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Identification of a Human T Cell Clone with the Cytotoxic T Lymphocyte and Natural Killer-like Cytotoxic Function against Autologous Mammary Carcinoma and K562 Line

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Pleural exudative lymphocytes (PLEL) from a 60-year-old female patient showed high cytotoxicity against the autologous mammary tumor line, HMC-2, and NK-susceptible K562 cells, although PLEL demonstrated only weak cytotoxic potentials against several allogeneic tumor lines. We successfully obtained seven cytotoxic T cell clones from PLEL bulk populations, and assessed the possibility that these lymphocytes are simply natural killer (NK)-like cells or have the dual cytotoxic activity of cytotoxic T lymphocytes (CTL) and NK-like cells. These clones, designated as T_{cHMC-2}, showed strong cytotoxicity against both HMC-2 and K562 cells. In contrast, allogeneic human peripheral blood-derived NK cells could not kill HMC-2 targets. Furthermore, a blocking study of T_{cHMC-2} cytotoxicity using monoclonal antibodies against CD3, CD8 and human MHC class I products showed that all of these antigen molecules were involved in the cytotoxicity of T_{cHMC-2} clone against autologous HMC-2 cells, indicating MHC class I recognitive cytotoxicity. These data indicate that the T_{cHMC-2} clone may have dual cytotoxicity with CTL- and NK-like activity against autologous HMC-2 mammary tumor and K562 cells, respectively.

Key words: Human cytotoxic T lymphocyte clone — Natural killer clone — Autologous tumor

There are many reports using animal tumor models that demonstrate host effector mechanisms against the tumors.[1)] It has been considered that the cytotoxic T lymphocytes (CTL) and natural killer (NK) cells are the most important effectors that are directly involved in the host's defense against tumors,[2–5)] although *in vitro* proliferated lymphokine-activated killer (LAK) cells may have a strong cytotoxic potential against tumors as well.[6)] Hercend *et al.*[3)] recently reported the characteristics and proposed a definition of NK cells. NK cells are CD3(−), T cell receptors (TCR) (α, β, γ, δ) (−), large granular lymphocytes which express commonly CD16 and NKH-1 (Leu19). They do not require expression of class I or class II molecules on the target cells for the cytolysis. Certain T lymphocytes expressing TCR may express a cytotoxic activity that resembles that of NK cells upon particular activation. These T lymphocytes could be termed either T lymphocytes displaying NK-like activity or non-MHC-requiring cytolysis. Furthermore, LAK cells have the characteristics of a mixture of IL-2 activated NK and IL-2 activated T lymphocytes with NK-like activity. None of these effector T lymphocytes requires MHC molecules on the target cells.

It is obviously important to use effector cloned cells, not bulk-cultured cells, for analyzing the details of these effector mechanisms against human autologous tumors. In our previous studies,[7,8)] we demonstrated a human CTL clone T_{cHMC-1} which shows specific cytotoxicity against an autologous mammary tumor cell line, HMC-1-8. In our present study, in connection with our continuing research on the clonal analysis of human autologous CTL against tumors, we established another cytotoxic T cell clone T_{cHMC-2} and tumor target HMC-2. These were derived from the metastatic pleural fluid of the breast carcinoma of a female patient. Pleural exudative lymphocytes (PLEL) of this patient from the beginning of cultivation showed a high cytotoxicity against both autologous HMC-2 tumor and K562 targets. It was demonstrated that T lymphocyte clones from PLEL showed cytotoxicity against both HMC-2 and K562 cells. The cytotoxicity against HMC-2 autologous targets involved CD3 and CD8 molecules under the restriction of MHC class I products.

There have been a large number of reports on the clonal analysis of CTL induced by allogeneic MLC.[9–13)] Some of these clones may have NK activity alone, and others have the dual activity with allospecific CTL and NK-like activity, although the majority of clones have been indicated to be CD3-positive and cytotoxic specifically to allotargets. However, there is no definitive report which demonstrates the dual cytotoxicity of CTL and NK-like cytotoxic function of a T cell clone derived from autologous tumor-associated lymphocytes.

