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Selectively Expanded of CD4$^+$ T Cells from Peripheral Blood Mononuclear Cells Induced by Interleukin-2 Plus Anti-CD3: Effects on the Cytotoxicity, the Proliferation and the Modulation of HLA Antigens and Intercellular Adhesion Molecule-1 against Human Malignant Melanoma Cell Lines

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ABSTRACT

We selected human CD4$^+$ T cells using immunomagnetic beads from peripheral blood mononuclear cells of 5 healthy volunteers, and cultured with recombinant interleukin-2 (RIL-2) plus anti-CD3 culture supernatants. After successful generation of the CD4$^+$ T cell cultures, we examined the therapeutic value for human malignant melanoma. Through flow cytometry analysis, ^{51}Cr-release assay and proliferation assay, we found that a) the total increase in 7 to 10 day cultures ranged from 30 to 100-fold; b) the expanded cells were mostly CD2$^+$, CD3$^+$, CD4$^+$, CD8$^-$, CD25$^+$, CD29$^+$, CD56$^-$ and T cell receptor $(\alpha/\beta)^+$ T cell; c) some of them possessed moderate levels of natural killer cell and lymphokine -activated killer cell activities; d) culture supernatants significantly inhibited the growth of cultured human melanoma cell lines; e) culture supernatants enhanced surface expression of class I, class II and intercellular adhesion molecule-1 on cultured human melanoma cell lines; and f) culture supernatants contained large amounts of cytokines such as IL-2, IL-6 and interferon-γ. These results suggested that selectively expanded CD4$^+$ T cells may be available as a new strategy in the future human adoptive immunotherapy.

Key Words: CD4, Melanoma, Anti-CD3, Immunomagnetic beads, Cytokine

INTRODUCTION

A number of clinical trials of adoptive immunotherapy using in selected patients lymphokine-activated killer (LAK) cells, cytotoxic T lymphocytes (CTL) and tumor infiltrating lymphocytes (TIL) have reported partial and com-

plete responses to these cells, but to a very limited extent(1-4). The current methods of adoptive immunotherapy owe many of their limitations to insufficient numbers and inappropriate tumor specific cytotoxicity of transferred cells and the absence of helper functions in the local and systemic immune system(5-7). Adoptive immunotherapy might give better results if helper T cells can be transferred into the tumor sites together with tumor specific killer cells.

It has been reported that $CD4^+$ helper T cells possess both helper and killer activities at the clonal level and interleukin-2 (IL-2) producing activity(8-10). Recently, Nakamura *et al.*(11) developed a large-scale culture system of human $CD4^+$ T cells using immunomagnetic beads, immobilized anti-CD3 monoclonal antibody (mAb) plus recombinant IL-2 (RIL-2). In the present study, we used soluble anti-CD3 culture supernatants, which were added only once at the beginning of the culture, instead of immobilized anti-CD3 purified mAb. Some of the selectively expanded $CD4^+$ T cells possessed moderate levels of natural killer (NK) and LAK activities. Culture supernatants obtained from $CD4^+$ T cells significantly inhibited the cultured human melanoma cell lines and variably enhanced the surface expressions of class I, class II and intercellular adhesion molecule -1 (ICAM-1) . Expanded $CD4^+$ T cells also secreted large amounts of IL-2, IL -6 and interferon-γ (IFN-γ). These results suggest that selectively expanded $CD4^+$ T cells have potential for in vivo anti-tumor activity and could enhance immunologic mechanisms against melanoma cells.

MATERIALS AND METHODS

Culture medium

Serum-free medium (AIM-V) was purchased from Gibco Laboratories (Grand Island, NY) and used for expansion of $CD4^+$ lymphocytes. Complete medium (CM) consisted of RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mg streptomycin/m*l* and 100 U penicillin/m*l* (all from Gibco).

Recombinant interleukin-2 (RIL-2)

RIL-2 was kindly supplied by Shionogi & Co., Ltd. (Settsu, Osaka, Japan).

Monoclonal antibodies (mAbs)

The mAbs employed in the phenotyping of lymphocytes were anti-Leu5b (CD2), anti-Leu4 (CD3), anti-Leu3a (CD4), anti-Leu2a (CD8), anti-Leu19 (CD56), anti-IL-2 receptor (IL-2R)-α (CD25), anti-IL-2R-β (Mik-β1), anti-HLA-DR (class II), anti-T cell receptor (TCR) α/β (WT31), anti-TCR-γ/δ (11F2), anti-2H4 (CD45RA), anti-UCHL1 (CD45RO) and anti-4B4 (CD29).

