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# Feasibility Study of Adoptive Immunotherapy for Metastatic Lung Tumors Using Peptide-pulsed Dendritic Cell-activated Killer (PDAK) Cells

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**Abstract.** *We have established a novel culture system to generate effector lymphocytes designated as peptide-pulsed dendritic cell-activated killer (PDAK) cells using cultured dendritic cells (DCs), synthetic peptide, peripheral blood lymphocytes, and interleukin-2 plus immobilized anti-CD3 antibody. A feasibility study of an adoptive immunotherapy trial using PDAK cells was conducted on HLA-A2 and HLA-A24 cancer patients with antigen-positive lung metastasis that was defined by serological analysis or PCR analysis. Eleven patients with lung metastasis participated in the study: 6 with colorectal cancer, 2 with pancreatic cancer, 1 each with breast and lung cancer, and 1 with melanoma. The patients received either Muc-1, CEA, gp100, Her-2 or SART-3-PDAK cells generated in vitro, intravenously in combination with 350,000 U IL-2 weekly for 9 weeks, together with a planned dose-escalation schedule of three transfers each of $1x10^7$, $3x10^7$ and $1x10^8$ PDAK cells/kg for 6 patients, and with a uniform dose of $3x10^7$ PDAK cells/kg for the remaining 5 patients. Peptide/HLA-specific cytotoxic activity and TCRVβ gene usage of PDAK cells were analyzed. All transfers of PDAK cells, which showed peptide/HLA-specific lysis, were well-tolerated in all patients, and adverse effects (elevation of transaminase, fever, and headache) were observed primarily at grade 1, but in no case greater than grade 2. The generation of sufficient cells to treat the patients with $3x10^7$ PDAK cells/kg was feasible using our culture system, but we were able to generate and administer the dose of $1x10^8$ PDAK cells/kg in only one patient. One partial response (PR) of lung metastasis occurred in a pancreatic cancer patient who received $3x10^7$ Muc-1-PDAK cells/kg. The cytolytic units of PDAK cells in this patient appeared to be substantially higher compared to those in PD patients. TCR gene usage analysis on PDAK cells revealed preferential usage of TCRVβ segments. These results suggest that adoptive immunotherapy using PDAK cells for cancer patients with antigen-positive lung metastasis is safe and feasible, and tumor response should be examined in a future clinical trial.*

*Abbreviations:* CEA, carcinoembryonic antigen; CD, cluster of differentiation; CTL, cytotoxic T-lymphocyte; FCM, flow cytometry; GM-CSF, granulocyte-macrophage colony stimulating factor; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; RT-PCR, reverse transcription-polymerase chain reaction; SART, squamous cell carcinoma antigen recognized by T cells; TCRV, T-cell receptor variable region; TNF, tumor necrosis factor.

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*Key Words:* Adoptive immunotherapy (AIT), peptide-pulsed dendritic cell-activated killer (PDAK) cells, T-cell receptor (TCR), lung metastasis.

The discovery and molecular cloning of the crucial lymphocyte growth factor, interleukin-2 (IL-2) (1), has facilitated the clinical application of adoptive immunotherapy (AIT) for cancer using autologous lymphocytes activated *in vitro* with IL-2. Disease-associated immunosuppression in patients with cancer can disturb the effective emergence of anti-tumor responses *in vivo* (2). Therefore, the adoptive transfer of effector lymphocytes which have been educated and activated *ex vivo* to recognize tumor cells would, theoretically, provide an effective treatment for cancer. Of the techniques developed to date, the use of lymphokine-activated killer (LAK) cells (3), autolymphocyte therapy (ALT) (4) and tumor-infiltrating lymphocytes (TILs) (5) have been the best studied. While these approaches have not yet consistently shown great benefit for metastatic cancer (6), the conditioning chemotherapy regimen that enhances tumor responses of TIL therapy has recently been published (7).

We have conducted *ex vivo* cell therapy for cancer treatment using activated autologous lymphocytes, including

LAK cells, TILs and tumor-sensitized lymphocytes (8). The clinical results of trials using these activated lymphocytes, however, have demonstrated their limited tumor response in a fraction of patients with lung metastasis of renal cell carcinoma by systemic administration of effector cells (CR+PR=9%) (8). Locoregional administration of TILs has shown favorable results (77%) to reduce malignant effusions (8). These results suggest that effector cells, which express stronger and more specific cytotoxic activity, may result in better clinical efficacy, and that clinical trials of AIT using effector cells may be planned for metastatic lung tumors or locoregional administration. The success of adoptive cellular therapy depends on the ability to optimally produce cells equipped with the desired antigenic specificity, and then induce cellular proliferation while preserving the effector function and trafficking abilities of the lymphocytes (9).

In developing new approaches to AIT for patients with metastatic cancer, the increasing molecular understanding of antigen presentation and recognition has highlighted the use of professional antigen-presenting dendritic cells (DCs) (6, 9). In a previous work, we discussed a novel system for generating cytotoxic effector lymphocytes using antigenic peptides and cultured DCs, designated as peptide-pulsed DC-activated killer (PDAK) cells (10). In the present paper, we report on a clinical study of AIT using PDAK cells for patients with antigen-positive metastatic lung tumors which provides evidences in favor of the safety, feasibility and anti-tumor activity of this type of AIT.

## Patients and Methods

*Patients.* Patients were eligible if they were HLA-A0201 or -A24 adults under 80 years old who had histologically-confirmed cancer with antigen-positive lung metastases that were refractory to standard therapy. Antigen expression of the tumor was ensured by serum carcinoembryonic antigen (CEA) levels, or reverse transcription-polymerase chain reaction using primers specific for Muc-1 (11), gp100 (12), Her-2 (13) and SART-3 (14) on the primary tumors and biopsy samples of metastatic lung tumors. They had Eastern Cooperative Group performance status 0 to 3 (15), and adequate bone marrow, hepatic and renal functions. Exclusion criteria included the following: uncontrolled infection; uncontrolled diabetes mellitus; overt autoimmune disease; concomitant use of corticosteroids; and history of interstitial pneumonia and pulmonary fibrosis. Patients who had received antitumor drugs within the preceding 4 weeks were also ineligible.

*Study design.* The study was an open-label, non-randomized, dose-escalation study, and was performed at Hiroshima University Hospital since 2000. The protocol was approved by the institutional review board, and all of the patients gave written informed consent. The patients received either Muc-1, CEA, gp100, Her2, SART-3-PDAK cells intravenously in combination with 350,000 U IL-2 (Shionogi, Japan) weekly for 9 weeks, together with a planned dose-escalation schedule of three transfers each of 1 x $10^7$, 3 x $10^7$ and 1 x $10^8$ PDAK cells/kg for 6 patients, and with a uniform dose of 3 x $10^7$ PDAK cells/kg for the remaining 5 patients. Adverse effects and tumor responses were carefully evaluated after every three transfers. Toxicity was assessed using the National Cancer Institute common toxicity criteria version 2.0. All patients were monitored clinically using imaging analysis such as chest X-ray and computed tomographic examinations and clinical efficacies were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) (16).

Table I. *Antigenic peptides used for PDAK cell induction.*

| Antigen | HLA | Peptide sequence | Reference |
|---|---|---|---|
| Muc-1 | A2 | STAPPAHGV | 19 |
| | A24 | GVTSAPDTRPAPGSTAPPAH | 20 |
| CEA | A2 | YLSGADLNL | 21 |
| | A24 | TYACFVSNL | 22 |
| gp-100 | A2 | VYFFLPDHL | 23 |
| Her-2 | A2 | KIFGSLAFL | 24 |
| SART-3 | A24 | AYIDFEMKI | 25 |

*DC preparation.* DCs were induced using a modification of Romani *et al.* (17). Briefly, peripheral blood mononuclear cells (PBMCs) were collected from patients by the centrifugation of 20 ml heparinized venous blood samples on Ficoll-Conray gradients. PBMCs were allowed to adhere to culture flasks (Sumitomo Berclight, Akita, Japan) for 2 h at 37°C in RPMI-1640 medium containing 2% autologous serum. After removal of the non-adherent cells, adherent cells were cultured in RPMI-1640 medium supplemented with 2% autologous serum, 800 U/ml GM-CSF (IBL, Gunma, Japan), and 500 U/ml IL-4 (IBL). On day 5 of the culture, 100 U/ml TNF-α (IBL) was added and cells were cultured for another 2 days. The floating cells were collected as DCs. DCs were analyzed for quality assurance, and the release criteria of cultured DCs were defined with typical morphology (>95% non-adherent veiled cells) and phenotype (>85% HLA class I+, >75% HLA-DR+, >95% CD80+, >75% CD86+, >65% CD83+ and <20% CD14+) (10, 18).

*Generation of PDAK cells.* PDAK cells were generated as mentioned in detail elsewhere (10, 18). In brief, PBMCs were collected and fractionated into adherent and non-adherent cells. DCs, which had previously been prepared as mentioned above, were inactivated with 50 μg/ml mitomycin-C (Kyowahakko, Tokyo, Japan) and pulsed with antigenic peptide (40 μg/ml) for 2 h in RPMI-1640 medium containing 2% autologous serum supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The peptides used in this study are shown in Table I (19-25). The IFN-γ response of the patient's PBMCs to the peptide was confirmed before generating the PDAK cells (26). After 4 washes of DCs, the non-adherent fraction of PBMCs ($10^7$) was stimulated with peptide-pulsed DCs at a responder-to-stimulator ratio of 10, and maintained in RPMI-1640/2% autologous serum medium containing 10 U/ml IL-7 (IBL). Two days later, cells were washed and 80 U/ml IL-2 (Shionogi,

Table II. *Patients enrolled in the study.*

| Case No. | Cancer | Age/Sex | Weight | HLA | P.S. | Metastasis | Prior therapy |
|---|---|---|---|---|---|---|---|
| 1 | pancreas | 52/M | 50 | A24 | 3 | lung, lymph nodes, pleuro-peritoneum | GEM |
| 2 | pancreas | 36/M | 78 | A2 | 1 | lung, liver | GEM |
| 3 | breast | 45/F | 54 | A24 | 0 | lung | CAF, TAX |
| 4 | colon | 59/M | 67 | A2 | 2 | lung, liver | LV, 5FU |
| 5 | melanoma | 76/F | 46 | A2 | 2 | lung, liver | - |
| 6 | colon | 68/M | 55 | A24 | 1 | lung, pleura, lymph nodes | LV, 5FU |
| 7 | colon | 48/M | 60 | A24 | 3 | lung, bone | LV, 5FU , RT |
| 8 | lung | 24/M | 55 | A2 | 0 | lung | CBDCA, TAX |
| 9 | colon | 50/F | 45 | A24 | 0 | lung | LV, 5FU, CPT-11 |
| 10 | rectal | 71/M | 64 | A24 | 0 | lung | LV, 5FU |
| 11 | colon | 78/M | 58 | A2 | 0 | lung, lymph nodes | LV, 5FU, CPT-11 |

GEM, gemcitabine; CAF, cyclophosphamide + adriamycine + 5-fluorouracil; TAX, taxol; LV, leucovorin; 5FU, 5-fluorouracil; CBDCA, carboplatin.

