Tumor Res. **26**, 29—41 (1991)

Heterogeneity of Human Breast Cancer Cell Clones with respect to Cytotoxic Susceptibility detected by Cytotoxic T-Lymphocytes and Natural Killer Cells

Takashi SATO[1], Noriyuki SATO*, Junmin CHO*,
Shuji TAKAHASHI*, Kazunori TODA, Kazuaki ASAISHI,
Koichi HIRATA, and Kokichi KIKUCHI*

*Department of Surgery and *Department of Pathology, Sapporo Medical College, S-1, W-16, Sapporo 060, Japan*

ABSTRACT

Clonal heterogeneity of human breast cancer cells, HMC-1, with respect to the cytotoxic susceptibility against autologous cytotoxic T-lymphocytes (CTL), TcHMC-1, and natural killer (NK) cells was demonstrated in a ^{51}Cr release cytotoxicity assay. We have established 8 tumor cell clones, HMC-1-1 through HMC-1-8, from HMC-1 cells and autologous TcHMC-1 clone that showed high cytotoxic activity as well. In the cytotoxicity assays, HMC-1-8 clone showed significantly high cytotoxic susceptibility by TcHMC-1, and conversely, HMC-1-7 clone showed low cytotoxic susceptibility. In case of autologous NK cells as effector, HMC-1-8 clone showed low cytotoxic susceptibility and HMC-1-7 clone did high. On the other hand, as to lymphokine-activated killer (LAK) cells, such heterogeneity as TcHMC-1 and NK cells demonstrated was not shown by either autologous or allogeneic LAK cells. These heterogeneous cytotoxic susceptibilities were further examined to determine whether they are related to the tumorigenicity of HMC-1 clones using a soft agar assay, showing that HMC-1-8 clone indicated significant high plating efficiency and HMC-1-7 showed low plating efficiency. The results of this study indicated that the heterogeneity of HMC-1 clones in cytotoxic susceptibility was shown by autologous CTL and NK cells but not by LAK cells, and the NK susceptibility alone seemed to be concerned with the tumorigenicity of tumor cells. To achieve more

[1] To whom requests for reprints and all related correspondence should be addressed: Department of Surgery, Sapporo Medical College, S-1, W-16, Chuo-ku, 060 Sapporo, Japan.

[2] The abbreviations used are: CTL, cytotoxic T-lymphocytes; HMC-1, a human breast cancer cell line; HMC-1-1 through HMC-1-8, clones of HMC-1; TcHMC-1, a cytotoxic T-lymphocyte clone; NK, natural killer; LAK, lymphokine-activated killer; MLTC, mixed-lymphocyte tumor cell culture; rIL-2, recombinant interleukin 2; E/T ratio, effector/target cell ratio; mAb, monoclonal antibody.

effective cancer immunotherapy, it is thought to be necessary to clarify such a new type of tumor heterogeneity in cytotoxic susceptibility.

Key word: Heterogeneity, Cytotoxic susceptibility, Human breast cancer cells, Cytotoxic T-lymphocytes, NK cells

INTRODUCTION

Patients with malignant neoplasma have been reported to have impairments in cell-mediated immunity (1, 2). Therefore, in order to attain progress in cancer therapy, it is important to explore the relationships between tumor cells and various effector lymphocytes such as cytotoxic T-lymphocytes (CTL)[2], NK cells, and LAK cells *in vivo* as well as *in vitro*. Our previous studies showed that autologous CTL may prove useful in adoptive immunotherapy for cancer (3, 4). By single cell cloning of CTL obtained from a breast cancer patient, noticeably high potentiated CTL clone, TcHMC-1, was established successfully. On the other hand, as other workers have reported (5, 6), heterogeneous CTL which showed differential cytotoxic activities against human autologous melanoma cells were demonstrated. As to the effector lymphocytes such as CTL not but LAK cells, the adoptive transfer of CTL, especially using autologous tumor-specific cytotoxic T-lymphocytes with more cytotoxic activities has been required to produce improvements of cancer immunotherapy.

