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Large-scale culture system of human CD4+ helper/killer T cells for the application to adoptive tumour immunotherapy

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Summary A simple method for the rapid expansion of human CD4+ T cells with both helper and killer functions was established. CD4+ T cells separated from peripheral blood mononuclear cells using immunomagnetic beads were stimulated with immobilised OKT-3 monoclonal antibody (mAb) plus recombinant interleukin 2 (rIL-2) in 96 well culture plates. After 6 day-culture, the CD4+ T cells were restimulated by immobilised OKT-3 mAb for an additional 24 h, then inoculated into concentrated rotary-tissue culture bag and cultured for further 9 days. This procedure yielded a 3000-fold increase in cell number (about $3-5 \times 10^9$ per bag). Most of the cells (over 96%) continued to express CD4+ antigen and retained their capacity to produce IL-2. The activated CD4+ T cells showed marked cytotoxicity against Fc receptor positive tumour cells in the presence of OKT-3 mAb. Moreover, we succeeded in a specific targeting of the expanded CD4+ helper/killer T cells to c-*erbB*-2 positive tumour cells by means of anti-CD3 × anti-c-*erbB*-2 bispecific antibody. These results suggested that our established simple system will be available for the expansion of large number of CD4+ helper/killer T cells which may provide an efficient strategy for adoptive tumour immunotherapy.

Recent work has demonstrated that introducing 'local help' at the tumour site is an important goal for the induction of anti-tumour activity in tumour-bearing hosts (Nishimura *et al.*, 1988; Fearon *et al.*, 1990; Bubenik *et al.*, 1990). It has been demonstrated that adoptive tumour immunotherapy using lymphokine-activated killer (LAK)[5] cells and cytotoxic T lymphocytes was effective in animal and clinical systems (Cheever *et al.*, 1982; Mule *et al.*, 1984; Nishimura *et al.*, 1986; Rosenberg *et al.*, 1985). However, adoptive immunotherapy might give better results if helper T cells were transferred into the locality of the tumour together with killer cells. To develop this helper/killer therapy, it is necessary first to establish a large-scale culture system for human CD4+ T cells.

It has been demonstrated that culture of peripheral blood mononuclear cells (PBMC) in the presence of interleukin 2 (IL-2) caused the predominant growth of CD8+ T cells (Gullberg *et al.*, 1983; Taylor *et al.*, 1985) and the selective *in vitro* growth of CD4+ T cells in the presence of IL-2 has been considered to be difficult. The different IL-2 responsiveness of CD8+ and CD4+ T cells was demonstrated to be derived from their differential expression of p75 IL-2 receptor (IL-2R) (Nakamura *et al.*, 1991; Ohashi *et al.*, 1989). Recently, however, we demonstrated that stimulation of FACStar-sorted CD4+ T cells with immobilised OKT-3 monoclonal antibody (mAb) induced p75 IL-2R expression and IL-2 responsiveness of CD4+ T cells. Moreover, CD4+ T cells have demonstrated to display both IL-2 producing activity and bispecific antibody-directed antitumour activity (Nishimura *et al.*, 1991, 1992*a*). Therefore, CD4+ helper/killer T cells could be available for adoptive tumour immunotherapy if we could develop a more simple method for the isolation and expansion of CD4+ T cells.

In this paper, we describe a simple method for the generation and expansion of CD4+ T cells with both helper and killer functions in the presence of immobilised anti-CD3 mAb plus IL-2. The large-scale expansion of activated CD4+ helper/killer T cells was achieved using a concentrated rotary tissue-culture (CRTC) bag. We also investigated *in vitro* targeting of the CD4+ helper/killer T cells by means of anti-CD3 × anti-c-*erbB*-2 bispecific antibody (BsAb).

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Materials and methods

Monoclonal antibodies

OKT-3 (anti-CD3), OKT-4 (anti-CD4) and OKT-8 (anti-CD8) mAbs were purchased from American Type Culture Collection (Rockville, Mayland, USA). The mAbs against CD45RA (Leu-18) and CD45RO (Leu-45RO) were purchased from Becton Dickinson (Mountain View, CA), and mAb against CDw29 (4B4) from Coulter Electronics (Hialeah, FL). SER-4 mAb recognising p185 kD extracellular domain of c-*erbB*-2 gene product (kindly donated by Dr T. Masuko, Tohoku University Pharmaceutical Institute, Sendai 980, Japan) was produced from the mice immunised with SK-BR-3 breast cancer cells as described previously (Masuko *et al.*, 1989).

