Effective Adoptive Immunotherapy by T-LAK Cells Retargeted with Bacterial Superantigen-conjugated Antibody to MUC1 in Xenografted Severe Combined Immunodeficient Mice

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INTRODUCTION

The prognosis of patients with BDC is known to be poor because of the difficulty of curative resection due to irregular tumor invasion to lymphatic tissues and/or perineural spaces (1). Even adjuvant chemotherapy or radiotherapy after surgery generally cannot achieve satisfactory results. To improve this situation, immunotherapy has attracted a great deal of attention. In a previous study of human BDC-grafted SCID mice, specific targeting therapy, consisting of i.v. administration of T-LAK cells sensitized with two kinds of bispecific antibodies, i.e., anti-CD3 × anti-MUC1 and anti-CD28 × anti-MUC1 antigen (2), demonstrated remarkable inhibition of tumor growth. 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 SAgs. SAgs, named by White et al. (3), are the most potent known activators of human T lymphocytes. They bind outside of the peptide-binding groove of MHC class II molecules and activate T cells expressing a certain Vβ type of TCR, unlike the case with conventional antigen recognition (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 in this study. 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. To test this hypothesis, in vitro and in vivo experimental studies were carried out. In this study, we report the first effective adoptive immunotherapy by human T-LAK cells with SEA-conjugated antibodies in BDC solid tumor-grafted SCID mice.

MATERIALS AND METHODS

mAbs. For induction of T-LAK cells and construction of SEA-MUSE11, the following mAbs were used: OKT-3 (anti-CD3, mouse IgG2a) and MUSE11 (anti-MUC1, mouse IgG1) produced by Hinoda (5). These mAbs were purified from sera and ascitic fluid of mice inoculated with hybridoma cells by caprylic acid treatment in combination with the ammonium sulfate method (6). For the flow cytometry analysis, W6/32 (anti-MHC class I), L243 (anti-MHC class II), WT31 (anti-TCR-β; Becton Dickinson, San Jose, CA), 11F2 (anti-TCR-γ; Becton Dickinson), and MUSE11 mAbs were used as the first antibodies, and FITC-conjugated goat anti-mouse IgG (6250, Biosource International, Camarillo, CA) was used as the second antibody.

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. Both were established in our laboratory (7) and cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 10 units/ml penicillin, and 100 μg/ml streptomycin (designated as culture medium below).

Reagents. SEA (AP101) was purchased from Toxin Technology, Inc. (Sarasota, FL). Rabbit anti-SEA Ab (S-7566) was purchased from Sigma Chemical Company (St. Louis, MO). Recombinant human IL-2 was kindly supplied by Shionogi Pharmaceutical Co. (Osaka, Japan).

Effector Cells. For induction of T-LAK cells, peripheral blood mononuclear cells, isolated by density gradient centrifugation from a healthy volunteer, were cultured for 48 h in culture medium supplemented with 100 IU/ml recombinant human IL-2 at a cell density of 1 × 10^6/ml in a culture flask (A/S NUNC, RosKilde, Denmark) precoated with OKT-3 mAb (10 μg/ml). The T-LAK cells were then transferred to another flask and expanded in culture medium containing 100 IU/ml IL-2 for 2–3 weeks.

Production and Purification of SEA-conjugated MUSE11 and F(ab')2 of the MUSE11 Ab. The MUSE11 mAb was purified from sera and ascitic fluid of mice inoculated with hybridoma cells and its F(ab')2 was obtained by digestion with papain in 0.1 M acetate buffer (pH 5.5) at 37°C for 6 h at a concentration of 15 mg/ml and diluted to 1:10 with 40 mM sodium phosphate/150 mM sodium chloride, pH 7.5. For conjugation, mAb or F(ab')2 of mAb (12 mg/ml) was mixed with a 3-fold molar excess of SPDP (Pierce Chemical Co.), which had been dissolved in DMSO at a concentration of 64 mg/ml and diluted 1:10 with 40 mM sodium phosphate/150 mM sodium chloride, pH 7.5 (8). The mixture was incubated at room temperature for 2 h with gentle rocking under sterile conditions, then applied to a Sephadex G25-prepacked PD-10 column.
Adoptive Immunotherapy with Sea-Conjugated Antibody