In recent years, special attention has been given to the influence of tumor cell heterogeneity on the response of tumor cells to treatment with radiation (7), endocrine agents (8), and cytotoxic drugs (9, 10). The identification of resistant subpopulations of tumor cells that determine the response of a tumor to treatment is vital to the optimization of that therapeutical modality in the clinic. Therefore, from a view of the tumor cell side, to assess the cytotoxic susceptibility of tumor cell against CTL may confer a great benefit upon cancer immunotherapy. In our previous publications (3, 4, 11), human breast cancer cell line designated as HMC-1 and its cloned cell lines. HMC-1-1 through HMC-1-8, were precisely demonstrated and the immunological analysis of autologous CTL clone (TcHMC-1) also examined. In the present study at the clonal level, in order to clarify the complicated tumor-lymphocyte interactions *in vivo* and delineate a more effective immunotherapeutic regimen, we scrutinized the data for significant differences in the cytotoxic susceptibility of the tumor cell clones against autologous TcHMC-1 clone, NK cells, and LAK cells *in vitro*, and for heterogeneity of the cell clones in tumorigenicity by using soft agar assays as well.

MATERIALS AND METHODS

Tumor cell culture and cloning

A human breast cancer cell line, HMC-1 (11), was derived from a metastatic pleural effusion in a 35-year-old female patient who had undergone radical mastectomy 2 years before attempting to produce a culture for infiltrating ductal carcinoma of the right breast. As the establishment of pancreatic cancer cell lines derived from malignant ascitic effusions was reported previously (12, 13), almost the same procedures for the cultivation of cells were utilized in the study. Briefly, 500-800 m*l* of metastatic pleural effusions were centrifuged at 250×g for 10 min. Resultant cell pellets were resuspended in 20 m*l* of RPMI-1640 medium containing 10% fetal calf serum (FCS) with 292 μg/m*l* of L-glutamine, 100 μg/m*l* of streptomycin and 100 units/m*l* of penicillin, and then the cell suspension was layered on a 20 m*l* of Ficoll-Conray density gradient (S. G. 1.082). After spinning down at 1,000×g for 25 min at room temperature, the interface in which tumor cells, macrophages, mesothelial cells, fibroblasts and lymphocytes were enriched was collected, and washed 3 times with RPMI-1640 medium. Then the cells were seeded in a 25 cm^2 culture flask (Falcon # 3013, Oxnard, CA.), and cultured at 37°C in a 5% CO_2 incubator. The supernatant of the flask was daily reseeded into other flasks, and in this manner, the newly seeded cells were enriched in tumor cells. One month after the beginning of culture, non-malignant cells were reduced and floating tumor cells began to adhere onto the bottom of the culture flasks. Propagating cells were serially transferred into other flasks by treating with 0.05% trypsin (Sigma Chemicals Co., St. Louis, MO.) plus 0.02% EDTA in a phosphate buffered saline (PBS) at weekly intervals. At approximately 5 months of a successful culture, a cell line designated as HMC-1 propagated constantly in an adherent style of monolayer. Then the HMC-1 was cloned twice *in vitro* by limiting dilution technique as reported previously (3, 4). Briefly, a single cell was implanted into 24-well culture plates (Costar # 3524, Costar, Cambridge, MA.) and maintained in RPMI-1640 medium containing 10% FCS. Two weeks later, those cultures showing cell growth were harvested and a second single cell cloning was undertaken for each clone. Eight HMC-1 clones were obtained and HMC-1-1 through HMC-1-8 clones were subsequently established. These cell lines were assessed for mycoplasma contamination by using a mycoplasma stain kit (Flow Laboratories Inc., VA.) and all cell lines were shown to be free of mycoplasma contamination.