Melanoma surface antigens were assessed with anti-HLA-A, B, C, (class I), anti- HLA-DR (class II) and anti-intercellular adhesion molecule-1 (ICAM-1) (CD54). Anti-IL-2R-α, anti-HLA-A, B, C, anti-TCR-α/β and anti-TCR-γ/δ were purified mAbs, and the others were conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE). All mAbs except for anti-IL-2R-β, anti-HLA-A, B, C, anti-ICAM-1, anti-2H4, anti-UCHL1 and anti-4B4 were obtained from Becton Dickinson (Mountain View, CA). Anti-IL-2R-β was purchased from Nichirei (Misaki, Tokyo), anti-HLA-A, B, C from Sera-Lab Ltd. (Sussex, England), anti-ICAM-1 from AMAC, Inc. (Westbrook, ME), anti-2H4 and anti-4B4 from Coulter Immunology (Hialeah, FL) and anti-UCHL1 from Dakopatts (Copenhagen, Denmark). FITC-conjugated F(ab')2 fragments of goat anti-mouse IgG antibody (Cappel Laboratories, Cochranville, PA) were used as second stage antibody for unlabeled primary antibodies. Simultest Control IgG1 FITC+IgG2aPE (Becton Dickinson) was used as a control.

The OKT3 and OKT4 murine hybridomas were purchased from American Type Culture Collection. Anti-CD3 and CD4 mAb containing supernatants were obtained by incubating 1×10^5 hybridomas cells/m*l* of CM for 3 days.

Cell lines

K562, a NK sensitive cell line established from human erythroleukemia cells, Daudi, a LAK sensitive cell line established from human Burkitt's lymphoma, and cultured human melanoma cell lines G361 (# CCL1424) and SK-MEL-28 (# HTB72) were purchased from the American Type Culture Collection. Cultured human melanoma cell line MeWo was kindly supplied by Dr. S. K. Liao (McMaster University, Hamilton, Canada). All cell lines were maintained in CM.

Cell preparation

Peripheral blood mononuclear cells (PBMC) from 5 healthy volunteers were isolated by Ficcoll-Conray (IBL, Fujioka, Japan) and resuspended in CM. For purification of $CD4^+$ T cell, lymphocytes were incubated with saturating amounts of anti-CD4 culture supernatants for 45 min at 4℃. After washing three times, cells were incubated with goat anti-mouse IgG-coated magnetic particles (Dynabeads-M450, Dynal A. S, Oslo, Norway) for 45 min and the bead-coated cells were removed by magnetic separation. $CD4^+$ T cells obtained above were adjusted to a density of 5×10^5 cells/m*l* of AIM-V medium supplemented with 500 U/m*l* of RIL-2 plus 5%v/v of anti-CD3 culture supernatants which were added only once at the beginning of the culture and not thereafter.Two m*l* of suspension were dropped into each well of 24 well plates (Linbro, Flow Laboratories,McLean,VA). These were maintained at 37℃ in a humidified 5% CO_2

atmosphere. On day 4, the rapidly expanded cells were washed with AIM-V media, counted, and placed back into culture at a concentration of 1×10^6 cells/m*l* in AIM-V media supplemented with 500 U/m*l* of RIL-2. Cells were fed and readjusted to 1×10^6 cell/m*l* every 2 or 3 days. On day 10, cells were harvested, and magnetic particles were removed by magnetic separation. Finally, phenotypic and cytotoxic activity analyses of expanded lymphocytes were performed.

Flow cytometry

Fluorescence analyses of the cell surface antign expressions of expanded $CD4^+$ T cells and cultured melanoma cell line (G361, SK-MEL-28 and MeWo) were carried out on an EPICS Profile II Analyzer (Coulter Electronics, Hialeah, FL). All procedures were carried out at 4℃. Results were expressed as a percentage of positive cells. The mean fluorescence intensity was calculated by integrating the fluorescence histograms for the positive cells as compared with control cells.

Surface analysis of selectively expanded $CD4^+$ T cells

Direct immunofluorescence was performed by incubating 100 μl of a cell suspension (1×10^6 cells) with a saturating amount of FITC-or PE-conjugated mAbs for 30 min. The cells were washed and then analyzed. Indirect immunofluorescence was performed by incubating 100 μl of a cell suspension (1×10^6 cells) with purified anti-IL-2R-α, anti-TCR-α/β and anti-TCR-γ/δ mAbs for 30 min. Goat anti-mouse IgG labeled with FITC was added as a second stage antibody, incubated for 30 min, and then analyzed.

Tumor targets and cytotoxicity assay

Cryopreserved K562, Daudi, and allogeneic melanoma cell lines G361, MeWo and SK-MEL-28 were used as targets in the cytotoxicity assays. Cryopreserved target cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham Japan Limited) for 60 min in 1.0 m*l* of CM at 37℃. The cells were then washed three time with CM and used as targets at 1×10^4 cells/well. Expanded $CD4^+$ lymphocytes were incubated with ^{51}Cr-labeled target cells in a 96-well U-bottom microplate (Nunc, Roskilde, Denmark) at four different effector to target ratios (5 : 1, 10 : 1, 20 : 1, 40 : 1) at 37℃ for 4 h. Cultures were harvested with the Skatron-Titertek system (Skatron, Sterling, VA). Spontaneous ^{51}Cr release was determined by incubation of target cells with CM and maximum release was determined by incubation with 0.1% NP-40. Percent specific lysis was calculated by the following formula: % of cytotoxicity=(experimental cpm-spontaneous release cpm)×100/

(maximum release cpm-spontaneous release cpm).

