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Specific Cytotoxicity of a Long-Term Cultured T-Cell Clone on Human Autologous Mammary Cancer Cells[1]

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ABSTRACT

We established an autologous specific T-cell killer clone, T_{cHMC-1}, that has been cultured and has retained its function for over 1 year. T_{cHMC-1} and target cells (HMC-1-8) were derived from the metastatic pleural effusion of a patient with mammary carcinoma. At culture initiation, pleural exudative lymphocytes (PLEL) already demonstrated a high cytotoxic activity against uncloned HMC-1 breast tumor cell targets but not against autologous fibroblasts and K562 targets, and phenotypically these cells showed 100 and 90% reactivity with OKT3 and OKT8 monoclonal antibodies, respectively. However, at the early phase of cultivation under interleukin 2, PLEL had a relatively high cytotoxicity against some allogeneic tumor cells. Furthermore, the longer these PLEL were cultured with interleukin 2 and stimulated with MMC-treated HMC-1, the less cytotoxic activity of PLEL against HMC-1 targets became. We then cloned PLEL as well as HMC-1 tumor cells, and an autologous pair of T_{cHMC-1} and a target cell clone, HMC-1-8, was successfully obtained. T_{cHMC-1} showed more than 60% specific cytotoxicity against HMC-1-8, and it was confirmed, using cold target inhibition assays, that T_{cHMC-1} did not demonstrate nonspecific cytotoxicity against allogeneic targets as well as the natural killer cell activity. Moreover, we examined the *in vivo* action of T_{cHMC-1} against HMC-1-8 cells by the Winn assay using nude mice. The data showed that s.c. injections with a mixture of T_{cHMC-1} and HMC-1-8 clearly resulted in a failure of tumor development in the nude mice even 12 weeks after injections, whereas mice given injections of HMC-1-8 and allogeneic T-lymphocytes cultured with interleukin 2 developed tumors.

The autologous pair of a killer T-cell clone and tumor line could be very useful for future investigations of the specific destruction of autologous tumor cells by cytotoxic T-lymphocytes, including analysis for tumor-specific antigens possibly of rejection type and clonotypic T-cell antigen receptors.

INTRODUCTION

The investigation of effector mechanisms of specific CTL[3] against human autologous tumor cells is crucially important for understanding of the immunobiological role of CTL on autologous tumor rejection and the immunological manipulation by these cells in cancer patients. These studies could also lead to approaches for the analysis of tumor-specific antigens and clonotypic receptors on CTL.

However, difficulties have been encountered in obtaining sufficient numbers of viable tumor cells, especially autologous tumor-specific CTL clones that can be maintained continuously while retaining specific activity. Recent reports showed that it was possible to obtain long-term cultured CTL with IL-2 (1–5). Furthermore, Vose *et al.* (6) and Vánky *et al.* (7–9) demonstrated that autologous tumor-specific CTL were detected when PBL from patients were stimulated with autologous tumor cells, but the cytotoxic activity of these CTL was observed only for the first 2 months after cultivation. On the other hand, nonspecific CTL such as lymphokine-activated killer cells (3, 4), phytohemagglutinin-activated killer cells (lectin-induced killer cells) (10–13), interferon-induced killer cells (14, 15), fetal calf serum-induced killer cells (16, 17), and bacterial extract-induced killer cells (18, 19) have been identified. It was shown that there could be differences in terms of the cytotoxic potential between the specific CTL and nonspecific CTL in the autologous tumor system (20), suggesting the loss of specific activity on autologous tumor cells during cultivation with IL-2. Although it might be due to a simple disappearance of specific killer T-cell clone during culture, there is the possibility that autologous specific killer T-cells might have only a short life span and could not be cultured long term even with the addition of IL-2. Moreover, it was shown that although PBL stimulated with mitomycin C-treated autologous tumor cells could have CTL activity specific for autologous tumor cells at the early phase of cultivation, the prolonged cultivation of these T-cells activated on autologous mixed lymphocyte-tumor cell culture with IL-2 resulted in the induction of CTL with the activity not only against autologous tumor cells but also against allogeneic tumor cells (21, 22). Mukherji *et al.* (23) referred to the cloned CTL obtained via a limiting dilution *in vitro*, demonstrating the successful culture of CTL clones with specific cytotoxic activity after the addition of IL-2. However, the clones were viable in the culture for only 2 months.