Culture and cloning of lymphocytes

As described previously (3), autologous cytotoxic T-lymphocytes were separ-

ated from the pleural effusion. In brief, approximately 20 m*l* of a pleural effusion were layered on 30 m*l* of Ficoll-Conray density gradient and centrifuged at 1,400×g for 25 min. Collected cells were washed 3 time in PBS. Then T-lymphocytes were purified from other cells by a Percoll discontinuous density centrifugation. Furthermore, these T-lymphocytes which showed high killer activity were cloned by a limiting dilution in 96-well microtiter plates (Costar # 3799). Expanding in 0.2 m*l* of RPMI-1640 medium containing rIL-2 (20 units/m*l*) which was kindly provided by Ajinomoto Central Research Laboratory (Tokyo, Japan), the CTL clones, TcHMC-1, was stimulated at weekly intervals with autologous MLTC using mitomycin C and was propagated under an addition of rIL-2 *in vitro*. The details of autologous MLTC were described in our previous reports (3, 14). NK cells were isolated from peripheral blood in the patient by using a Percoll discontinuous density centrifugation as demonstrated previously by others (15, 16), collecting cells which showed homogeneous population of large to medium-sized lymphocytes with abundant neutrophilic cytoplasm containing localized azurophilic granules by staining of May-Crünwald-Giemsa technique were frozen in liquid nitrogen and stored at −80°C until use. In addition, LAK cells were generated from peripheral blood of the patient and a normal healthy volunteer by using the same procedure of CTL-separation described as above and stored. LAK cells were also propagated in RPMI-1640 medium with 10% FCS containing rIL-2 (20 units/m*l*) for 4 days. In the present study, the viabilities of these effector lymphocytes were examined by trypan blue exclusion test and adjusted to E/T ratio of 50 : 1, and employed for cytotoxicity assay.

Cytotoxicity assay

The ^{51}Cr release assay for the determination of cytotoxic activity of TcHMC-1, NK cells and LAK cells were carried out as described previously (3, 4). Briefly, target cells were labeled by 100 μCi of ^{51}Cr Sodium 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 m*l* RPMI-1640 medium were seeded into U-bottomed microtiter plates (Costar # 3799). Thereafter, 0.1 m*l* of the effector cell suspension as a predetermined dose was added, and the plates were centrifuged at 200×g for 5 min. After 6 to 12 h incubations at 37°C in 5% CO_2 incubator, 0.1 m*l* of culture supernatant was harvested and the radioactivity was counted with a liquid scintillation counter (Packard Auto-Gamma scintillation spectrometer). To assay of NK activity, we used NK-sensitive K562 target cells, of human erythroleukemia origin, as described previously (14). The percent of lysis was calculated as follows: % specific lysis = (Experimental release−Spontaneous release)×100/ (Maximal release−Spontaneous

release). To determine the maximal release, 0.1 m*l* of 1% Nonidet P-40 (Nakarai Chemical Co., Kyoto, Japan) 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% in the experiment. All determinations were made in triplicate, and the data were represented as the mean ± SE.

Surface phenotype of lymphocytes

Surface markers of TcHMC-1, NK cells and LAK cells were detected by indirect immunofluorescence using saturating amounts of monoclonal antibodies (mAbs) such as OKT3 (CD3) reactive with a T-lymphocytes; OKT4 (CD4) reactive with a subpopulation of T-lymphocytes including inducer and helper cells; OKT8 (CD8) reactive with a subpopulation of T-lymphocytes including cytotoxic and suppressor cells and NKH-1 (CD56) reactive with a large proportion of NK cells. The first three mAbs were purchased from Ortho Pharmaceutical CO., Ratin, NJ., and NKH-1 were obtained from Becton Dickinson and Co., Mountain View, CA.

Tumorigenicity of tumor cell clones

In order to estimate the tumorigenicity of HMC-1's clones *in vitro,* an anchorage-independent growth was examined by using a soft agar assay as described previously (11-13). Briefly, 10^3 HMC-1-1 through HMC-1-8 cells were plated in dishes (Falcon # 3002) containing 0.3% bacto-agar (Difco, Detroit, NJ.), and incubated at 37°C under 5% CO_2 incubator. As the positive control of anchorage-independent growth of cells, a highly tumorigenic murine colon cancer cell line C-C 36 (17, 18) was employed. NIH 3T3 and BALB 3T3 which have been shown to be completely low tumorigenic in nature *in vitro* (13) were both employed for negative controls in the present assay. The number of colonies in soft agar were scored with % plating efficiency after 2 and 3 weeks of cultivation as follows: % Plating efficiency = (Number of clusters − Number of original cell aggregates) × 100/Number of viable nucleated cells plated. On the other hand, the cell clones were also employed for the detection of tumorigenicity *in vivo* using nude mice. HMC-1 and its clones growing *in vitro* were harvested by trypsinization, and 10^6 cells per mouse were inoculated subcutaneously into the back of five BALB/c nude mice obtained from CLEA Japan Co., Shizuoka, Japan. The mice were observed weekly for the tumor growth until their termination.

Statistics

Statistical differences in the present assay of HMC-1 cells and their clones

were determined by the Student's t test. A probability value less than 0.05 was considered statistically significant.