Reactivities of TFK-1, HT-17, and T-LAK cells to various antibodies and SEA. These results were obtained by flow cytometry using W6/32 (anti-class I), L243 (anti-class II), WT31 (anti-TCRαβ), 11F2 (anti-TCRγδ), MUSE11 (anti-MUC1), and SEA-F(ab')2 of MUSE11 (anti-MUC1) as the first Ab and FITC-conjugated goat antimouse IgG as the second Ab. Binding of SEA, SEA-MUSE11, and SEA-F(ab')2 to these cells was determined. Cells were first incubated with SEA, SEA-MUSE11, or SEA-F(ab')2. After washing, rabbit anti-SEA as the first Ab and FITC-conjugated goat antimouse IgG as the second Ab were applied for staining. With binding tests of SEA-MUSE11 and SEA-F(ab')2, similar results were obtained when FITC-conjugated goat antimouse IgG Ab was used for determination, instead of rabbit anti-SEA as the first Ab and FITC-conjugated goat antirabbit IgG as the second Ab. Figures in the table are percentages of positively staining cells. Figures in the parentheses are relative mean fluorescence intensities, calculated as the ratios of mean fluorescence intensities of test cells to negative controls. Data are from triplicate determinations.

Table 1  Flow cytometric analyses of the reactivities of TFK-1, HT-17, and T-LAK cells to antibodies

<table>
<thead>
<tr>
<th>Antigen examined</th>
<th>mAb</th>
<th>TFK-1</th>
<th>HT-17</th>
<th>T-LAK</th>
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<tr>
<td>MHC class I</td>
<td>W6/32</td>
<td>NDa</td>
<td>ND</td>
<td>99.9 (99.6)</td>
</tr>
<tr>
<td>MHC class II</td>
<td>L243</td>
<td>4.03 (2.1)</td>
<td>1.13</td>
<td>96.0 (95.0)</td>
</tr>
<tr>
<td>TCRαβ</td>
<td>WT31</td>
<td>ND</td>
<td>ND</td>
<td>93.3 (42.4)</td>
</tr>
<tr>
<td>TCRγδ</td>
<td>11F2</td>
<td>ND</td>
<td>ND</td>
<td>9.8 (9.7)</td>
</tr>
<tr>
<td>MUC1</td>
<td>MUSE11</td>
<td>92.2 (36.4)</td>
<td>1.65</td>
<td>0.84</td>
</tr>
<tr>
<td>MUC1</td>
<td>MUSE11 F(ab')2</td>
<td>88.7 (18.8)</td>
<td>1.25</td>
<td>0.10</td>
</tr>
<tr>
<td>SEA binding</td>
<td></td>
<td>2.33 (1.2)</td>
<td>0.82</td>
<td>99.9 (15.3)</td>
</tr>
<tr>
<td>SEA-MUSE11 binding</td>
<td></td>
<td>72.2 (42.4)</td>
<td>1.84</td>
<td>92.7 (10.0)</td>
</tr>
<tr>
<td>SEA-F(ab')2 binding</td>
<td></td>
<td>70.1 (21.8)</td>
<td>ND</td>
<td>70.5 (7.1)</td>
</tr>
</tbody>
</table>

a ND, not done.

Fig. 1. TN of T-LAK cells with various concentrations of Abs and SEA against TFK-1 cells. Various concentrations of MUSE11 mAb, SEA, SEA plus MUSE11 mAb (SEA-MUSE11, 1:1), SEA-MUSE11, or SEA-F(ab')2 were added to T-LAK cells. The TN index was determined by 48-h MTS assay at an E:T ratio of 5:1. Columns show the results (means; bars, SD) of multiplicate determinations; *, P < 0.001 for 0.01-1 µg/ml versus 0 µg/ml for each group (by Student's t test).

Analysis by Flow Cytometry. Flow cytometric analysis was performed on a FACScan flow cytometer using CellQuest software (Becton Dickinson, San Jose, CA).

Binding of SEA, SEA-MUSE11, and SEA-F(ab')2 to TFK-1, HT-17, or T-LAK Cells. Cells (5 × 10⁵) were first incubated with 50 µl (5 µg/ml) of SEA, SEA-MUSE11, or SEA-F(ab')2. After washing, they were exposed to rabbit anti-SEA as the first Ab and then to FITC conjugated goat anti-rabbit IgG (H + L, 0833, Immunotech Inc., Westbrook, ME) as the second Ab. The stained cells were analyzed by flow cytometry.