Culture supernatants obtained from expanded CD4$^+$ T cells

Ten days after successful generation of the CD4$^+$ T cell cultures, cells were washed 3 times with AIM-V media and placed back into culture at a concentration of 1×10^6 cells/m*l* in AIM-V media supplemented with 500 U/m*l* of RIL-2. After incubation for 3 days the culture supernatants were harvested and immediately frozen in liquid nitrogen for storage at-80℃ for further processing. AIM-V media supplemented with 500 U/m*l* of RIL-2 without CD4$^+$ T cells were also incubated for 3 days as a control.

Assays for proliferation of human melanoma cell lines

In each well of a flat-bottomed 96-well microtiter plate (Nunc), melanoma cell lines G361 and MeWo (5×10^3) were cultured with serial dilutions of culture supernatants (50%, 25% and 12.5% at the final concentration). After 72 h of incubation, cultures were pulsed overnight with 0.5 μCi/well of [^{3}H] -thymidine (Amersham Japan Limited), treated with EDTA (0.01 M final concentration) for 10 min to assure removal of adherent cells, and harvested onto glass filters with a PhD cell harvester (Cambridge Technology, Cambridge, MA). Radioactivity was assessed by liquid scintillation on a β counter and the results were expressed as cpm $\pm$ standard error of the mean for triplicate cultures. The percent growth inhibition of (^{3}H) thymidine incorporation was calculated by the following formula: % growth inhibition=(1-cpm from target cells treated with test conditioned medium/cpm from target cell treated with control medium)X 100%.

Surface analysis of cultured melanoma cells treated with CD4$^+$ T cell culture supernatants

Five m*l* of cell suspensions which contained either melanoma cell lines G361 or SK-MEL-28 (3×10^5) in the AIM-V media were placed in 25 cm^2 tissue culture flasks (Becton Dickinson) for 1 h. After confirming adherence of the cells to the flask bottom, serial dilutions of culture supernatants (50%, 25% and 12.5% at the final concentration) obtained from 3 healthy volunteers (cases A, B and D) were added and incubated for 18 h. As a control 5 m*l* AIM-V media was supplemented with 500 U/m*l* of RIL-2 in place of the culture supernatants. After incubation, cells were harvested with EDTA (0.01 M at the final concentration) for 10 min to assure removal of adherent cells. Direct immunofluorescence was performed by incubating 100 μl of a cell suspension (1×10^6 cells) with a saturating amount of FITC-or PE-conjugated anti-HLA-DR and anti-ICAM-1 mAbs for 30 min. The cells were washed and then analyzed. Indirect

immunofluorescence was performed by incubating 100 μl of a cell suspension (1×10^6 cells) with purified anti-HLA-A, B, C mAb for 30 min. Goat anti-mouse IgG labeled with FITC was added as a second stage antibody, incubated for 30 min, and then analyzed.

Cytokine assays

Cytokine concetrations (IL-2, IL-4, IL-6 and IFN-γ) in the culture supernatants of 3 healthy volunteers (caseA, B and D) and control culture supernatants were measured with commercially available enzyme immunoassay and radioimmunoassay systems. The IL-2 assay kit was purchased from Medgenix (Brussels, Belgium). The IL-4 and IL-6 assay kits were obtained from Research and Diagnostics Systems (Minneapolis, MN). The IFN-γ assay kit was purchased from CENTOCOR (Malvern, PA).

RESULTS

In vitro expansion of CD4$^+$ T cells

CD4$^+$ T lymphocytes separated by immunomagnetic beads were cultured in medium containing 500 U/ml of RIL-2 plus anti-CD3 culture supernatants. Since 5% (v/v) anti-CD3 mAb culture supernatants reproducibly activated the lymphocytes, this culture condition was also used in this study. After overnight cultures, small colonies of CD4$^+$ T cells could be seen among the immunomagnetic beads, and these colonies grew rapidly until day 2. After 3 days, monomorphous blastoid lymphocytes covered the bottom of the 24-well plates (Figure 1). Cultures were passaged every 2 or 3 days, or as dictated by the rate of cellular proliferation. The total fold increase (expansion index), which was calculated from the original number of plated cells seen in 7 to 10 day cultures, ranged from 30 to 100-fold. After 10 day cultures, the expansion rate continued to maintain at the average of 4 to 10-fold weekly. The maximum fold expansions were 7×10^{12} by 135 days in the case of E (data not shown).

