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High Sensitivity to Peripheral Blood Lymphocytes and Low HLA-class I Antigen Expression of Small Cell Lung Cancer Cell Lines with Diverse Chemo-radiosensitivity

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Three cell lines of small cell lung cancer (SCLC), which were established from specimens of untreated primary tumors biopsied by diagnostic bronchofiberscopy, were analyzed for immunological characteristics. These cell lines showed considerable heterogeneity in chemo-radiosensitivity, which was well correlated with clinical responses of the respective tumors, but their HLA-class I antigen expressions were equally depressed and they were susceptible to peripheral blood lymphocytes (PBL) and lymphokine-activated killer (LAK) cells, irrespective of their diverse chemo-radiosensitivity. Treatment of the cell lines with recombinant immune interferon (rIFN-γ) increased their HLA-class I antigen expression and conversely depressed PBL sensitivity but not LAK sensitivity. This inverse relationship between HLA-class I expression and PBL susceptibility was also demonstrated using other pairs of autologous PBL and SCLC cell lines. rIFN-γ changed neither HLA-class II antigen nor SCLC-specific antigen expression under the same experimental conditions. *In vitro* immunization of allogeneic peripheral blood lymphocytes with rIFN-γ-treated SCLC cells induced allo-specific killer cells which lysed rIFN-γ-treated more strongly than non-treated SCLC cells. These results suggest that reduced HLA-class I antigen expression of SCLC could protect the cancer from attack of killer T cells in spite of the higher sensitivity to PBL or LAK cells.

Key words: Small cell lung cancer — Chemo-radiosensitivity — Peripheral blood lymphocyte — HLA-class I — Interferon-γ

Small cell lung cancer (SCLC)[4] is clinically characterized by rapid growth and early metastasis, and few cases are operable at diagnosis. Although most SCLCs are sensitive to initial chemo-radiotherapy, they soon relapse and become refractory to any therapy. Thus the 3 year-survival of SCLC patients is still only about 10%. It is urgently necessary to devise an alternative therapy for such refractory SCLC. Immunotherapy may be one of the more promising approaches but an effective one for SCLC has not been established.

Doyle *et al.*[1)] reported that HLA-class I antigen expression was markedly depressed in SCLC, compared to non-SCLC, and suggested the existence of escape mechanisms of SCLC from host immune surveillance. This reduced expression of HLA-class I antigen has been shown to be reversed by interferon (IFN) in several kinds of tumors including SCLC. Recombinant interferons (rIFN) are widely available and their immunomodulatory effects have been elucidated,[2)] i.e., stimulation of natural killer (NK) cells, lymphokine-activated killer (LAK) cells and macrophages, enhancement of both HLA-class I and class II antigen expression[3)] and some tumor-associated antigen expression.[4)] These favorable effects have led to clinical trials against renal cell carcinoma, malignant melanoma, and other malignancies.[5, 6)]

On the other hand, HLA-class I antigen expression was shown to be inversely associated with NK susceptibility in some kinds of tumor cells.[7)] NK cells are not restricted to major histocompatibility complex (MHC) and are supposed to play an important role in host defense mechanisms against malignancy and infection.[8, 9)]

In order to study the immunobiology of SCLC and devise an effective immunotherapy for refractory SCLC, we have established SCLC cell lines from biopsied specimens of untreated primary tumors by diagnostic bronchofiberscopy.[10)] Our cell lines were shown to be representatives of the original tumors by the close correlation between *in vitro* chemo-radiosensitivity and clinical responses: OS1 was resistant to both drugs and irradiation, OS2 was sensitive to both and OS3 was sensitive only to

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[4] Abbreviations used are: SCLC, small cell lung cancer; PBL, peripheral blood lymphocytes; NK, natural killer; LAK, lymphokine-activated killer; rIFN-γ, recombinant immune interferon; rIL-2, recombinant interleukin 2; MLTC, mixed lymphocyte tumor culture.

high-dose irradiation. In the present study, we analyzed the immunological characteristics of the SCLC cell lines with diverse chemo-radiosensitivity, including HLA antigen expression and susceptibility to immune competent cells. We suggest the possibility of adoptive immunotherapy of killer T cells against refractory SCLC in the light of the inverse correlation between HLA-class I antigen expression and the sensitivity of SCLC cell lines to peripheral blood lymphocytes (PBL).