Our present study considers the ontogeny of human CTL against autologous tumor cells, since the T cells

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with these dual cytotoxic functions may generate the major cytotoxic T cell population and participate in confronting autologous tumor cells in the tumor-infiltrated tissue of some patients.

MATERIALS AND METHODS

Culture and cloning of breast cancer-derived line HMC2 HMC-2 mammary tumor cell line was obtained from a 60-year-old female who had undergone a redical mastectomy 3 years prior to cultivation for infiltrating ductal carcinoma of the left breast. The procedure of establishment of the line was described in our previous reports.[7, 12] At approximately 5 months after a successful culture, single cell cloning was performed, using 60 mm petri dishes (Falcon #3002).

Allogeneic tumor cell lines Fourteen allogeneic cell lines were used as targets in the cytotoxicity assays of T-lymphocytes. PANC-1[15] and HCG-25[16] were of a pancreatic cancer origin and a gift from Dr. Suzuki, Department of Pathology, Niigata University School of Medicine, Niigata. HPC-1, -3 and -4 were established in our own laboratory,[17] and these were derived from the metastatic ascitic fluid of human pancreatic adenocarcinoma of ductal cell origin, which grew continuously as monolayer cultures in RPMI1640 containing 10% FCS. MKN45, a gastric cancer line, and M7609, a line obtained from metastasis to the skin of colon cancer, were also gifts from Dr. Suzuki. Two mammary cancer cell lines, ZR-75-1 and ZR-75-30,[18] were purchased from Flow Laboratories, Inc., Bethesda, Md., and mammary cancer HMC-1-8 was established in our own laboratory using the same procedure as established for HMC-2. A chronic myeloid leukemia line K562 and B lymphoma cell line Daudi cells were maintained in a suspended culture of RPMI1640 plus 10% FCS. These were used as the NK-sensitive and insensitive targets, respectively. As one of the large-scale target cell panels, we also employed autologous fibroblasts derived from the same metastatic pleural fluid of the patient as that from which HMC-2 was established.

Separation, culture and surface phenotype of lymphocytes Approximately 20 ml of a pleural effusion and peripheral blood (PBL) were layered on 30 ml of Ficoll-Conray density gradient and centrifuged at 1400*g* for 25 min. Lymphocyes collected from the interface were then washed three times in PBS. Furthermore, T lymphocytes were purified from cells containing tumor cells and other mononuclear cells by Percoll discontinuous density centrifugation at 2000*g* for 30 min, as previously described by Gutierrez *et al.*[19] Approximately 10^4 lymphocytes (pleural exudative lymphocytes, PLEL) were collected from a pleural effusion, and they were resuspended in RPMI1640 containing 10% FCS at 37°C in a 5% CO_2 humidified atmosphere.

Surface markers of PLEL were detected by indirect immunofluorescence or with a FACStar (Beckton Dickinson Co., Mountain View, Ca.) using saturating amounts of monoclonal antibodies (mabs) OKT3 (CD3), 4 (CD4) and 8 (CD8), and MHC class II products. The first three mabs were purchased from Ortho Pharmaceutical Co., Raritan, N. J., and anti-HMC-class II mab was obtained from Becton Dickinson Co. OKT 3, 4 and 8 mabs were also used in the cytotoxicity assays of $T_{cHMC\text{-}2}$ clones against a HMC-2 autologous tumor target.

IL-2 Human recombinant IL-2 (rIL-2) was provided by the Ajinomoto Central Research Laboratory, Tokyo.

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 (Coster #3424) in 2 ml of RPMI-1640 supplemented with 10% FCS. These activated lymphocytes were separated by a Percoll discontinuous density gradient centrifugation at 2000*g* for 30 min. After 3 washes with FCS-free medium, the lymphocytes were cultured with 20 units/ml of IL-2.