Separation of CD4+ T cells by immunomagnetic beads

Human PBMC were isolated from two breast cancer patients and two colon cancer patients by Ficoll/Conray (1.077 g ml^{-1}) gradient centrifugation. The cells were incubated at 37°C for 30 min on a nylon-wool column (Wako Pure Chemicals, Osaka, Japan) to remove macrophages and B lymphocytes. Nylon-passed T enriched cells (10^7 cells) were incubated with 10 μg of OKT-4 mAb on ice for 15 min. The cells were washed twice with RPMI-1640 medium containing 10% human AB serum. The separation of CD4+ T cells was performed by a positive selection technique (Gaudernack *et al.*, 1986) using sheep anti-mouse IgG-coated immunomagnetic beads (Dynabeads M-450, Dynal Inc., Oslo, Norway). Dynabeads were washed three times with RPMI-1640 medium containing 10% human AB serum to remove sodium azide. The OKT-4 mAb-treated cell pellets (10^7) were mixed with Dynabeads (4×10^7) at 4°C for 30 min with gentle shaking every 5 min. CD4+ T cells bound to the Dynabeads were recovered with a Dynal MPC-1 magnet (Dynal Inc.). The purity of CD4+ T cells in recovered cells was over 98%. The isolated CD4+ T cells consisted of 46% of CD45RO and 54% of CD45RA.

Generation and expansion of CD4+ helper/killer T cells by culture with immobilised OKT-3 mAb plus recombinant human IL-2 (rIL-2)

CD4+ T cells ($1-3 \times 10^6$) were isolated from 20 ml of blood of tumour patient using Dynabeads as described above. The CD4+ T cells bound to Dynabeads were suspended in RPMI-1640 medium containing 10% AB human serum, 2 mM glutamine, 1 mM sodium pyruvate, 25 mM Hepes buffer, 100 U ml^{-1} of penicillin and 100 $\mu g\ ml^{-1}$ of streptomycin. OKT-3 mAb was immobilised in 96 well flat-bottomed culture plates by incubating 100 μl of OKT-3 mAb (5 $\mu g\ ml^{-1}$) in each well for 2 h at 37°C. Then, CD4+ T cells ($2-3 \times 10^5$ cells) were added to each well and incubated at 37°C in the presence of 2000 U ml^{-1} of rIL-2. After 3 day-culture, Dynabeads were detached from the cells by vigorous pipetting and were eliminated from the culture using a Dynal magnet. Then, the cells were further expanded in 96 well plate in the presence of 2000 U ml^{-1} of IL-2. The activated CD4+ cells increased to 10^8 levels in cell number using 96 well-plates at 6–7 days after the initiation of culture. A large-scale expansion of CD4+ T cells were further carried out using concentrated rotary tissue-culture (CRTC) bags (CC5100 E; Kawasumi Laboratories Inc., Tokyo, Japan) (Nakamura *et al.*, 1989; Noto *et al.*, 1989). As illustrated in Figure 1, the CRTC bag has two compartments, an inner compartment separated from an outer compartment by a semipermeable membrane (Union Carbide Corp., pore size = 2.4 nm). At 6–7 days after the initiation of culture, the CD4+ helper/killer T cells were restimulated with immobilised OKT-3 mAb for 24 h at 37°C using 96 well plates ($3-5 \times 10^5$ cells/well). Then, the activated cells (2×10^8) were harvested, resuspended in 500 ml of RPMI medium containing 20% human AB serum and 2000 U ml^{-1} of rIL-2, and were poured into an inner compartment of the CRTC bag. Two litres of RPMI medium were placed into an outer compartment. The bag was rotated at an angle of 45° between 0.5 and 1.0 r.p.m., in a 37°C incubator for 8–9 days as described previously (Nakamura *et al.*, 1989).