MATERIALS AND METHODS

Cell lines Six SCLC cell lines (OS1, OS2, OS3, OS2-R, OS6, OS7) were established in our laboratory; the biological properties of the first 3 cell lines were previously characterized.[10] OS2-R was derived from the relapsed tumor of the same patient as OS2. The karyotype of OS2-R was quite similar to that of OS2, but OS2-R was more resistant to chemo-radiotherapy. The result of a comparative study will be presented elsewhere. OS6 and OS7 were newly established from the biopsy specimens of a cervical lymph node and a primary tumor in the left B3 bronchus, respectively. Both cell lines were characteristic of SCLC in terms of neuroendocrine features and morphology. The clinical courses of patients from whom the 6 SCLC cell lines were derived are summarized in Table I, including *in vitro* chemo-radiosensitivity of OS1, OS2, OS2-R and OS3. A549, Lu65 and PC9 were non-SCLC cell lines which were kindly given by Dr. Shimosato. Daudi was a Burkitt lymphoma cell line which was originally established by Dr. Klein and K562 was a chronic myelogenous leukemia cell line. Both cell lines were serially passaged in our laboratory and used as NK-resistant and NK-sensitive cell lines, respectively. Cell lines (A549, Lu65, PC9, Daudi, K562) were cultured in RPMI-1640 (Nissui Pharmaceutical Co., Tokyo) supplemented with penicillin (PC; 100 U/ml), streptomycin (SM; 100 μg/ml), 2 m*M* fresh L-glutamine (L-Gln) and 10% heat-inactivated fetal bovine serum (FBS, General Scientific Laboratories, Los Angeles, CA), which was designated as complete medium, and other cell lines (OS1, OS2, OS2-R, OS3) in RPMI-1640 supplemented with PC (100 U/ml), SM (100 μg/ml), 2 m*M* L-Gln, 5% FBS, hydrocortisone (10 n*M*), insulin (5 μg/ml, Shimizu Pharmaceutical Co., Osaka), transferrin (100 μg/ml), 17β-estradiol (10 n*M*), and sodium selenite (30 n*M*), which was designated as HITES medium, containing 5% FBS. Hydrocortisone, trans-

Table I. Clinical Responses and *in vitro* Chemo-radiosensitivity of SCLC Cell Lines Used in the Present Study[10]

Cell line	Tumor source	Clinical response[a)]		*In vitro* sensitivity	
		Chemotherapy	Radiotherapy	Drugs[b)]	Radiation[c)]
OS1	Primary lung	NC	NC	Multiple resistant	Resistant
OS2	Primary lung	CR	CR	Sensitive	Sensitive
OS2-R	Relapsed primary lung	NC	Not done	Less sensitive	Resistant
OS3	Primary lung	NC	PR	Multiple resistant	Less sensitive
OS6	Metastatic lymph nodes	PR	Not done	Not done	Not done
OS7	Primary lung	PR	PR	Not done	Not done

a) NC, no change: the tumor did not regress to less than 50% of the initial tumor mass which was measured from X-ray films after the scheduled course of chemotherapy or radiotherapy. CR, complete response: tumor disappeared on the basis of routine tests including bronchofiberscopic examination. PR, partial response: tumor regressed to less than 50% of the initial tumor mass but did not disappear.
b) "Resistant" means that less than 50% reduction of colonies was observed by clonogenic cell assay at 1/10 of the peak plasma concentration after the administration to humans of the standard dose of drugs including cyclophosphamide, adriamycin, vincristine, cisplatin and etoposide. Multiple drug resistance was defined as the situation that SCLC cell lines were resistant to all 5 drugs. The term "less sensitive" was used when cell lines were resistant to more than one of the 5 drugs.
c) In radiosensitivity assay, "resistant" was used when less than 50% reduction of colonies was observed at 4 Gy. OS3 was judged "less sensitive" as it gave such a reduction at 2 Gy.

ferrin, 17β-estradiol and sodium selenite were obtained from Sigma Chemical Co., St. Louis, MO. Cells were incubated in a humidified atmosphere of 5% CO_2:95% air at 37°C. Cell lines were free of mycoplasma contamination as tested on Bacto PPLO Agar (Difco Laboratories, Detroit, MI).

