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Antitumor Responses of Adoptively-Transferred Tumor-Specific T-Cell Cultures in a Murine Lymphoma Model

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Abstract: The purpose of this study was to establish an *in vitro* culture method of tumor-specific T cells, and determine the efficacy of the cultured tumor-specific cytotoxic T-lymphocytes (CTL) as an agent of anti-tumor immunotherapy against a murine lymphoma, TIMI.4. Tumor-specific T-lymphocytes derived from C57BL/6 mice (thy-1.2) immune to TIMI.4 were activated by *in vitro* stimulation with the irradiated TIMI.4 cells, and expanded by restimulation with TIMI.4 in the presence of the concanavalin A-stimulated rat spleen culture supernatant, and splenic antigen-presenting cells. *In vitro* restimulation enhanced markedly the proportion of $CD8^+$, a predominant surface marker of CTL, and the cytotoxic activity in the cultured immune T cell population. The resulting TIMI.4-specific T cells were adoptively transferred into nude mice. The tumor cells residing in the host after 7 days of adoptive transfer to B6.PL (thy-1.1) mice were quantified by use of an antibody directed to the thy-1.2 allele. The TIMI.4 cells in the recipient nude mice were decreased in a dose-dependent manner. Anti-tumor activity of the TIMI.4-specific T cells was also demonstrated by a survival test, where the tumor-bearing nu/nu mice which received the activated T-cells survived about 30% longer than the control mice which received the tumor cells alone. These suggest that adoptive transfer of TIMI.4-specific T cells could be a candidate for effective therapy of the murine lymphoma.

Key words: adoptive transfer, cytotoxic T-lymphocytes, immunotherapy, lymphoma.

It has been generally speculated that tumor-specific T-cells cultured *in vitro* in a large quantity and transferred to tumor-bearing mice would attack and abolish the tumor (Byrne and Oldstone, 1984; Lukacher *et al.*, 1984; Moskophidis and Pircher, 1992; Huneycutt and Cao, 1993; Welsh, 1993). It has not, however, clearly demonstrated the significance of such a speculation. The major obstacle in elucidating the question lies in the way how to culture the antigen-specific effector T cells, CTL and/or helper T cells.

The MHC molecule has a single antigen binding cleft that has the capacity to bind a large number of diverse peptides with limited specificity. However, the specific antigen of general tumor cells was rarely found, and it is difficult to utilize them as a target antigen even if they are available. It is therefore desirable to use the whole tumor cell as the source of antigen instead of isolated specific antigens. It could then be anticipated that both helper T cells stimulating the production of antibodies and CTL having the ability to attack tumor cells are induced in immunized animals or humans (Bruno *et al.*, 1987; Hoegen *et al.*, 1989; Kern *et al.*, 1990).

Murine tumors induced by either RNA or DNA tumor viruses can be successfully eradicated by adoptive transfer of cloned $CD8^+$ or $CD4^+$ T cells (Cheever *et al.*, 1980; Fernandex-Cruz *et al.*, 1980; Cerundolo, 1987) $CD8^+$ CTL clones are particularly effective usually when used in combination with interleukin-2 (IL-2) (Donahue *et al.*, 1984; Melief *et al.*, 1991). A complete eradication of large tumor masses has been achieved in the case of Friend virus-induced leukemia and human adenovirus type 5 (Ad5) early region 1 (E1)-induced tumors in mice by treating with cloned $CD8^+$ CTL and IL-2 (Kaplan *et al.*, 1984; Andrew *et al.*, 1986; Kast and Melief, 1991). In another *in vitro* model, CTL cleared the human immunodeficiency virus

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and Epstein-Barr virus from cultured cells (Moss *et al.*, 1978; Walker *et al.*, 1986; Brinchmann *et al.*, 1990; Kannagi *et al.*, 1990).

In this study, attempts were made to culture T cells in a large quantity and to determine the condition in which T cells could be transferred to other mice where they could grow and act as an effector. TIMI.4 cells which express the leukemia G-virus antigen, and lymphoma originated from C57BL/6 were employed as a tumor model in this work. C57BL/6 mice (thy-1.2) of H-2b MHC specificity and B6.PL (74NS) mice (thy-1.1) of the same MHC background were used as the donor and recipient of T cells, respectively; in this system the immune response between host and donor does not occur and trace mapping of transferred T cells was possible through the medium of thy-1 antigen.