Osaka, Japan) was added. Responder cells were re-stimulated with peptide-pulsed DCs on days 7 and 14 of the culture. Peptide-pulsed DC-activated killer (PDAK) cells ($10^6$ /ml) were further expanded on an anti-CD3 antibody (Janssen-Kyowa, Tokyo, Japan)-coated flask in the presence of 80 U/ml IL-2 (IL-2/CD3 system). The culture medium was half-changed with fresh medium containing IL-2 every 3-4 days. This culture system could usually yield more than $10^9$ PDAK cells per flask. The above procedure was repeated for weekly administrations of PDAK cells in separate cultures. On day 21 or 28, the cells were counted, washed 3 times by saline, filtered through 50 μm mesh, and resuspended in 100 ml saline before administration. Bacterial and endotoxin examinations were made 3 days before and on the day of the administration.

*Peptide/HLA-specific cytotoxic activity of PDAK cells.* To examine the peptide/HLA-specific cytotoxic activity of PDAK cells, a conventional 4-h $^{51}$Cr release assay was performed. The target cells used were T2 (10) and TISI (18) cells for HLA-A2 and A24 patients, respectively, maintained in RPMI-1640 medium supplemented with 10% FCS, 50 units/ml penicillin and 2 mM L-glutamine. Target cells were pulsed with or without 40 μg/ml peptide, which had been used for PDAK cell generation, for 18 h at 37°C and labelled with $^{51}$Cr (100 μCi) for 2 h. Target cells were washed 3 times and plated onto round-bottomed, 96-well microtiter plates at a density of $1x10^4$ cells /0.1 ml. PDAK cells were added over the target cells at various densities in a final volume of 0.2 ml. After 4-h incubation at 37°C, release of $^{51}$Cr in the supernatant was measured by an automated γ counter (Aloka, Tokyo, Japan). The mean percentage of the peptide/HLA-specific lysis of the triplicate wells was calculated by the following formula: ((release by PDAK against peptide-pulsed target) – (release by PDAK against peptide-un-pulsed target))/((maximum release) – (spontaneous release)) x100. The spontaneous release was obtained from the wells of peptide-pulsed target cells alone and was around 15% of the maximum release, which was obtained from wells added with 2% Triton X-100 over the peptide-pulsed target cells instead of PDAK cells. In some experiments, PDAK cells were incubated for 1 h at 4°C with 10 μg/ ml of anti-TCRαβ, TCRVβ6, 12 mAbs (Ortho Diagnostic System, Raritan, NJ, USA), then cytotoxicity assays were carried out. Killer units were the killing activity of PDAK cells multiplied by transferred cell numbers and was calculated at each transfer by the following formula: ((peptide/HLA-specific killing activity of PDAK at an E/T=20) x (transferred cell number) / (body weight $x10^7$).

*Antibody and flow cytometry.* The DCs ($10^5$) and PDAK cells ($5x10^5$) were stained with antibodies, washed, and then analyzed on FACSCan (Becton Dickinson, San Diego, CA, USA). The antibodies used were anti-class I, anti-HLA-DR, anti-CD80, anti-CD86, anti-CD-83, anti-CD14 antibodies for DCs, and anti-CD3, anti-CD4 and anti-CD8 antibodies for PDAK cells. All antibodies used were purchased from Becton Dickinson.

*T-cell receptor gene usage analysis.* Total RNA was extracted from 5 x $10^5$ cells of PBMCs and PDAK cells and reverse-transcribed with random hexamer, as described previously (27). Aliquots of the cDNA were amplified by PCR in separate tubes, using Vβ-specific oligonucleotides and Cβ reverse primer on a DNA thermal cycler (Perkin Elmer, Norwalk, CT, USA). The amplified DNA was confirmed by Southern blot analysis using a Cβ probe with luminol reaction. The light output detected on X-ray film was quantified using NIH-imaging software and a Macintosh personal computer.

*Diagnostic single-strand conformation polymorphism.* To detect the clonotype of the complementarity determining region (CDR) 3 in the PCR product of each TCRβ band, the diagnostic single-strand conformational polymorphism (SSCP) technique was performed (27). In brief, 5 μl of the asymmetric PCR product, which was mixed with 5 μl of 95% formamide containing xylene cyanol and bromophenol blue, was heated at 95°C for 5 min, cooled on ice, and then loaded onto a 10% acrylamide gel. This was run at 100 V for 4 h in a cold room (4°C). The gel was then silver-stained (Silver Stain Plus, BioRad, Hercules, CA, USA).

*Statistical analysis.* Statistical evaluations for experimental values were analyzed using the non-parametric Student's *t*-test.

Table III. *Characteristics of PDAK cells, adverse effects and clinical responses.*

| Case | Disease | Peptide | Step | Total cell No. (x $10^9$) | CD4/CD8[a] | Killer unit (mean units in step 2) | Adverse effects (grade) | Response | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | lung (duration) | tumor marker[b] | total |
| 1 | pancreas | Muc-1 | 1,2 | 10.5 | 36/62 | 71, 65,–, 243, 237, 208, 189, 168, 204 (208) | – | PR[c](4[d]) | stable | PR |
| 2 | pancreas | Muc-1 | 1,2 | 16.4 | 41/76 | 34, 28, 30, 72, 75, 54, 62, 57, 70 (65) | – | SD(3) | increase | PD |
| 3 | breast | CEA | 1,2 | 11.3 | 25/81 | 25,–,–, 54, 66, 64, 57, 54, 69 (61) | hepatic (1) | SD(9) | stable | SD |
| 4 | colon | CEA | 1,2 | 14.1 | 44/69 | 35, 24, 29, 73, 67, 77, 58, 64, 64 (67) | – | SD(3) | increase | PD |
| 5 | melanoma | gp100 | 1 | 0.9 | –/– | –,– | – | NE | - | NE |
| 6 | colon | CEA | 1,2,3 | 23.1 | 28/80 | 60,–, 54, 33, –,75, 169,–, 231(54) | – | SD(3) | decrease | PD |
| 7 | colon | CEA | 2 | 5.4 | 35/42 | 6, 4, 3 (4) | – | PD | increase | PD |
| 8 | lung | Her-2 | 2 | 14.9 | 39/88 | 14, 21,–, –, 15, 18, 9, 11, 8 (14) | – | PD | - | PD |
| 9 | colon | CEA | 2 | 12.2 | 48/36 | 7, 11,–, 10,–, 19,–, 25, 18 (15) | headache (1) | PD | increase | PD |
| 10 | rectum | SART-3 | 2 | 17.3 | 20/77 | 55, 62, 57,–, 83, 77, 72, 68, 66 (68) | – | SD(4) | decrease | SD |
| 11 | colon | CEA | 2 | 15.7 | 32/86 | 93,–, 88, 73, 75, 82, 80, 93,–(83) | fever (1) | SD(4) | decrease | SD |

Patients were administered intravenously with PDAK cells as indicated (step 1, $1x10^7$/kg; step 2, $3x10^7$/kg; step 3, $1x10^8$ PDAK cells /kg), and adverse effects and tumor responses were evaluated. Killer unit was measured at every transfer, as described in Materials and Methods.

[a], mean percentage of PDAK cell phenotypes in step 2.

[b], CA19-9 for pancreatic cancer, and CEA for colon and breast cancers.

[c], PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable.

[d], month for response duration.

## Results

*Patients.* Eleven patients with various types of cancer (6 colon, 2 pancreatic, 1 breast, 1 lung and 1 malignant melanoma) participated in the present study (Table II). There were 8 males and 3 females, and their mean age was 55, with a range from 24 to 76. Five patients had HLA-A2 haplotype and 6 had HLA-A24 haplotype. The patients' Eastern Cooperative Oncology Group (ECOG) performance status was 0, 1, 2 and 3 in 5, 2, 2 and 2 patients, respectively. Seven patients had distant metastases in addition to lung metastases, and all but the melanoma patient had previously been treated with chemotherapy or radiotherapy, which had failed to inhibit tumor growth. Antigen peptides used for generating PDAK cells are shown in Table III. CEA was used for patients who had high serum CEA levels. The other antigen expression was confirmed by RT-PCR analysis for biopsy samples of lung metastasis from cases 1, 5 and 8, but not done in cases 2 and 10. Muc-1 peptide was chosen for case 2 because most pancreatic cancer has been shown to express Muc-1 antigen (11), and SART-3 peptide was chosen for case 10 because SART-3 has been reported to be ubiquitously expressed (14). Before generating PDAK cells, all peptides used were confirmed *in vitro* to stimulate IFN-γ production from patient's PBMCs (data not shown).

*PDAK cells.* PDAK cells could be generated in all patients enrolled (Table III). The total number of PDAK cells infused varied from 0.9 to $23.1x10^9$ cells depending on the patients' body weight and dose steps of the study. PDAK cells generated from 9 out of 10 patients tested showed predominant expansion of CD8 phenotype. In the dosage of step 2, PDAK cells from 7 patients expressed <50, but those from 3 patients showed ≥15 peptide/HLA-specific killer units, which mean peptide/HLA-specific activity of PDAK cells was connected with patient's body weight and cell numbers infused. Mean values of the killer units in the step 2 dosage varied from 4 to 208 among the patients tested.

*Feasibility and toxicities.* Feasibility and toxicity are also shown in Table III. Dose escalation of PDAK cell transfer was performed in 6 patients, with doses of $1x10^7$ and $3x10^7$ PDAK cells/kg given to 6 and 5 patients, respectively, but the planned dose of $1x10^8$ PDAK cells/kg could be administered to only 1 patient. The treatment was stopped in a melanoma patient with 2 transfers of gp-100-PDAK cells and in a colon cancer patient with 3 transfers of CEA-PDAK cells due to rapid disease progression. Grade 1 toxicities, including an increase of transaminase, headache and fever, were found in the breast cancer patient and 2 colon cancer patients who were treated with CEA-PDAK cells. No correlation was observed between toxicities and killer units or total numbers of PDAK cells transferred.