Phenotypic characterization of the selectively expanded CD4$^+$ T cells

The surface phenotypes of selectively expanded CD4$^+$ T cells in each experiment are summarized in Table 1. The vast majority of expanded lymphocytes was CD2$^+$, CD3$^+$, CD4$^+$, CD25$^+$, CD29$^+$ and TCR $(\alpha/\beta)^+$ T cell. There were few cells bearing the NK and LAK associated marker CD56. There was negligible staining for CD8, TCR (γ/δ), and IL-2R-β. All experiment groups showed relatively high reactivity with CD45RA and CD45RO, although the percentage of cells stained with the mAb varied from 36% to 90%. The reactivity of HLA-DR was variable. Representative profiles of fluorescence analysis of

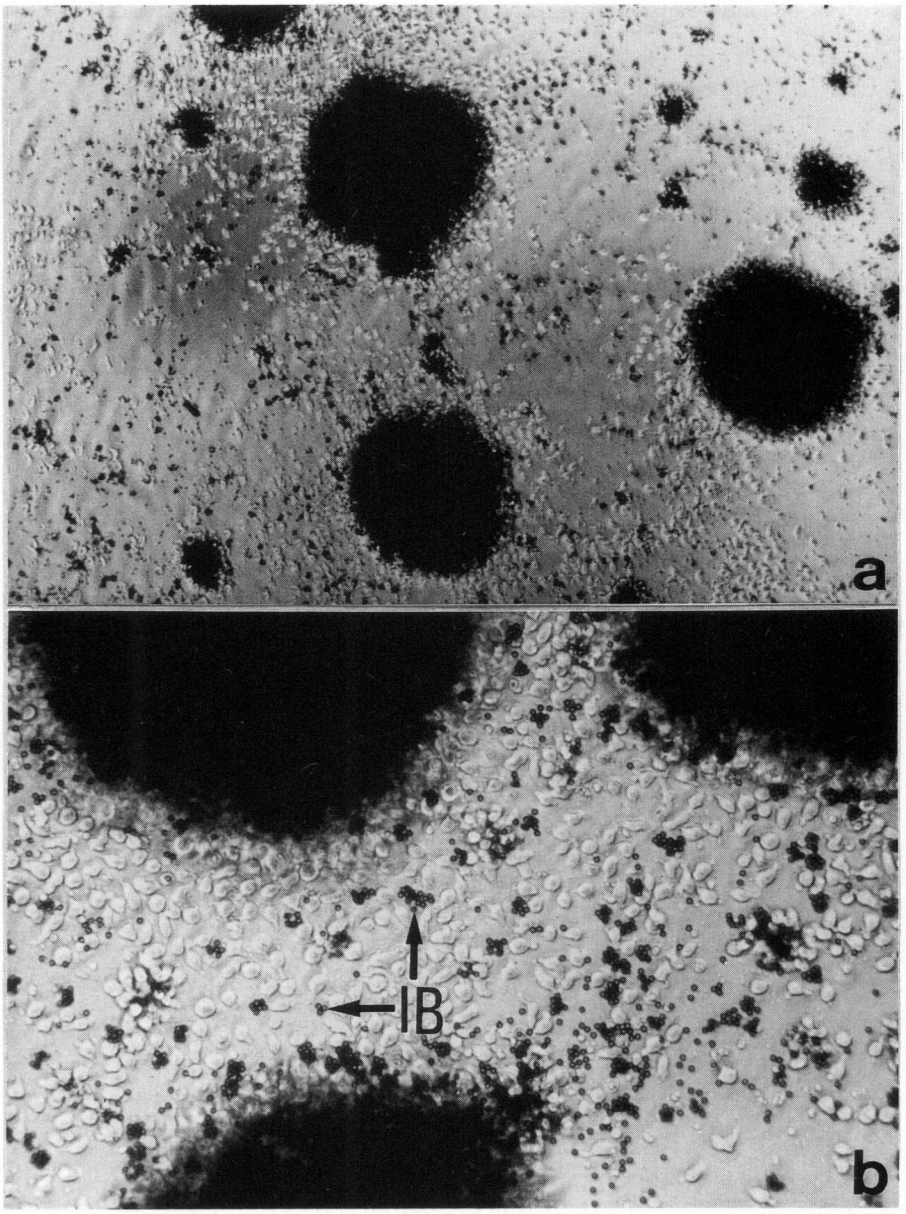

Fig. 1 Phase-contrast microscopic findings of $CD4^+$ T cells with immunomagnetic beads and cultured by RIL-2 plus anti-CD3. **a.** After 3 days of culture showing various sized colony formations(×85) **b.** Higher magnification of figure 1a(×30) immunomagnetic beads (IB).

Table 1 *Phenotype of lymphocytes expanded by RIL-2 plus anti-CD3*

CD2	98	98	99	98	97
CD3	100	95	98	99	97
CD4	99	99	99	99	96
CD8	3	3	2	4	19
CD25	66	90	98	98	94
IL-2R(β)	1	1	1	1	1
CD56	1	3	2	2	2
CLA-DR	54	14	17	14	26
TCR-α/β	99	98	99	98	98
TCR-γ/δ	1	0	0	0	1
CD29	99	100	100	1000	99
CD45RA	40	74	65	81	68
CD45RO	63	36	90	84	52