Materials and Methods

Mice and tumor cells

Six to eight week old C57BL/6 mice, B6.PL (74NS) mice and nude (nu/nu) mice were obtained from The Korea Research Institute of Bioscience and Biotechnology (Taejon, Korea). TIMI.4, a lymphoma of C57BL/6 (H-2b) origin that expresses G surface antigen induced by leukemia type G (Gross) virus was purchased from the Americal Type Culture Collection (Rockville, USA).

Preparation of Con-A activated rat spleen culture supernatant (CAS)

Spleen was removed, teased into single cell suspension and washed twice in Hank's balanced salt solutions (HBSS). Red blood cells were lysed with a hemolytic buffer (Sigma Chem. Co., St. Louis, USA) and washed. Cells were suspended at 1×10^7 cells/ml in RPMI 1640, 10% fetal bovine serum plus concanavalin A (Con A) at 10 μg/ml. Supernatant was collected after 24 h at 37℃ and cells were resuspended in the same medium. After another 24 h incubation the supernatant was collected, pooled, filtered and frozen. Finally α-methymannoside (500 μg/ml) was added to remove the remaining Con A. CAS was used as a source of murine lymphokines, including IL-2.

Preparation of the tumor-specific donor T cells

Tumor specific donor T cells were prepared from spleens of C57BL/6 mice previously immnunized with TIMI.4. Donor mice were immunized with TIMI.4 by two i.p. inoculations, separated by 2 weeks, of the 1x 107 irradiated (9,000 rad) TIMI.4 cells, and the immune cells were obtained 2 to 6 weeks later. Two million lymphocytes from spleen or lymph nodes of mice were cultured for 5 days in the presence of 2.5×10^5 irradiated TIMI.4 cells in wells of 24-well plates. The cells (2×10^5/ml) were then expanded during a subsequent 7-day culture in a medium containing CAS (10 %), irradiated TIMI.4 (2.5×10^4/ml), and irradiated C57 BL/6 spleen cells (1×10^6/ml) as accessory cells. Cells were cultured for 5 days before they were used in adoptive transfer experiments.

Measurement of CTL activity using a flow cytometer

CTL activities were measured according to the methods described by Grooth *et al.* (1990) with a slight modification. Briefly, 100 μl of target cells (5×10^6/ml) in a FACS tube was stained with octadecanoylaminofluorescein (OAF) by incubation for 30 min at 37℃. After washing, effector cells of serial dilutions were added and centrifuged at 800 rpm for 5 min. After a second incubation for 3 h in a CO_2 incubator, cells were stained by the addition of propidium iodide (PI), washed, and the fluorescence was measured on a flow cytometer. Live gates were set for FL-1-positive populations so that the population that was not labeled with OAF was removed from the analysis. The resulting populations were analyzed for PI staining to discriminate between the live cells and dead cells, from which cytotoxicity could be calculated.

Adoptive transfer immunotherapy using nude mice

TIMI.4 cells and T cell cultures were injected into a peritoneal cavity of nu/nu mice to examine the anti-tumor activity of the T-cell cultures *in vivo*. After 7 days, the cells prepared from peritoneal cavity, spleen, and lymph nodes were incubated with anti-Thy-1.2 mAb (J1j) to detect TIMI.4 and stained with goat anti-rat Ig-FITC.

A survival test was also performed to examine anti-tumor activity of the T cells transferred. Nude mice were divided into 3 groups and injected i.p. with live 5×10^6 TIMI.4 cells. After 6 weeks, 2.5×10^7 and 1.25×10^8 T cells were adoptively transferred i.p. and the survival rates of the mice in a specific pathogen-free state were observed.