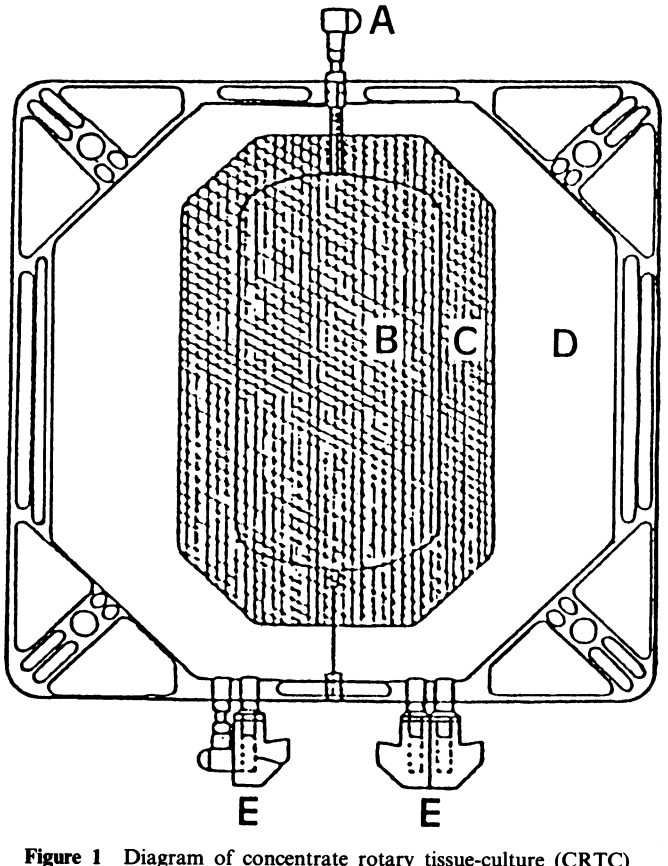

Figure 1 Diagram of concentrate rotary tissue-culture (CRTC) bag. **A**, Outlet of inner space; **B**, Inner bag (semipermeable membrane), **C**, Protection net; **D**, Outer bag; **E**, Outlet of outer space.

Flow cytometry

The analysis of cell surface markers was carried out by FACScan (Becton Dickinson) using the Consort 30 program. The detailed procedure for staining was described in a previous paper (Nishimura *et al.*, 1990). Fluorescence data were collected with logarithmic amplification. For each sample, data from 10,000 volume-gated viable cells were collected.

The production of IL-2 by expanded CD4+ helper/killer T cells

The production of IL-2 was determined using IL-2-dependent murine T cell clone, HT-2. CD4+ T cells (2×10^5) were stimulated with 20 ng ml^{-1} of phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO), 500 ng ml^{-1} of calcium ionophore (A23187, Sigma), PMA plus A23187, OKT-3 mAb-coated Dynabeads (8×10^5) or PMA plus OKT-3 mAb-coated Dynabeads for 24 h at 37°C. After incubation, the culture supernatants were harvested and their IL-2 activity were measured using HT-2 cells. Briefly, 10^4 HT-2 cells/well were cultured with IL-2 samples or standard IL-2 for 20 h, then pulsed with ^{3}H-TdR (0.5 μCi/well) for 4 h. The radioactivity of the labeled cells was measured by the usual liquid scintillation technique (Chiba *et al.*, 1985). As demonstrated previously (Maeda *et al.*, 1988), human IL-2, but not human IL-4, could stimulate the proliferation of mouse T cell lines. Therefore, only human IL-2 activity could be determined using HT-2 cells.

*Preparation of bispecific antibody (BsAb) containing anti-CD3 and anti-c-*erbB-*2*

For the targeting of CD4+ helper/killer T cells, BsAb containing anti-CD3 and anti-c-*erbB*-2 were prepared. OKT-3 mAb was chemically conjugated with anti-c-*erbB*-2 mAb (SER-4) by incubation with a thiol activating reagent 5, 5'-dithio-bis-2-nitrobenzoic acid (DTNB, Sigma, St. Louis MO) as described previously (Nitta *et al.*, 1990*a*; Nitta *et al.*, 1990*b*; Nishimura *et al.*, 1991).

Cytotoxicity assay

The cytotoxicity of the expanded CD4+ T cells was determined by 4 h–^{51}Cr release assay as described previously (Nishimura *et al.*, 1987). Briefly, 2.5×10^3 ^{51}Cr-labeled target cells were incubated with 5×10^4 effector cells in the presence or absence of OKT-3 mAb (5 $\mu g\ ml^{-1}$) or BsAb (0.5 $\mu g\ ml^{-1}$) for 4 h. After 4 h, 100 μl of culture supernatants were removed to determine the cytotoxicity of effector cells. Percent cytotoxicity was determined by the following formula; % cytotoxicity = ^{51}Cr release with effector cells – spontaneous ^{51}Cr release/^{51}Cr release with 0.1 N HCl – spontaneous ^{51}Cr release. OKT-3, OKT-4 and OKT-8 hybridoma cell, Fc-receptor (FcR) positive Daudi (Barkitt lymphoma), K562 (preerythroblastic cell), U937 (monocyte-related cell) and FcR negative IMR-32 glioma cells were used as target cells. NIH-3T3, SV11 (NIH-3T3 transfected with normal c-*erbB*-2 gene), A4–15 (NIH-3T3 cells transfected with mutated c-*erbB*-2 gene), RAScD (NIH-3T3 cells transfected with EJ *ras* gene) and KATOIII (human gastric cancer) were used for the targeting of CD4+ helper/killer T cells. The oncogene transfectants were kindly donated by Dr T. Yamamoto (Tokyo University School of Medicine, Tokyo, Japan) (Masuko *et al.*, 1989).