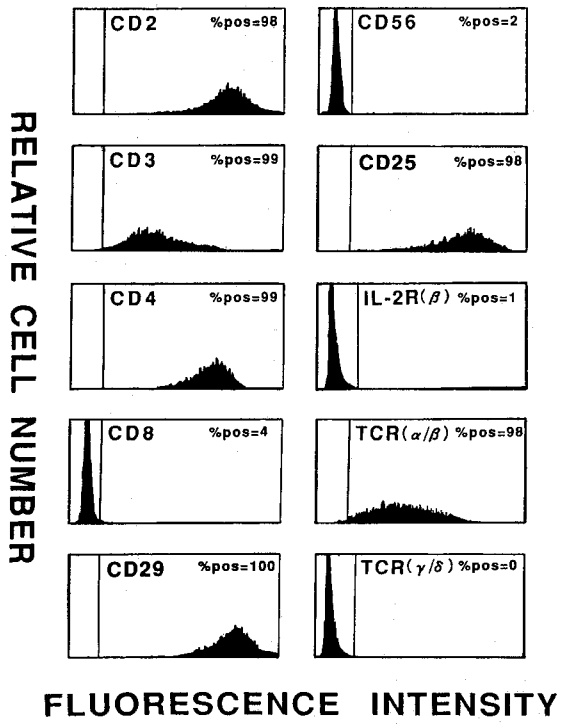

Fig. 2 Representative profiles of surface phenotypes of selectively expanded $CD4^+$ T cells in case D. The surface phenotypes were determined by flow cytometry on day 10. The vast majority of expanded lymphocytes was $CD2^+$, $CD3^+$, $CD4^+$, $CD8^-$, $CD25^+$, $CD29^+$ and TCR $(\alpha/\beta)^+$ T cell.

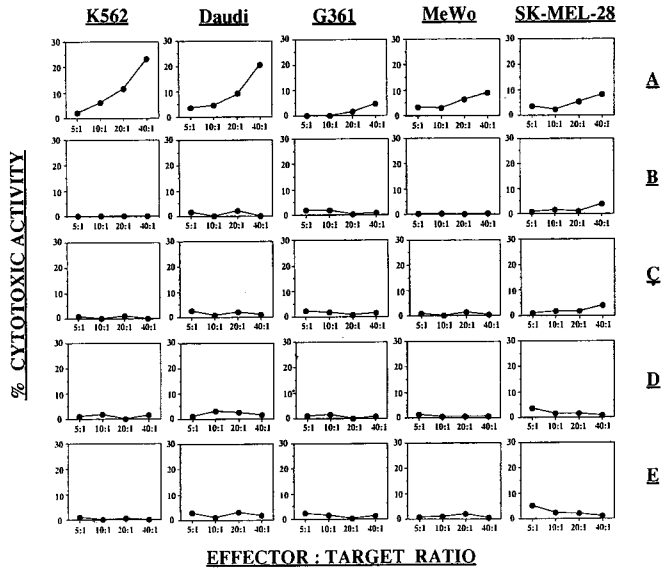

Fig. 3 Cytotoxic activity of selectively expanded CD4+ T cells cultured by RIL-2 plus anti -CD3 after 10 days of culture. Five cell lines including K562, Daudi, and the allogeneic melanoma cell lines G361, MeWo and SK-MEL-28 were used as targets in the cytotoxic assay. Percent cell lysis was compared at several effector-to-target ratios. Only in case A, expanded CD4+ lymphocytes possessed moderate levels of NK and LAK activities and low levels of cytotoxic activities against allogeneic melanoma cell lines.

case D are shown in Figure 2.

Cytotoxic activity of expanded CD4+ T cells

Expanded CD4+ T lymphocytes after 10 day cultures were assayed for activity against 5 target cell lines, including K562, Daudi, and the allogeneic melanoma cell lines G361, MeWo and SK-MEL-28. Percent cell lysis was compared at several effector-to-target ratios (E : T) (5 : 1, 10 : 1, 20 : 1, 40 : 1). Figure 3 shows the results of each experiment. Only in case A, expanded CD4+ lymphocytes possessed moderate levels of NK and LAK activities and low levels of cytotoxic activities against allogeneic melanoma cell lines.

Effects of cytokines released from selectively expanded CD4+ T cell on the in vitro proliferative response of cultured human melanoma cell lines

In order to determine if cytokines released from selectively expanded CD4+ T cells could inhibit the proliferative response of cultured human melanoma cell lines (G361, MeWo), we performed a [^{3}H] thymidine incorporation assay after 3

