERIALS AND METHODS**

**MAbs.** For construction of BsAb, the following MAbs were used: two Mabs, against CD3 (OKT-3, mouse IgG2a) and against CD28 (15E8, mouse IgG1), directed at effector cells, and MUSE11 (mouse IgG1), directed at the MUC1 antigen on tumor cells. The MUSE11 MAb was produced by Dr. Hinoda (Sapporo Medical University, Sapporo, Japan; Ref. 6). For inhibition of BsAb-dependent cytotoxicity, OKT3, MUSE11, and L243 (mouse IgG2a, directed at class II antigen) MAbs were used. These MAbs were purified from sera and ascitic fluid of mice inoculated with hybridomas by caprylic acid treatment in combination with the ammonium sulfate method (7). For flow cytometry analysis, TS2/18.1.1 (anti-CD2), OKT-3 (anti-CD3), OKT-8 (anti-CD8), 3-G-8 (anti-CD16), 15E8 (anti-CD28), and NE-150 (anti-CD56) MAbs were used. 15E8 (8) and NE-150 were provided kindly by Dr. R. A. van Lier (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands) and Dr. Ueda (Aichi Cancer Center, Nagoya, Japan), respectively.

**Cell Lines.** Three human BDC cell lines (TFK-1, OCUCH-LM1, and HuCC-T1) were used as target cells, and a human HCC cell line (HT-17) was used as a control. TFK-1 (9) and HT-17 were established in our laboratory. OCUCH-LM1 was used in combination with Dr. Yamada (Osaka City University School of Medicine, Osaka, Japan), and HuCC-T1 was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan).

**Effector Cells.** PBMCs, isolated from heparinized blood of a healthy volunteer by density gradient (Lymphoprep) centrifugation, were cultured for 48 h in RPMI 1640 supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 µg/ml), and 100 IU/ml recombinant human IL-2 (IL-2, Shionogi Pharmaceutical Co., Osaka, Japan) at a cell density of 1 × 10⁶/ml, in a culture flask (178891; A/S NUNC, DK-4000, RosKilde, Denmark) precoated with OKT-3 MAb (10 µg/ml) for induction of LAK cells. The LAK cells were expanded in culture medium containing 100 IU/ml IL-2. For generation of IL-12-LAK cells, PBMCs were cultured for 24 h in RPMI 1640 supplemented with 10% FBS, penicillin, streptomycin, and human IL-12 (supplied kindly by Dr. M. Kobayashi, Genetics Institute, Cambridge, MA) at a cell density of 2 × 10⁶/ml, in a culture flask. The cells were then transferred to another flask and cultured with 20 units/ml of IL-12 and 300 IU/ml of IL-2. The scale of culture was increased gradually with the same medium in line with the increase of cell number.

**Preparation of BsAbs.** First, the OKT-3 MAb was digested to F(ab')₂ with trypsin in 0.1 M citrate buffer (pH 4.1) at 37°C for 2 h. Then, F(ab')₂ fragments were reduced by DTT (0.5 mm), the reaction was stopped by addition of...
DTNB, and OKT-3 Fab')-S-NB fragments were separated from excess reagent by gel filtration. The MUSE11 MAb was digested with preactivated papain in 0.1 M acetate buffer (pH 5.5) at 37°C for 6 h to generate Fab’-SH. After reduction, again with DTT, the thus-generated Fab-SH fragments were separated by gel filtration (3, 4). Fab’-SH fragments of OKT-3 MAB and Fab-SH fragments of MUSE11 MAB were then mixed at a molar ratio of 1:1 and incubated at room temperature for 4 h. The resulting preparation was applied to the TSK-2000SW column (Toosoh, Tokyo, Japan) of a fast protein liquid chromatography system (Pharmacia Biotech, Uppsala, Sweden) to remove the unreactive fragments, allowing MUC1 × CD3 BsAbs to be obtained. MUC1 × CD28 BsAbs were prepared as follows. Fab’-SH fragments of 15E8 MAB were obtained by digestion with preactivated papain and reduced with DTT. The reduction was stopped by addition of DTNB and the Fab-SH fragments were separated by gel filtration chromatography. Fab-SH (15E8) and Fab’-SH (MUSE11) were then mixed in a 1:1 ratio and incubated for 4 h. The reconstituted MUC1 × CD28 BsAbs were purified by fast protein liquid chromatography.