Cloning of cultured T-cells The cloning of T-cells from PLEL, which had been stimulated previously for 4 days with autologous MLTC, was carried out by limiting dilution in 96-well microtiter plates (Coster #3799). The cells were immediately expanded in 0.2 ml of 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, 7 clones had grown in the wells, and the cytotoxic potentials of these clones against HMC-2 and K562 were assessed.

Cytotoxicity assays The ^{51}Cr release cytotoxicity assays for the determination of cytotoxic activity of PBL, PLEL and T cell clones were described previously.[7] Briefly, target cells were labeled with 100 μCi of sodium ^{51}Cr chromate (New England Nuclear, Boston, Mass.) and were incubated for 3 h at 37°C. The cells were washed five times with PBS, and 1×10^4 target cells in 0.1 ml of medium were seeded into U-bottomed microtiter plates (Costar #3799). Thereafter, 0.1 ml of the effector cell suspension as a predetermined dose (usually 5×10^5–1×10^6 effector cells) was added, and the plates were centrifuged at 200*g* for 5 min. After 6 to 12 h incubations at 37°C, 0.1 ml of the culture supernatant was harvested and counted with a liquid scintillation counter (Packard Auto-Gamma scintillation spectrometer). The percent of lysis was determined as: % specific lysis = (experimental release − spontaneous release) × 100/(maximal release − spontaneous release). To determine the maximal release, 0.1 ml of 1% Nonidet P 40 (Nakarai Chemical Co., Kyoto) was added to the appropriate wells. Spontaneous release was assessed by incubation of target cells with the

medium alone, and it was usually below 15%. All determinations were made in triplicate, and the data gathered are given as the mean±SE.

In a separate experiment, the effects of mabs OKT3, OKT4, OKT8, HH-1 and TC-8B1 on the cytotoxicity of a T_{cHMC-2} clone were assessed. Mabs HH-1 and TC-8B1 could react against the framework portions of the human MHC class I and class II products, respectively. T_{cHMC2} clone no. 1 was treated with saturating amounts of mabs OKT3, 4 and 8 at 4°C for 60 min. The cells were washed twice with PBS, and then used in cytotoxicity assays against HMC-2. HH-1 and TC-8B1 was used to pretreat HMC-2 targets. HMC-2 cells treated with saturating amounts of these mabs were washed twice with PBS, and were used as targets in cytotoxicity assays.

Preparation of NK cells Human peripheral blood from healthy randomly selected donors was obtained from our laboratory. Mononuclear cells were separated by Ficoll-Conray centrifugation as described above. After washing, the cells were incubated in plastic flasks (Falcon #3024) for 3 h to remove adherent cells, after which the nonadherent cells were run through a nylon wool column (1 h at 37°C) and collected.[20] These cells were used as the NK cells against K562, Daudi, HMC-1-8 and HMC-2 targets at predetermined effector/target ratios in the cytotoxicity assays against various targets, as described above.

RESULTS

The cytotoxicity of PLEL against autologous tumor cells HMC-2 The cytotoxicity of autologous MLTC-stimulated PLEL and PBL against HMC-2 mammary tumor cells was determined at various effector/target ratios. As shown in Fig. 1, PLEL but not PBL were cytotoxic to HMC-2. The optimal PLEL effector/target ratio was 50, and the use of a higher ratio resulted in reduced PLEL cytotoxicity. This may be due to the culture conditions of the cytotoxicity assay.

We assessd the surface phenotypes of PLEL that had been stimulated by autologous MLTC and cultured for 30 days after separation from a patient in the presence of rIL-2 by a FACS analyzer using mabs OKT3, 4 and 8, and anti-human MHC class II mab. The data demonstrated that all PLEL were OKT3 (CD3) and 8 (CD8)-positive and OKT4 (CD4)-negative. These PLEL also strongly expressed MHC class II products.