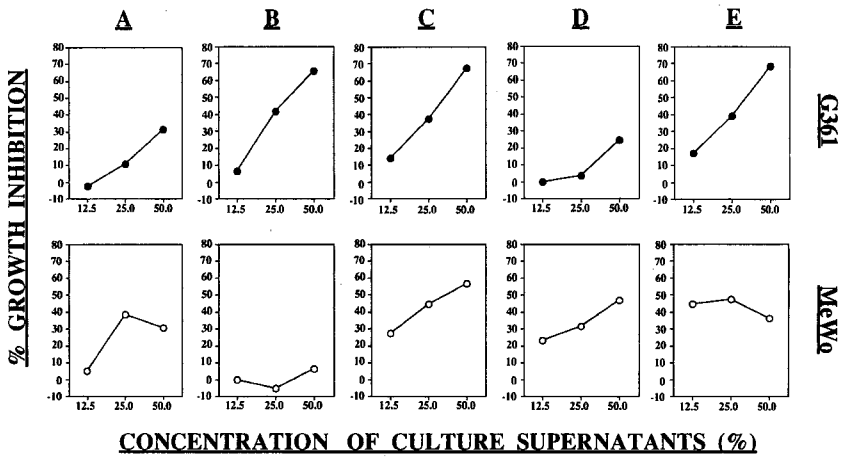

Fig. 4 Effects of cytokines released from selectively expanded CD4+ T cells on the in vitro proliferative response of cultured human melanoma cell lines G361 and MeWo. Ten days after successful generation of the CD4+ T cell cultures, the culture supernatants were obtained through the incubation of 1×10^6 cells/ml in AIM-V media supplemented with 500 U/ml of RIL-2 for 3 days. Melanoma cells (5×10^3) were cultured with serial dilutions of the culture supernatants. After 72 h of incubation, 0.5 μCi/well of [^{3}H]-thymidine was added overnight and the incorporated radioactivity was measured using a β counter. The culture supernatants significantly inhibited growth of human allogeneic melanoma cell lines; however, the results obtained differed with case and cell line.

days of culture with culture supernatants. Figure 4 summarizes the results of each experiment. Although proliferative response of G361 was inhibited in a dose dependent manner, that of MeWo was variably inhibited in each case. Proliferation of MeWo in case E was inhibited by a low concentration of culture supernatants, whereas that in case B was not significantly inhibited even at a high concentration of culture supernatants. Culture supernatants in cases A and C showed almost the same % growth inhibition against both cell lines. Culture supernatants in case B showed significant inhibition of the proliferative response for the cell line G361, but not MeWo. In contrast proliferation of MeWo in case D was more highly inhibited by culture supernatants as compared to that of G361.

Effects of cytokines released from selectively expanded CD4+ T cells on the expression of class I, class II and ICAM-1 antigens on cultured human malignant melanoma cell lines

In an attempt to identify whether cytokines released from selectively expanded CD4+ T cells could alter cell surface molecule expressions of melanoma cell

lines (G361, SK-MEL-28), we analyzed the cell surface antigens by mAbs against class I, class II and ICAM-1 antigens. The results were consistent for each experiment. Class I antigen expression on G361 and SK-MEL-28 was slightly enhanced by culture supernatants in a dose dependent manner; however, there were no statistically significant differences between 25% and 50% culture

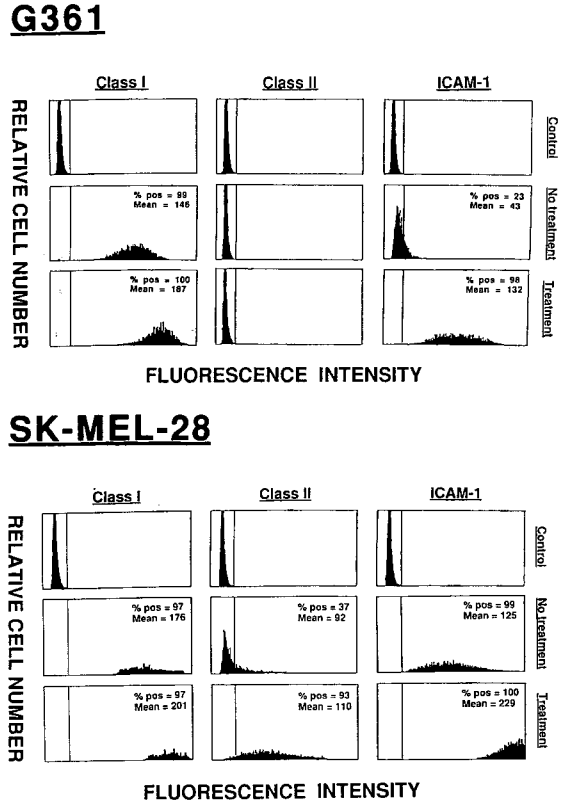

Fig. 5 Effects of cytokines released from selectively expanded $CD4^+$ T cells on the expression of class I, class II and ICAM-1 antigens on cultured human melanoma cell lines G361 and SK-MEL-28. Serial concentrations of culture supernatants obtained as mentioned in the materials and methods were added and incubated for 18 hr with melanoma cells. The results were consistent for each experiment. Representative results of case D (50% culture supernatants) are shown in this figure. Class I antigen expression on G361 and SK-MEL-28 was slightly enhanced by the culture supernatants. Class II antigen expression on SK-MEL-28, nearly 40% of which were class II positive, was moderately enhanced by them. Culture supernatants had no effect on the expression of class II on G361, which was originally class II negative. ICAM-1 expression of both melanoma cell lines was significantly enhanced.

supernatants treatment group (data not shown). Each culture supernatant had no effect on the expression of class II in melanoma cell line G361, which was originally class II negative, even at a high concentration of culture supernatants. Class II antigen expression on SK-MEL-28, nearly 40% of which were class II positive, was moderately enhanced by culture supernatants. ICAM-1 expression of both melanoma cell lines was enhanced by a low concentration of culture supernatants. Representative results of fluorescence analysis of case D (50% culture supernatants) are shown in Figure 5. We also observed that surface expression and intensity of these molecules on melanoma cell lines were enhanced in time dependent manners (data not shown).