**Cytotoxicity Assay.** Cytotoxicity was determined by the MTT assay according to the method described in previous papers, with a slight modification (4, 11, 12). Target culture cells at subconfluent phase, detached gently with 0.02% EDTA-PBS solution, were suspended in RPMI 1640 supplemented with 10% FBS. Five thousand cells (100 μl cell suspension) were distributed to each well of a half-area (A2) 96-well flat-bottomed plate (3696, Costar Corp., Cambridge, MA) and cultured overnight to promote adherence to the plate. After removing the culture medium by aspiration, BsAb and effector cells suspended in RPMI 1640 plus 10% FBS were added to each well. For this, 50 μl of various concentrations of antibody solution and 50 μl of effector cell suspension were added. E:T ratios tested in this study were 1, 2.5, and 5. All determinations were carried out in quadruplicate. After culture for 48 h at 37°C, plates were washed with 100 μl of PBS/well three times to remove effector cells and killed target cells. Then, 50 μl/well of the culture medium containing 1 mg/ml MTT were added and the plates incubated at 37°C for 4–5 h. Crystalline formazan was dissolved by adding acid-isopropanol (0.04 n HCl in isopropanol, 50 μl/well), followed by overnight incubation at 37°C. Then, the plates were agitated thoroughly and read on a microplate reader (Bio-Rad model 3530) at 595 nm. As previously (4), percentage of cytotoxicity was calculated as follows:

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\text{Cytotoxicity (\%)} = \left(1 - \frac{A_{595 \text{nm of experiment}}} {A_{595 \text{nm of control}}} \right) \times 100
\]

where A is absorbance. LAK cells used for MTT cytotoxicity testing were cultured for 4–14 days and IL-12-LAK cells for 14–21 days.

**Blocking Test.** MUC1-specific cytotoxicity was confirmed by competitive inhibition using three kinds of MABs. Namely, cytotoxicities of LAK cells were determined by 48-h MTT assay in the presence of MUC1 × CD3 BsAbs (0.5 μg/ml) at E:T = 5:1, and 5.0 or 20.0 μg/ml Fab’-SH of MUSE11, intact OKT3, or L243 MABs were added to the assay system.

**Tumor Inoculation and Treatment Regimens.** SCID mice 6–8 weeks old (Fox CHASE C.B. 17/ler-Scid Jcl) obtained from Japan Clea (Tokyo, Japan) were inoculated with 5 × 106 TFK-1 cells obtained from tissue culture, into the dorsal thoracic wall s.c. on day 0 of each experiment. Treatment started on day 10 and consisted of various permutations of LAK cells, BsAbs, and IL-2. On days 10–13, for 4 consecutive days, the mice received i.v. 2 × 107 LAK cells via the tail vein, together with IL-2 (500 IU/mouse) in 0.15 ml PBS. The LAK cells given to the SCID mice were sensitized with BsAbs before old (Fox CHASE C.B. l7IIcr-Scid Jcl) obtained from Japan Clea (Tokyo, Japan) were suspended in RPMI 1640 supplemented with 0.02% EDTA-PBS solution, were suspended in RPMI 1640 supplemented with 10% FBS. Five thousand cells (100 μl cell suspension) were distributed to each well. For this, 50 μl of various concentrations of antibody solution and 50 μl of effector cell suspension were added. E:T ratios tested in this study were 1, 2.5, and 5. All determinations were carried out in quadruplicate. After culture for 48 h at 37°C, plates were washed with 100 μl of PBS/well three times to remove effector cells and killed target cells. Then, 50 μl/well of the culture medium containing 1 mg/ml MTT were added and the plates incubated at 37°C for 4–5 h. Crystalline formazan was dissolved by adding acid-isopropanol (0.04 n HCl in isopropanol, 50 μl/well), followed by overnight incubation at 37°C. Then, the plates were agitated thoroughly and read on a microplate reader (Bio-Rad model 3530) at 595 nm. As previously (4), percentage of cytotoxicity was calculated as follows:

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**Statistical Analysis.** Results obtained for the various experimental groups were compared using the Student’s t test.