Results

Proliferation of freshly isolated human CD4+ T cells by immobilised OKT-3 mAb plus rIL-2

Human CD4+ T cells which were freshly isolated from PBMC of breast cancer patient using FACStar or Dynabeads were plated into 96 well plates and their proliferative responses to IL-2, immobilised OKT-3 mAb or IL-2 plus immobilised OKT-3 mAb were determined. As shown in Figure 2, FACStar-sorted CD4+ T cells did not respond to IL-2 or immobilised OKT-3 mAb alone. However, they showed a marked proliferative response in the presence of IL-2 plus immobilised OKT-3 mAb. In contrast to FACStar-sorted CD4+ T cells, CD4+ T cells separated with Dynabeads showed a marked responsiveness to immobilised OKT-3 mAb alone. The OKT-3 mAb-induced proliferation of Dynabeads-separated CD4+ T cells was further enhanced by adding rIL-2 into the culture. It was also notable that Dynabeads-separated CD4+ T cells revealed higher level of proliferative responses to immobilised OKT-3 mAb plus rIL-2 than CD4+ T cells isolated by FACStar.

Large-scale expansion of CD4+ T cells

CD4+ T cells were isolated from 20 ml of blood of two breast and two colon cancer patients by Dynabeads. Then, the separated CD4+ T cells ($1-3 \times 10^6$) were cultured with immobilised OKT-3 mAb plus rIL-2. After 6–7 day-culture, the activated CD4+ T cells (2×10^8) were restimulated with immobilised OKT-3 mAb and inoculated into CRTC bags. As illustrated in Figure 3, CD4+ T cells showed rapid growth in CRTC bag in the presence of rIL-2, and their numbers increased to over $3-5 \times 10^9$ within 14–16 days. This large-scale culture yielded an approximate 3000-fold increase in cell numbers after 16 day-culture.

Helper and killer functions of the expanded CD4+ T cells

The functions of the expanded CD4+ T cells were examined after 16 days of culture. The cells produced large amounts of IL-2 after stimulation with PMA plus A23187, but not with either alone (Table I). The OKT-3 mAb-coated Dynabeads also stimulated CD4+ T cells to produce IL-2. Addition of PMA into the culture enhanced OKT-3 mAb-induced IL-2 production of CD4+ T cells. Thus, the CD4+ T cells expanded by our culture system retained their ability to produce IL-2, which was triggered through T cell receptor-

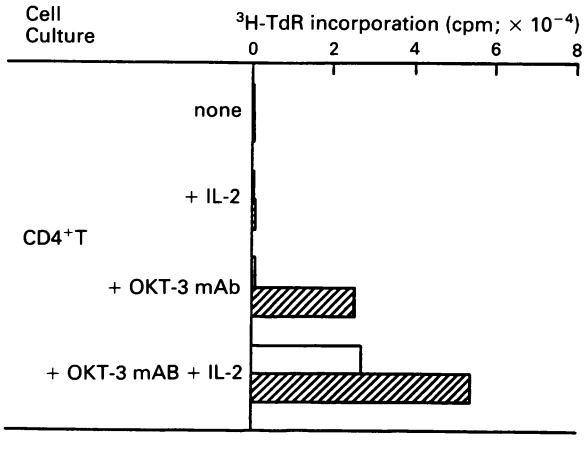

Figure 2 Proliferative responses of human CD4+ T cells isolated with FACStar or Dynabeads. Human CD4+ T cells were isolated using FACStar (open bars) or Dynabeads (hatched bars) as described in Materials and methods. The isolated CD4+ T cells were cultured in 96 well plates with medium, 2000 U ml^{-1} of rIL-2, immobilised OKT-3 mAb or immobilised OKT-3 mAb plus rIL-2. The proliferative responses of the cells were determined by pulsing with ^{3}H-TdR for 4 h at 3 days after culture.