RESULTS

Cytotoxicity of autologous cytotoxic T-lymphocyte clone, TcHMC-1, against HMC-1 cells and their clones

The standard chromium release assay was used to measure cytotoxic susceptibility of tumor cell clones, HMC-1 and HMC-1-1 through HMC-1-8, against TcHMC-1. As shown in Fig. 1, HMC-1-8 clone showed a significantly high % cytotoxicity (65.6 ± 3.7; $P<0.001$) at 12 h incubation and an E/T ratio of 50. Other tumor cell clones, HMC-1-1 through HMC-1-6 did not show a clear significant difference with HMC-1 cells. In contrast, HMC-1-7 clone showed a statistically significant low cytotoxicity (28.4 ± 3.0; $P<0.001$). The phenotypic surface marker of TcHMC-1 was enriched to 100% of OKT3 (CD3) and OKT8 (CD8) positive and further OKT4 (CD4) and NKH-1 (CD56) negative. In case of NK susceptable K562 target cells, considerably low cytotoxicity was obtained at 6 h incubation and an E/T ratio of 50.

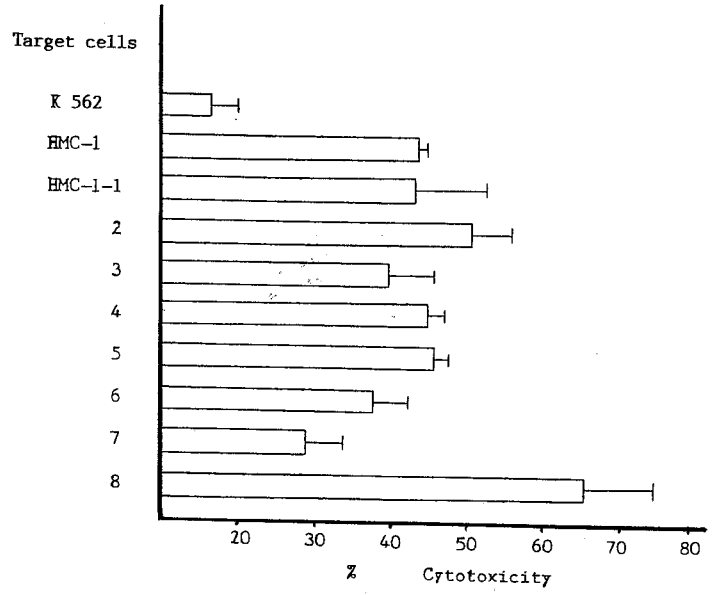

Fig. 1 Cytotoxicity of CTL clone, TcHMC-1, obtained from pleural effusion against HMC-1 target cells and their clones. HMC-1 cells were cloned twice by a single cell cloning at their 23 rd passage generation. and 8 clones, HMC-1-1 through HMC-1-8, were established. TcHMC-1 cells were stimulated at weekly intervals with autologous MLTC, and the culture in the cytotoxicity assays were carried out for 12 h incubation at an E/T ratio of 50. Columns indicate cytotoxicity in percentage and bars represent SE.

Cytotoxicity of autologous NK cells against HMC-1 cells and their clones

As shown in Fig. 2, cytotoxic susceptibilities of tumor cell clones against autologous NK cells were assessed in cytotoxicity assay at 6 h incubation and an E/T ratio of 50. Large to medium-sized lymphocytes isolated from the patient's peripheral blood were cultured with rIL-2 for a week and proved to be NK cells cytologically and phenotypically; OKT8 (CD8) and NKH-1 (CD56) positive and OKT3 (CD3) and OKT4 (CD4) negative. The results of the assays indicated that the highest cytotoxic susceptibility of all was found in HMC-1-7 (42.3 ± 1.8; $P<0.005$) and statistically significant low % cytotoxicity was seen in HMC-1-8 (14.8 ± 2.4; $P<0.01$). Under the same conditon, % cytotoxicity of K562 was 41.3 ± 3.2 ($P<0.005$) and this was nearer to HMC-1-7 clone.