We describe here the establishment of an autologous specific killer T-cell clone that has been cultured and has continuously functioned *in vitro* for over 1 year. This autologous pair of a killer T-cell clone and breast cancer line as the target could be very useful for investigations analyzing the tumor-specific antigen and its clonotypic T-cell receptors.

MATERIALS AND METHODS

Culture of Breast Cancer-derived Line HMC-1. The cells were derived from a malignant pleural effusion of a 35-year-old female who had undergone left radical mastectomy 3 years previously for an infiltrating scirrhous carcinoma of the left breast. Approximately 500 ml of pleural effusion were centrifuged at 250 × *g* for 10 min. The cell pellet, containing 1×10^9 of total cell numbers, was layered on a Ficoll-Conray density gradient and centrifuged at 1000 × *g* for 15 min. The interface containing tumor cells was collected and washed in PBS three times, and 1×10^7 cells/culture flask were cultured in the plastic culture flasks (Costar Nos. 3275 and 3150; Costar, Cambridge, MA) with RPMI 1640 containing 10% FCS. The cells were fed every day with the replacement of one-half of the medium. In this manner, the newly seeded cultures were enriched with slowly attaching tumor cells. In contrast, other more rapidly attaching cells, particularly macrophages, fibroblasts, and mesothelial cells, were selectively reduced. At that time, autologous fibroblasts were successfully passed into other culture flasks and maintained in RPMI 1640 with 10% FCS. Propagated adherent tumor cells were cultured and passaged weekly by trypsinization (0.05% trypsin plus 0.02% EDTA). Continuous tumor cell lines were established about 1 month after initiation of the culture and were named

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[3] The abbreviations used are: CTL, cytotoxic T-lymphocytes; PLEL, pleural exudative lymphocytes; PBL, peripheral blood lymphocytes; HMC-1, a human breast cancer line; HMC-1-1 through HMC-1-8, clones of HMC-1; T_{cHMC-1}, a cytotoxic T-cell clone; IL-2, interleukin 2; PBS, phosphate-buffered saline; FCS, fetal calf serum; NK, natural killer; MLTC, mixed-lymphocyte tumor cell culture; E/T ratio, effector/target cell ratio.

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HMC-1. HMC-1 had an epithelioid nature with polygonal configurations in confluent monolayer culture. Then we tested the *in vitro* and *in vivo* tumorigenicity of HMC-1. An anchorage-independent cell growth test using 0.3% soft agar clearly demonstrated that the HMC-1 line was tumorigenic. When 10^3 HMC-1 cells were seeded in soft agar (Falcon NO. 3002; Falcon Plastics, Oxnard, CA), approximately 24% of cells formed colonies of cells. HMC-1 also resulted in a 100% tumor incidence when 10^5 cells were inoculated into nude mice.

Allogeneic Target Cell Lines. Thirteen allogeneic cell lines were used as targets in the cytotoxic assays of T-lymphocytes. PANC1 (24) and HGC25 (25) were of pancreatic cancer origin and were gifts from Dr. Suzuki, Department of Pathology, Niigata University School of Medicine, Niigata, Japan. HPC1, 3, and 4 were established in our laboratory, and these were derived from the metastatic ascitic fluid of human pancreatic adenocarcinoma of ductal cell origin, which grew continuously as monolayer cultures in RPMI 1640 containing 10% FCS over 6 months. MKN45, a gastric cancer line, M7609, a line obtained from a metastasis to the skin of colon cancer, and PC10, derived from a lung cancer, were also gifts from Dr. Suzuki. Two allogeneic mammary cancer cell lines, ZR-75-1 and ZR-75-30 (26), were purchased from Flow Laboratories, Inc., Bethesda, MD, and mammary cancer HMC-2 was established in our laboratory using the same procedure of establishment as for HMC-1. A K562 cell line, established by Lozzio and Lozzio (27) from a patient with chronic myeloid leukemia and shown to have erythroleukemic characteristics (28), was maintained in suspension culture in RPMI 1640 plus 10% FCS. It was used as the NK sensitive target.