*Tumor response.* Tumor response is shown in Table III. When focused on lung metastasis, growth arrest of the lung tumor, including PR or SD responses, was observed in 7

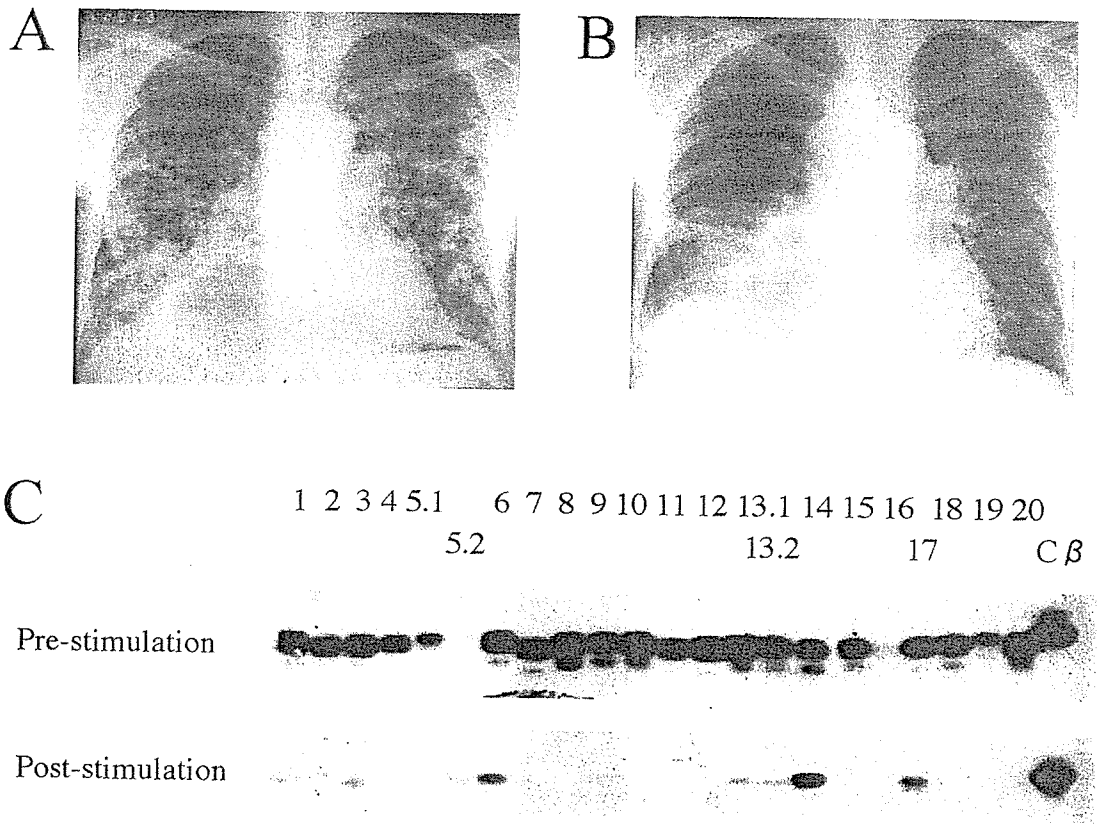


Figure 1. *A pancreatic cancer patient treated with Muc-1 PDAK cells. A 52-year-old male patient with pancreatic cancer who had lung, peritoneal, paraaortic lymph node metastases (case 1) was administered intravenously with Muc-1 PDAK cells according to the dose-escalation schedule, and chest X-ray examination was performed prior to (A) and after (B) the treatment. T-cell receptor gene analysis for Muc-1-PDAK cells was performed prior to and after the stimulation (C).*

out of 11 patients, although 4 of the 7 patients showed tumor progression in distant metastases other than the lung. A pancreatic cancer patient (case 1, Figure 1), who received $3x10^7$ Muc-1-PDAK cells/kg, showed <30% tumor reduction in the lung; in this patient, other distant metastases to the pleuro-peritoneum and lymph nodes were stable for a period of 4 months. A decrease of serum CEA levels was observed in 3 of the 7 colorectal cancer patients, one of whom (case 6, Figure 2) showed growth arrest of lung metastasis with $23.1x10^9$ CEA-PDAK cells, but showed no other tumor response in the pleura or lymph nodes. In an overall assessment, 1 PR (a pancreatic cancer patient), 3 SDs (1 breast and 2 colorectal cancer patients) and 6 PDs were observed.

There was no relationship between tumor response and total numbers of PDAK cells infused. Regarding the killer units, 7 patients with PR or SD response at lung lesions had mean value ± standard deviation of 87±54 activity, while 3 PD patients had that of only 11±6 activity, in the dose level of step 2 (Figure 3). There was a significant difference between these values ($p<0.05$).

*T-cell receptor analysis.* A 52-year-old male patient with pancreatic cancer (case 1) showed a partial response in lung metastasis by Muc-1-PDAK cell transfer (Figure 1A, B). The PBMCs of the patient demonstrated a diverse expression of TCRVβ gene usage before stimulation, while the transferred Muc-1-PDAK cells showed preferential usage of TCRVβ3, 6, 13.1, 13.2, 14 and 17 (Figure 1C).

A 68-year-old male patient with colon cancer (case 6) showed growth arrest of lung metastasis with a decrease in serum CEA level due to CEA-PDAK cell transfer (Figure 2A, B). TCRVβ gene usage analysis clearly demonstrated a difference in TCRVβ expression before and after stimulation, and the preferential usage of TCRVβ1, 2, 3, 5.2, and especially of TCRVβ6 and 12 was indicated (Figure 2C). The CEA-PDAK cells killed T2 target cells pulsed with the CEA peptide, whose cytotoxicity was significantly abrogated in the presence of anti-TCRVβ12 antibody ($p<0.05$), but this did not occur in the presence of anti-TCRVβ6 antibody or the irrelevant control antibody (Figure 2D). SSCP analysis showed clonotypic band pairs of the TCRVβ12 (Figure 2E).

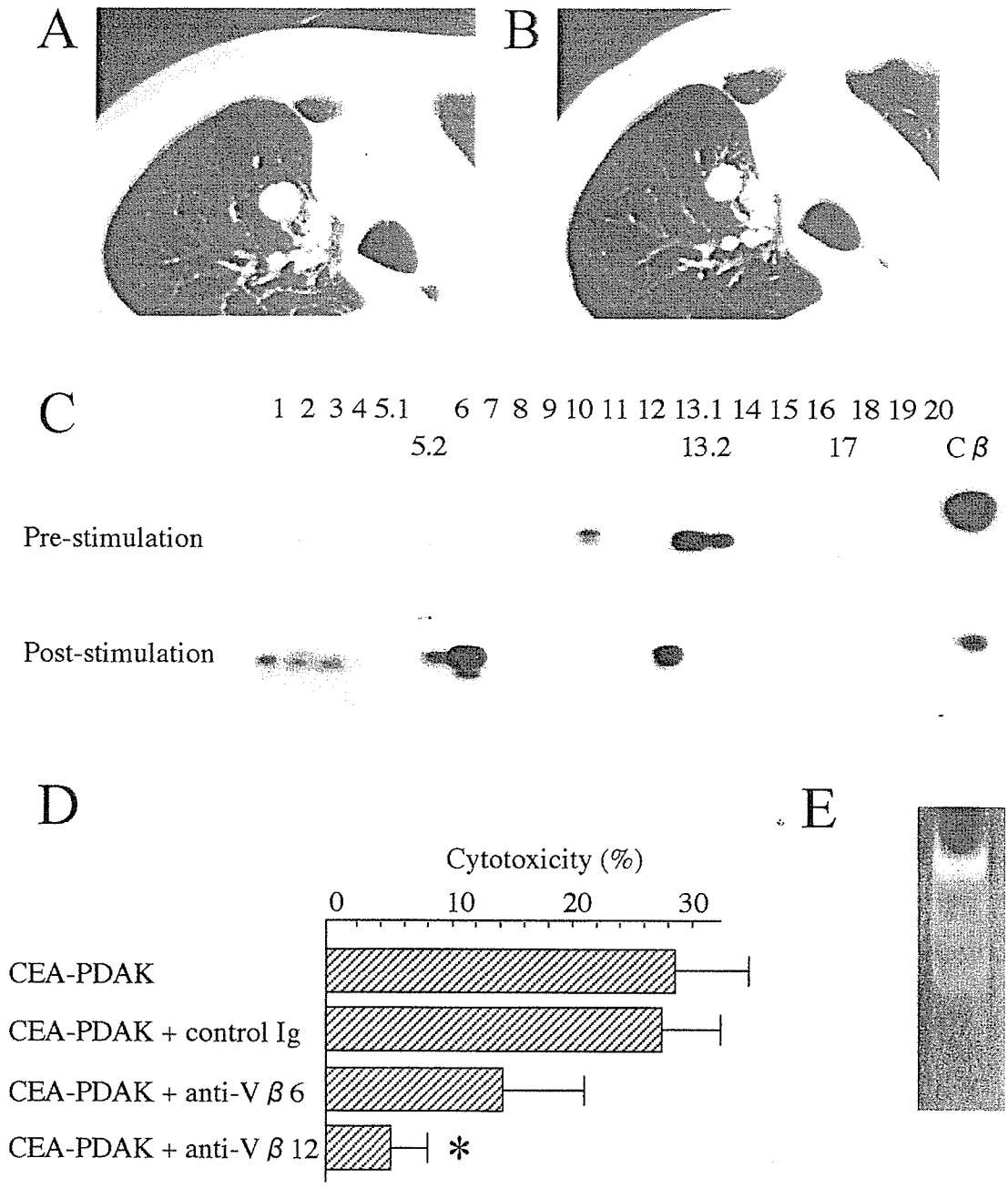


Figure 2. *A colon cancer patient treated with CEA-PDAK cells. A 68-year-old male patient with colon cancer who had lung, pleural and mediastinal lymph node metastases (case 6) was administered intravenously with CEA-PDAK cells, and CT scan examination was performed prior to (A) and after (B) the treatment. Serum CEA levels decreased from 40.5 to 21.8 ng/ml by the treatment. T-cell receptor gene analysis for CEA-PDAK cells was performed prior to and after the stimulation (C). The cytotoxicity of CEA-PDAK cells was determined against CEA peptide-pulsed T2 cells at an E/T ratio of 25 in the presence of the antibodies indicated (D). SSCP analysis was performed for detecting clonotypes of TVRVβ12 (E).*

## Discussion

In the present study, we demonstrated the safety of AIT using PDAK cells for metastatic lung tumors. This is to be expected since AIT using activated lymphocytes has been shown to be essentially safe except in combined administration of high dose IL-2 (28, 29). We also showed that our method of AIT using PDAK cells was feasible at an administration of $3x10^7$ cells/kg, but not at a dose of $1x10^8$ PDAK cells/kg. Importantly, one partial response was observed in a

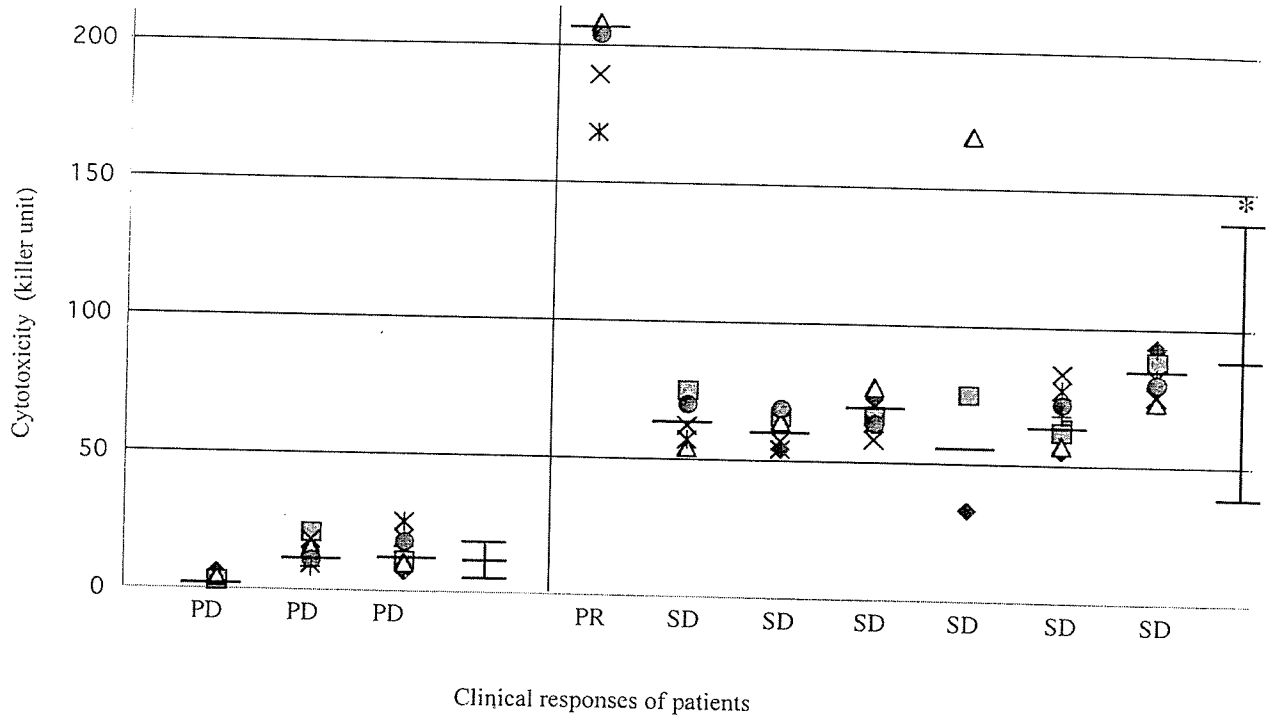


Figure 3. *Cytotoxic activity of PDAK cells in relation to the clinical responses. Killer units were determined before every transfer of PDAK cells, and those at the step 2 dosage of PDAK cells were plotted. Symbols indicate the killer units of PDAK cells at each transfer. A significant difference, *p<0.05.*

pancreatic cancer patient with a dose of $3x10^7$ Muc-1-PDAK cells/kg. Although Simon *et al.* state that dose-finding studies may not be necessary in cell therapy such as tumor vaccine trials (30), our results suggest that the dose of $3x10^7$ PDAK cells/kg is the limitation of our culture system and may be the least dose required for tumor responses.