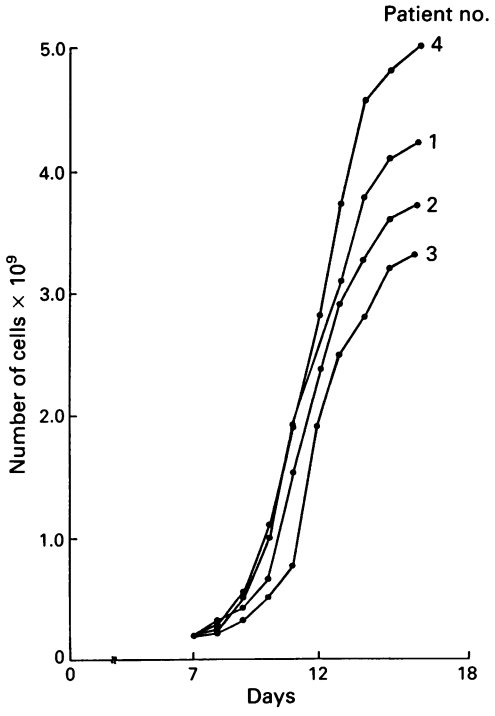

Figure 3 The growth curve of CD4+ helper/killer cells in a CRTC bag. CD4+ T cells were isolated from colon cancer patients (No 1 and No 3) or breast cancer patients (No 2 and No 4) using Dynabeads. The isolated CD4+ T cells ($1-3 \times 10^6$) were cultured with immobilised OKT-3 mAb plus IL-2. At 3 days after culture, Dynabeads were removed from the culture using Dynal magnet. When the cells were increased to $1-2 \times 10^8$ in cell number (6–7 days after culture), the cells were restimulated with immobilised OKT-3 mAb in 96 well plates for 24 h, and then inoculated into CRTC bag. The cell numbers in CRTC bag was counted until 16 days after the initiation of culture.

Table I Production of IL-2 by expanded CD4+ helper/killer T cells

	Patient No.			
Stimulator	*1*	*2*	*3*	*4*
None	<2	<2	<2	<2
PMA alone	<2	<2	<2	<2
A23187 alone	<2	<2	<2	<2
PMA + A23187	64.0	73.5	73.8	79.0
OKT-3 mAb bound Dynabeads	58.0	46.0	58.0	20.0
OKT-3 mAb bound Dynabeads + PMA	68.0	56.0	80.0	78.6

At the end of culture, the cells were stimulated with various stimulators and their ability to produce IL-2 was measured using IL-2-dependent HT-2 cells, as described in Materials and methods. IL-2 activity is expressed by a unit per 2×10^6 cells. The unit of IL-2 was defined by the reciprocal dilution of IL-2 sample that evoked 50% of the maximum proliferation (c.p.m.) of HT-2 cells. Recombinant IL-2 which unit was already defined by NIH standard sample was used for the determination of IL-2 unit of sample.

CD3 complex or intracellular signals. The IL-2 activity in the culture supernatants was completely neutralised by anti-IL-2 polyclonal antibody (data not shown).

The killer functions of the activated CD4+ T cells were also determined. As shown in Figure 4, the activated CD4+ T cells lysed anti-CD3 mAb-producing hybridoma cells, but not anti-CD4 or anti-CD8 mAb-producing hybridoma cells. These results indicated that CD4+ T cells lysed hybridoma cells in anti-CD3 mAb-dependent manner. Therefore, we also examined the cytotoxicity of expanded CD4+ T cells against various tumour cell lines in the presence or absence of OKT-3 mAb. OKT-3 mAb-induced cytotoxicity has been demonstrated to be only achieved by direct binding of the FcR on