Separation and Culture of Lymphocytes. Approximately 20 ml of a pleural effusion and peripheral blood were layered on 30 ml of Ficoll-Conray density gradient and centrifuged at $1400 \times g$ for 25 min. Lymphocytes collected from the interface were then washed three times in PBS. Furthermore, T-lymphocytes were purified from PLEL containing tumor cells and other mononuclear cells by Percoll discontinuous density centrifugation at $2000 \times g$ for 30 min, as described previously by Gutierrez *et al.* (29). Approximately 10^4 and 10^6 lymphocytes were collected from a pleural effusion and peripheral blood, respectively, and were resuspended in RPMI 1640 containing 10% FCS at 37°C in a 5% CO_2 humidified atmosphere.

Detection of PLEL Surface Markers. Surface markers of PLEL were detected by indirect immunofluorescence using saturated amounts of monoclonal antibodies OKT3, OKT4, OKT8, HNK1, and HLA-DR. The first three monoclonal antibodies were purchased from Ortho Pharmaceutical Co., Raritan, NJ. HNK1, which reacts with a subpopulation of NK cells, and anti-Ia (HLA-DR) were obtained from Becton Dickinson and Co., Mountain View, CA.

IL-2. The human recombinant IL-2 was kindly provided by Dr. J. Hamuro (Ajinomoto Central Research Laboratory, Tokyo, Japan).

MLTC. PLEL (1×10^5/well) were stimulated with 1×10^4 mitomycin C-treated autologous tumor cells for 4 days at 37°C in a 5% CO_2 incubator. Cultures were grown in 24-well plates (Costar No. 3424) in 2 ml RPMI 1640 supplemented with 10% FCS. These activated lymphocytes were separated by a Percoll discontinuous density gradient centrifugation at $2000 \times g$ for 30 min. After 3 washes with FCS-free medium, the lymphocytes were cultured with IL-2 (20 units/ml).

Cloning of Cultured T-Cells and HMC-1. The cloning of T-cells from PLEL, which had been stimulated already for 4 days with autologous MLTC, was carried out by a limiting dilution in 96-well microtiter plates (Costar No. 3799). The cells were immediately expanded in 0.2 ml medium containing IL-2 (20 units/ml). In this experiment, we used 120 wells each seeded with one T-cell. At 2 weeks of cultivation, 6 clones were successfully grown in the wells, and their cytotoxic potentials against HMC-1 were assessed. Simultaneously, these clones were transferred to 24-well plates (Costar No. 3424). Clone 1 was most cytotoxic, and during 2 weeks after cultivation, clone 1 cells were growing well. Therefore, the T-cell clone from this well was restimulated at weekly intervals with autologous MLTC and was expanded under the addition of IL-2. This T-cell clone described here in detail has been propagated for over 1 year in the culture and was designated $T_{cHMC\text{-}1}$.

In addition to the cloning of killer T-cells, we also tried cloning of HMC-1 in order to obtain a sensitive target for T-cell clones, because the cytotoxic potential of T-cells from PLEL against autologous HMC-1 cells were gradually decreasing during cultivation. Briefly, a single cell suspension of HMC-1 at 23rd passage after ample trypsinization was implanted in tissue culture dishes (Falcon No. 3002). A second single cell cloning was done for each clone obtained from the first cloning described above. Eight HMC-1 clones, namely HMC-1-1 through HMC-1-8, were obtained and used as target cells for the cytotoxic assays. In the experiment, the cells from 5th to 10th passages were used.

Cytotoxicity Assay. The ^{51}Cr release assay described elsewhere (30) was used for the determination of T-cell cytotoxic activity. Briefly, target cells were labeled by 100 μCi sodium [^{51}Cr]chromate (New England Nuclear, Boston, MA) and were incubated for 3 h at 37°C. The cells were washed 5 times with PBS, and 1×10^4 target cells in 0.1 ml medium were seeded into U-bottomed microtiter plates (Costar No. 3799). Thereafter, 0.1 ml of effector cell suspension at a predetermined dose was added, and the plates were centrifuged at $200 \times g$ for 5 min. After 6 or 12 h incubation at 37°C, 0.1 ml of culture supernatants was harvested and counted with a liquid scintillation counter (Packard Auto-Gamma scintillation spectrometer). The percentage of lysis was determined as

$$\% \text{ of specific lysis} = (\text{Experimental release} - \text{spontaneous release}) \times 100/\text{Maximal release} - \text{spontaneous release}$$

To determine maximal release, 0.1 ml of 1% Nonidet P-40 (Nakarai Chemical Co., Kyoto, Japan) was added to appropriate wells. A spontaneous release was assessed by incubation of target cells with medium alone, and it was usually below 15% in the experiments. All determinations were made in triplicate, and the data were represented as the mean ± SE.