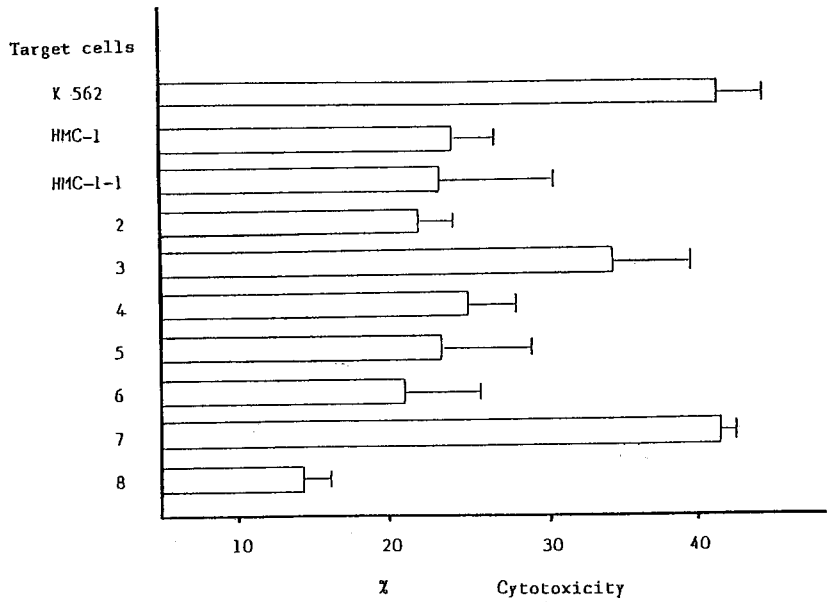

Fig. 2 Cytotoxicity of autologous NK cells obtained from peripheral blood against HMC-1 target cells and their clones. NK cells were cultured with rIL-2 for 7 days before the cytotoxicty assays. Incubation time was 6 h at 50 E/T ratio. Columns indicate percent cytotoxicity and bars represent SE.

Cytotoxicity of autologous and allogeneic LAK cells against HMC-1 cells and their clones

Both autologous and allogeneic LAK cells derived from the patient and a non-patient respectively, were generated by culturing with rIL-2 for 4 days.

LAK cells of the patient were stored at −80°C and recovered completely on the day before cytotoxicy assay, on the other hand, LAK cells normal volunteers were employed for the assay without freezing storage. Viabilities of cells were over 98% by trypan blue exclusion tests and phenotypically both cells showed OKT3 (CD3) and OKT8 (CD8) positive and OKT4 (CD4) and NKH-1 (CD56) negative. As schemed in Fig. 3, the results of this assay showed that there was no statistically significant difference in cytotoxic susceptibility among tumor clones against LAK cells generated from the patient as well as a non-patient. The range of % cytotoxicity of LAK cells from the patient showed 23.3−27.3% and that from a non-patient was 22.0—26.9% with no statistically significance, respectively.

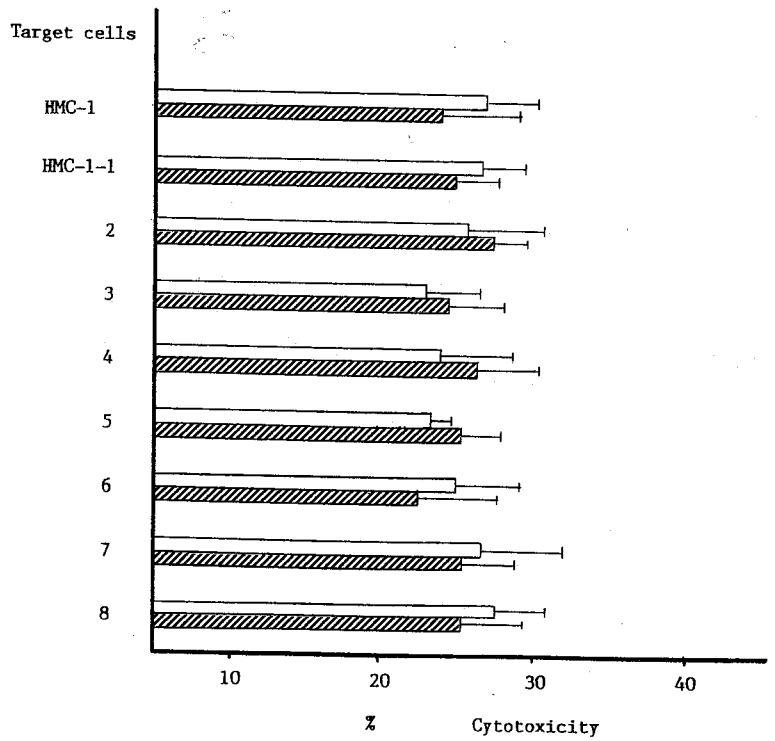

Fig. 3 Cytotoxicity of autologous (□) and allogeneic (▨) LAK cells against HMC-1 target cells and their clones. Lymphocytes were generated from peripheral blood of the patient and healthy volunteers, culturing with rIL-2 for 4 days before the assays, respectivery. The incubation was conducted for 12 h at an E/T ratio of 50. Columns indicate percent cytotoxicity and bars represent SE.