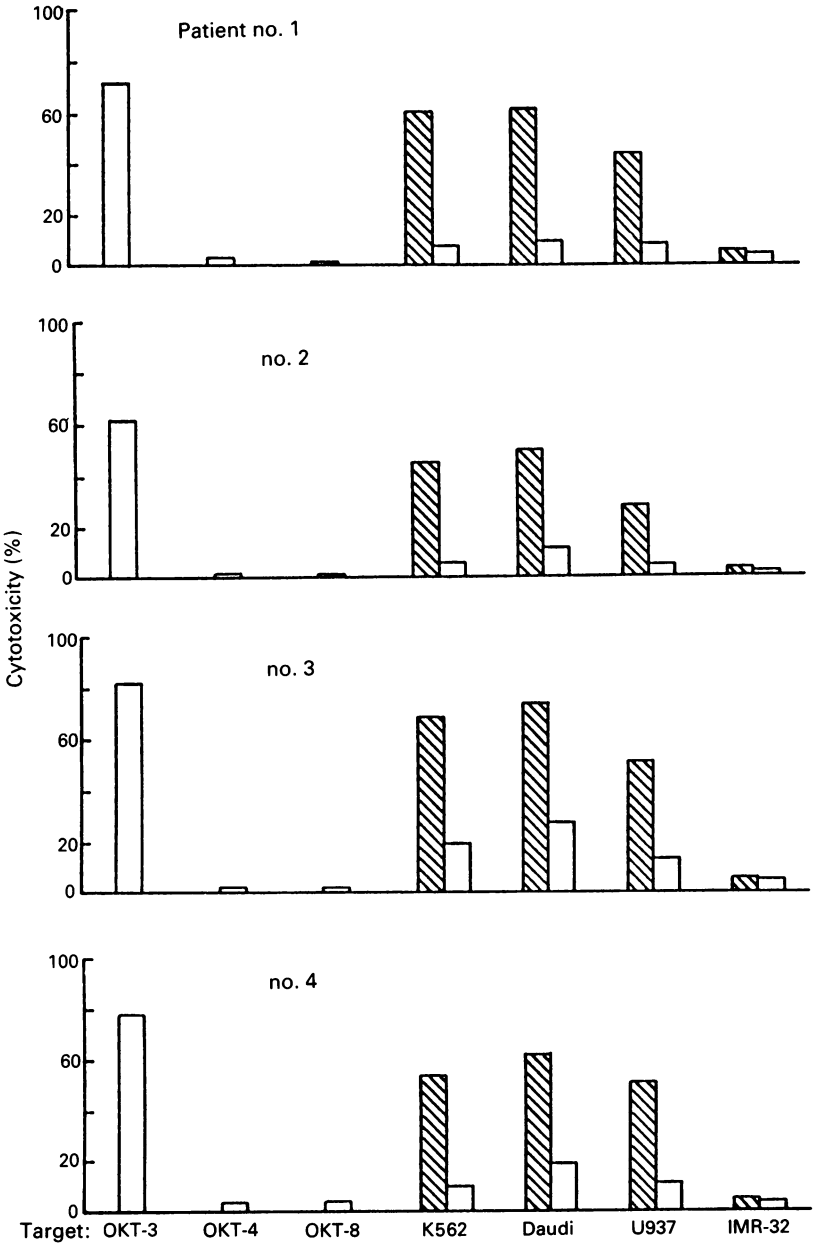

Figure 4 OKT-3 mAb-induced cytotoxicity against a variety of tumour cells by expanded CD4+ helper/killer T cells. CD4+ helper/killer cells were induced from four different tumour patients described in the legend of Figure 3. Cytotoxicity against various tumour cells was measured by a ^{51}Cr release assay in the presence (hatched bars) or absence (open bars) of 5 $\mu g\ ml^{-1}$ of OKT-3 mAb. Effector to target cell ratio was 20:1. No exogenous OKT-3 mAb was added into the culture when hybridoma cells (OKT-3, OKT-4, OKT-8) were used as target cells, but each hybridoma cell could produce anti-CD3, anti-CD4 or anti-CD8 mAb, respectively, during cytotoxicity assay.

target cells to Fc posion of OKT-3 mAb (Seventer *et al.*, 1987). Judging from their sensibility to anti-CD3 mAb induced cytotoxicity, K562, Daudi and U937 cells, but not IMR-32 glioma cells, have demonstrated to express FcR on their cell surface (Seventer *et al.*, 1987 and Nitta *et al.*, 1990*b*). Inconsistent with these results, the activated CD4+ T cells lysed K562. Daudi and U937 cells in the presence of OKT-3 mAb. However, IMR-32 glioma cells were resistant to OKT-3 mAb-induced cytotoxicity of CD4+ T cells (Figure 4). The resistance of IMR-32 against CD4+ T cells-mediated killing was not derived from their unresponsiveness to killing because IMR-32 were lysed by CD4+ T cells by adding anti-CD3 × anti-glioma bispecific antibody (Nishimura *et al.*, 1992*a*). From these results, it was demonstrated that the activated CD4+ T cells with immobilised OKT-3 mAb plus rIL-2 had both helper and killer functions. Such killer function of CD4+ T cells were not detected in freshly isolated CD4+ T cells as reported previously (Nishimura *et al.*, 1992*a*).

In vitro *targeting of the expanded CD4+ helper/killer T cells by using anti-CD3 × anti-c-*erbB*-2 BsAb*

We tried *in vitro* targeting of the expanded CD4+ helper/killer T cells to various kinds of tumour cell lines by using anti-CD3 × anti-c-*erB*-2 BsAb. As shown in Figure 5, the expanded CD4+ helper/killer T cells showed slight cytotoxicity against tumour cell lines in the absence of BsAb. However, addition of BsAb into the culture resulted in the induction of a strong cytotoxicity against c-*erbB*-2 transfectants (A4–15 and SV-11) and c-*erbB*-2 expressing tumour cell line, KATOIII. In contrast, native NIH-3T3, EJ *ras*-transfectant (RAScD) or c-*erbB*-2 negative tumour cell line, Daudi B lymphoma cells were resistant to CD4+ helper/killer cells even in the presence of BsAb. Thus, these results indicated that it was possible to develop an efficient strategy for the targeting of CD4+ helper/killer T cells to tumour cells using BsAb.