Winn Assay. Continuously cultured 10^4 $T_{cHMC\text{-}1}$ per well of 24-well plates (Costar No. 3424) were expanded as described above. After 10 to 14 days of cultivation, approximately 10^6 $T_{cHMC\text{-}1}$ were obtained from each well. A mixture of 1×10^6 HMC-1-8 cells and 5×10^7 autologous killer T-cell clone, $T_{cHMC\text{-}1}$, was inoculated in the backs of five BALB/c nude mice (CLEA Japan Co., Shizuoka, Japan). Simultaneously, a mixture of allogeneic T-cells cultured with IL-2 and HMC-1-8 cells at the same effector/target ratio and HMC-1-8 cells alone were injected for controls. Tumor development in the mice was observed at weekly intervals after inoculations.

Cytotoxicity of $T_{cHMC\text{-}1}$ Cultured *in Vitro* for 1 Year against HMC-1 Target Clones. We determined whether the target specificity of killing by $T_{cHMC\text{-}1}$ that was cultured *in vitro* for 1 year in the presence of IL-2 and autologous MLTC stimulation was changed. Two HMC-1 clones (HMC-1-8 and HMC-1-7) and K562 were utilized as targets. In this experiment, we examined the cytotoxic activity of PLEL against uncloned HMC-1 cells which were cultured for about 7 months (35 to 40 passages). PLEL obtained from a patient were cultured *in vitro* for 1 month and were then cryopreserved for 6 months. These PLEL were recultured *in vitro* for 5 months with stimulation by autologous MLTC in the presence of IL-2 and were used for the cytotoxicity assay.

RESULTS

Cytotoxicity of Autologous PLEL and PBL against HMC-1. To determine the optimal culture conditions of incubation time and E/T ratio for T-cell cytotoxicity against autologous HMC-1 cells which were cultured *in vitro* for 1 month, we examined the cytotoxicity of PLEL and PBL from the patient. These cells were cultured with recombinant IL-2 and stimulated with autologous MLTC. The data shown in Fig. 1 demonstrate that PLEL had optimal cytotoxic activity at 12 h incubation and an E/T ratio of 100. Therefore, the subsequent cytotoxic assay using PLEL was performed under this experimental condition, unless otherwise mentioned. Fig. 1 also demonstrates that PBL

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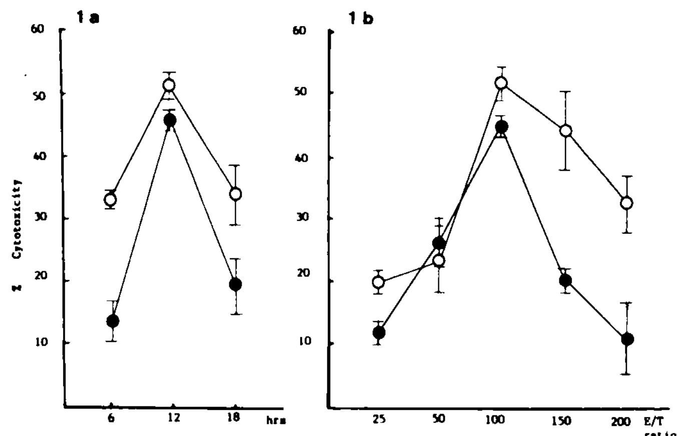

Fig. 1. Cytotoxicity of autologous PLEL (○) and PBL (●) against HMC-1 breast tumor. The PLEL and PBL obtained from the patient were cultured for 2 weeks in the presence of human recombinant IL-2 (20 units/ml) and with stimulation for 4 days by autologous MLTC and were used for the cytotoxicity assays to HMC-1 cells. *a*, optimal duration (h) for the cytotoxicity assays at an E/T ratio of 100; *B*, optimal E/T ratio at 12 h incubation time of culture. *Points*, percentage of cytotoxicity; *bars*, SE.