Anchorage-independent growth of HMC-1 cells and their clones

The growth capability of HMC-1 cells and their clones, HMC-1-1 through HMC-1-8 in an anchorage-independent manner was assessed using 0.3% soft agar. As shown in Table 1, HMC-1-8 clone showed a significantly high % plating efficiency ($P<0.001$) at 2nd and 3rd week of cultivation, respectively. HMC-1-7, in contrast, was significantly low in both cultivations ($P<0.01$). As a highly tumorigenic murine colon cancer line C-C 36, showed a high plating efficiency ($P<0.001$). On the other hand, there were no colony formations for NIH 3T3 and BALB 3T3 cells used as negative controls.

Table 1 *Anchorage-independent growth in 0.3% agar of HMC-1 cells and their clones.*

cells	% Plating efficiency	
	2nd week	3rd week
HMC-1	10.7±2.1	12.0±2.2
HMC-1-1	11.5±0.5	12.7±1.7
2	13.3±2.5	14.3±2.5
3	11.0±2.2	12.6±2.6
4	9.7±0.5	13.3±2.1
5	13.9±2.0	15.0±1.6
6	11.6±3.4	13.0±2.9
7	4.3±0.9*	6.3±1.2*
8	25.7±1.7**	32.7±3.7**
C-C 36	60.3±3.7	74.7±5.4
NIH 3T3	0	0
BALB 3T3	0	0

10^2 cells per dish were inoculated into a seeder layer containing 0.3% agar. % plating efficiency was expressed as follows: (The number of clusters-The number of original cell aggregates) ×100/The number of viable cells plated. All determinations were made in tripricate, and the data gathered were give as the mean ± SE. Significant differences from parental HMC-1 cells were indicated by Studen't test: *$P<0.01$, **$P<0.001$.

Tumorigenicity in nude mouse

In order to examine the *in vivo* tumorigenicity of these tumor clones, the heterotransplantation of clones into nude mice were carried out, resulting that 100% (5/5) of HMC-1-8 cells growing and 14.3% (1/7) of HMC-1-7 cells were shown, while parental HMC-1 cells was 57.1% (4/7) in 3 months. No tumor growing mice were investigated up to 6 months after cells inoculations.

DISCUSSION

For the desirable clinical use of adoptive immunotherapy with effector T-lymphocytes, it is important to clarify the function of CTL, especially against autologous tumor cells, at the clonal level. In conection with our continuous works (3, 4, 14), the understanding of the existence of heterogeneous effector lymphocytes is a very meaningful other issue and the heterogeneity of tumor cells in the cytotoxic susceptibility to effector lymphocytes as well. More recently, Anichini *et al.* (6) demonstrated the cytotoxic heterogeneity of CTL clones and simultaneously stressed the evidence of heterogeneous cytotoxic susceptibility to autologous CTL clones in human melanoma cells. Furthermore, many investigators reported on the cytotoxic heterogeneity of cloned NK cells in human (19, 20). In addition, as to LAK cells, no different cytotoxic activity of LAK cells against various tumor cells was documeted by Rosenberg and his co-workers (21), but not analyzed at the clonal level. Taking a cancer immunology into consideration, the utilization of cytotoxic effector cells, especially autologous cloned cytotoxic T-lymphocytes, seemed to be more effective in adoptive immunotherapy rather than using LAK cells and NK cells which may have moderately high cytotoxic potentials, since such CTL were thought to have antigen-specific recognitions against tumor cells and possess high killing activities. The *in vivo* antitumor efficacy of autologous cytotoxic T-lymphocyte clone. TcHMC-1, was clearly demonstrated in our previous study (3). TcHMC-1 clone was unique in that it had the character of autologous tumor killer specific.