The cytotoxicity of PLEL against autologous fibroblasts and allogeneic tumor targets The cytotoxicity of PLEL that had been stimulated by MLTC and cultured in the presence of rIL-2 for 30 days after separation from the pleural cavity was assessed against autologous pleural fibroblasts and various allogeneic tumor target cells. These were K562, HMC-1, ZR-75-1, ZR-75-30, M7609,

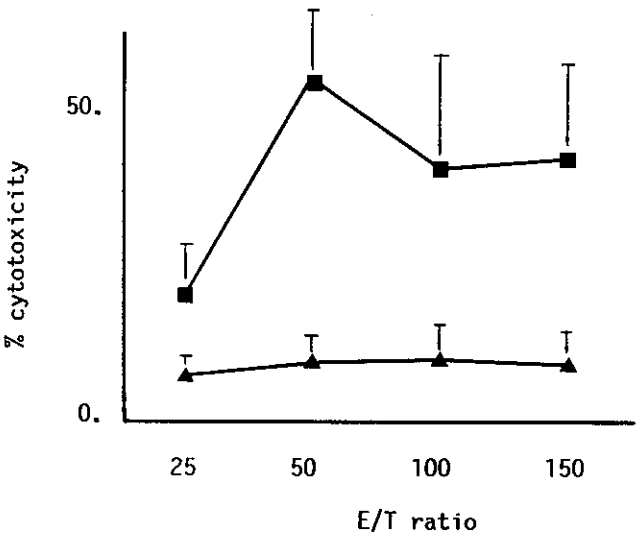

Fig. 1. Cytotoxicity of PLEL (■) and PBL (▲) against autologous HMC-2 mammary tumor cells. The cytotoxicities of PLEL and PBL against HMC-2 were assayed after a 12 h incubation at various E/T ratios. Bars represent SE.

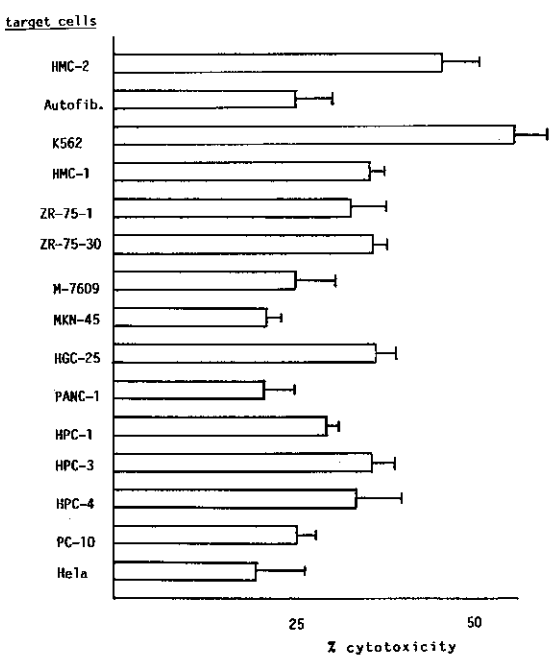

Fig. 2. Reactivity of PLEL to an autologous pleural fibroblast and human allogeneic tumor lines. PLEL obtained from the patient were cultured for 30 days stimulated with autologous MLTC for 4 days before utilization in the cytotoxicity assays with various target cells. The cultures were performed for 12 h at an E/T ratio of 50. Columns indicate percent cytotoxicity and bars represent SE.

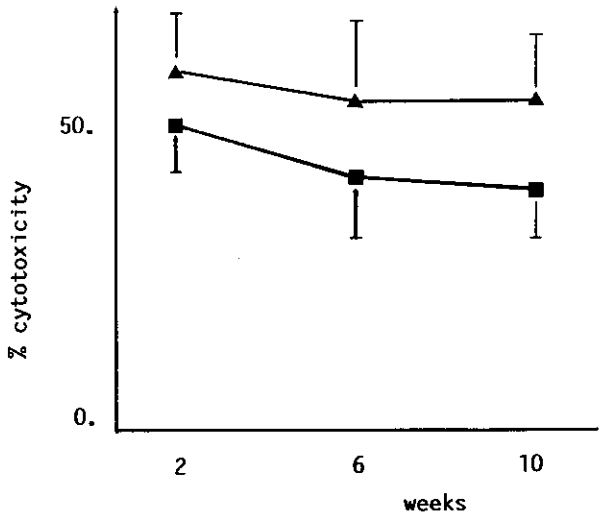

Fig. 3. Time course of cytotoxic activity of PLEL that were cultured from 2 to 10 weeks after separation from the patient against an autologous HMC-2 tumor (■) and K562 (▲) cells. The cytotoxicities of these PLEL against HMC2 and K562 were assayed after a 12 h incubation at an E/T ratio of 50. Bars represent SE.