TN Assay. TN assay was determined with an MTS assay kit (CellTiter 96AQEOUS nonradioactive cell proliferation assay; Promega Co. Madison, WI; Ref. 9). Target cells, detached with 0.02% EDTA-PBS solution, were adjusted to 10⁵/ml with culture medium, and 10,000 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 adhesion to the wells of the plate, and after removing the culture medium by aspiration, 50 µl of various concentrations of SEA-MUSE11, SEA-F(ab')2, SEA, or MUSE11 mAb in culture medium and 50 µl of effector cell suspension were added to each well. E:T ratios tested in this study were 2.5 and 5. After culture for 48 h at 37°C, each well was washed with 100 µl of PBS three times to remove effector cells and dead target cells. This was followed by the addition of 95 µl/well of culture medium and 5 µl/well of a fresh mixture of MTS/phenazine methosulfate solution (Promega Co.). The plates were read on a microplate reader (Bio-Rad model 3550) at 490 nm. The plates were read on a microplate reader (Bio-Rad model 3550) at 490 nm.
ADOPTIVE IMMUNOTHERAPY WITH SEA-CONJUGATED ANTIBODY

Fig. 2. TN of T-LAK cells prestimulated by SEA. T-LAK cells were incubated at 37°C for 6 h with 0.1 µg/ml or 0.5 µg/ml of SEA, washed, resuspended in culture medium, and applied to the TN assay (48-h MTS assay; E:T, 5:1) of TFK-1 cells. Columns show the results (means; bars, SD) of multiple determinations.

nm after incubation for 1–3 h at 37°C. The TN index (2) was calculated as follows: TN index (%) = \{1 - (A_{490} of experiment/A_{490} of control)\} × 100.

Blocking Tests. To confirm the specificity of the SEA-MUSE11 enhanced TN index, blocking tests using MUSE11 and anti-SEA antibodies were performed. For this, T-LAK cells in the presence of SEA-MUSE11 (0.5 µg/ml) underwent 48-h MTS assays at an E:T ratio of 2.5, with rabbit anti-SEA Ab (0–30 µg/ml) or MUSE11 mAb (0–50 µg/ml) added for blocking.

In Vivo Tumor Models. SCID mice (Fox CHASE C.B.17/Icr-Scid Jcl), 6–8 weeks of age, purchased from Japan Clea (Tokyo, Japan), were inoculated with 5 × 10⁶ TFK-1 cells s.c. into the dorsal thoracic wall on day 0. The treatments were initiated on day 10 after tumor inoculation. For 4 consecutive days, the mice received adoptive immunotherapy, i.e., 2 × 10⁷ T-LAK cells s.c. into the dorsal thoracic wall on day 0. Then each xenografted mouse received i.v. via the tail vein 500 IU (2 µl) IL-2 and T-LAK cell suspension (2 × 10⁷/mouse/day) in 0.15 ml of PBS without washing out antibodies and SEA. Tumor size was measured with a caliper weekly for 10 weeks, and tumor weight (W) in mg was calculated from linear measurements of the width (A) in mm and the length (B) in mm as follows (10):

\[ W = \frac{A^2 \times B}{2} \]

Statistical Analysis. Results were compared using Student’s t test.

RESULTS

Reactivities of TFK-1, HT-17, and T-LAK Cells. Data for reactivities of TFK-1, HT-17, and T-LAK cells to various antibodies, SEA, and SEA-conjugates, as assessed by flow cytometry, are summarized in Table 1. TFK-1 cells had strong reactivities with MUSE11, SEA-MUSE11, and SEA-F(ab')2 but were not reactive with SEA. HT-17 cells, used as a control, had no reactivity with SEA and SEA-MUSE11 because of their lack of expression of the MUC1 antigen and MHC class II molecules. On the other hand, most T-LAK cells in this study expressed MHC class I, class II, and TCRαβ but demonstrated only negligible MUC1 antigens. Consequently, T-LAK cells had strong reactivities with SEA, SEA-MUSE11, and SEA-F(ab')2. The results suggest that both SEA-MUSE11 and SEA-F(ab')2 are able to bind well to TFK-1 and T-LAK cells but not HT-17 cells, indicating that SEA-conjugates are appropriate for retargeting T-LAK cells.