Results

Generation and characterization of tumor-specific cytotoxic T lymphocytes

T cells specific for TIMI.4 cells were obtained by culturing lymph nodes or spleen cells from C57Bl/6 mice immunized with irradiated TIMI.4 cells. The cultured cells were expanded by repeated *in vitro* stimulation as described in Materials and Methods. The relative amount of antibody specific to TIMI.4 in sera of immunized C57BL/6 mice was examined to confirm success-

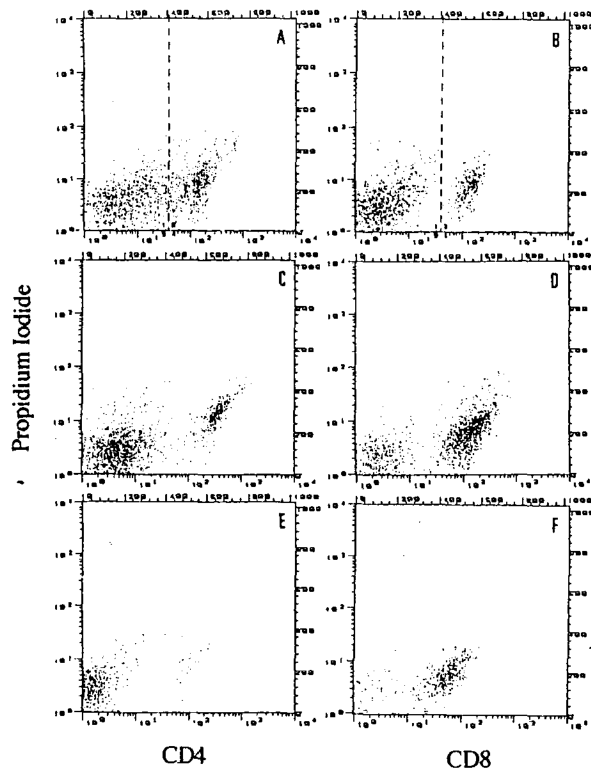

Fig. 1. Subset analysis of TIMI.4-specific T cells prepared from lymph nodes. T cell cultures of primary culture (A and B), secondary culture (C and D), and tertiary culture (E and F) were incubated with GK 1.5 (anti-CD4; A, C and E) or 3.168 (anti-CD8; B, D and F), and subsequently stained with goat anti-rat Ig-FITC. Each samples were also stained with propidium iodide. The fluorescences were measured by a flowcytometer.

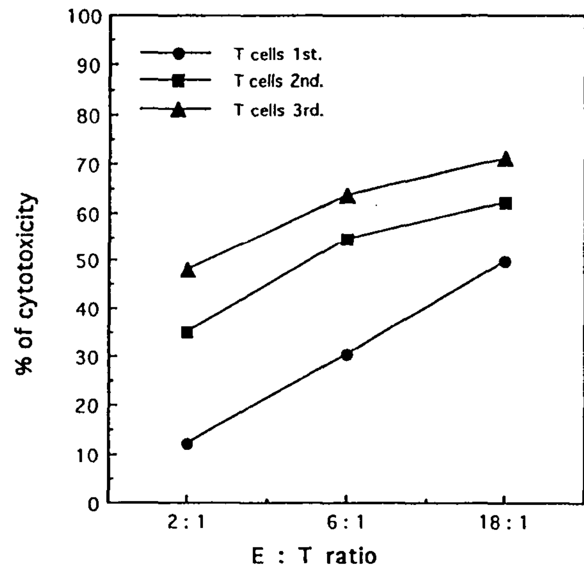

Fig. 2. Cytotoxic activity of TIMI.4-specific T cells against TIMI.4. The cytotoxic activities of cultures LN cells of primary culture (T cells 1st.), secondary culture (T cells 2nd.), tertiary culture (T cells 3rd.) were measured by a flow cytometer.

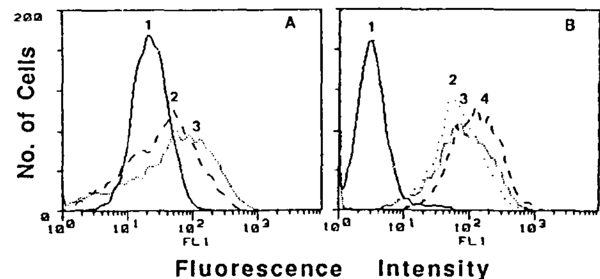

Fig. 3. T helper activity in T cell cultures. Panel A: Sera were prepared from B6.PL mice immunized with none (1), TIMI.4 cells only (2), or TIMI.4 cells plus T cell cultures (3). Appropriately diluted sera were added to TIMI.4, incubated for 45 min on an ice bath. The antibody bound TIMI.4 cells were stained with goat anti-mouse Ig-FITC. The fluorescences were detected by a flow cytomeetr. Panel B: Sera were prepared from preimmune B6.PL mice which were subsequently received with none (1), TIMI.4 cells only (2), TIMI.4 cells plus T cell cultures (1×10^7; 3), or TIMI.4 cells plus T cell cultures (3×10^7; 4). The sera were treated and analyzed as described in Panel A.