We could observe only one PR and 6 SDs in metastatic lung lesions and no response in other lesions including liver, lymph node and effusion, by the intravenous systemic administration of PDAK cells. It has been reported that the trafficking of effector cells toward the tumor site is critical for tumor response (5-9). For example, other researchers have attempted to address hepatic tumors by arterial infusion of effector cells through the hepatic artery (31). By intravenous administration, 100% effector cells can reach lung lesions. Moreover, PDAK cells have been shown to express chemotactic chemokine receptor 5 (18), which is reported to be required for locoregional trafficking of effector lymphocytes (32). However, these may not explain the unsatisfactory results of lung lesions in this study by PDAK cell transfer. To augment the efficacies, we may pay more attention not only to generating the effector cells of high quality, but also to conditioning the host immune regulation systems before AIT using PDAK cells. Dudley *et al.* (7) reported the remarkable enhancement of the clinical efficacy of AIT against malignant melanoma using TILs by pre-treating patients with non-myeloablative lympho-depleting chemotherapy.

We measured the peptide/HLA-specific killing activity of PDAK cells and calculated the killer units upon transfer; the number of killer units indicates the total of peptide/HLA-specific killing activity of transferred PDAK cells. It is an interesting question whether or not this parameter of killing activity correlates with clinical responses to AIT using PDAK cells. In the present trial, the number of killer units in PR and SD patients was significantly higher than that in PD patients. In previous AIT trials using LAK cells, it has been reported that neither tumor reduction nor clinical toxicity correlates with dose or with the cytolytic activity of LAK cells, nor are there any correlations with other laboratory parameters including base-line lymphocyte count and IL-2-induced lymphocytosis (29). However, Kawakami *et al.* (23) have reported that tumor regression is correlated with the recognition of gp100 epitopes by the adoptively administered TILs in treating patients with melanoma. Like TILs, but unlike LAK cells, PDAK cells have been shown to recognize tumor cells in a peptide/HLA-specific manner (10, 18). Therefore, the peptide/HLA-specific killing activity of PDAK cells may be involved in clinical results of tumor responses. In addition,

measurement of peptide/HLA-specific killing activity is also important for the quality control of PDAK cells used in the trials (30). This issue for the quality control of the effector cells remains to be addressed in future clinical trials.

In order to generate PDAK cells of high quality, the selection of appropriate peptides may be critical. We have previously reported that the CEA peptide, which can stimulate CTL precursors to produce IFN-γ, differs in individuals among HLA-A24 healthy donors and colorectal cancer patients when tested with a whole blood assay using a CEA peptide panel (26), although CEA652 has been shown to have the most potent binding affinity for HLA-A24 molecules and to induce CEA-reactive CTLs (22). Kedl *et al.* (33) have reported the affinity maturation of a secondary T cell response and shown that high-affinity T cells out-compete lower affinity T cells during a response to antigenic challenge *in vivo*. This suggests that, when generating PDAK cells, inappropriate peptides may induce the affinity maturation of a secondary T cell response that may not be the best for an appropriate CTL generation. In a study by Mine *et al.*, notable tumor responses were realized in peptide vaccine trials, in which only those peptides that were able to stimulate patients' PBMCs to produce IFN-γ were administered (34). We suggest that the peptides to be used in cancer immunotherapy should be selected not only according to the HLA binding affinity of the peptide, but also depending on the patient's CTL precursor status. Thus, the host-oriented peptide evaluation (HOPE) approach may augment tumor responses to AIT using PDAK cells (26).

A more important issue to be addressed may be the identification of the TCRs that are involved in tumor eradication. We have shown preferential usage of TCRs in the PDAK cells in one patient (case 6) whose TCRVβ12 was involved in the recognition of peptides pulsed on experimental target cells. However, we failed to confirm whether or not the TCRs were involved in autologous tumor-specific recognition. In our communicating investigation using Her2-specific CTL clones, Her2 peptide-pulsed T2 cells, and Her2-expressing tumor cells, we have observed that there are 2 types of TCRs, one involved only in peptide/HLA recognition and one which is also involved in tumor recognition; the latter is more important in antigen-based immunotherapy (unpublished data). Identification of the TCR genes which are involved in tumor recognition permits us to produce TCR gene-modified effector cells that are rendered specifically to be reactive with antigen-expressing tumors (35). This approach may accelerate the development of tumor antigen-specific AIT.

In conclusion, AIT for antigen-positive metastatic lung tumors using PDAK cells was found to be both safe and feasible. Based on the present data on dosages, tumor response should be examined in a future clinical trial. Large-scale clinical trials are on-going to prove the efficacy of AIT using PDAK cells against metastatic lung tumors.

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# Generation of Antigen-Presenting Cells Using Cultured Dendritic Cells and Amplified Autologous Tumor mRNA

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**Key Words**
Dendritic cells · Tumor mRNA · Cytotoxic T cells · Peptide · Malignant melanoma of the esophagus

**Abstract**
Novel antigen-presenting cells (APCs) were generated using cultured dendritic cells (DCs) and amplified tumor mRNA, and the potential of tumor antigen-reactive T cell induction by the tumor RNA-introduced DCs (DC/tumor RNA) was analyzed in a patient with melanoma antigen-encoding gene (MAGE3)-positive malignant melanoma of the esophagus. DCs were generated from an adherent fraction of peripheral blood mononuclear cells in the presence of granulocyte macrophage colony-stimulating factor and interleukin-4. Tumor mRNA was purified from tumor tissue, amplified in vitro using a T7 RNA polymerase system, and then introduced into DCs by electroporation (150 V/150 μF or 100 V/200 μF). The gene introduction efficiency was 44–55% as measured by enhanced green fluorescent protein reporter gene expression, and the viability of RNA-introduced DCs was approximately 80%. DC/tumor RNA could induce tumor antigen-reactive cytotoxic T lymphocytes (CTLs) in an mRNA-specific manner, but had no effect on the self-antigen-reactive T cells. DC/tumor RNA could induce the polyspecific antigen-reactive CTL responses mediated by both human leukocyte antigen class I and class II molecules, whereas MAGE3 peptide-pulsed DCs induced only the monospecific MAGE3-reactive CTL responses mediated by human leukocyte antigen class I molecules, showing the superiority of the DC/tumor RNA over the DC/peptide. It is suggested that the use of DC/tumor RNA as antigen-presenting cells may be more effective, convenient and practical for the DC-based anti-cancer immunotherapy.



## Introduction

The identification of the melanoma antigen-encoding gene (MAGE) by Van der Bruggen et al. [1] has contributed greatly to the molecular understanding of antigen presentation and recognition in the immune system [2]. When the immune system recognizes the tumor, tumor-associated antigens (TAAs) are internalized, processed and presented on antigen-presenting cells (APCs) as antigenic epitope peptides in the context of human leukocyte antigen (HLA) molecules [3]. Recently, dendritic cells (DCs) have been the focus of anti-tumor immunotherapy, because they are professional APCs and can ini-

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tiate a primary immune response by recruiting and activating naive T cells [4, 5]. Moreover, functional DCs can be easily generated in vitro from progenitors in the peripheral blood using granulocyte macrophage colony-stimulating factor and interleukin-4 (IL-4) [6]. Many clinical trials employing DCs in anti-tumor immunotherapy have been reported [7–16]. In these trials, available sources of TAAs include identified peptides, native or modified proteins, tumor cell lysates and tumor cells. Previously, we reported using tumor antigen-specific CTLs induced by autologous DCs interacting with identified peptides in a clinical trial [17]. Results of these trials provided strong evidence for the ability of the DCs to induce autologous tumor-specific CTL responses in vivo and in vitro and to stimulate clinically beneficial anti-tumor immune responses.

However, a wider use of DC therapy for tumor patients is limited by the availability of identified TAAs and HLA phenotypes or sufficient tumor tissues for TAA preparation. Gilboa et al. [18, 19] have shown that murine and human DCs transfected with mRNA encoding antigens can stimulate potent CTL responses in vitro and in vivo. Treatment of tumor-baring mice with DCs transfected with tumor RNA led to a significant reduction in metastases or survival benefit [20]. Use of the RNA form as TAAs has one significant advantage since it can be amplified in sufficient amounts from only a few tumor cells by polymerase chain reaction (PCR) [21]. In addition, the transfection of autologous unfractionated tumor mRNA into DCs has the clinical benefit that we must not identify TAAs and HLA phenotypes when educating naive T cells to tumor-specific CTLs in vitro and in vivo.

In this study, we attempted to generate novel APCs by introducing cultured DCs with amplified RNA encoding antigens by electroporation and analyzed them for anti-tumor immune responses in vitro. First, the optimal electroporation conditions for introducing cultured immature DCs with mRNA encoding enhanced green fluorescent protein (EGFP) were tested. Secondly, we tested whether the functional APCs could be generated using in vitro amplified mRNA and cultured DCs in healthy volunteers. Finally, we generated the novel APCs using in vitro amplified autologous tumor-extracted mRNA and cultured autologous DCs in a patient with malignant melanoma of the esophagus, in whom MAGE3 peptides were identified as TAAs. The tumor mRNA-introduced DC system was compared with the MAGE3 peptide-pulsed DC system for the potential to induce anti-tumor immune responses in vitro.

## Materials and Methods

*Cells and Tissue Materials*

Peripheral blood mononuclear cells (PBMCs) from 3 healthy volunteers and a HLA-A24 patient with malignant melanoma of the esophagus were obtained, after receiving written informed consent, by the Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, N.J., USA) density gradient separation method. Subjects underwent subtotal esophagectomy, and tissue materials (malignant melanoma and normal esophageal mucosa) were obtained and snap frozen in liquid nitrogen. A human breast cancer cell line BT-474 from the American Type Culture Collection was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Paisely, UK).

