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Bispecific Antibody-directed Antitumor Activity of Human CD4$^+$ Helper/Killer T Cells Induced by Anti-CD3 Monoclonal Antibody plus Interleukin 2

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Freshly isolated human CD4$^+$ T cells can not respond to recombinant interleukin 2 (rIL-2) because of their lack of p75 IL-2 receptor expression. However, we succeeded in inducing a marked proliferation of purified CD4$^+$ T cells by activation with rIL-2 plus anti-CD3 monoclonal antibody (mAb) cross-linked to a plastic plate. The proliferated CD4$^+$ T cells produced a significant amount of IL-2 upon stimulation with phorbol ester plus A23187. Interestingly, CD4$^+$ T cells activated with anti-CD3 mAb plus rIL-2 revealed a strong cytotoxic activity against Fc receptor (FcR)-positive tumor cells in the presence of anti-CD3 mAb. Moreover, the CD4$^+$ T cells could lyse FcR-negative glioma cells by targeting with bispecific mAb containing anti-CD3 mAb and anti-glioma mAb. Thus, we demonstrated that rIL-2 and immobilized anti-CD3 mAb allowed the rapid generation of human CD4$^+$ helper/killer T cells, which may be useful for the development of a new adoptive tumor immunotherapy.

Key words: Bispecific antibody — CD4$^+$ T cells — Interleukin 2 — Anti-CD3 monoclonal antibody — Human

Adoptive tumor immunotherapy using cytotoxic T lymphocytes specific for tumor cells has been demonstrated to be effective to eradicate *in vivo* established tumor.[1-3] The major difficulty in the application of this approach to the treatment of human cancer has been the inabillity to induce sufficient numbers of autologous human cytotoxic T cells against tumor antigen. To overcome this problem, adoptive tumor immunotherapy using LAK[6] cells has been investigated.[4-7] For the application of LAK cells to immunotherapy, the *in vivo* infusion of large amounts of rIL-2 is essential because LAK cells are IL-2-dependent cells.[8] However, *in vivo* administration of large amounts of rIL-2 causes several side effects, even though rIL-2 is rapidly catabolized *in vivo*.[9,10] Therefore, it is necessary to develop a new method to augment "local help" which facilitates the activation and proliferation of antitumor effector cells at the tumor site. The ideal method to enhance local help is to transfer helper T cells to the tumor site. However, it has been considered to be difficult to expand freshly isolated CD4$^+$ helper T cell population in the presence of IL-2.[11]

As reported previously,[12] freshly isolated human CD4$^+$ T cells could not respond to rIL-2 because of their lack of p75 IL-2 receptor expression. However, we found that stimulation of isolated CD4$^+$ T cells by immobilized anti-CD3 mAb in the presence of rIL-2 resulted in a striking proliferation of CD4$^+$ T cells (Fig. 1). Such proliferation was not observed upon stimulation with rIL-2 alone or immobilized anti-CD3 mAb alone at any time during culture. CD4$^+$ T cells expressed p55, but not p75 IL-2R before culture. However, culture of CD4$^+$ T cells with anti-CD3 mAb plus rIL-2 caused a significant expression of p75 IL-2R (data not shown). Therefore, induction of high-affinity IL-2R by immobilized anti-CD3 mAb may be the major cause of the augmented proliferation of CD4$^+$ T cells in the presence of IL-2 and immobilized anti-CD3 mAb.

Over 98% of the proliferating cells continued to express CD4$^+$ antigen 10 days after culture (data not shown). Moreover, the proliferating CD4$^+$ T cells maintained their helper function and could produce significant amounts of IL-2 by stimulation with PMA plus A23187, in accordance with previous results (Fig. 2).[13] CD4$^+$ T cells also produced IL-2 upon stimulation with anti-CD3-conjugated beads (data not shown). Recently, it has been

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[6] Abbreviations: LAK, lymphokine-activated killer; rIL-2, recombinant interleukin 2; IL-2R, IL-2 receptor; PMA, phorbol 12-myristate 13-acetate; mAb, monoclonal antibody; CD4$^+$ helper/killer T cells, CD4$^+$ T cells which have both helper and killer functions.