Enzyme treatment For fluorescence-activated cell sorter (FACS) analysis and cytotoxicity assay, densely packed SCLC cell lines were treated with 0.01% DNase I (Sigma Chemical Co.) and 0.05% collagenase S-1 (Nitta Gelatine Co., Osaka) for 1 h at 37°C. After washing with complete medium 3 times, cells were suspended in HITES medium supplemented with 5% FBS and again incubated overnight in a humidified atmosphere of 5% CO_2:95% air at 37°C. On the day of assay, cells were suspended in fresh medium and gently pipetted. Cell suspension in a plastic tube was kept in an upright position for several minutes and a portion of single cells was used for the assays. This enzyme treatment did not reduce cell surface HLA-class I antigens after overnight culture (data not shown).

FACS analysis HLA-class I and class II and SCLC-specific antigens were measured with FACScan (Becton Dickinson, Mountain View, CA) and monoclonal antibodies (MoAb). IOT2 is a murine MoAb directed against a monomorphic determinant of human class I HLA molecules (HLA-A, B, C) associated with β2 microglobulin. IOT2a is a murine MoAb directed against a monomorphic determinant of human class II HLA molecules (HLA-DR). Both were obtained from Cosmo Bio Co. Ltd., Tokyo. ITK-2, a murine anti-SCLC MoAb, was raised by immunizing BALB/c mice with OS1 in our laboratory.[11] This antibody specifically reacted with SCLC and not with non-SCLC. It also reacted with neuroblastoma cell lines and neuroendocrine organs. Competitive inhibition assay, however, showed that the MoAb reacted with a different epitope from those identified by MOC-1 (Bio-Science Products, Emmenbrucke, Switzerland) and NKH-1 (Coulter Immunology, Hialeah, FL). WAS 05, used as a control antibody, was obtained from Serotec, Oxford, England. The second antibody was a fluorescein-labeled goat F(ab′)2 anti-mouse IgG, which was obtained from Tago, Inc., Burlingame, CA. All 3 cell surface antigens of SCLC cell lines were monitored the day after the enzyme treatment with DNase I and collagenase S-1. This enzyme treatment did not change either viability or cell surface antigen expression (data not shown). After passage through nylon mesh, cells (10^6) were incubated with the first antibody for 20 min on ice, washed twice and incubated with the second antibody for 20 min on ice. Cells were washed in 8 μg/ml propidium iodide (Sigma) to stain dead cells. Complete medium containing 0.1% NaN_3 was used for each dilution and wash. Fluorescence-stained cells suspended in phosphate-buffered saline (PBS: 0.68% NaCl; 10.4 m*M* Na_2HPO_4; 3.2 m*M* KH_2PO_4; pH 7.2) containing 0.1% NaN_3 were analyzed with FACScan.

Cytotoxicity assay PBL were isolated from normal human blood by using Ficoll-Paque (Pharmacia, Piscataway, NJ). For the first experiments on PBL sensitivity, a part of PBL was passed through nylon wool to deplete adherent cells. Since the cytotoxic activities of whole PBL and nylon wool-passed PBL were in parallel, whole PBL were used as effector cells in the following experiments. Representative FACS data of whole PBL were as follows: OKT3 37.7%, OKT4 28.4%, OKT8 27.0%, T4/T8 1.05, Leu7 15.7%. For LAK cytolysis assay, whole PBL were cultured for 4 days at 10^6 cells/ml in complete medium supplemented with recombinant interleukin 2 (rIL-2) at 1 U/ml. Representative FACS data of LAK were as follows: OKT3 59.8%, OKT4 42.2%, OKT8 47.7%, T4/T8 0.88, Leu7 30.0%, Leu11 31.2%. Human rIL-2 was kindly provided by Takeda Chemical Industries, Ltd., Osaka. All effector cells were washed 3 times before use. SCLC cell lines were treated with DNase I and collagenase S-1 for 1 h at 37°C, washed 3 times and resuspended in fresh medium one day before the assay. This procedure did not change either viability or sensitivity to effector cells. On the day of assay, a portion of single cells was labeled with ^{51}Cr for 45 min and washed 3 times. Five thousand ^{51}Cr-labeled target cells suspended in 100 μl of complete medium were added to each well of a microculture plate (Linbro, McLean, VA) containing 0.375 to 5×10^5 effector cells suspended in 100 μl of complete medium. Plates were centrifuged at 800 rpm for 1 min and incubated at 37°C for 4 h. After incubation, supernatants were harvested and counted for radioactivity with a gamma counter. The percentage of specific cytolysis in triplicate was calculated by using the formula: % specific cytolysis = (experimental cpm − spontaneous cpm) / (total cpm − spontaneous cpm) × 100. Tests in which spontaneous cpm exceeded 30% were not evaluated.