In the present study, in order to explore the tumor defense mechansisms, we used cytotoxic T-lymphocyte clone, TcHMC-1, showing phenotypically OKT3 (CD3) and OKT8 (CD8) positive and OKT4 (CD4) and NKH-1 (CD56) negative as well as autologous tumor cell clones, HMC-1-1 through HMC-1-8, derived from a human breast cancer patient, and examined the cytotoxic susceptibility of the tumor cells against not only TcHMC-1 but NK cells and LAK cells by using ^{51}Cr release cytotoxicity assay. On the other hand, NK cells showing OKT8 (CD8) and NKH-1 (CD56) positive and OKT3 (CD3) and OKT4 (CD4) negative were obtained from the peripheral blood of the patients, while two types of LAK cells, autologous and allogeneic, were obtained from the peripheral blood of the patient and a healthy volunteer, respectively. The obtained results using cytotoxicity assays showed that the cytotoxic susceptibility of HMC-1 clones against TcHMC-1 and NK cells was clearly revealed. Of eight HMC-1 clones examined, HMC-1-8 clone alone showed significantly high cytotoxic susceptibility against TcHMC-1 (65.6%) but low cytotoxic susceptibilities against autologous NK cells (14.8%). On the contrary, HMC-1-7 clone showed significantly low

cytotoxic susceptibility against TcHMC-1 (28.4%) but showed considerably high cytotoxic susceptibility against NK cells (42.3%). On the other hand, however, both autologous and allogeneic LAK cells showing phenotypically the same expression of mAbs, OKT3 (CD3) and OKT8 (CD8) positive and OKT4 (CD4) and NKH-1 (CD56) negative did not show any cytotoxic heterogeneity against HMC-1 clones, HMC-1-1 through HMC-1-8, still the reason remains unknown. The results of these cytotoxicity assays at the clonal level indicated that the cytotoxic susceptibility of human autologous CTL and NK cells were heterogeneous, suggesting that such heterogeneity may be due to variations in the levels of differentiation and/or activation *in vivo*.

To elucidate the relationship between cytotoxic susceptibility of the tumor cells and their tumorigenicity, a soft agar assay *in vitro* was employed additionally, indicating that HMC-1-8 clone alone showed signgficant high plating efficiency. In contrast, HMC-1-7 clone showed fairly low plating efficiency in spite of other homogeneous tumorigenicity of the clone including of parental HMC-1 cell clone. Moreover, the *in vivo* tumorigenicity of HMC-1 cells and their clones were assessed by the heterotransplantation of these cells into nude mice, suggesting that HMC-1-8 cells had a markedly high tumorigenicity, while HMC-1-7 cells showed rather low value. On the other hand, these HMC-1 cells and their clones showed almost the same growth characteristics (doubling time; 26 h) and the negative for 17 β-estradiol and progesterone hormone receptors in spite of their original receptor showed positive, and morphologically the same light and electron-microscopic features as well (data not shown). Moreover, as to HMC-1 cells and their contrasting two clones, HMC-1-7 and HMC-1-8, these clones also showed almost the same modal chromosome number of 72 by a conventional chromosome analysis. Interestingly, the heterogeneity of HMC-1 clones, MHC-1-7 and HMC-1-8, in tumorigenicity seemed to be related with the cytotoxic susceptibility of clones against NK cells but CTL cells, if so, this evidence may be concerned with the metastatic mechanism of tumor cell *in vivo*. Actually, as other investigators mentioned previously (22, 23), NK cells, surculating or not, may have not a small responsibility for tumor metastasis. Furthermore, we have previously demonstrated by clonal analysis that an another human cytotoxic T-lymphocyte clone, TcHMC-2, could show specific cytotoxicity against the autologous breast cancer cell clone HMC-2 as well as K562 target cells; such a clone possessing dual cytotoxic function as TcHMC-2 may contribute to the evaluation of the role of T cell receptor molecules in effector lymphocytes against NK susceptible and resistant targets, and therefore the next steps for recognition of cytotoxic heterogeneity in T cell receptor mechanism will be an another special problem to solve.

In a widely complicated tumor-lymphocyte immunological field, utilizing these autologous pairs of effector lymphocyte clones and cloned tumor cells, further endeavor to clarify the interaction of tumors and lymphocytes and heterogeneity of tumor cells with respect to cytotoxic susceptibility seemes to be more encouraged toward various cancer therapies as well as prevention of tumor recurrence and metastasis. Further studies along this line are in progress.