*Reagents*

The following HLA-A24 restricted synthetic peptides (>90% pure) purchased from TaKaRa (Shiga, Japan) were used, i.e. $MAGE3_{76-84}$ peptides (NYPLWSQSY), $MAGE3_{113-121}$ peptides (VAELVHFLL), carcinoembryonic antigen $(CEA)_{10-19}$ peptides (RWCIPWQRLL) and $CEA_{101-108}$ peptides (IYPNASLLI) [22, 23]. Mouse monoclonal antibodies to human HLA-ABC (HLA class I), HLA-DR (HLA class II) and control immunoglobulin were from Pharmingen (San Diego, Calif., USA).

*RNA Extraction, Amplification and in vitro Transcription*

Total RNA from the tissue materials or PBMCs was extracted using the RNeasy kit (Qiagen, Valencia, Calif., USA) according to the manufacturer's protocol. Total RNA was reverse transcribed using the Smart Race cDNA amplification kit (Clontech, Calif., USA). Briefly, first-strand full-length cDNA synthesis was primed with a modified oligo(dT) primer (5′-AAGCAGTGGTATCAACGCAGAGTAC $(T)_{30}N_{-1}$ N-3′, N = A, C, G or T; $N_{-1}$ = A, G or C) and a Smart II A oligonucleotide (5′-AAGCAGTGGTATCAACGCAGA TACGCGGG-3′) and reverse transcribed using Power Script reverse transcriptase for 1.5 h at 42°C. For the full-length cDNA amplification, Universal Primer Mix A (long: 5′-CTAATACGACTCACTCACTATAGGGAAGCAGTGGTATCAACGCAGA-3′; short: 5′-CTAATACGACTCACTATAGGGC-3′; underline indicates T7 promoter sequence), Nested Universal Primer Mix A (5′-AAGCAGTGGTATCAACGCAGAGT-3′), the Advantage DNA Polymerase Mix and the following cycling parameters were used: 95°C for 60 s × 1 cycle, 95°C for 15 s/65°C for 30 s/68°C for 6 min × 20 cycles, and 4°C hold. The quality of cDNA was evaluated on ethidium bromide-stained 1.2% agarose gels. In vitro transcription was performed using the mMessage mMachine high-yield capped RNA transcription kit (T7 Kit; Ambion, Tex., USA). Briefly, the transcription mix, ribonucleotide mix, amplified cDNA and T7 RNA polymerase were mixed and incubated at 37°C for 4 h. The DNA template was degenerated by incubating with DNase I at 37°C for 15 min [24, 25]. Total RNA (1 μg) was extracted from about 5 mg of tissue materials and used for the synthesis of 50 μg of first-strand full-length cDNA, and the latter was stored at –20°C. The full-length cDNA was used to synthesize 500 mg of mRNA by PCR amplification and in vitro transcription.

*Preparation of mRNA-Encoding EGFP Reporter Gene*

A pEGFP-N1 Vector (Clontech) was doubly digested with restricted enzymes *Hind* III and *Not* I (both from TaKaRa) into 0.8- and 3.9-kbp fragments. The 0.8-kbp digested fragment (EGFP ds-

DNA) was amplified with EGFP forward primer (5′-CGGAACAAGGGAGCTTCGAATTCTGC-3′), EGFP reverse primer (5′-TGAGTCAAGGGCTAGCTTTACTTGTACAG-3′) and DNA polymerase, using the following cycling parameters: 94°C for 2 min × 1 cycle, 94°C for 60 s/33°C for 60 s/72°C for 60 s × 8 cycles, and 94°C for 60 s/61°C for 60 s/72°C for 60 s × 25 cycles. The fragments were ligated with the T7 promoter sequence (5′-GACTCGTAATACGACTCACTATAGGGCCCT-3′) at the 5′-end and with poly(dA) sequence (5′-GACTCAAAGGGA$(A)_{24}$CCTAAATCGTATGTGTATGATACATA-3′) at the 3′-end using Topo tools (Invitrogen, Calif., USA). The resulting product was amplified by PCR and followed by in vitro mRNA transcription using the mMessage mMachine high-yield capped RNA transcription kit (T7 Kit; Ambion). The final product was used as mRNA encoding EGFP reporter gene (EGFP mRNA).

*Generation of DCs from Peripheral Blood Progenitors*

Human DCs were generated according to Romani et al. [6] with minor modifications. PBMCs were cultured in serum-free RPMI-1640 at 37°C and 5% $CO_2$. After 2 h, the nonadherent cells were removed. The adherent cells were resuspended in RPMI-1640 medium supplemented with 1 m*M* *L*-glutamine, 2% autoserum, 800 U/ml granulocyte macrophage colony-stimulating factor (Osteogenetics GmbH, Germany), and 500 U/ml IL-4 (Osteogenetics GmbH) in a humidified incubator at 37°C and 5% $CO_2$ for 5 days. This immature DC preparation was used for subsequent RNA introduction in order to generate APCs.

*Generation of APCs*

RNA was introduced into the cultured immature DCs either by passive pulsing or electroporation. The passive pulsing procedure was modified from the report of Heiser et al. [25]. Briefly, 10 μg of RNA was added to $2 \times 10^5$ cells in 200 μl of serum-free RPMI-1640 medium and incubated for 45 min at 37°C and 5% $CO_2$ in a humidified incubator. Electroporation was done using Gene Pulser II, as directed (Bio-Rad, Calif., USA). Approximately $2 \times 10^5$ cells ($1 \times 10^6$ cells/ml) in 200 μl of serum-free RPMI-1640 medium were placed in a 4-mm gap chamber along with 10 μg of RNA. The mixture was placed in the Gene Pulser II and electroporated at various electrical settings [26, 27]. Subsequently, RNA-introduced DCs (DC/RNA) were allowed to maturate in the presence of 1,000 U/ml tumor necrosis factor-α in RPMI-1640 complete medium for 2–3 days. Phenotypic analysis on the matured DCs showed >85% HLA class I+, >75% HLA-DR+, >95% CD80+, >75% CD86+, >65% CD83+ and <20% CD14+. These mature DC/RNA were treated with 50 μg/ml mitomycin C (Kyowa Hakkou Pharmaceutical Co., Ltd., Tokyo), washed three times with RPMI-1640 medium and used as APCs. In some experiments, peptide-pulsed DCs (DC/peptide) were used as APCs. DC/peptide were generated from mature DCs by pulsing them with 20 μg/ml of an antigenic epitope peptide for 2 h [17].

*Flow Cytometric Analysis*

EGFP mRNA and Cellstain Double Staining Kit (Dojindo, Kumamoto, Japan) were used to evaluate the RNA introduction efficiency into immature DCs [24]. Briefly, the EGFP expression rate in EGFP mRNA-introduced DCs (DC/EGFP mRNA) was assessed 48 h after electroporation by flow cytometric analysis using FACSCalibur (Becton-Dickinson, N.J., USA). The cell viability rate was assessed by calcein and propidium iodide (PI) double staining and flow cytometric analysis. Immediately after electroporation, the DCs were stained using the Cellstain Double Staining Kit according to the manufacturer's protocol. Prior to flow cytometric analysis, calcein-acetyoxymethyl (calcein-AM) and PI were added, at a final concentration of 2 and 4 μ*M*, respectively, directly into the DCs suspended in RPMI-1640 complete medium and incubated for 15 min at 37°C. The fluorescence of calcein in viable cells was read at 490 nm excitation and 530 nm emission setting. The fluorescence of PI in the dead cells was read at 530 nm excitation and 590 nm emission setting. The percentage of the EGFP mRNA introduction efficiency was calculated according to the following formula: (EGFP expression rate) × (cell viability rate) × 100.

*Induction of Effector Cells*

In order to induce effectors, the nonadherent cells of PBMCs that had been cultured in RPMI-1640 complete medium supplemented with 20 U/ml IL-2 (Genzyme, Cambridge, UK) were stimulated with APCs, which were prepared as above, for 5–7 days in a responder:stimulator ratio of 10:1. This stimulation process was repeated three times every 7 days.

*Cytotoxicity Assay*

The calcein-AM cytotoxicity assay was used to determine cytotoxicity [28]. Briefly, target cells (approximately $10^6$ cells/ml) were incubated with 10 μ*M* calcein-AM in RPMI-1640 complete medium for 30 min at 37°C with occasional shaking. Only the live target cells can produce insoluble fluorescent product calcein from calcein-AM in cytoplasm. Therefore, the live target cells were labeled, treated with 50 μg/ml mitomycin C for 30 min, and washed three times with RPMI-1640 medium prior to cytotoxicity assays. Effectors and calcein-labeled targets with various effector/target (E/T) ratios were cocultured in U bottom 96-well plates in triplicates for 4 h at 37°C in a total volume of 200 μl. Supernatant samples were measured using Fluoroskan Ascent (Labsystems, Chesire, UK; exciting filter: 485 ± 9 nm; band-pass filter: 530 ± 9 nm). Data were expressed as arbitrary fluorescent units. Specific lysis (%) of the cells was calculated as follows: [(test release – spontaneous release)/(maximum release – spontaneous release)] × 100. The maximum and spontaneous release represents calcein release from the targets in medium with and without 2% Triton X-100, respectively. Each measurement was done in at least six replicate wells.

*Enzyme-Linked Immunosorbent Assay for Interferon-γ*

Antigen recognition of CTL precursors in PBMCs was detected by interferon (IFN)-γ secretion after stimulation with generated APCs. PBMCs ($1 \times 10^5$ cells) were cocultured with the APCs ($5 \times 10^3$ cells) in 96 flat bottom plates in triplicates for 72 h at 37°C in a total volume of 200 μl. In some experiments, effector cells were stimulated with APCs in the presence of 10 μg/ml anti-HLA class I, class II antibodies or control IgG. Supernatant samples were tested for IFN-γ secretion by enzyme-linked immunosorbent assay (ELISA) (Quantikine human IFN-γ; R&D Systems, Inc., Minn., USA) according to the manufacturer's protocol. Measurements are presented as picogram/milliliter IFN-γ released by $10^5$ PBMCs per 72 h.

*Statistics*

Results are expressed as the mean ± SD. Statistical analysis was conducted by unpaired Student's t test using StatView software (version 5) on a Macintosh computer. A p value <0.05 was considered statistically significant.

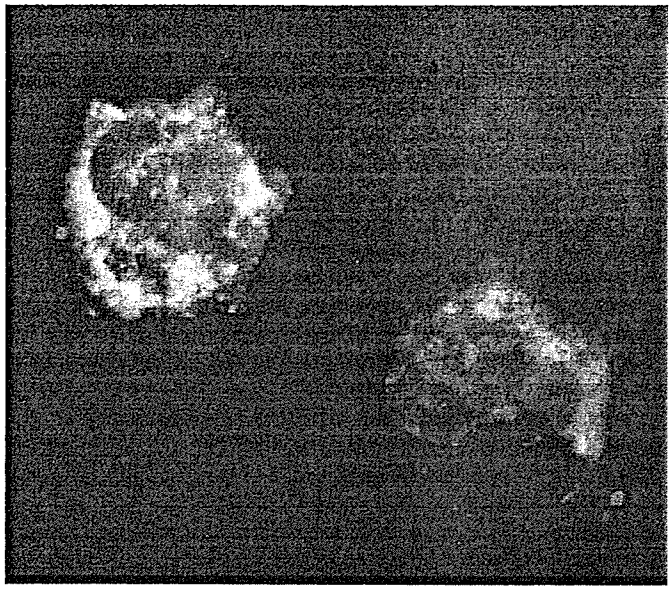

**Fig. 1.** Fluorescent microscopic images of cultured dendritic cells introduced with EGFP mRNA. Cultured DCs were introduced with mRNA encoding EGFP using electroporation at a capacitance of 150 μF and a voltage of 150 V, and fluorescent microscopy was examined.