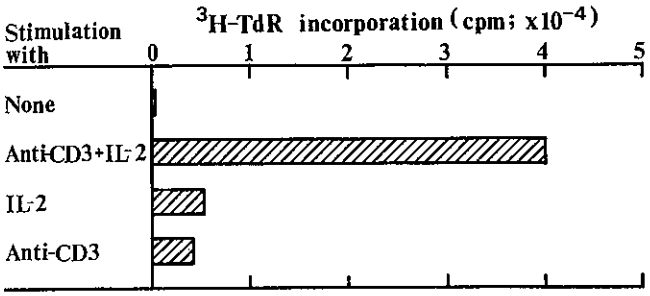

Fig. 1. Proliferation of freshly isolated human CD4$^+$ T cells by immobilized anti-CD3 mAb plus rIL-2. Peripheral blood mononuclear cells were obtained from healthy donors by Ficoll Hypaque centrifugation. Human CD4$^+$ T cells were isolated by FACstar after staining with FITC-conjugated OKT4. The isolated cells suspended in RPMI1640 medium containing glutamine, penicillin, streptomycin, HEPES buffer and 10% heat-inactivated human AB serum were cultured in 96-well plates at the cell concentration of 2×10^5/well with immobilized anti-CD3 mAb plus rIL-2 for 4 days at 37°C. Immobilized anti-CD3 mAb was prepared by incubation of the 96-well flat-bottomed plates with 100 μl per well of anti-CD3 mAb (5 μg/ml) for 1 h at 37°C. After culture, the cells were pulsed with ^{3}H-TdR (0.5 μCi/well) for 4 h and their ^{3}H-TdR incorporation was measured by the method described in "Materials and Methods."

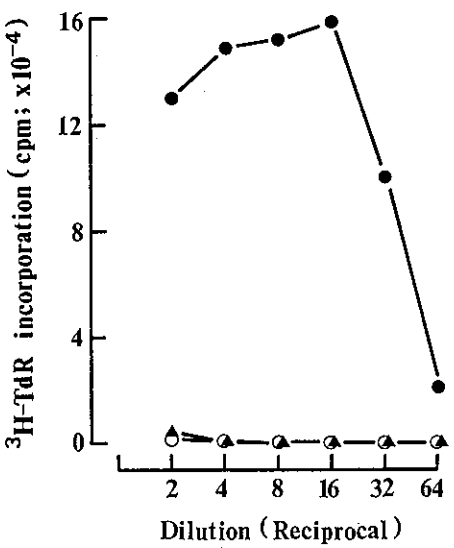

Fig. 2. The production of IL-2 in the expanded CD4$^+$ T cells by stimulation with PMA plus A23187. CD4$^+$ T cells (10^6/ml), which were cultured for 10 days *in vitro* in the presence of immobilized anti-CD3 mAb plus rIL-2, were stimulated with PMA (20 ng/ml) (○), A23187 (500 ng/ml) (▲) or PMA plus A23187 (●) for 24 h. After culture, the IL-2 activity of culture supernatant was measured using the IL-2-dependent HT-2 cell line.

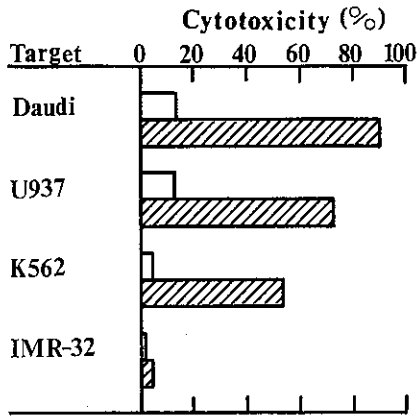

Fig. 3. CD4$^+$ T cells expanded with anti-CD3 mAb plus rIL-2 can lyse various tumor cells in the presence of anti-CD3 mAb. CD4$^+$ T cells were cultured with immobilized anti-CD3 mAb plus rIL-2 for 10 days. After culture, their cytotoxicity against Daudi, U937, K562 or IMR-32 was determined by 4-h ^{51}Cr-release assay in the presence (▨) or absence (□) of 5 μg/ml of anti-CD3 mAb. The E/T ratio was 10:1.

demonstrated that the majority of cloned CD4$^+$ T cells showed both helper and killer functions at clonal levels.[14,15] Jung *et al.*[16] also reported that anti-CD3 mAb-activated CD4$^+$ T cells revealed cytotoxicity in the presence of anti-CD3 mAb. To investigate the cytotoxic potential of activated CD4$^+$ T cells, we carried out a reverse antibody-dependent cell-mediated cytotoxicity assay, which enabled us to measure the cytotoxicity of CTL without knowing the antigen specificity.[17] As shown in Fig. 3, the proliferated CD4$^+$ T cells showed marginal cytotoxicity in the absence of anti-CD3 mAb. However, they showed strong cytotoxic activity against FcR-positive Daudi, U937 and K562 tumor cells in the presence of anti-CD3 mAb. In contrast, FcR-negative IMR-32 glioma cells were resistant to CD4$^+$ killer T cells even in the presence of anti-CD3 mAb. Thus, we demonstrated that activation of CD4$^+$ T cells with immobilized anti-CD3 mAb plus rIL-2 allowed the rapid generation of a CD4$^+$ T cell population with both helper and killer functions.