ful immunization before the mice were sacrificed; the titer of TIMI.4-specific antibody increased with repeated immunization (data not shown). Although the number of T cells obtained from the cultures were decreased with repeated *in vitro* stimulation, the cell numbers remained constant after the 4th restimulation. Mixtures of cytokines (in this study, CAS) were essential after culture of the second restimulation and thereafter.

Cell populations stained by mAb 3.168 in the cultured T cells increased during repeated restimulation (Fig. 1), indicating that the $CD8^+$ T cell population was enriched during repeated cultures. Similar results were obtained with the cultured T cells of spleen origin (data not shown). The cytotoxic activity of the T cell cultures, as measured by a flow cytometer, increased with repeated stimulations. An example of the T cell culture of LN origin was shown in Fig. 2, and those obained with the cuturєs of spleen origin resulted in a similar pattern (data not shown).

Helper activity of cultured T cells

Although it was clear that the cultured T cell possessed a strong cytotoxicity against TIMI.4, it was also probable that the culture carried the activity of helper T cells. The titer of the antibody was evaluated by comparing B6.PL mouse immunized with TIMI.4 alone or TIMI.4 plus cultured T cells in normal or TIMI.4-immune mice. Sera obtained at 6 days after injection were subjected to incubation with TIMI.4 and the amount of mouse immunoglobulin bound to the cell surface was measured by subsequent staining with goat anti-mouse immunoglobulin-FITC using a flow cytometer. It was observed that the antibody titer in the sera of mice that received T cells along with TIMI.4 cells

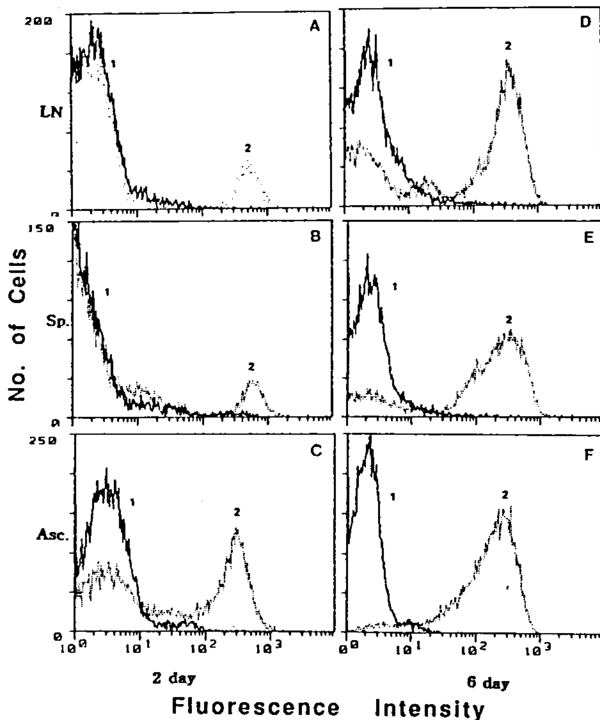

Fig. 4. Distribution of adoptively-transferred T cells from C57BL/6 to C57BL/6 PL mice. Lymph nodes (A and D), spleen (B and E), ascites (C and F) of B6.PL mice that received the T cells were prepared after 2 days (A, B, C) or 6 days (D, E, F) of the adoptive transfer. Cells were stained with normal mouse serum (1) or J1j.10 (2), and subsequently stained with goat anti-rat Ig-FITC. The fluorescences were determined by a flow cytometer.

was greater than that in mice that were given only TIMI.4 cells (Fig. 3). The difference of antibody level between the control and T cell-transferred mice was even greater when mice previously immune to TIMI.4 cells were used as recipient mice (data not shown). This result implies that the cultured T cells include T cells that have helper activity for antibody production.