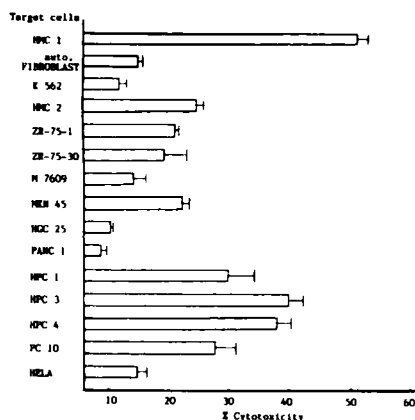

Fig. 2. Reactivity of PLEL to an autologous fibroblast and human allogeneic tumor lines. PLEL obtained from the patient were cultured for 1 month and were stimulated with autologous MLTC for 4 days before utilization in the cytotoxicity assays to various target cells. The cultures were performed for 12 h at an E/T ratio of 100. *Columns*, percentage of cytotoxicity; *bars*, SE.

were cytotoxic to HMC-1, although their cytotoxic potential was less than that of PLEL at each incubation or E/T ratio. The specific cytotoxicity of PLEL as well as PBL was reduced at 18 h incubation or at an E/T ratio of more than 150. We believe that this was due to a poor culture condition caused by too many effectors and targets in the culture wells.

Phenotypic Analysis of PLEL by Monoclonal Antibodies. Because the possibility remained that the PLEL population might be affected markedly during culture, we determined the phenotype of PLEL at the initiation of culture and at 1 month of cultivation *in vitro* including stimulation with autologous MLTC in the presence of IL-2. There were no overt changes in the PLEL population; rather it was enriched to 100% of OKT3-, OKT8-, and HLA-DR-positive cells during the 1 month culturing, whereas OKT4- and HNK1-positive cells disappeared from the culture.

Specificity of PLEL Cytotoxicity. In order to assess the target specificity of PLEL, we examined the cytotoxicity of PLEL against autologous fibroblasts and other allogeneic tumor cell lines including K562. PLEL obtained from the patient were cultured for 1 month. As shown in Fig. 2, PLEL demonstrated clearly the highest cytotoxic activity against an autologous HMC-1 target and a reduced cytotoxicity to autologous fibroblasts and K562. However, some of the targets such as HPC-3 and HPC-4 showed relatively high sensitivity to PLEL, although this was less than that of HMC-1. In contrast, it is noteworthy that the allogeneic mammary cancer lines HMC-2, ZR-75-1, and ZR-75-30 indicated less sensitivity to PLEL cytotoxicity.

We examined the time course activity of PLEL against K562. PLEL were continuously cultured *in vitro* in the presence of IL-2 with stimulation by autologous MLTC and were assayed for a NK-sensitive K562 target. PLEL before cultivation were less cytotoxic than those of third or seventh week, and their cytotoxicity against a K562 target was usually less than 15% (data not shown).

Cytotoxicity of a Killer T-Cell Clone from PLEL, T_{cHMC-1}, against HMC-1. Although PLEL have shown continuously the highest cytotoxic activity to HMC-1 among the various target cells used in the assays, the longer PLEL were cultured *in vitro*, the less their cytotoxicity against HMC-1 became. Therefore, we cloned effector as well as target cells. The cloning of killer T-cells was carried out by a limiting dilution of PLEL, and the cells were expanded with IL-2. Of 120 wells, each seeded with one T-cell, we could successfully expand only 6 clones in the culture (*i.e.*, 5% cloning efficiency). The cytotoxic activity of all of these clones against HMC-1 was examined. The data shown in Fig. 3 indicate the differences in cytotoxicity among these clones and show that clone 1 was most cytotoxic against HMC-1. Moreover, this clone was growing well in the culture in the presence of IL-2. Therefore, this clone, designated as T_{cHMC-1}, was then stimulated with autologous MLTC and expanded in the presence of IL-2 for further assays.

We cloned HMC-1 tumor cells twice and obtained 8 clones, HMC-1-1 through HMC-1-8. Then we studied the cytotoxicity of T_{cHMC-1} against each of these HMC-1 clones (Fig. 4). The data demonstrate that the HMC-1-8 clone was highly sensitive to T_{cHMC-1}. In contrast, T_{cHMC-1} showed less than 30% cytotoxicity against the HMC-1-7 clone. Hence, we utilized the autologous pair of T_{cHMC-1} and HMC-1-8 for further analysis of cytotoxicity.