In order to apply such a CD4$^+$ helper/killer T cell population to adoptive tumor immunotherapy, it is necessary to develop some method to target these effector cells to the tumor site. Recent studies have demonstrated that bispecific antibody containing anti-CD3 and anti-target antibody is useful for the targeting of antitumor effector cells.[18-20] Therefore, we next examined whether CD4$^+$ T cells expanded by anti-CD3 mAb plus rIL-2 could be targeted to FcR-negative IMR-32 glioma cells *in vitro* using bispecific antibody consisting of anti-CD3 mAb×

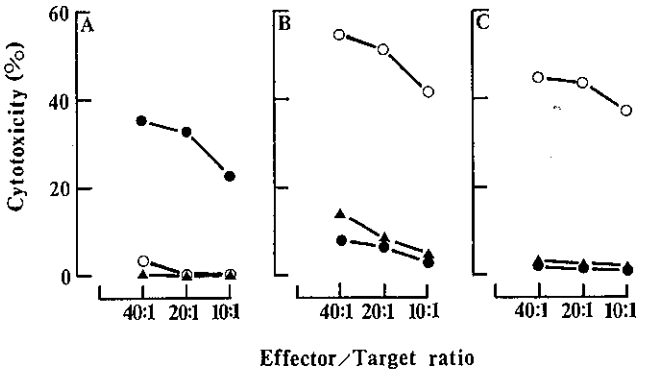

Fig. 4. *In vitro* targeting of $CD4^+$ helper/killer T cells by anti-CD3 × anti-tumor bispecific antibody. The cytotoxic activity of $CD4^+$ helper/killer T cells against FcR-negative IMR-32 glioma cells (A), FcR-positive Daudi B lymphoma cells (B) or FcR-positive K562 chronic myeloid leukemia cells (C) was measured by 4-h ^{51}Cr-release assay in the presence of none (▲), anti-CD3 mAb (5 μg/ml) (○), or anti-CD3 × anti-glioma bispecific antibody (5 μg/ml)(●). Bispecific antibody was prepared as described previously.[19,20] Target cells (2500 cells/well) were cultured with effector cells at various effector-to-target (E/T) ratios as indicated in the illustration. Over 98% of effector cells were $CD3^+CD4^+$ T cells.

anti-glioma specific mAb. As shown in Fig. 4, the $CD4^+$ helper/killer T cells showed little cytotoxicity against IMR-32 glioma cells even in the presence of anti-CD3 mAb. However, addition of bispecific antibody into the culture caused a dramatic increase of cytotoxic antivity of $CD4^+$ T cells against IMR-32 glioma cells (Fig. 4A). In contrast, the cytotoxicity of $CD4^+$ helper/killer cells against Daudi (Fig. 4B) and K562 cells (Fig. 4C), both of which were highly sensitive to $CD4^+$ helper/killer cells in the presence of anti-CD3 mAb, was not augmented by the addition of anti-CD 3 × anti-glioma bispecific antibody. These results demonstrated that $CD4^+$ helper/killer T cells could be specifically targeted to tumor cells by combination with bispecific antibody. Because over 98% of effector cells used for the experiment were $CD4^+$ T cells (data not shown) and NK-sensitive K562 cells were not lysed by the effector cells, $CD8^+$ T cells or NK cells were not involved in the bispecific antibody-directed cytotoxicity against IMR-32.

It is possible to maintain the growth and function of the activated $CD4^+$ helper/killer T cells for over 50 days by weekly stimulation with immobilized anti-CD3 mAb plus rIL-2 (data not shown). Therefore, we can easily expand sufficient numbers of $CD4^+$ helper/killer T cells to use for adoptive tumor immunotherapy. If the activation of $CD4^+$ helper/killer T cells were induced at the tumor site by bispecific antibody, it may be possible that the activated $CD4^+$ T cells act as both killer and helper cells which facilitate tumor destruction and IL-2 production at the local tumor site. Taking together these results and the facts that $CD4^+$ helper/killer cells were induced from all tumor patients tested so far (data not shown), it is strongly suggested that $CD4^+$ helper/killer therapy may provide a new strategy for the treatment of human cancer. We are now investigating *in vivo* targeting of $CD4^+$ helper/killer T cells to human tumor inoculated into nude mice.

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