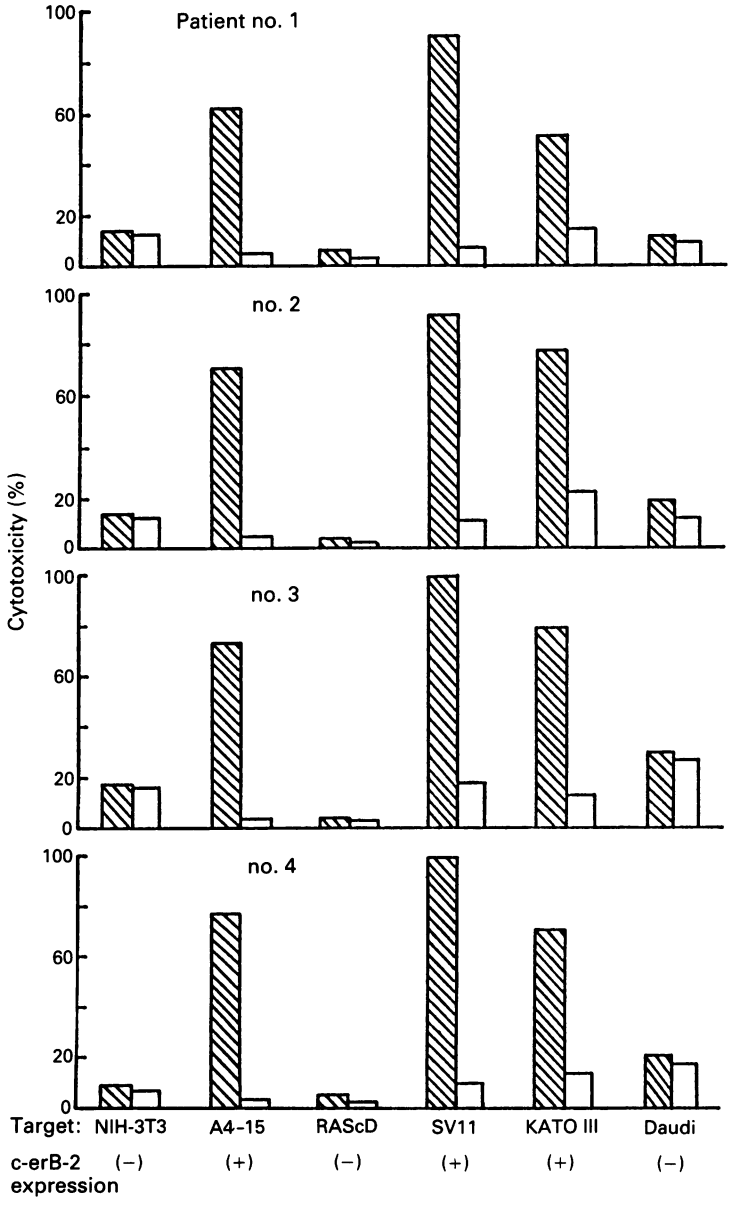

Figure 5 Specific *in vitro* targeting of expanded CD4$^+$ helper/killer T cells by using anti-CD3 × anti-c-*erbB*-2 BsAb. CD4$^+$ helper/killer cells were induced from four different tumour patients described in the legend of Figure 3. The cytotoxicity was determined by 4 h-^{51}Cr release assay in the presence (hatched bars) or absence (open bars) of anti-c-*erbB*-2 BsAb (0.5 μg ml^{-1}). Effector to target cell ratio was 20:1. Oncogene transfectant (SV11, A4–15, RAScD) and human tumour cells (KATOIII, Daudi B lymphoma) were used as target cells. The cell surface expression of c-*erbB*-2 was determined by FACScan.

Flow cytometry analysis of CD4$^+$ helper/killer T cells

The surface markers of the expanded CD4$^+$ helper/killer T cells were analysed by flow-cytometry technique at the end of culture. As shown in Table II, the activated cells continued to express CD4 antigen. Moreover, it was demonstrated that most of the CD4$^+$ T cells expressed CDw29 and CD45RO, though a small percentage of the cells also expressed CD45RA antigen. These results indicated that stimulation of CD4$^+$ T cells with OKT-3 mAb and rIL-2 resulted in a selective expansion of helper-inducer subpopulation of CD4$^+$ T cells.

Table II Cell surface markers of expanded CD4$^+$ helper/killer T cells

Patient No.	*CD4/CD8*[a]	*CD4, CDw29*[b]	*CD45RO/CD45RA*[c]
1	98/1	99	96/1
2	95/2	97	89/10
3	97/1	98	90/6
4	96/1	99	98/3

[a]Percentage of cells expressing either CD4 or CD8 was determined by flow cytometric analysis as described in Materials and methods. [b]Percentage of cells expressing both CD4 and CDw29. [c]Percentage of cells expressing either CD45RO or CD45RA.