Distribution of the transferred T-cells *in vivo*

Attempts were made to examine the trafficking of the adoptively-transferred T cells, and we prepared Con A-stimulated C57BL/6 splenocytes and injected them into B6.PL mice, and the transferred T cells were traced. C57BL/6 mouse spleen cells were made into a single cell suspension, activated with Con A and injected i.p. to B6.PL mice. After several days, the presence of donor T-cells was examined from a cell preparation of several organs by staining with mAb J1j 10, and it was observed that Thy-1.2$^+$ T cells were distributed in lymph nodes, spleen and ascites, lymph nodes being the most predominant organ of the three. When the flow cytometric analyses were carried out in 2 or 6 days after the transfer, the prevalence in lymph nodes was most distinct in 6 days after the transfer (Fig. 4).

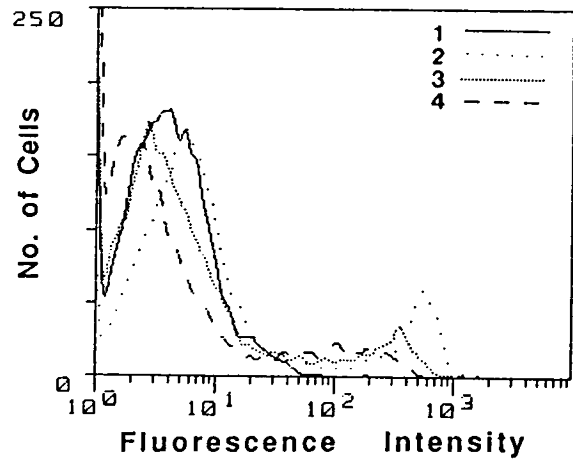

Fig. 5. Effect of adoptive transfer of TIMI.4-specific T cells in nude mice. Lymph nodes of group I that recieived 3×10^6 T cells (2), group II that recieived 1×10^7 T cells (3), group III that recieived 3×10^7 T cells (4) were incubated with normal rat serum (1) or J1j.10 (2, 3 and 4). The antibody bound T cells were stained with goat anti-rat Ig-FITC. The fluorescences were determined by a flow cytometer.

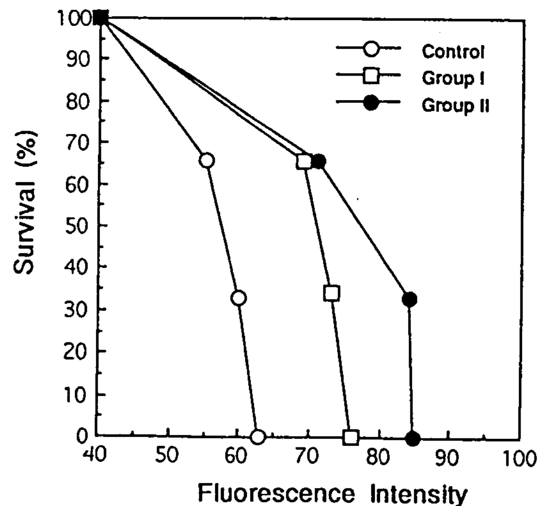

Fig. 6. Survival rates of tumor-bearing nude mice that were subsequently received withthe cuture T cells. Nude mice were divided into 3 groups. Mice were injected with 5×10^6 live TIMI.4 cells on day 0. Cultured T cells (2.5×10^7) were adoptively transferred into Group I mice after 6 weeks, and 1.25×10^8 cultured T cells into Group II mice at the same time as Group I. Each group consists of 3 mice.

***In vivo* anti-tumor efficacy of the adoptively-transferred T cell cultures in a nude mice model**

Since the cultured T cells appeared to have cytotoxicity against TIMI.4 tumor cells when examined *in vitro* and helper T cell functions as manifested by enhancement of antibody production, it was important to demonstrate the T cells also have in vivo anti-tumor activity. Thus, TIMI.4 cells or TIMI.4 cells along with the cultured T cells were injected into a peritoneal cavity.