To assess whether T_{cHMC-1} has greater specificity than uncloned PLEL for an HMC-1-8 target, we carried out a cytotoxic assay using two cold (unlabeled) targets, HPC-3 and HPC-4,

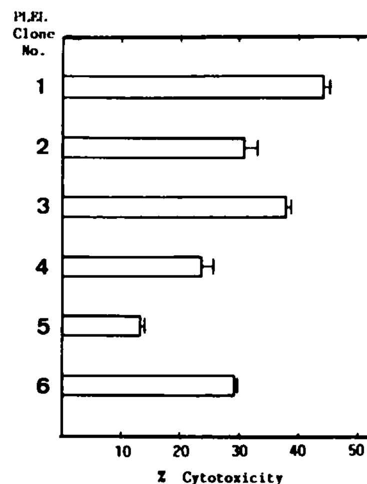

Fig. 3. Cytotoxic potential of each PLEL clone. The cloning of PLEL which were stimulated for 4 days with autologous MLTC was carried out by limiting dilution. The cells were immediately expanded in the presence of IL-2 (20 units/ml). About 2 weeks after seeding of PLEL to the plates, six T-cell clones were successfully grown in the wells, and their cytotoxic potentials against HMC-1 were assessed. The cytotoxicity assays of these T-cell clones were performed for 6 h incubation at an E/T ratio of 100. *Columns*, percentage of cytotoxicity; *bars*, SE.

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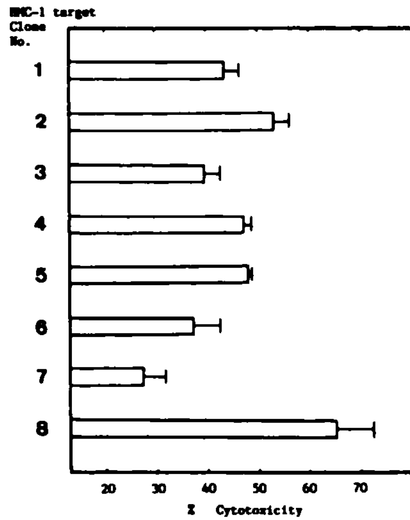

Fig. 4. Cytotoxic sensitivity of HMC-1 target clones by a T_{cHMC-1} killer T-cell clone. HMC-1 cells were cloned twice by a single cell cloning at their 23rd passage generation, and eight clones, HMC-1-1 through HMC-1-8, were obtained. The cytotoxicities of T_{cHMC-1} against these target clones were assayed for 12 h incubation at an E/T ratio of 50. *Columns*, percentage of cytotoxicity; *bars*, SE.

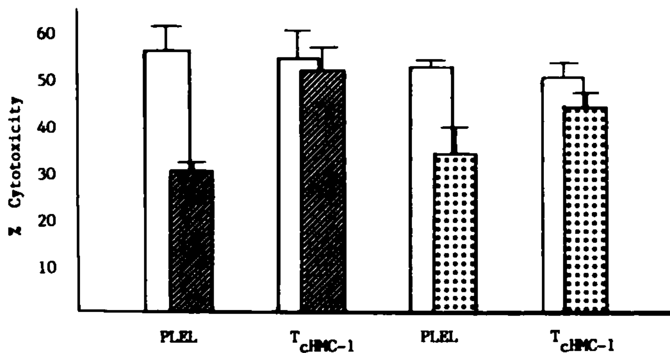

Fig. 5. Inhibition assay of PLEL and T_{cHMC-1} killing activity by HPC-3 and HPC-4 unlabeled cold targets. To examine whether T_{cHMC-1} could react more specifically to HMC-1-8 targets than uncloned PLEL, the cytotoxic assays were carried out using two cold (unlabeled) targets, HPC-3 (■) and HPC-4 (▩). A mixture of 5×10^5 T_{cHMC-1}, 10^4 ^{51}Cr-labeled HMC-1-8 cells, and unlabeled 10^4 HPC-3 or HPC-4 cells was cocultured for 12 h, and the cytotoxicity of each mixture of cells was determined. *Columns*, percentage of cytotoxicity; *bars*, SE.

Table 1 *Augmented cytotoxicity against a HMC-1-8 target clone of a killer T-cell clone, T_{cHMC-1}, that has been cultured in vitro for 1 year*

Target	Effector	% of cytotoxicity ± SE[a]
HMC-1	PLEL	23.5 ± 6.1
HMC-1-7	T_{cHMC-1}	28.4 ± 3.0
HMC-1-8	T_{cHMC-1}	65.6 ± 3.7
K562	T_{cHMC-1}	14.8 ± 4.3

[a] The cytotoxicity assay was done at an E/T ratio of 50 for 12 h incubation.

which showed the relatively high sensitivity to PLEL in the previous experiment, as in Fig. 2. The data in Fig. 5 indicate that the specific cytotoxicity of PLEL against an HMC-1-8 target was affected by the addition of HPC-3 as well as HPC-4 cold targets. However, that of T_{cHMC-1} was not affected by HPC-3 or presumably by HPC-4 cold targets and was specific for HMC-1-8.