SEA-Antibody-mediated T-LAK Cell TN. Various concentrations of MUSE11 mAb, SEA, SEA plus MUSE11 mAb (mixture of SEA and SEA-MUSE11 mAb at 1:1 by molar ratio), SEA-MUSE11, or SEA-F(ab')2 were added to TFK-1 target cells and T-LAK effector cells in the MTS TN assay (Fig. 1). The TN index of T-LAK cells alone was ~20%, and this was much enhanced (to ~60%) by the addition of SEA-MUSE11 or SEA-F(ab')2, even at 0.01 µg/ml, indicating applicability for in vivo experiments in which the dose of SEA should be kept as low as possible. Addition of the mixture (SEA plus MUSE11 mAb) did not enhance TN index statistically, although the mean value was slightly elevated.

TN of T-LAK Cells Prestimulated by SEA. To confirm the effect of SEA on T-LAK cells, the TN due to T-LAK cells prestimulated by

Fig. 3. Blocking tests with various concentrations of MUSE11 and anti-SEA Abs. TN of T-LAK cells was determined by 48-h MTS assay in the presence of SEA-conjugated MUSE11 Ab (0.5 µg/ml) at an E:T of 2.5:1, with various concentrations of rabbit anti-SEA or MUSE11 Abs added. Columns show the results (means; bars, SD) of multiplicate determinations.

Table: Blocking Tests with Various Concentrations of MUSE11 and Anti-SEA Abs

<table>
<thead>
<tr>
<th>E/T=2.5</th>
<th>0</th>
<th>0.5</th>
<th>0.5</th>
<th>0.5</th>
<th>0.5</th>
<th>0.5</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEA-MUSE11</td>
<td>0</td>
<td>0.1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>anti-SEA</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
<td>1.0</td>
<td>10</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>MUSE11</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
<td>1.0</td>
<td>10</td>
<td>20</td>
<td>50</td>
</tr>
</tbody>
</table>

(µg/ml)
ADOPTIVE IMMUNOTHERAPY WITH SEA-CONJUGATED ANTIBODY

Sea was measured (Fig. 2). T-LAK cells were preincubated at 37°C for 6 h with 0.1 or 0.5 μg/ml Sea and then washed and applied for the TN assay against TFK-1 cells. The direct TN index of T-LAK cells was slightly increased by this Sea prestimulation, indicating that the significant enhancement of T-LAK cell TN in the presence of Sea-MUSE11 or Sea-F(ab')2 (Fig. 1) was dependent not only on increased targeting ability but also on direct Sea stimulation of T-LAK cells, although this increase of T-LAK cell TN with Sea was nonspecific.

Specificity of TN. To verify the specificity of the TN in this study, blocking tests were performed. When MUSE11 or anti-Sea antibodies were added to the assay system, significant dose-dependent reduction of the TN index was observed (Fig. 3). Enhancement of T-LAK cell TN by Sea-MUSE11 was not obtained, even at 1.0 μg/ml, when the hepatocellular carcinoma cell line HT-17 was used as a MUC1-negative target (Fig. 4). These results indicate that the effects of Sea-MUSE11 or Sea-F(ab')2 on T-LAK cells are dependent on the specificity of the MUSE11 antibody.

Attachment of T-LAK Cells Mediated by SEA-MUSE11. TFK-1 cells and T-LAK cells were observed during the TN assay using a phase-contrast microscope (Fig. 5). When Sea-MUSE11 was added to TFK-1 and T-LAK cells in coculture, T-LAK cells started to surround TFK-1 cells after only 1 h. This phenomenon did not occur in the absence of Sea-MUSE11.

Experimental Treatments of Xenografted SCID Mice. Experimental treatments were initiated 10 days after s.c. inoculation of 5 × 10⁶ TFK-1 cells into the dorsal thoracic walls of SCID mice. For 4 consecutive days, xenografted SCID mice received injections i.v. of IL-2 (500 IU) and T-LAK cells (2 × 10⁷ cells) preincubated with 2 μg of Sea-MUSE11, Sea-F(ab')2, MUSE11 mAb, F(ab')2 of MUSE11 mAb, or Sea at 4°C for 1 h in 0.15 ml PBS. Each group consisted of 15–25 mice. The tumor size was measured weekly for 10 weeks (Fig. 6). With both Sea-MUSE11 and Sea-F(ab')2, marked inhibition of tumor growth was observed from an early phase and continued throughout the experimental period. The average inhibition was ~70%, but no complete disappearance of tumors was noted.