**Table 1.** Efficiencies of RNA introduction by electroporation into dendritic cells and their viabilities

| Capacitance, μF | | Voltage, V | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 100 | 150 | 200 | 250 |
| Expt. 1 | | | | | | |
| 0 | efficiency, % | 0.4 | | | | |
| | viability, % | 90.1 | | | | |
| 150 | efficiency | – | 33.9 | 55.6 | 57.3 | 44.5 |
| | viability | – | 80.0 | 78.7 | 59.8 | 42.0 |
| 200 | efficiency | – | 54.7 | 42.5 | 28.6 | – |
| | viability | – | 86.4 | 73.1 | 46.2 | – |
| Expt. 2 | | | | | | |
| 0 | efficiency, % | 0.5 | | | | |
| | viability, % | 91.5 | | | | |
| 150 | efficiency | – | 27.1 | 43.8 | 34.3 | 18.7 |
| | viability | – | 79.0 | 82.1 | 64.5 | 45.5 |
| 200 | efficiency | – | 44.0 | 31.1 | 13.2 | – |
| | viability | – | 83.7 | 76.6 | 51.2 | – |

Cultured DCs were introduced with mRNA encoding EGFP using electroporation at various electrical settings. Efficiencies of RNA introduction into DCs and their viabilities were investigated as described in 'Materials and Methods'. A setting of 0 V and 0 μF indicates the passive pulsing.

## Results

*Optimization of mRNA Electroporation into Cultured DCs*

In order to optimize the mRNA-based electroporation, we used cultured immature DCs from healthy volunteers and an EGFP mRNA reporter gene. Following electroporation at various electrical settings, EGFP expression rate and cell viability rate in the electroporated DCs were assessed as described in 'Materials and Methods'. Of all the electrical settings tested, a voltage of either 150 or 100 V combined with a capacitance of 150 or 200 μF were found to be optimal. At these settings, introduction efficiencies of 55.6 and 54.7% and 43.8 and 44.0%, with cell viabilities of 78.7 and 86.4% and 82.1 and 83.7% were observed (table 1). On the other hand, cultured immature DCs, which were passively pulsed with EGFP mRNA, demonstrated an introduction efficiency of only 0.4 and 0.5% (table 1). Figure 1 shows the finding of fluorescent microscopy of the EGFP expression in DC/EGFP mRNA.

*Antigen-Presenting Capacity of RNA-Introduced DCs*

To evaluate the antigen-presenting capacity of the RNA-introduced DCs (DC/RNA), PBMCs from healthy volunteers were stimulated in vitro with the DC/RNA, and IFN-γ secretion in the supernatant was determined. First, we compared IFN-γ secretion by stimulating PBMCs with DCs introduced with four different RNAs: (1) autologous total RNA from PBMCs (auto-tRNA), (2) allogeneic total RNA from the BT474 cell line (BT474 tRNA), (3) allogeneic amplified mRNA from the BT474 cell line (BT474 mRNA), and (4) xenogenic amplified EGFP mRNA. Here, passive pulsing was used as an RNA delivery system into cultured DCs. Experiments were performed independently in 3 volunteers and repeated three times. Representative data of similar results are shown in figure 2. It was observed that stimulation of PBMCs with DC/BT474 tRNA, DC/BT474 mRNA, and DC/EGFP mRNA resulted in significant IFN-γ secretions from PBMCs. On the other hand, DC/auto-tRNA, as well as mock DCs, failed to stimulate PBMCs to secrete IFN-γ (fig. 2a). Next, to test whether the RNA delivery system could influence the antigen-presenting capacity of DC/RNA, two different delivery systems of passive pulsing and electroporation were compared. BT474 mRNA was used as a transgene. PBMCs secreted twice as much IFN-γ when stimulated with electroporation than pas-

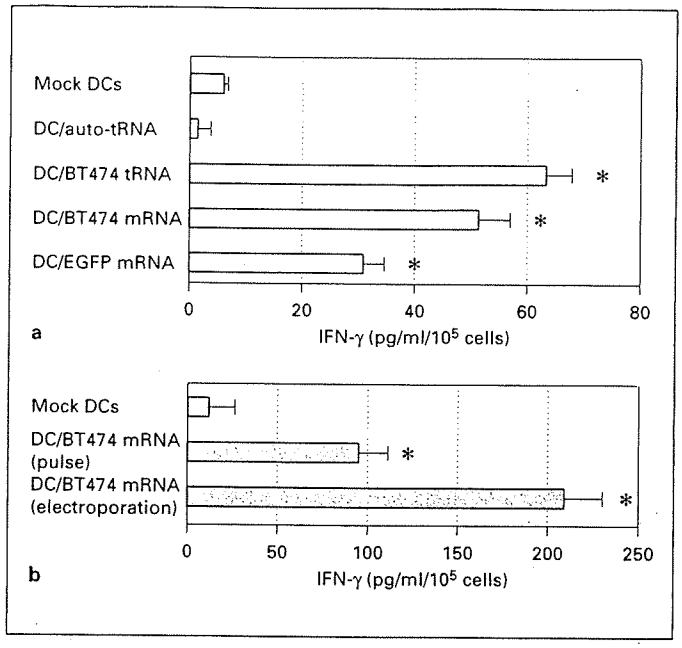


**Fig. 2.** Priming of CTL precursors in PBMCs using DC/RNA in vitro. PBMCs from healthy volunteers were stimulated with autologous mock DCs (control), DC/auto-tRNA, DC/BT474 tRNA, DC/BT474 mRNA or DC/EGFP mRNA (**a**), and with mock DCs (control), passive pulsing-based DC/BT474 mRNA (pulse) or electroporation-based DC/BT474 mRNA (electroporation) (**b**), for 72 h. The responder (PBMCs):stimulator (DC/RNA) ratio was 20:1. Supernatants were tested for IFN-γ secretion by ELISA. Experiments were performed independently in 3 volunteers and repeated three times. Representative data of similar results are shown. * $p < 0.05$, significant differences from the value of mock DC.

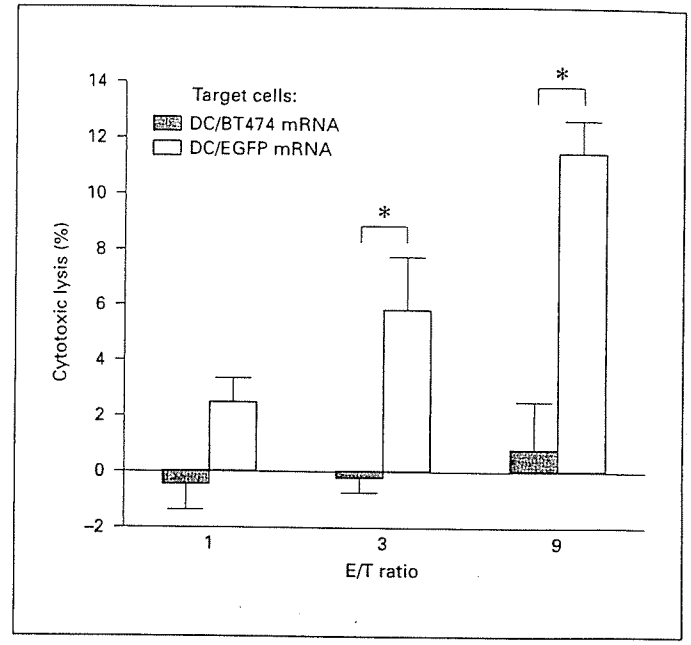


**Fig. 3.** RNA-specific cytotoxic activity of CTLs induced with DC/RNA. CTLs were induced with DC/EGFP mRNA and cytotoxicity assay was performed against DC/EGFP mRNA or DC/BT474 mRNA as targets at various E/T ratios. Cytotoxicity assay was assessed by calcein-AM release assay. Experiments were repeated three times, and representative data of similar results are shown. * $p < 0.05$.

sively pulsed DC/BT474 mRNA, showing a significant difference ($p < 0.05$) (fig. 2b).

*EGFP-Specific CTL Induction by DC/EGFP mRNA*

To assess whether DC/RNA can induce CTL responses specific to introduced RNA, cytotoxicity assays were conducted using one CTL line as an effector and two different DC/RNAs as targets. The effector CTLs (CTL/EGFP) were induced by stimulating PBMCs from healthy volunteers with DC/EGFP mRNA. Target cells used were DCs introduced with DC/EGFP mRNA and DC/BT474 mRNA. Experiments were repeated three times, and representative data of similar results are shown in figure 3. It was observed that the effector CTL/EGFP was capable of recognizing and lysing only DC/EGFP mRNA in a dose-dependent manner. However, CTL/EGFP did not at all recognize or lyse DC/BT474 mRNA.

*Tumor-Specific CTL Induction by Autologous DC/Tumor RNA*

To demonstrate whether DCs that were introduced with amplified autologous tumor mRNA can induce the tumor-specific CTL responses, effector CTLs were induced in a patient with malignant melanoma of the esophagus. Effector cells, designated as control CTL, CTL/muc and CTL/mel, were generated by stimulating patient's PBMCs with patient's mock DCs, patient's normal mucosa mRNA-introduced DCs (DC/muc-RNA) and patient's melanoma mRNA-introduced DCs (DC/mel-mRNA), respectively. The DC/mel-mRNAs were substituted for the tumor cells as the target cells. Experiments were repeated three times, and representative data of similar results are shown in figure 4. It was observed that the cytotoxic activity of CTL/mel showed 73% at an E/T ratio of 80 against the target cells and decreased in a dose-dependent manner. However, the cytotoxic activities of control CTL and CTL/muc were only 13 and 11% at an E/T ratio of 80 (fig. 4a). There were significant differences in the cytotoxic activities between control CTL, CTL/muc and CTL/mel ($p < 0.05$).