**RESULTS**

**Reactivity of MUSE11 with Various Cell Lines.** The reactivities of MUSE11 MAB (anti-MUC1) with two extrahepatic BDC cell lines (TFK-1 and OCUC-hLM1), one intrahepatic BDC cell line (HuCC-T1), and a HCC cell line (HT-17) were examined by flow cytometry. MUSE11 MAB showed strong reactivity with both extrahepatic BDC cell lines (TFK-1 and OCUC-hLM1) and weak reactivity with the intrahepatic BDC cell line, HuCC-T1. It did not react with HT-17 cells (Fig. 1).

**Characterization of BsAbs.** Using a chemical conjugation method, we obtained two BsAbs, MUC1 × CD3 BsAb, and MUC1 × CD28 BsAb. These BsAbs obtained by thiol-disulfide interchange using DTNB each showed a molecular weight (M,) of 100,000 by SDS-PAGE analysis (data not shown). The reactivities of the BsAbs were examined by flow cytometry (Fig. 2). Both BsAbs reacted well with TFK-1 cells, although a slight decrease of reactivity was found compared with that of parental MUSE11 MAB (Fig. 2, A and C). Almost all LAK cells reacted with MUC1 × CD3, and MUC1 × CD28 BsAbs (Fig. 2, B and D), an increase of reactivity being noted especially with MUC1 × CD28 BsAb when compared with the parental MAB (anti-CD28, Fig. 2D). MUC1-negative HT-17 did not react with the two BsAbs, indicating good specificity (data not shown).

**Effect of BsAb Concentration on Cytotoxicity.** To determine the optimal BsAb concentration for the in vitro cytotoxicity test, graded concentrations of MUC1 × CD3 BsAbs were added to the assay system in which TFK-1 cells and LAK cells were used as target and effector cells, respectively. About 20% cytotoxicity was achieved with LAK cells alone. Cytotoxicity increased depending on the concentration of BsAbs added but reached a plateau at 0.5 μg/ml BsAb (Fig. 4). From these results, the BsAb concentration for the following experiments was fixed at 0.5 μg/ml.

**BsAb-dependent Attachment of LAK Cells.** Cells in the cytotoxicity assay observed under a phase-contrast microscope are shown in Fig. 5. Cultured target cells (TFK-1) showed an epithelial-like appearance (Fig. 5A). When LAK cells and TFK-1 cells (E:T = 1:1) were cocultured in the presence of (MUC1 × CD3) BsAb (0.5 μg/ml) for 24 h, TFK cells became attached to the surrounding LAK cells (Fig. 5B). Fig. 5C shows cocultivation without BsAb in the microplate, LAK cells being scattered diffusely. BsAb-dependent attachment of LAK cells was inhibited partly by adding OKT3 (Fig. 5D) or MUSE11 (Fig. 5E) MABs.

**MUC1-specific Cytotoxicity with BsAbs.** The specificity of cytotoxicity in this assay was examined using various tumor cell lines as target cells (Fig. 6). Two MUC1-positive cell lines (TFK-1, OCUC-hLM1) were effectively killed in this assay when MUC1 × CD3 BsAb and LAK cells were added (Fig. 6A). Low MUC1-expressing HuCC-T1 and MUC1-negative HT-17 cells were examined in the same assay system (Fig. 6B); cytotoxicity against HuCC-T1 cells was low, and that against HT-17 was negligible.

To confirm the specificity of the assay system, a blocking test was performed with addition of various concentrations of either Fab’-SH of MUSE11, the intact form of OKT3, or L243 MABs to the culture (Fig. 7).
Cytotoxicity with a Combination of the Two BsAbs. To examine possible combination effects of the BsAbs, various pairs were tested for cytotoxicity. In the control study where MUC1 × CD3 BsAb was added to the assay, about 60% cytotoxicity was observed. When MUC1 × CD28 BsAb (0.5 μg/ml) was added, about 20% cytotoxicity was observed, which was similar to that obtained by LAK cells without BsAb. When MUC1 × CD3 BsAb and MUC1 × CD28 BsAb were added, cytotoxicity similar to MUC1 × CD3 BsAb (60%) was obtained (Fig. 8).