Discussion

This paper presents an efficient method for the large-scale culture of human CD4$^+$ helper/killer T cells from Dynabeads-isolated CD4$^+$ T cells. As reported previously (Nakamura *et al.*, 1991), freshly isolated CD4$^+$ T cells by FACStar can not respond to rIL-2 because of their lack of p75 IL-2R expression. However, stimulation of CD4$^+$ T cells with immobilised OKT-3 mAb induced p75 IL-2R expression and their marked proliferative response to rIL-2 (Nishimura *et al.*, 1992*a*). As shown in Figure 2, FACStar sorted CD4$^+$ T cells showed no significant proliferative response to

immobilised OKT-3 mAb in the absence of IL-2. In contrast, $CD4^+$ T cells isolated with Dynabeads revealed a significant proliferative response to immobilised OKT-3 mAb in the absence of rIL-2 (Figure 2). Crosslinking of cell-bound OKT-4 mAb with anti-mouse Ig coupled-Dynabeads resulted in the conversion of OKT-4 mAb from soluble to solid phase. Therefore, Dynabeads-separated $CD4^+$ T cells may be activated through both CD3 and CD4 molecules. Our results are supported by the results of Emmrich *et al.* (1987) who reported that double immobilisation of anti-CD3 and anti-CD4 induced higher proliferation of $CD4^+$ T cells compared with single immobilisation of anti-CD3 mAb. Thus, the separation procedure of $CD4^+$ T cells using Dynabeads has two advantages over a method using a cell sorter; (1) The procedure is simple and rapid; (2) Dynabeads preparation causes augmented proliferation of $CD4^+$ T cells against immobilised anti-CD3 plus IL-2.

As reported previously (Noto *et al.*, 1989), we demonstrated that large numbers of LAK cells could be expanded in CRTC culture bag which was developed by us. We have now shown that the CRTC culture bag is suitable for the expansion of $CD4^+$ T cells. It has been reported that culture of PBMC by culture plate in the presence of rIL-2 alone yielded about 10–20 fold increase in cell numbers, and culture with immobilised anti-CD3 mAb plus rIL-2 yielded a 500 fold increase in cell numbers (Anderson *et al.*, 1988). We also demonstrated that culture of isolated $CD4^+$ T cells with immobilised OKT-3 mAb plus rIL-2 caused 200 fold increase from 10^6 to 2×10^8 cells in 6–7 days culture. As previously reported by us, $CD4^+$ T cells could be expanded for over a month in a split culture using 96 well plate. Because $CD4^+$ T cells grew in 96 well plates at the same growth rate as in the CRTC bags, it is theoretically possible to expand 10^9 levels of $CD4^+$ T cells by general tissue culture method if we use hundreds of culture plates and huge volumes of culture medium. As shown in Figure 3, we demonstrate that 10^9 levels of $CD4^+$ T cells can be expanded using a simple CRTC culture bag system without changing medium. Our established large-scale culture system, which consists of initial culture using 96 well plates and late culture using the CRTC bag, yielded a 3000 fold increase in cell number within 16 days of culture. Therefore, it is possible to obtain 10^{10} isolated $CD4^+$ T cells in less than 20-day culture. We believe that our established culture system is the simplest method for the large-scale expansion of $CD4^+$ helper/killer T cells. In this paper, we showed four representative results using $CD4^+$ T cells isolated from tumour patients. Generally, tumour-bearing hosts reveal suppressed immune responses (Burgar *et al.*, 1984), whereas the separated $CD4^+$ T cells from tumour patients showed the same level of proliferative responses to immobilised OKT-3 plus rIL-2 as $CD4^+$ T cells isolated from healthy donors (Nishimura *et al.*, 1992*a*). Actually, $CD4^+$ T cells obtained from both tumour patients and healthy donors showed the same growth curve in CRTC bags (data not shown).

As shown in Table I and Figure 4, the $CD4^+$ T cells expanded in CRTC bag also showed both IL-2 producing activity and OKT-3 mAb-mediated cytotoxicity. Triggering of the expanded $CD4^+$ T cells through CD3 molecule stimulated both helper and killer functions. Therefore, if we can target the $CD4^+$ helper/killer cells to tumour cells using anti-CD3 × anti-tumour bispecific antibody, the targeted $CD4^+$ T cells act as both killer and IL-2 producer at tumour local site. As shown in Figure 5, $CD4^+$ helper/killer cells expanded by our established methods were specifically targeted to c-*erbB*-2 positive tumour cell lines *in vitro* using anti-CD3 × anti-c-*erbB*-2 BsAb. We have an evidence that $CD4^+$ helper/killer cells showed a strong therapeutic efficacy against human colon cancer cells implanted into nude mice by combination with BsAb (Nishimura *et al.*, 1992*b*). Recent results demonstrated that $CD4^+$ tumour infiltrating lymphocytes were specifically targeted to tumour site compared with LAK cells (Grimm *et al.*, 1991). Several animal experiments also demonstrated that adoptive tumour immunotherapy using $CD4^+$ T cells was effective (Bookman *et al.*, 1987). The therapeutic advantage of $CD4^+$ helper/killer cells is that they may function both as effector cells and helper cells at local site of tumour. We believe that our established simple method for the large-scale culture of $CD4^+$ helper/killer will be available for new trials of adoptive immunotherapy (helper/killer therapy).

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