ACKNOWLEDGEMENTS

This work was supported in part by a Grant-in-Aid for a Special Research Project in the field of biotechnology of Japan. The authors thank Ms. Akemi Kitamura for preparing the manuscript.

REFERENCES

1. KERBEL, R. S.: **Nature 280**, 358-359 (1979).
2. OLD, L. J.: **Cancer Res. 41**, 361-375 (1981).
3. SATO, T., SATO, N., TAKAHASHI, S., KOSHIBA, H. and KIKUCHI, K.: **Cancer Res. 46**, 4384-4389 (1986).
4. SATO, T., SATO, N., TAKAHASHI, S., OKUBO, M., YAGIHASHI, A., TORIGOE, T., TAKAHASHI, N., OKAZAKI, M., ASAISHI, K. and KIKUCHI, K.: **Cancer Res. 48**, 3892-3897 (1988).
5. ANICHINI, A., FOSSATI, G. and PARMINI, G.: **Int. J. Cancer 35**, 683-689 (1985).
6. ANICHINI, A., FOSSATI, G. and PARMINI, G.: **J. Exp. Med. 163**, 215-220 (1986).
7. BARENDSEN, G. W., ROELSE, H., HERMENS, A. F., MADHUIZEN, H. T., van PEPERZEEL, H. A. and RUTGERS, D. H.: **J. Natl. Cancer Inst. 51**, 1521-1526 (1973).
8. OSBONE, C. K.: **Semin. Oncol. 12**, 317-326 (1985).
9. YUNG, W-K. A., SHAPIRO, J. R. and SHAPIRO, W. R.: **Cancer Res. 42**, 992-998 (1982).
10. SATO, T., SATO, N., KIKUCHI, K. and LeGRUE, S. J.: **Tumor Res. 23**, 73-82 (1988).
11. SATO, T., SATO, N., TAKAHASHI, S., OKUBO, M., YAGIHASHI, A., OKAZAKI, A., OKAZAKI, Y., TODA, K., HIRATA, K., OKAZAKI, M., ASAISHI, K., HAYASAKA, H. and KIKUCHI, K.: **Tumor Res. 22**, 27-39 (1987).
12. SATO, T., SATO, N., TAKAHASHI, S., KOSHIBA, H., USUI, T., HIRATA, K., SHIRAMATSU, S., HAYASAKA, H. and KIKUCHI, K.: **Tumor Res. 20**, 33-41 (1985).
13. SATO, T., SATO, N., TAKAHASHI, S., OKUBO, M., YAGIHASHI, A., KOSHIBA, H., USUI, T., KATSURAMAKI, T., YAMADA, T., HIRATA, K., SHIRAMATSU, K., HAYASAKA, H. and KIKUCHI, K.: **Tumor Res. 21**, 9-19 (1986).
14. SATO, T., OKUBO, M., WADA, Y., SATO, N. and KIKUCHI, K.: **Jpn. J. Cancer Res. 80**, 655-661 (1989).
15. GUTIERREZ, C., BERNABE, R. R., VEGA, J. and KREISLER, M.: **J. Immunol. Methods 29**, 57-63 (1979).
16. TIMONEN, T. and SAKSELA, E.: **J. Immunol. Methods 36**, 285-291 (1980).
17. SATO, N., MICHAELIDES, M. C. and WALLACK, M. K.: **Cancer Res. 41**, 2267-2272 (1981).
18. SATO, N., SATO, T., TAKAHASHI, S. and KIKUCHI, K.: **Cancer Res. 46**, 362-366 (1986).

19. VOSE, B. M.: **Cancer Immunol. Immunother. 17**, 73-75 (1984).
20. ZAGURY, D., MORGAN, D. A., FOUCHARD, M., BERNARD, J., FELDMAN, M. and HERBERMAN, R. B.: **J. Natl. Cancer Inst. 74**, 553-562 (1985).
21. PAPA, M. Z., MULE, J. J. and ROSENBERG, S. A.: **Cancer Res. 46**, 4973-4978 (1986).
22. HANNA, N.: **Cancer Metastasis Rev. 1**, 45-64 (1982).
23. BARLOZZARI, T., LEONHARDT, J., WILTROUT, R. H., HERBERMAN, R. B. and REYNORDS, C. W.: **J. Immunol. 134**, 2783-2789 (1985).