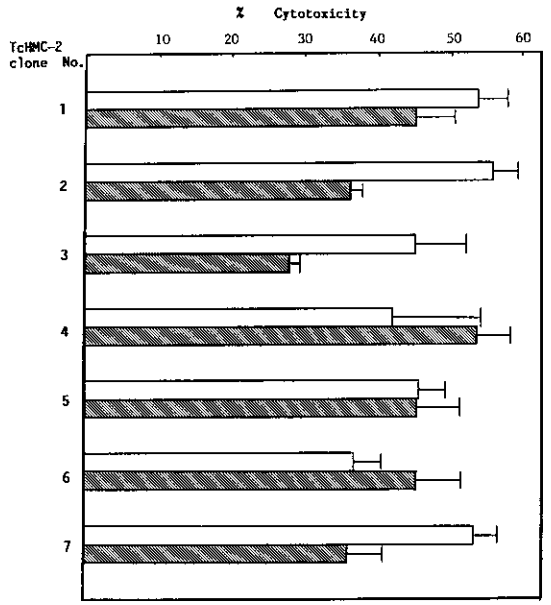

Fig. 4. Cytotoxicity of T_{cHMC-2} cytotoxic T cell clones against an autologous HMC-2 mammary tumor cell line (□) and NK-sensitive K562 line (▨). The cloning of cytotoxic T cells was carried out by limiting dilution of PLEL, and the cells were expanded with rIL-2. Seven different single cell clones (no. 1 through 7) were successfully obtained, and the cytotoxic activity of all these clones against HMC-2 and K562 was examined. Lymphocytes were stimulated with autologous MLTC for 4 days before utilization in the cytotoxicity assays, and the cultures were performed for 12 h at an E/T ratio of 50. Columns indicate percent cytotoxicity and bars represent SE.

MKN-45, HGC-25, PANC-1, HPC-1, -3, and -4, PC-10 and Hela cells. These target cells were cultured for 12 h with PLEL at an effector/target ratio of 50. As can be seen in Fig. 2, PLEL clearly showed high cytotoxicity against autologous tumor target HMC-2 and K562. Although some lines such as HMC-1, ZR-75-30, HGC-25 and HPC-3 showed susceptibility to PLEL cytotoxicity, the cytotoxicity of PLEL to these lines was not more than 30%. The cytotoxicity against these lines seemed to be within the range of background cytotoxic activity of PLEL, since the cytotoxicity of these PLEL against autologous pleural fibroblasts was also 20–25%, and there was no statistically significant difference among them.

We further assessed the time course of the cytotoxic activity of PLEL that were cultured from 2 through 10 weeks after separation from the patient's pleural cavity. These PLEL were stimulated by mytomycin C-treated HMC-2 for 4 days per week and were continuously cultured in the presence of rIL-2. This means that PLEL at 2, 6 and 10 weeks of cultivation had been stimulated 2, 6 and 10 times, respectively, by autologous MLTC. As demonstrated in Fig. 3, PLEL showed high cytotoxicity against the K562 target at every point of the cultivation, although these PLEL showed a slightly decreased cytotoxic potential against the autologous HMC-2 target (statistically not significant). However, PLEL at 10 weeks of cultivation still showed nearly 40% cytotoxicity against HMC-2.