Tumor cells residing in the host after 7 days of adoptive transfer were identified and quantified by use of mAb to the thy-1.2 allele in a flow cytometry with a live gate setting in such a way that TIMI.4 cells could be discriminated by forward scattering and side scattering channels. TIMI.4 in the adoptively-transferred nude mice were decreased in a dose-dependent manner (Fig. 5). In the survival test, as another anti-tumor activity test, nude mice which received 1.25×10^8 T cells, 25 times as much as tumor cells, survived 35% longer than the control mouse, 85 days and 63 days respectively (Fig. 6).

Discussion

Experimental animal data and circumstantial evidence from clinical observations indicate that T cells in general and class I MHC-restricted CTL, in particular, seem to play an important role in tumor eradication (Melief and Kast, 1991; Koeppen *et al.*, 1993). A prerequisite for effective tumor eradication by CTL is the induction or passive transfer of a sufficient number of tumor-specific CTL (Naito *et al.*, 1987; Greenberg *et al.*, 1988). The methods of *in vitro* stimulation of antigen-specific CTL with antigenic peptides have been described (Carbone *et al.*, 1988; Kos, 1992). In this report attempts were made to set up the culture method of immune T cells using whole tumor cells as the source of antigen since identification of tumor-specific antigens expressed on a given tumor cell type requires a very long and elaborate process and therefore it is usually regarded as almost impossble. Moreover, identification of the antigenic epitope of the antigen, even if the whole molecule of the antigen can be isolated at all, is even more difficult.

IL-2 has been considered to play an important role in culturing T cells because $CD8^+$ T cells are largely IL-2 dependent both *in vitro* and *in vivo* (Crossland *et al.*, 1991; Jicha and Rosenberg, 1990; Quentmeier *et al.*, 1992). CAS, a cocktail of cytokines, was used in this work as a source of IL-2 in the T cell culture. The T cell culture was examined for its surface expression of CD4 and CD8 makers to monitor the changes in T cell subsets; after a third restimulation, most of the cell population had CD8 molecules, representative surface marker of CTLs.

As a method of assaying cytotoxicity, flow cytometry was employed in this work, replacing the most commonly used assay for cytotoxic activity, ^{51}Cr release assay. In this assay target cells were tagged with OAF that make it possible to discriminate targets from effectors on the flow cytometry, and then they were induced to lysis by effector cells. The cytotoxicity was determined by the amount of targets which were stained by propidium iodide that specifically stains dead cells. This assay appeared to be economical in addition to the advantage of being free of hazardous radioisotopes.

Complete and permanent eradication of large established tumors by tumor-specific CTL have been achieved in a limited number of tumor systems (Kast *et al.*, 1989; Melief and Kast, 1990). We tested the efficiency of the cultured CTL in two different ways. When it was evaluated on day 7 after an adoptive transfer, the number of tumor cells in the mice decreased markedly in the lymph nodes, demonstrating that the cultured T cells were effective *in vivo*. Although many early workers have used a syngeneic mouse with tumor cells in the survival test, nude mice were used in this work, since we found that TIMI.4 cells are readily rejected when injected into C57BL/6 mice.

When the survival rates were evaluated following transfer of TIMI.4-specific CTLs into nude mice which received TIMI.4 cells 6 weeks earlier, a partial elimination of tumor cells was observed. Perhaps, if mice in which the tumors were less developed had been used or if larger doses of T cells had been given, the effect of CTL might has been more drastic. In addition, when $CD4^+$ subsets of the cultured T cells were adoptively transferred to preimmune B6.PL mice, splenocytes in the recipient mice also appeared to have a high cytotoxic activity (unpublished observation). It might be due to the fact that there already existed a $CD8^+$ population in the preimmune mouse and administration of $CD4^+$ T cells and IL-2 helped this population to show a high cytotoxicity. The argument that $CD4^+$ cells are essential in cytotoxic activity by $CD8^+$ populations has previously been rasied (Hoegen and Schirrmacher, 1989).

Taken together, these data suggest that adoptive transfer of TIMI.4-specific CTLs can be an effective candidate for immunotherapy of the murine lymphoma, TIMI.4. However, to define the best conditions for the therapy, to elucidate the role of CTL in tumors of major importance, and to learn how to manipulate them for prophylaxis and therapy still remains a challenge.

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