Cytokine assays

In an attempt to identify what kinds of cytokines were released from selectively expanded $CD4^+$ T cells in vitro, we analyzed the cytokine concentrations (IL-2, IL-4, IL-6 and IFN-γ) in the culture supernatants with commercially available enzyme immunoassay and radioimmunoassay systems. The results are summarized in Table 2. The concentration of IL-2 in the culture supernatants of case A, B and D were 456 U/m*l*, 308 U/m*l* and 364 U/ml, respectively. Control culture supernatants contained 120 U/m*l* of IL-2. IL-2 production in each case was estimated at 336 U/m*l*, 188 U/ml and 244 U/m*l*, (mean ± standard deviation [SD] , 265±75 U/m*l*), respectively. IL-4 productions were below the detection limit (<31 pg/m*l*). Significant amounts of IL-6 and IFN-γ were detected in

Table 2 *Production of cytokines by $CD4^+$ cells expanded by RIL-2 pius anti-CD3*

	A	B	D	detection limit
IL-2 [U/ml]	336	188	244	< 0.8
IL-4 [pg/ml]	< 31	< 31	< 31	< 31
IL-6 [pg/ml]	103	131	140	< 4.0
IFN-γ [U/ml]	43	39	160	< 0.1

each case(mean ± SD, 125±19 pg/m*l* and 81±69 U/m*l*, respectively).

DISCUSSION

Many recent reports have described adoptive immunotherapy with LAK cells, CTL and TIL in conjunction with RIL-2 for the treatment of advanced cancer. Despite extensive clinical trials, partial or complete responses have only been demonstrated in a limited number of patients with certain types of cancer(1-4).

The success of adoptive immunotherapy may depend on the generation of a sufficient number of tumor-specific lymphocytes. In addition, introduction of helper functions at local sites of tumors may be beneficial for the maintenance of cytotoxic activity of transferred cells and to accelerate the generation of antitumor effector cells in tumor-bearing hosts(5-7). In an attempt to overcome helper function deficiency, we selected human $CD4^+$ T cells using immunomagnetic beads from PBMC of 5 healthy volunteers, and cultured them with RIL-2 plus soluble anti-CD3 culture supernatants. In the present study, we describe these selectively expanded $CD4^+$ T cells with regards to their surface phenotypes, cytotoxic activities, released cytokines, as well as the growth inhibitory effects of cultured human melanoma cell lines and the immunomodulation of tumor associated antigens expressed on their surfaces.

Anti-CD3 mAb has a mitogenic effect and produces large increases in cell number when PBMC and TIL are cultured with both RIL-2 and anti-CD3(12-17). Unlike previous reports(11-18), we used soluble anti-CD3 culture supernatants to simplify the culture system, which we added only once at the beginning of culture. When $CD4^+$ T cells separated by immunomagnetic beads were cultured with RIL-2 alone, a sufficient number of the cells for further analyses were not obtained (data not shown). When they were cultured with RIL-2 plus anti-CD3, high cell yields were easily obtained. Emmrich *et al.*(19) reported that double immobilization of anti-CD3 and anti-CD4 induced higher proliferation of $CD4^+$ T cells as compared to single immobilization of anti-CD3. In our culture system, $CD4^+$ T cells were cross-linked with magnetic beads and activated through RIL-2 plus anti-CD3. These results suggest that our culture procedure is the simplest available method to expand $CD4^+$ T cells from a small amount of starting materials.

The vast majority of expanded lymphocytes seen in 10 day cultures was $CD2^+$, $CD3^+$, $CD4^+$, $CD8^-$, $CD25^+$, $CD29^+$, $CD56^-$ and TCR $(\alpha/\beta)^+$T cells. On the basis of various isoforms of CD45, human $CD4^+$ T cells can be divided into two populations, i. e., memory T cells and naive T cells. While naive T cells are confined to the $CD45RA^+$ subset, memory T cells are identifiable by CD45RO expression(20,21). In the early phase of cultures, selectively expanded $CD4^+$ T cells contained an admixture of $CD45RO^+$ cells and $CD45RA^+$ cells. However, in continuing in vitro expansion, there was an increase in $CD45RA^+$ cells and a decrease in $CD45RO^+$ cells. This was demonstrated particularly well for case D, where after 10 days of culture, 81% of the expanded cells were $CD45RA^+$, and 84% of them were $CD45RO^+$. After 28 days of culture the $CD45RA^+$ subpopulation had increased to 97%, whereas the $CD45RO^+$ subpopulation had decreased to 5% (data not shown).