The cytotoxicity of T_{cHMC-2} clones against HMC-2 and K562 PLEL showed a continuous cytotoxic activity toward both HMC-2 and K562 during approximately 3 months of PLEL cultivation. In order to analyze in more detail the cytotoxic mechanisms of the effector cells, we cloned these PLEL. The cloning of cytotoxic T cells was carried out by limiting dilution of PLEL, and the cells were expanded with rIL-2. Of 120 wells, each seeded with one T cell, we could successfully expand only 7 clones (i.e. 5.8% cloning efficiency). The cytotoxic activity of all of these clones against HMC-2 and K562 was examined. The data shown in Fig. 4 indicate that five out of seven clones showed almost the same cytotoxic potential against HMC-2 and K562 target cells. Clones 2 and 3 had a much stronger cytotoxic capability against HMC-2 autologous cells than K562 cells, suggesting that these clones were tumor-specific CTL. However, the cytotoxicity of these clones against K562 was relatively high (28–36%). Therefore, it seemed to us that these

clones have basically the same specificity to the target cells as the other five clones. In other words, the cultures may have already been "auto-cloned" and the clones derived may have actually been progeny from a single cell. These data indicate that the cytotoxic T cell clones, designated as T_{cHMC-2}, have cytotoxic potentials against both HMC-2 and K562 targets, suggesting directly that PLEL were not a mixture of effector cells such as autologous tumor-specific CTL and NK cells.

Mechanism of T_{cHMC-2} cytotoxicity against HMC-2

T_{cHMC-2} clones exhibited direct cytotoxic activity against HMC-2 and K562. This fact suggests two possibilities. The first is that T_{cHMC-2} has the dual cytotoxic potential of CTL and NK cells against HMC-2 and K562, respectively. The second is that T_{cHMC-2} acts only as a human NK clone against these target cells. Therefore, we assessed the susceptibility of HMC-2 cells to NK lysis. It was clearly indicated that HMC-2 is resistant to NK lysis even under experimental conditions in which more than 50% of K562 cells were lysed by NK cells. HMC-2 cells showed almost the same resistance as the Daudi line which is used as an NK-resistant line. These data strongly suggest that T_{cHMC-2} clones have the dual cytotoxic activity of both CTL and NK cells (data not shown).

We further studied the mechanism of T_{cHMC-2} cytotoxicity against autologous HMC-2 as well as K562 tumor cells using mabs OKT3, 4 and 8, anti-human MHC class I products mab HH-1 and anti-human MHC class II products mab TC-8B1. As shown in Table I, the cytotoxicity by T_{cHMC-2} (clone no. 1 in Fig. 4) against HMC-2 was blocked by pretreatment of T_{cHMC-2} with OKT3 and 8, and was also inhibited by pretreatment of HMC-2 traget cells with HH-1 but not with TC-8B1. The T_{cHMC-2} cytotoxicity against K562 was also inhibited by OKT3 but not by HH-1 pretreatment. OKT8 mab pretreatment of T_{cHMC-2} clone resulted in slight inhibition of the cytotoxicity, but this inhibition was not statistically significant. We have preliminary data showing that this cytotoxicity was not blocked by several mabs reacting against non-MHC class I antigens that are expressed on HMC-2 cells (data not shown). These data suggest that the cytotoxicity of this clone against the autologous tumor target is MHC class I-restricted, and that antigen-specific T cell receptors on T_{cHMC-2} clone were involved in the cytotoxicity of this specific cytotoxic T cell clone.

Table I. Effect of Mabs on the Cytotoxicity of T_{cHMC-2} Clone no. 1 against HMC-2 Autologous Mammary Tumor and K562 Lines

Mabs treatment[a]	% Cytotoxicity[b] target cells	
	HMC-2	K562
Medium	27.5±2.5	25.7±3.2
OKT3	7.1±2.8	10.1±2.2
OKT4	21.0±4.4	ND
OKT8	8.8±1.9	16.5±5.9
HH-1	9.5±2.3	21.6±4.8
TC-8B1	24.5±4.8	ND

a) T_{cHMC-2} clone no. 1 cells were treated with saturating amounts of mabs OKT3, 4, and 8 at 4°C for 60 min and were used in the cytotoxicity assays against HMC-2 cells. HH-1 (anti-MHC class I) and TC-8B1 (anti-class II) mabs were used to treat HMC-2 target cells at 4°C for 60 min.
b) The cytotoxicity assays were performed at an E/T ratio of 50 for 12 h at 37°C in a 5% CO_2 incubator. The data represent mean±SE. ND means not determined.