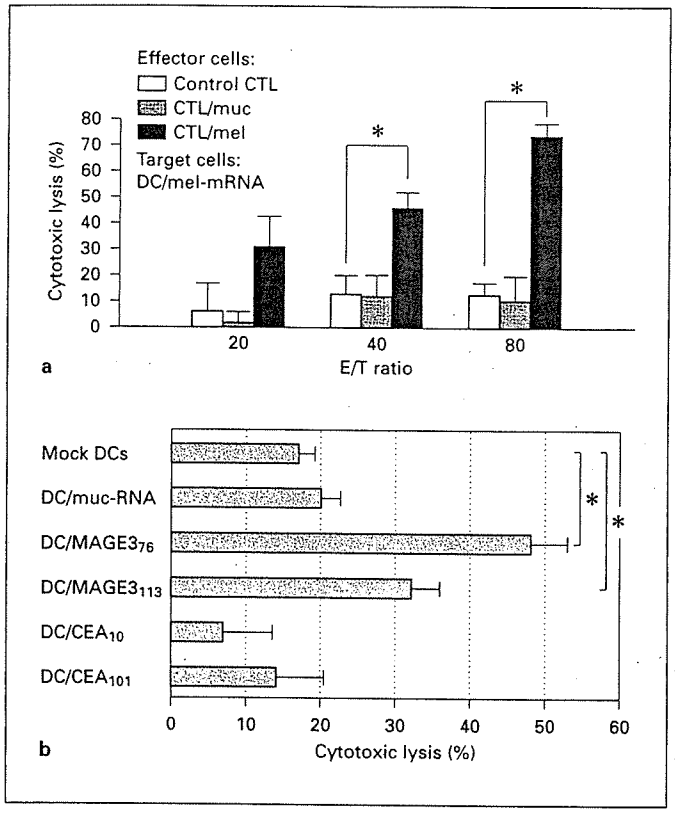


**Fig. 4.** Cytotoxic activity of CTLs induced with DC/mel-mRNA. Effector CTLs were induced from patient's PBMCs with autologous mock DCs, autologous DC/muc-RNA or autologous DC/mel-RNA. Effector CTLs were described as control CTL, CTL/muc or CTL/mel, respectively. DC/mel-RNA were used as the target cells. Cytotoxicity assay was performed at various E/T ratios (**a**). Cytotoxic activity of CTL/mel was tested against mock DCs, DC/muc-RNA, MDC/MAGE3$_{76}$, DC/MAGE3$_{113}$, DC/CEA$_{10}$ and DC/CEA$_{101}$. The E/T ratio was 40/1 (**b**). Experiments were repeated three times, and representative data of similar results are shown. * $p < 0.05$

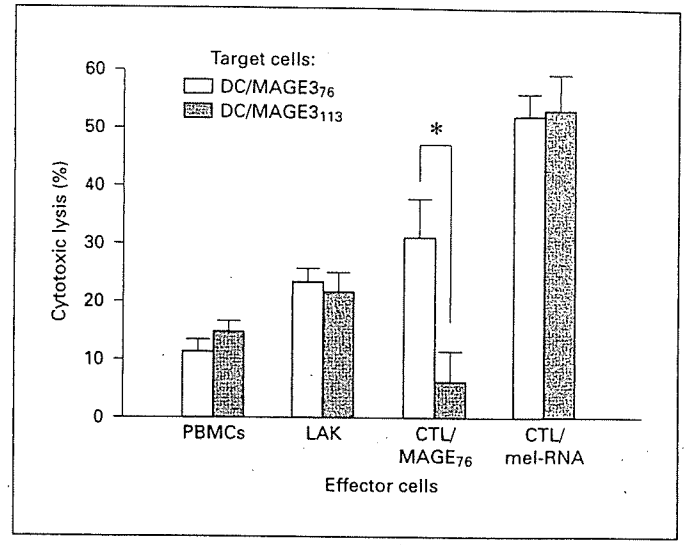


**Fig. 5.** Comparison of cytotoxic activity between CTL/peptide and CTL/RNA. Four different effector cells were used: (1) unstimulated PBMCs, (2) LAK cells induced with 400 U/ml IL-2, (3) CTL/peptide induced with DC/MAGE3$_{76}$ and (4) CTL/RNA induced with DC/mel-RNA. DC/MAGE3$_{76}$ and DC/MAGE3$_{113}$ were used as targets. Cytotoxicity assay was performed at an E/T ratio of 50/1. Experiments were repeated three times, and representative data of similar results are shown. * $p < 0.05$.

Next, antigen peptide specificity of the CTL/mel generated in the patient was analyzed. In this melanoma, the epitope peptides of TAAs were MAGE3$_{76-84}$ and MAGE3$_{113-121}$, but not the CEA$_{10-19}$ or CEA$_{101-108}$ peptides, when identified using the host-oriented peptide evaluation approach described in our previous report (data not shown) [29]. Therefore, we evaluated the cytotoxic activity of the CTL/mel against target cells of mock DCs, DC/muc-RNA, MAGE3$_{76-84}$ peptide-pulsed DCs (DC/MAGE3$_{76}$), MAGE3$_{113-121}$ peptide-pulsed DCs (DC/MAGE3$_{113}$), CEA$_{10-19}$ peptide-pulsed DCs (DC/CEA$_{10}$), and CEA $_{101-108}$ peptide-pulsed DCs (DC/CEA$_{101}$). It was observed that the CTL/mel showed 47 and 32% cytotoxic activity against DC/MAGE3$_{76}$ and DC/MAGE3$_{113}$, respectively. However, the CTL/mel showed only 16, 18, 7, and 14% cytotoxic activity against the mock DCs, DC/muc-RNA, the DC/CEA$_{10}$, and DC/CEA$_{101}$, respectively (fig. 4b). There were significant differences between the cytotoxic activities against mock DCs and DC/MAGE$_{76}$ or DC/MAGE$_{113}$ ($p < 0.05$).

*Polyspecific CTL Induction by DC/Tumor RNA*

Next, the TAA-presenting potential of CTLs generated using DC/tumor RNA was evaluated. The patient's PBMCs were stimulated with the DC/MAGE3$_{76}$ and DC/mel-RNA to generate the effector CTLs that were designated as the CTL/MAGE3$_{76}$ and the CTL/mel-RNA, respectively. Patient's PBMCs alone and the lymphokine-activated killer (LAK) cells that were stimulated with 400 U/ml recombinant human IL-2 were used as the control effector cells. Patient's DC/MAGE3$_{76}$ and DC/MAGE3$_{113}$ were used as the target cells. Experiments were repeated three times, and representative data of similar results are shown in figure 5. It was observed that the PBMCs showed only 11 and 14% cytotoxic activity, and the LAK cells showed 23 and 21% cytotoxic activity

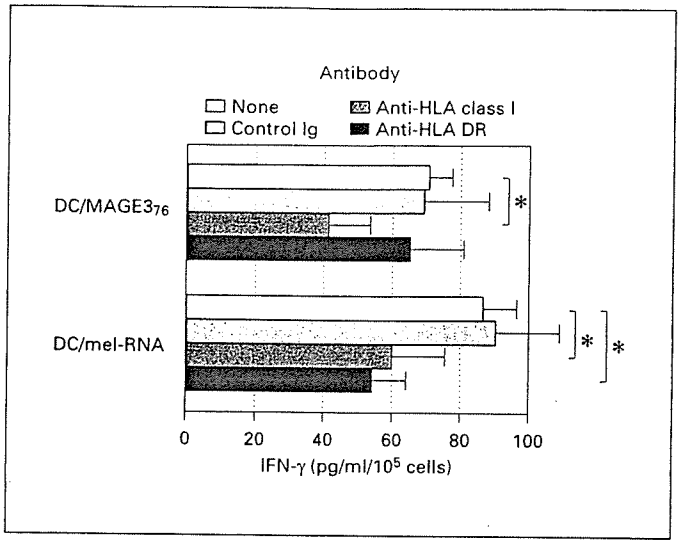


**Fig. 6.** Involvement of HLA class I and class II pathways in the effector cell induction with DC/peptide and DC/RNA. PBMCs were stimulated with DC/MAGE3$_{76}$ and DC/mel-mRNA in the presence of various antibodies indicated: (1) no immunoglobulin, (2) control IgG, (3) anti-HLA class I and (4) anti-HLA-DR antibodies. The responder:stimulator ratio was 20:1. Each supernatant sample was tested for IFN-γ secretion by ELISA. Experiments were repeated three times, and representative data of similar results are shown. * $p < 0.05$.

against the DC/MAGE3$_{76}$ and the DC/MAGE3$_{113}$, respectively. The CTL/MAGE3$_{76}$ showed a 31% cytotoxic activity against the DC/MAGE3$_{76}$, but only a 6% cytotoxic activity against the DC/MAGE3$_{113}$. The cytotoxic activity of the CTL/MAGE3$_{76}$ was significantly higher against the DC/MAGE3$_{76}$ than against the DC/MAGE3$_{113}$, whereas the CTL/mel-RNA demonstrated cytotoxic activity against both the DC/MAGE3$_{76}$ and the DC/MAGE3$_{113}$, showing an activity of 51 and 52%, respectively. There was no significant difference between the cytotoxic activity of CTL/mel-RNA against DC/MAGE3$_{76}$ and DC/MAGE3$_{113}$.

*Involvement of HLA Class I and II Pathways in Stimulation of PBMCs by DC/mel-RNA*

To determine the involvement of HLA class I or class II pathways when stimulating PBMCs with two different APCs, blocking studies with monoclonal antibodies were performed. The patient's PBMCs were stimulated with the DC/MAGE3$_{76}$ and the DC/mel-RNA in the presence or absence of the antibodies indicated, and IFN-γ secretion in the supernatant was determined. Experiments were repeated three times, and representative data of similar results are shown in figure 6. The control immunoglobulin had no effect on the IFN-γ secretion of PBMCs when stimulated with DC/MAGE3$_{76}$ or DC/mel-RNA. The addition of anti-HLA class I, but not the anti-HLA-DR monoclonal antibodies resulted in a significant inhibition of IFN-γ secretion of PBMCs stimulated with DC/MAGE3$_{76}$. However, when stimulated with DC/mel-RNA, not only the addition of anti-HLA class I but also of anti-HLA-DR monoclonal antibodies resulted in a significant inhibition of IFN-γ secretion of PBMCs.

## Discussion

In this study, we have shown the generation of tumor antigen-reactive CTL using the tumor RNA-introduced DCs. Tumor mRNA that was obtained from tiny tumor mass could easily be amplified in vitro and efficiently introduced into DCs by an electroporation-based mRNA delivery system. Here, laser capture microdissection (LCM) may be of value to be introduced into our system, because LCM can avoid the contamination of normal cells when tumor RNA is extracted [30]. The LCM may augment the CTL induction in our system by obtaining pure tumor RNA, although no induction of CTLs reactive with normal tissue mRNA-introduced DCs was observed in this study even when normal RNA was used. The benefit of LCM remains to be addressed.

The approach using amplified tumor RNA and an electroporation-based mRNA delivery system has several advantages: (1) DCs can be introduced to levels comparable with transduction by recombinant viruses, such as poxviruses [31] or adenoviruses [32], without the problems associated with viral vectors [33, 34]; (2) DCs can be introduced with the total antigenic spectrum using mRNA extracted from the tumor tissues without prior identification of TAAs; (3) RNA can be amplified by PCR to provide an unlimited supply of TAAs from an often small amount of clinical tumor tissues [24], and (4) RNA has a short cellular half-life and lacks the potential to integrate into the host genome, and thereby, we can avoid the potential safety hazard in the context of clinical therapeutic trials [35, 36]. Our results showed that the electroporation-based mRNA delivery system had sufficient mRNA introduction efficiency and low cell toxicity against cultured immature DCs under optimal electrical settings (table 1). This observation is consistent with other reports showing that the electroporation-based mRNA delivery system is superior to the commonly used techniques of

lipofection or passive pulsing in providing better RNA transfection efficiency [26, 27].