The IL-2R is composed of at least two subsets, the p55 (IL-2R-α) and p75 (IL-2R-β) glycoprotein(22-24). The low-affinity IL-2R is composed of the p55 subunit, while the intermediate-affinity IL-2R is composed of the p75 subunit, and the high-affinity IL-2R is a complex of both subunits. An increase in CD25 (IL-2R-α) expression on $CD4^+$ T cells was observed during the culture. Particularly in the cases of C and D, 98% of expanded $CD4^+$ T cells possessed CD25 antigen. Because a high population of $CD4^+/CD25^+$ T cells could not be obtained by culture using IL-2 plus anti-CD3 (data not shown), it is conceivable that cross-linking of cell bound CD4 with anti-mouse IgG coupled immunomagnetic beads may play a key role for the enhanced expression of CD25 antigen. Nishimura *et al.* (18) demonstrated that $CD4^+$ T cells cultured with RIL-2 plus immobilised anti-CD3 induced p75 (IL-2R-β) expression. However, we could not induce p75 on $CD4^+$ T cells in our culture system. This discrepancy may have been caused by the different method of anti-CD3 we used.

It has been well known that the majority of Th cell clones exhibit both helper and killer functions(8-10). Akbar *et al.*(25) demonstrated that $CD4^+/CD45RO^+$ and $CD4^+/CD45RA^+$ T cells showed alloantigen-specific cytotoxic activity although the cytotoxic activity of these cells was weaker than that of $CD8^+$ T cells. Some $CD4^+$ T cells are also known to release significant amounts of perforin and serine esterase to destroy target cells(18). Therefore, introduction of helper functions at local sites of tumors may be useful to generate antitumor effector cells in tumor-bearing hosts.

A number of studies have examined the ability of naive and memory $CD4^+$ T cells to synthesize cytokines. Naive T cells synthesize greater amounts of IL-2 than memory T cells(26,27). IL-4, IL-6 and IFN-γ are also preferentially synthesized by memory T cells rather than by naive T cells(28-29). These cytokine production patterns suggest that adoptive immunotherapy might be more effective if activated memory T cells are transferred into the tumor sites together with tumor specific killer cells. Selectively expanded $CD4^+$ T cells consisted of various populations of $CD45RA^+$ and $CD45RO^+$ cells; however, almost all of them expressed CD25 antigen. Recently, Kanegane *et al.*(30) demonstrated that $CD4^+/CD45RA^+$ T cells expressing CD25 might already acquire memory cell-like function with respect to cytokine production and provide help for B cell differentiation into Ig-producing cells. As expected, although IL-4 production was not detected, large amounts of IL-2,IL-6 and IFN-γ productions were observed in the present cytokine assays. These results suggested that selectively expanded $CD4^+$ T cells by immunomagnetic beads in a culture with RIL-2 plus anti-CD3 can induce memory T cell-like function.

The current investigations indicate that melanoma cells can be affected by

multiple cytokines acting on cell proliferation and on antigenic profile. Different classes of antibody-defined melanoma antigens such as HLA class I, II antigens and ICAM-1 could predict the interaction of tumor cells with immune cells, thus they would function as determinants of antitumor responses. IFN-γ revealed a dose-dependent anti-proliferative effect on melanoma cells and induced a dedifferentiated and biologically aggressive phenotype of melanoma cells(31). The frequent co-modulation of HLA antigens and ICAM-1 by IFN-γ noted by Mortarini *et al.* suggests that exposure of melanoma cells to IFN-γ might contribute significantly to T cell mediated responses(32). IL-2 has no direct cytotoxic effects on melanoma cells in vitro, but it transforms precursor lymphocytes into LAK cells and stimulates the capacity to lyse tumor cells resistant to NK cells(31). Although there have been few reports about IL-6, recently Kirnbauer *et al.*(33) reported that it significantly upregulated G361 melanoma cell ICAM-1 expression.

These anti-proliferative and immunomodulatory effects may contribute to the antitumoral activity of cytokines in vivo. Assays for proliferation of human melanoma cell lines showed that culture supernatants obtained from $CD4^+$ T cells significantly inhibited the growth of human melanoma cell lines. In the flow cytometry analyses, surface expression and intensity of class I, class II and ICAM-1 on human melanoma cell lines were prominently enhanced by the culture supernatants. Particularly, case D, who produced the highest level of IFN-γ, showed the most significant enhancement in all three antigen expressions. One possible explanation for these results is that the surface phenotypes of melanoma cells might be changed in a highly complex way following exposure to IFN-γ or synergistic effects of combinations of IFN-γ and other defined cytokines such as IL-2 and IL-6. However, that alone does not fully explain the considerable changes seen in the surface phenotypes. Other unknown cytokines containing culture supernatants probably play important roles in these phenomena. Further studies are necessary to clarify these observations.

It was possible to maintain the growth and function of selectively expanded $CD4^+$ T cells for over 120 days in AIM-V medium supplemented with 500 U/m*l* of RIL-2 (data not shown). In the present study, we could expand $CD4^+$ T cells from small numbers of starting materials to numbers sufficient for human adoptive immunotherapy. These results strongly suggested that expanded $CD4^+$ T cells have potential for in vivo applications against tumors and to enhance immunologic mechanisms against melanoma cells.

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