Currently, T_{cHMC-1} has been continuously cultured over 1 year in the presence of IL-2 and with stimulation by autologous MLTC. We tested whether T_{cHMC-1} killer activity has changed with respect to target specificity, including that of K562 and a less sensitive HMC-1-7 target clone. The data shown in Table 1 indicate augmented cytotoxicity (65.6%) by T_{cHMC-1} against HMC-1-8 but rather reduced activity to HMC-1-7. Moreover, T_{cHMC-1} showed less than 15% cytotoxicity against K562, suggesting no increase of NK-like activity by T_{cHMC-1} during cultivation over 1 year. It should be mentioned that cytotoxicity of PLEL was markedly reduced after long-term cultivation *in vitro*.

Influence of T_{cHMC-1} on HMC-1-8 Tumor Development *in Vivo* in the Winn Assay. We examined the influence of T_{cHMC-1} *in vivo* on HMC-1-8 tumor development in nude mice. A mixture of 1×10^6 HMC-1-8 and 5×10^7 T_{cHMC-1} was injected s.c. into the backs of five BALB/c nude mice. For controls of the effector and tumor, mice were given injections of a mixture of allogeneic T-cells cultured in the presence of IL-2 and HMC-1-8 at the same E/T ratio, or HMC-1-8 alone. The HMC-1-8 tumor development in these mice was observed weekly. The data showed the marked *in vivo* effect of T_{cHMC-1} on HMC-1-8 tumor development, as compared with tumor development seen in two control groups of mice (Fig. 6). Even at 12 weeks after injection, mice receiving a mixture of T_{cHMC-1} and HMC-1-8 did not develop tumors.

DISCUSSION

Approaches to clarification of the destructive mechanisms of autologous cancer cell killing by specific cytotoxic T-lymphocytes are of particular importance for tumor immunologists (31), since these approaches could lead directly to immunotherapeutic trials in patients. There have been many reports (32–36) in animal models that showed successful therapeutic effects by uncloned or cloned CTL of syngeneic origins, and the theory and experience obtained from these experiments could be applied to human trials. Obviously, there is no question that the well-characterized autologous killer T-cell clone should be used in these trials. Furthermore, it is crucially important to obtain massive amounts of this specific killer T-cell clone expanded *in vitro* for investigation of effector mechanisms of CTL against tumor cells. The homogeneous population of monospecific killer T-cells is basically required to analyze the molecular structure of the autologous tumor-specific antigen that is recognized by CTL and to investigate of the T-cell clonotypic receptor molecules on their surface.

Several investigators have reported the human killer T-cell clones against autologous as well as allogeneic targets (23, 37, 38) in short-term cultures. Spits *et al.* (39) demonstrated that the specific killer T-cell clones against Epstein-Barr virus-trans-

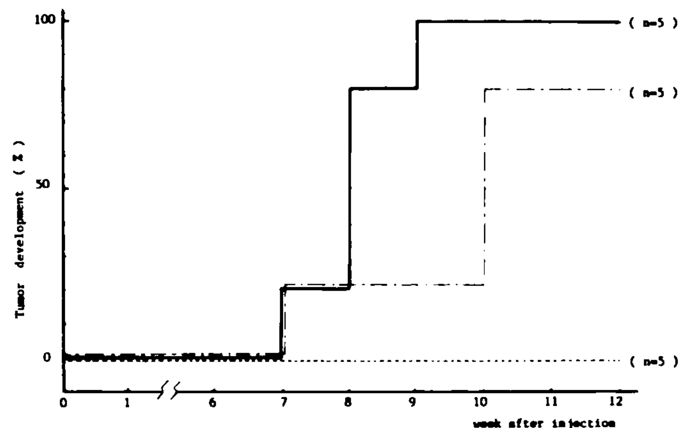

Fig. 6. Effect of T_{cHMC-1} on HMC-1-8 tumor development *in vivo*. A mixture (- - - - -) of 10^6 HMC-1-8 and 5×10^7 T_{cHMC-1} was injected s.c. into the backs of five BALB/c nude mice. Another group of mice were given injections of a mixture (– – –) of allogeneic T-cells cultured in the presence of IL-2 and HMC-1-8 at the same cell number or HMC-1-8 alone (——). The HMC-1-8 tumor development was observed at weekly intervals.