DISCUSSION

Bacterial Sags are the most potent known activators of human T lymphocytes; they bind to the outside of the peptide-binding groove of MHC class II molecules and present to T cells in an unprocessed form. Only T cells expressing a certain Vβ of the TCR recognizing Sags are activated, unlike the case with conventional antigen recognition (4).
T-LAK cells were incubated with 0.15 ml PBS containing 2 μg of SEA-MUSE11 (A). Specific targeting ability against MUC1 antigen on the target cells was demonstrated using polyclonal rabbit anti-human CD3 antibody, were more numerous after injection with SEA-MUSE11 than in the control groups (T-LAK alone, T-LAK injected with SEA, and T-LAK injected with MUSE11 Ab). This correlated with results for adoptive immunotherapy in xenografted mice.

Marked reduction of tumor growth was observed for T-LAK cells with SEA-MUSE11 or SEA-F(ab')2 in the tumor-xenografted model. Although identical TN indexes were obtained for these two groups in vivo (Fig. 1), the degree of tumor regression did vary slightly. It might be expected that SEA-F(ab')2 would confer higher cytotoxicity and tumor infiltration due to its smaller size than SEA-MUSE11, but the affinity difference between SEA-MUSE11 and SEA-F(ab')2 (Table 1) probably caused the difference in the in vivo results. To reduce the side effects of SAg's, SEA-F(ab')2 were administered to TFK-1-xenografted SCID mice. For this purpose, use of mutated SEA (26), which has minimum class II binding, though maintaining the potential for T cell activation, should be considered for future study.

The reasons for selection of the MUC1 antigen as the target antigen in this study were its specificity and broad overexpression in various epithelial cancers, such as those arising in the pancreas, stomach, ovaries, and bile ducts (27–29). Our previous study (2) demonstrated that specific targeting therapy with T-LAK cells and two kinds of bispecific antibodies (anti-CD3 × anti-MUC1 and anti-CD28 × anti-MUC1) inhibited the growth of xenografted human BDC, with the percentage cytotoxicity found to be dependent on the degree of the MUC1 expression on the target cells. It has been reported that the breast cancer-associated antigen, DF3/MUC1, deletes activated T cells by induction of apoptosis and thus contributes to the paucity of the anti-breast cancer immune response (30). However, successful induction of anti-MUC1 cytotoxic T cells has been reported (31). Recently, we obtained killer cells with remarkable cytotoxicity by mixed lymphocyte tumor cell culture of peripheral blood mononuclear cells with autologous MUC1 vaccine cells. The mechanism of induction of killer cell apoptosis by MUC1 antigen is still controversial. With regard to this problem, administration of activated T-LAK cells re-targeted with SEA-MUSE11 may be an effective approach to avoid this inhibition of the antitumor immune response by MUC1.

The injection of SAg into mice first induces strong T-cell activation, followed by functional inactivation and deletion of T cells (32).
It has been described that SEA-induced hypersensitiveness involves CD4+ cell deletion and a failure to produce cytokines in the remaining CD4+ T-cell compartment, whereas IFN-γ production and cytotoxicity by the CD8+ T-cell compartment remain relatively intact (33). However, IL-2 enhances and prolongs SEA-conjugated CTL activity after injection of SEA, and exogenous IL-2 prevents the reduction in cytotoxic activity after repeated injections with SEA (34, 35). Therefore, we consider IL-2 administration in combination with SEA-conjugated Ab as essential to obtain the best advantage of SEA for satisfactory antitumor effects. Although we used only T-LAK cells as effector cells in this study, cytokine production from local T cells in the tumor area by stimulation of SEA-MUSE11 would be expected to cause synergistic effects on T-LAK cell activity in clinical treatment.

For application of SEA-conjugated Ab for clinical trials, single-chain Fv antibody reconstituted from MUSE11 Ab gene may be useful because it lacks the Fc portion. Single-chain Fv antibody causes only a low immunogenic response in the host due to its fast clearance from the circulation and therefore may not present a problem, even after repeated administration (36).

In conclusion, this is the first report of effective adoptive immunotherapy by human T-LAK cells with SEA-conjugated antibodies in human s.c. tumor-grafted SCID mice. Although there are various limitations, the results point to potential application in clinical therapy for patients with BDC or MUC1-positive carcinomas.

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REFERENCES


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