By using DC/RNA, we could generate excellent effector cells, CTL/RNA, which were capable of recognizing the DC/RNA. This was also evidenced using the samples from a patient with malignant melanoma of the esophagus. Moreover, CTL/EGFP mRNA could only recognize DC/EGFP mRNA but not DC/BT474 mRNA, indicating the RNA-specific target recognition of CTL/RNA effector cells. This is consistent with other reports showing that exogenous DC/mRNA can prime precursors and induce antigen-specific CTLs in an introduced mRNA-specific manner [37, 38]. It indicates that tumor RNA which may contain numerous tumor antigen-coding genes must be able to stimulate numerous CTL precursors that have T cell receptors reactive with each tumor antigen, and suggests that DC/RNA are superior to DC/peptide in terms of tumor-reactive T cell activation. Actually, our results showed that the DC/mel-RNA induced polyspecific CTLs that were reactive with both $MAGE3_{76}$ and $MAGE3_{113}$, whereas the DC/$MAGE3_{76}$ induced monospecific CTLs reactive only with $MAGE3_{76}$. Since the malignant tumors have heterogeneity, CTLs induced with the DC/tumor RNA can reduce the chance of clonal tumor escape more effectively than CTLs induced with the TAA peptide-pulsed DCs. Heiser et al. [25] demonstrated that the amplified prostate tumor RNA-transfected DC-stimulated T cell responses were directed against the multiple TAAs, including the prostate-specific antigen and the telomerase reverse transcriptase. They also suggested that tumor RNA-transfected DCs might minimize the risk of clonal tumor escape [25].

Interestingly, IFN-γ response of PBMCs stimulated with DC/mel-RNA was inhibited not only with anti-HLA class I antibody but also with anti-HLA class II antibody, indicating that DC/tumor RNA cannot only stimulate potent CTL responses but also antigen-reactive CD4+ T cell responses. Nair et al. [19] and Weissman et al. [38] demonstrated that the antigenic mRNA transfection of DCs delivers encoded antigen to major histocompatibility complex class I and class II molecules; on the other hand, DC/TAA peptide can only stimulate potent CTL responses, but not CD4+ T cell responses, suggesting that the DC/tumor RNA is superior to the DC/peptide for a potent induction of the antigen-reactive CTLs and the antigen-reactive helper T cells. Furthermore, Zhao et al. [39] demonstrated that a short incubation of mRNA-transfected DCs with the antisense oligonucleotides (against the invariant chain) enhances the presentation of mRNA-encoded class II epitopes and the activation of CD4+ T cell responses in vitro and in vivo, and that immunization of mice with antisense oligonucleotide-treated DCs stimulates potent and longer-lasting CTL responses and enhances the anti-tumor efficacy of DC-based tumor vaccination protocols. More recently, Bonehill et al. [40] reported the presentation of MAGE-A3 antigen simultaneously in HLA class I and class II molecules by mRNA-electroporated DCs. The induction of CD4+ T cell responses plays an important role in the induction and persistence of HLA class I-restricted antigen-reactive CTLs. These observations indicate the importance of activating class II as well as class I pathways by DC/tumor RNA in DC-based tumor immunotherapy.

If tumor RNA contains numerous antigen genes and if DC/tumor RNA can stimulate numerous CTL precursors, the application of DC/tumor RNA for immunotherapy could potentially have several drawbacks. For example, unfractionated tumor mRNA contains self-antigen-encoding RNAs, and the use of DC/tumor mRNA in clinical trials can induce autoimmune toxicity by reducing tolerance to self-antigens. However, our results demonstrated that there was no induction of CTLs reactive with normal tissue mRNA-introduced DCs as targets. Several phase I clinical trials showed no apparent adverse effects or dose-limiting toxicities including autoimmune toxicity [41, 42]. Therefore, there is a strong possibility that the DC/tumor RNA may not stimulate the forbidden clones that react with self-antigens. This may augment the possible clinical application of DC/tumor RNA in tumor immunotherapy.

In summary, the use of autologous tumor mRNA-introduced DCs can stimulate the induction of anti-tumor immune responses against the multiple tumor-derived antigens without inducing autoimmunity against self-antigens. An immunotherapeutic approach using DC/tumor RNA permits broad applicability against various tumor-bearing patients without prior identification of HLA phenotypes and TAAs. This approach offers unlimited supply of tumor mRNA by in vitro amplification from a limited source of tumor tissue. Collectively, the approach using DC/mRNA offers novel possibilities for DC-based antigen-specific immunotherapy of cancer.


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# Exosomes secreted from monocyte-derived dendritic cells support in vitro naive CD4$^+$ T cell survival through NF-κB activation☆

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**Abstract**

We investigated the effect of exosomes secreted from human monocyte-derived dendritic cells (Mo-DCs), which are generated from PBMCs in response to treatment with GM-CSF and IL-4, on naive CD4$^+$ T cell survival in vitro. Exosomes isolated from culture supernatants of Mo-DCs (>90% purity) were purified with anti-HLA-DP, -DQ, -DR-coated paramagnetic beads. Purified exosomes prolonged the survival of naive CD4$^+$ T cells (>98% purity) in vitro. Treatment with neutralizing mAb against HLA-DR significantly decreased the supportive effect of purified exosomes on CD4$^+$ T cell survival. Exosomes increased nuclear translocation of NF-κB in naive CD4$^+$ T cells, and NF-κB activation was significantly suppressed by anti-HLA-DR mAb or NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC). In addition, PDTC inhibited the effect of exosomes on naive CD4$^+$ T cell survival. Thus, exosomes secreted by Mo-DCs appear to support naive CD4$^+$ T cell survival via NF-κB activation induced by interaction of HLA-DR and TCRs.


*Keywords:* Human monocyte-derived dendritic cells; Multivesicular body; Small membrane vesicle; TCR and MHC interaction

## 1. Introduction

Prolonged survival of naive CD4$^+$ T cells requires direct contact with self-MHC class II ligands in vivo [1–3]. CD8$^+$ T cells also require exposure to specific self-MHC class I proteins for prolonged survival [4]. Thus, interaction between TCR and MHC molecules plays an important role in supporting naive T cell survival in vivo [5,6]. However, there are few reports concerning the role of TCR and MHC interaction in short-term survival of naive CD4$^+$ T cells in vitro [7].

Exosomes were initially described as microvesicles containing 5′-nucleotidase activity and released from neoplastic cell lines [8,9]. Electron microscopy has shown that exosomes have a characteristic saucer-like morphology of a flattened sphere limited by a lipid bilayer. They range from 30 to 100 nm in diameter [10]. The most common procedure for purifying exosomes from cell-culture supernatants involves a series of centrifugations to remove dead cells and large debris, followed by a final high-speed ultracentrifugation to pellet the exosomes [11,12]. It is generally believed that exosomes are

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membrane vesicles that form within late endocytic compartments, multivesicular bodies (MVBs), and are secreted upon fusion of these compartments with the plasma membrane of living cells. As a result, all exosomal proteins reported up to now have been found in the cytosol, in the membrane of endocytic compartments, or at the plasma membrane. Various cell types secrete exosomes. APCs such as dendritic cells (DCs)[2] and B cells also secrete exosomes. Recent advances in biotechnology have made it possible to generate DC-like cells, monocyte-derived DCs (Mo-DCs), in vitro from PBMCs upon treatment with GM-CSF and IL-4 [13], and Mo-DCs secrete exosomes [14]. MHC class II proteins are very abundant in exosomes from Mo-DCs as well as other APCs [15]. In addition, APC-derived exosomes contain specific proteins, such as CD86 and integrins, which are involved in antigen presentation, suggesting a role of exosomes in T cell stimulation [16–18]. In fact, it has been shown that EBV-transformed B cell-derived exosomes stimulate human $CD4^+$ T cell clones in an antigen-specific manner [10]. T cell stimulation by exosomes produced by rat mast cells engineered to express mouse or human MHC class II proteins has been reported [19]. Interestingly, exosomes produced by tumor peptide-pulsed DCs induce T cell-dependent tumor rejection in vivo [14].

NF-κB is a transcription factor that is activated in T cells by interaction between TCRs and MHC class I or class II proteins [20–22] and has been shown to play an important role in the expression of anti-apoptotic genes [23]. In most resting cells, NF-κB is located in the cytoplasm as a heterodimer of the structurally related proteins p50, p52, RelA, c-Rel, and RelB. All of these are noncovalently associated with the cytoplasmic inhibitor IκB [24]. The most common NF-κB is the p65/p50 heterodimer. Activation of NF-κB is preceded by phosphorylation of IκB by IκB kinase, which is followed by proteolytic removal of IκB and movement of NF-κB to the nucleus. Nuclear translocation of NF-κB is thought to reflect its activation [25]. Zheng et al [26] reported a critically important function of NF-κB in TCR-induced regulation of $CD4^+$ T cell survival in p50−/− cRel−/− mice. In addition, survival of antigen-stimulated T cells requires NF-κB-mediated inhibition of p73 expression [22]. Thus a role of NF-κB in T cell survival appears to be important. However, a role of the NF-κB-activating pathway in naive $CD4^+$ T cell survival has not been identified in human cells.

Here, we report for the first time that Mo-DC-derived exosomes support naive $CD4^+$ T cell survival in vitro through interaction between TCRs and human leukocyte antigen (HLA)-DR, and that TCR-dependent NF-κB activation may contribute to this survival.

## 2. Materials and methods

### 2.1. Reagents

Pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB nuclear translocation, was purchased from Sigma Chemical (Deisenhofen, Germany).

### 2.2. Preparation of human Mo-DCs and naive $CD4^+$ T cells

Mo-DCs were generated from the adherent fraction of PBMCs from healthy volunteers, as previously described but with minor modifications [13]. Briefly, PBMCs were isolated from heparinized peripheral blood by Ficol–Paque (Life Technologies, Gaithersburg, MD, USA) density gradient centrifugation. PBMCs were resuspended in RPMI 1640 basal medium (Sanko Pure Chemicals, Tokyo Japan) supplemented with 1% human albumin (Mitsubishi Pharma, Osaka, Japan), 100 μg/ml penicillin (Meijiseika, Tokyo, Japan), and 100 μg/ml streptomycin (Meijiseika) (RPMI medium), plated at a density of $2 \times 10^6$ cells/ml, and allowed to adhere overnight at 37 °C in 24-well plates (Nalge Nunc International, Chiba, Japan). Nonadherent cells were removed, and adherent cells were cultured in RPMI medium containing GM-CSF (100 ng/ml, North China Pharmaceutical Group, Shijiazhuang, China) and IL-4 (50 ng/ml, Osteogenetics, Wurzburg, Germany). On day 7, nonadherent fractions were collected as Mo-DCs. Mo-DCs were further purified by negative selection with magnetic beads coated with mouse monoclonal anti-CD2, anti-CD3, and anti-CD19 antibodies (Dynabeads, Dynal Biotech, Oslo, Norway). This depletion procedure yielded over 90% $CD14^-$, $CD80^+$, and $HLA\text{-}DR^+$ Mo-DCs as assessed by fluorescence-activated cell sorting (FACS) (FACS Calibur flow cytometer, Becton–Dickinson Immunocytochemistry Systems, Franklin Lakes, NJ, USA) and analyzed with CELLQuest software (Becton–Dickinson).

Seven days after the initial culture of nonadherent cells, PBMCs were collected again from the same healthy volunteer. $CD4^+$ T cells were purified from fresh human PBMCs with a CD4-positive isolation kit (Dynabeads, Dynal Biotech) according to the manufacturer's instructions. This positive-selection process yielded over 98% $CD4^+$ T cells.

Fresh $CD4^+$ T cells and Mo-DCs isolated from the same healthy volunteer were used throughout this study.

[2] *Abbreviations used:* DC, dendritic cell; Mo-DC, monocyte-derived dendritic cell; PDTC, pyrrolidine dithiocarbamate; MVB, multivesicular body; CB, cacodylate buffer; MW, molecular weight; FSC, forward scatter; SSC, side scatter.