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formed autologous targets could be obtained from peripheral T-lymphocytes by repetitive stimulation with allogeneic Epstein-Barr virus-transformed B-cell line JY in a serum-free culture medium. These clones grew well *in vitro* over 5 months and maintained their cytotoxic functions and phenotypes with the same target specificity they showed at the initiation of culture in the presence of crude IL-2. In a mouse model, Gillis *et al.* (40) reported the long-term culture of murine tumor-antigen-specific cytotoxic T-cell line. Thus, in fact, the technological advance using growth factors like IL-2 allows the performance of studies that were previously impossible (41–44).

However, it still has been difficult to maintain long-term *in vitro* T-cell clones that show specific killing activity against human autologous targets even in the presence of IL-2 and with continuous stimulation by autologous tumor cells. The results in recent literature have suggested that there might be a tendency for killer T-cells to have not only cytotoxic activity to autologous targets but also NK activity to K562 during *in vitro* cultivation with IL-2 (3, 5, 6, 45–47). Moreover, it has been suggested that the killer T-cell clones, even if these were successfully cultured from autologous tumor materials, had only a short life span and could multiply for only 2 months after cultivation (23).

We describe in this paper a successful establishment of a T-cell clone, T_{cHMC-1}, that is specifically cytotoxic against an autologous cancer line, HMC-1-8, in the presence of IL-2 with repeated tumor cell stimulation. Both the T-cell clone and tumor cell line were derived from a patient with a metastatic mammary cancer in the pleural cavity. The T_{cHMC-1} clone has retained its specific cytotoxicity for over 1 year after the initiation of culture. These *in vitro*-maintained CTL are capable of preventing the growth of autologous tumors in nude mice. The evidence presented in this report confirms that CTL specific for an autologous tumor are present among tumor-associated lymphocytes in malignant pleural effusions.

The autologous tumor cells were cultured for at least 1 month before any cytotoxicity tests were performed. We did not determine whether PLEL and T_{cHMC-1} were also cytotoxic against fresh or cryopreserved, *i.e.*, not cultured, tumor cells, since the tumor cell suspensions separated from a pleural effusion with Ficoll-Conray density gradient contained a fairly large amount of different populations of cells such as mesothelial cells and macrophages. However, the fact that the longer PLEL and tumor cells were cultured *in vitro* the less their cytotoxic potential was observed may suggest that these killer T-cells were also cytotoxic to patient tumor cells. Although the population of PLEL became enriched to 100% of OKT3 and OKT8-positive T-cells during *in vitro* cultivation of PLEL, these cells showed eventually a gradual loss of its specific cytotoxicity against HMC-1 cells. This suggests the presence of heterogeneity among PLEL in terms of their cytotoxicity against HMC-1 cells. Furthermore, a T_{cHMC-1} clone showed augmented cytotoxicity and a proliferative response when an adequate target cell clone, HMC-1-8, was separated by cloning of HMC-1 cells. These data indicate that the effective cloning of both effectors and targets is critical to gain the killer T-cell clones which are specific to autologous tumor cells and are amenable to long-term *in vitro* culture.

With respect to the particular longevity of successful culture, this autologous pair of a killer T-cell clone and a target line is very unique and is useful for the future investigation of the effector mechanisms of killer T-cells against the neoplastic cells. Our preliminary study demonstrates that the cytotoxic activity of T_{cHMC-1} was inhibited by pretreatment of T_{cHMC-1} with OKT3 or OKT8 monoclonal antibodies. This inhibition was recovered by the addition of concanavalin A to the culture, suggesting that T_{cHMC-1} may have clonotypic antigen receptor molecules, possibly directed to the HMC-1-8 tumor-specific antigen, on the cell surface that could be associated with T3 molecules (48, 49). This approach may clarify the nature of the tumor-specific antigens, presumably of the rejection type of human tumor cells recognized by CTL, and may lead to immunobiological manipulation by these cells in cancer patients.

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