DISCUSSION

It has been demonstrated that the CTL separated from cancer patients can be cytotoxic towards autologous tumor cells.[21-27] These studies have suggested that the clones of some particular lymphocytes were generated in the tumor-bearing hosts, and responded to the corresponding specific antigens that might be expressed on the neoplastic targets. We have previously demonstrated by clonal analysis that the human cytotoxic T cell clone T_{cHMC-1} could be specifically lytic to an autologous mammary tumor HMC-1-8 target, but not to many allogeneic tumor targets including the K562 line.[7,8] The direct evidence now shows that there exist clones of CTL generated in cancer patients to fight specifically against tumorigenic autologous cells which are transformed in the same hosts.

In our present report, we describe the clonal analysis of the cytotoxic T cell clones T_{cHMC-2} which show cytotoxicity against the autologous tumor HMC-2 as well as K562 target cells. The cytotoxicity of this clone against autologous HMC-2 target cells was inhibited by OKT3 (CD3), OKT8 (CD8), and anti-MHC class I mabs. It was also demonstrated that HMC-2 was not susceptible to human PBL-derived NK cells. These data suggest that the T_{cHMC-2} clone has a dual cytotoxic function within a single cytotoxic T cell. The first is as CTL which lyzes specifically an autologous tumor and the second is as NK-like cells which act against K562.

There have been several reports demonstrating the dual cytotoxic function at the single cell level of T lymphocytes.[9-11] All of these clones consist of MLC-induced allospecific cytotoxic T cells. Moretta *et al.*[9] obtained dual cytotoxic clones that are lytic to a specific allotarget and K562. The cytotoxicity of these clones to K562 targets was not blocked by OKT8 mab, but the cytotoxicity to PHA blasts was clearly inhibited by this mab, suggesting that these clones may have different

recognition structures on their cell surfaces. In fact, K562 cells are devoid of MHC class I products on the cell surface. The density of MHC class I antigens on HMC-2 cells is not decreased (data not shown), and the fact that the cytotoxicity of $T_{cHMC\text{-}2}$ clone against HMC-2 was blocked by anti-MHC class I mab suggests that $T_{cHMC\text{-}2}$ clone may use different recognition structures for each of the autologous mammary tumors and K562 tumor cells.

Our present demonstration of dual specificity in the $T_{cHMC\text{-}2}$ clone seems, so far, one of very few reported examples of a human autologous CTL clone concomitantly having NK cell function. A more detailed study of the autologous tumor and effector lymphocytes especially as to the generation of these dual specific cytotoxic clones and the mechanisms of cytotoxicity against autologous epithelial tumor cells and NK sensitive-K562 cells is very important, since such studies may directly lead to a most effective method of immunotherapy in cancer patients. It was suggested that the cytotoxicity of $T_{cHMC\text{-}2}$ clone against K562 was blocked by pretreatment with OKT3 mab. There are several reports demonstrating that some but not all of the cytotoxic clones with dual cytotoxic function may use the CD3 molecule to generate the cytotoxicity in MLC-induced cytotoxic human T cell clones.[10, 11, 28] The importance and involvement of the CD3 molecule, and possibly T cell receptor molecules in $T_{cHMC\text{-}2}$, against NK-susceptible targets remains to be elucidated, until techniques are available to proliferate these cytotoxic lymphocyte clones to large amounts, sufficient for use in experiments. It seems to us that the immortalization of T cells with retention of their cytotoxic function would be another important issue.[29] It would provide opportunities for us to investigate the details of the cytotoxic mechanisms of human CTL against autologous neoplastic cells, and to apply the most effective protocol to cancer patients.

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