IL-12-induced LAK Cell Activity. Cytotoxicity was examined using IL-12-LAK cells after 14 days of cultivation with TFK-1 target cells. Without BsAb, cytotoxicity was approximately 50%, and clear increase was noted in the presence of MUC1 × CD3 BsAb (Fig. 9). No incremental increase was obtained with a combination of MUC1 × CD3 with MUC1 × CD28 BsAb.

STT in the Xenografted SCID Mice. SCID mice xenografted with human tumors have been used widely in studies of in vivo immunotherapy (5, 13, 14). In the present experiment, TFK-1 cell-xenografted SCID mice were administered LAK cells preincubated with BsAbs (MUC1 × CD3 BsAb alone, MUC1 × CD3 BsAb plus MUC1 × CD28 BsAb) together with IL-2. For STT, LAK cells preincubated with MUC1 × CD3 BsAb plus MUC1 × CD28 BsAb were introduced on day 10 after tumor inoculation when tumor size was 5 mm in diameter. This caused remarkable retardation of tumor growth as compared to the control group (no therapy), or treatment with LAK cells without BsAb or after preincubation with MUC1 × CD3 BsAb alone (Fig. 10).

DISCUSSION

The present study provides strong evidence in support of the efficacy of the BsAb approach for cancer immunotherapy. Both the in vitro and the in vivo results suggest applicability for control of BDC. CTL epitopes of human cancer cells have not yet been character-
ized fully, but several immunological determinants such as MAGE-1 (15), MART-1 (16), and gp100 (17) on melanomas have been well defined. Although CTL generation to these determinants has recently become easier, expansion is still time consuming, and the efficiency is not satisfactory. In contrast, substantial quantities of LAK cells can be obtained readily, and therefore, LAK cell therapy has been introduced for clinical immunotherapy. However, the clinical results obtained thus far have been disappointing (18, 19), with limitations of LAK therapy being attributable to a lack of effective contact with tumor cells and a loss of cytotoxic activity in vivo.

Use of BsAbs is one approach to overcome these defects, and examples reactive with TCR/CD3 and tumor antigens have been shown capable of efficient targeting of LAK cells (20, 21). Our previous in vitro study (12) also demonstrated that BsAb reactive with HCC and CD3 greatly enhanced specific LAK cytotoxicity.

When considering BsAb therapy for BDC, the selection of a target antigen on the tumors is of prime importance. As a target molecule, MUC1 (22–24) was selected for BsAb in the present study because of

Fig. 3. Phenotypes of effector cells analyzed by FACSort. A, results for LAK cells after 7 days of cultivation; B, results for IL-12-induced LAK cells after 14 days of cultivation.

Fig. 4. Dose dependence of LAK cell cytotoxicity with MUC1 × CD3 BsAb. The cytotoxicity of LAK cells, activated with immobilized OKT-3 MAb plus IL-2, was determined by 48-h MTT assay in the presence of BsAb (0.05–1.0 μg/ml). Target cells (5000 cells/well) were cultured with effector cells (E:T = 5:1) at various concentrations of BsAb. Columns, mean percentage of cytotoxicity; bars, SE.
the specificity and broad overexpression by neoplasms arising from the bile ducts. It has a unique extracellular domain consisting of tandem repeats of 20 amino acids and many O-linked glycosylation sites where the state of glycosylation varies between normal and malignant cells. It has been found that the tumor-associated MUC1 epitopes are exposed by underglycosylation (25) and are featured on various epithelial cancers (26), such as pancreas, stomach, and ovarian cancer (27). Immunohistochemical studies using anti-MUC1 MAb (MUSE11; Refs. 28 and 29) demonstrated most BDC tissues to be MUC1 positive. Most normal cells are negative, although pancreas, kidney, and breast tissues are exceptionally very weakly positive. The expression and specificity of MUC1 antigen are thus suitable for a target antigen for adoptive immunotherapy, especially for construction of BsAbs against BDC.

The MUC1 × CD3 BsAb described here reacts with both LAK cells and MUC1-positive tumor cells (Fig. 2), and examination of cytotoxicity by MTT assays revealed MUC1 × CD3 BsAb augmentation of LAK activity in a dose-dependent manner (Fig. 4). BsAb-induced enhancement was clearly restricted by the BsAb specificity, as shown in Fig. 6, because percentage of cytotoxicity was found to be dependent on the degree of expression of MUC1 antigen on the target cells (Fig. 1). The specificity of the cytotoxicity using BsAb could be confirmed by the blocking test (Fig. 7), where parental MUSE11 MAb was added to the cytotoxicity assay.

The microscopic examination (Fig. 5) demonstrated that LAK cells are indeed directed to attach to tumor cells in the presence of BsAb. With our previous results for HCC, slight in vivo growth inhibition

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Fig. 5. Phase-contrast (inverted) micrographs of cells in the cytotoxicity assay. A, control target cell culture (TFK-1). LAK cells and TFK-1 cells (E:T = 5:1) were cocultured for 24 h with MUC1 × CD3 BsAb (0.5 μg/ml; B) and without BsAb (C). Inhibition of BsAb-dependent attachment of LAK cells was examined by adding OKT3 (10 μg/ml; D) or MUSE11 (10 μg/ml; E) MAb to cocultures with BsAb.

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4 S. Saijo, unpublished observation.
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cells were sensitized with BsAbs before injection, because antitumor activity of the sensitized LAK cells has been demonstrated to be maintained in vitro for more than 24 h (34). As shown in Fig. 10, tumor growth inhibition was obtained by LAK cells sensitized with both kinds of BsAbs (MUC1 × CD3 BsAb plus MUC1 × CD28 BsAb) together. To our knowledge, this growth inhibition is the first documented for the solid epithelial tumor-grafted SCID mouse model, providing evidence that this STT using a combination of BsAbs is a very hopeful one.

In the previous paper (5) describing complete cure of xenografted human tumors, effector cells were generated by stimulating PBMNCs with allogeneic tumor cells (Hodgkin's-derived LS4OCY) in the presence of BsAb (CD3 × CD30; Ref. 5), and adoptive immunotherapy was performed against allogeneic target tumor cells (LS4OCY). In contrast, the effector cells employed in our study are typical LAK cells. In this respect, our model might be more applicable to clinical therapy.

The fact that we obtained different results with in vitro (Figs. 8 and 9) and in vivo (Fig. 10) tests with combination use of BsAbs (MUC1 × CD3 BsAb plus MUC1 × CD28 BsAb) requires explanation. Thus, only the combination of MUC1 × CD3 and MUC1 × CD28 BsAbs was effective in vivo, although it induced similar cytotoxicity (60%) to MUC1 × CD3 BsAb alone in the in vitro case. One possible explanation might have been the difference in concentration of BsAbs used in the in vitro test (0.5 μg/ml) as compared to the in vivo case, where 2.0 μg/ml of the BsAb were used for sensitization of LAK cells. In an additional in vitro test, MUC1 × CD28 BsAb at 2.0 μg/ml demonstrated enhancement of cytotoxicity (47%), which was not induced at 0.5 μg/ml (20%).

Experiments are now ongoing with variation of the MTT assay conditions to cast light on how the two kinds of BsAbs in combination may cause better targeting of effector cells. Concerning another question, why MUC1 × CD3 BsAb did not inhibit in vivo tumor growth in spite of enhanced in vitro cytotoxicity (Figs. 8 and 9), we have no definite answer. However, it is possible that LAK cells sensitized with MUC1 × CD3 BsAb were not able to survive and maintain activity long enough to inhibit tumor growth. To obtain good in vivo effects, costimulatory signals through TCR-CD3 and CD28 are essential. For in vivo analysis of the effectiveness of treatment with the two kinds of

Fig. 6. In vitro targeting of LAK cells to various cancer cell lines by MUC1 × CD3 BsAb. Cytotoxicity was determined by 48-h MTT assay in the presence of BsAbs (0.5 μg/ml) and different numbers of LAK cells. Target cells (5000 cells/well) were cultured with effector cells at various E:T ratios. Cytotoxicity against MUC1-positive cell lines is illustrated in A. □, TFK-1 in the presence of BsAb; ○, OCUC-LM1 in the presence of BsAb; ■, TFK-1 without BsAb; ●, OCUC-LM1 without BsAb. Cytotoxicity of LAK cells against MUC1-weak or -negative cell lines is shown in B. □, HuCCT1 in the presence of BsAb; ○, HT-17 in the presence of BsAb; ●, HuCCT1 without BsAb; ●, HT-17 without BsAb.

by a BsAb with LAK cells was obtained only when BsAb-sensitized LAK cells were inoculated simultaneously with tumor cells, s.c., in Winn's assay. This observation triggered us to determine the combination effects of BsAbs on effector cell activity, and construct more potent effector cells by stimulation with cytokines, to overcome the limits of adoptive immunotherapy.

In addition to OKT-3 (anti-CD3), the MAb to effector cells used for construction of BsAbs in the present study was 15E8 (anti-CD28). CD28, a homodimeric Mr 44,000 molecule belonging to the immunoglobulin superfamily, is a ligand of B7/BB-1 (30). Stimulation by cross-linking between B7/BB-1 and CD28, or anti-CD28 MAb, gives a costimulatory signal to T cells (5, 31–33). This is the reason why this 15E8 was chosen for construction of the second BsAb in the present case.

To assess the potential in vivo action of adoptive immunotherapy, STT of BDC (TFK-1) tumors in SCID mice was performed. LAK

Fig. 7. MUC1-specific cytotoxicity confirmed by inhibition assay. The cytotoxicities of LAK cells were determined by 48-h MTT assay in the presence of MUC1 × CD3 BsAb (0.5 μg/ml) at E:T = 5:1, where 5.0 or 20.0 μg/ml F(ab')2 of MUSE11 MAb were added. Intact OKT3 or L243 MAb (5.0 or 20.0 μg/ml) were also used for the inhibition assay. Columns, mean percentages of cytotoxicity; bars, SE.
BsAbs, the distribution and survival of sensitized LAK cells inoculated into xenografted SCID mice now requires careful examination.

When IL-12-LAK cells were used as effector cells, about 50% of the target cells (TFK-1) were killed in the absence of BsAb in our 48-h MTT assay (Fig. 9). The figure was far larger than for LAK cells without BsAb, which showed a 20% cytotoxicity. Thus, IL-12-LAK cells possess much stronger cytotoxicity than LAK cells, as also suggested earlier (35, 36). Addition of MUC1 × CD3 BsAb to IL-12-LAK cells greatly enhanced cytotoxicity so that almost 80% of target cells were killed, in comparison to the 75% achieved with LAK cells. However, IL-12-LAK cells showed only very slow proliferation under our culture conditions. We therefore experienced considerable difficulty in obtaining enough numbers of IL-12-LAK cells for inoculation into xenografted SCID mice. This is the reason why we used regular LAK cells for the in vivo experiment in the present case.

In the future, we hope to enhance antitumor effects in this model by using IL-12-LAK cells generated by improved culture conditions, or other cytokine-stimulated cells. In vivo administration of IL-12 (36–39) along with BsAbs featuring anti-CD28 MAb or superantigen-conjugated antitumor MAb (40) may also allow more potent antitumor effects to be achieved. In addition, administration of MUC1

Fig. 8. Cytotoxicity of LAK cells with combinations of BsAbs against TFK-1, determined by 48-h MTT assay. Percentages of cytotoxicity demonstrated are in the absence of BsAb (BsAb(−)); in the presence of MUC1 × CD3 BsAb (0.5 μg/ml), MUC1 × CD28 BsAb (0.5 μg/ml), and in the presence of MUC1 × CD3 BsAb plus MUC1 × CD28 BsAb (0.5 μg/ml for each BsAb). Columns, mean percentage of cytotoxicity; bars, SE). E:T = 5:1.

Fig. 9. Cytotoxicity of IL-12-LAK cells with BsAbs against TFK-1, determined by 48-h MTT assay without BsAb (BsAb(−)) and with MUC1 × CD3 BsAb alone or combined with MUC1 × CD28 BsAb. Columns, mean percentage of cytotoxicity; bars, SE). E:T = 5:1.

vaccine cells (MUC1 gene-transfected cells) might be an alternative to enhance adoptive immunotherapy of BDC.

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