Dose-dependent effect of rIFN-γ on HLA-class I expression and sensitivity of SCLC cells to PBL Six aliquots of target cells (8×10^4/ml) were cultured for 4 days in HITES medium supplemented with 5% FBS. To each aliquot 0.1 to 1,000 U/ml of rIFN-γ was added. Human rIFN-γ was kindly provided by Shionogi & Co. Ltd., Osaka. One day before the assay, SCLC cells were treated with DNase I and collagenase S-1 at 37°C for 1 h, washed 3 times and resuspended in fresh medium supplemented or not with rIFN-γ at the same concentration. Two identical sets of target cells were cultured for PBL cytolysis assay and FACS analysis.

Kinetics of HLA-class I expression and sensitivity of SCLC cells to PBL after rIFN-γ treatment To make

negligible the influence of culture conditions, initial cell concentration was lower than usual (2.5×10^4/ml). Four aliquots of target cells were cultured with or without 100 U/ml of rIFN-γ, starting 11 days before the assay. The first aliquot was cultured with rIFN-γ for 4 days, washed 3 times and again cultured without rIFN-γ for 7 days. The second aliquot was cultured in IFN-free medium for 3 days and then with rIFN-γ for 4 days, washed 3 times, and recultured without rIFN-γ for 4 days. The third aliquot was cultured in IFN-free medium for 5 days and then with rIFN-γ for 4 days, washed 3 times, and resuspended in IFN-free medium for 2 days. A control aliquot was cultured in IFN-free medium for 11 days. All aliquots of target cells were simultaneously treated with enzyme one day before the assay. Two identical sets of target cells were cultured for PBL cytolysis assay and FACS analysis.

Mixed lymphocyte tumor culture (MLTC) Tumor cells (5×10^6/ml) suspended in RPMI-1640 medium were mixed with 200 μg/ml of mitomycin C (MMC, Wako Pure Chemical Industries, Ltd., Osaka) and cultured at 37°C for 1 h. After washing 3 times, cells were suspended (10^5/ml) in complete medium supplemented with 50 μM 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd.), 5 mM HEPES buffer (Gibco, Grand Island, NY), 0.1 mM non-essential amino-acids (Gibco), and 1 mM sodium pyruvate solution (Gibco). PBL as responder cells were suspended in the same medium (10^6/ml) and incubated with or without MMC-treated cells (10^5/ml) at a responder:stimulator ratio of 10:1 in 24-well tissue culture plates (Costar, Cambridge, MA). After 3 days, rIL-2 was added to each well at 1 U/ml and again cultured for 4 days. This cycle was repeated 4 to 6 times. Cytotoxicity assay was performed after 1, 4 and 6 cycles by the same methods as PBL or LAK cytolysis assay. The FACS data of control and OS3-IFN in Experiment 1 of Table II were as follows: (control) OKT3 88.9%, OKT4 85.7%, OKT8 14.4%, T4/T8 5.95, Leu11 16.7%, (OS3-IFN) OKT3 88.8%, OKT4 79.3%, OKT8 48.1, T4/T8 1.65, Leu11 47.8%.

Statistical analysis Student's t test was used to compare the sensitivity of SCLC cell lines to cytotoxic cells. When the P value was 0.05 or smaller, the difference between two groups was considered significant.

RESULTS

PBL and LAK susceptibility of SCLC cells All 3 SCLC cell lines, which had different chemo-radiosensitivity, were sensitive to fresh nylon wool-passed PBL as compared to non-SCLC cell lines (A549, Lu65, PC9) or Daudi, although A549 was slightly sensitive (Fig. 1). Since 4-day-cultured PBL did not lyse either SCLC cells or K562, fresh PBL were necessary in mediating cyto-

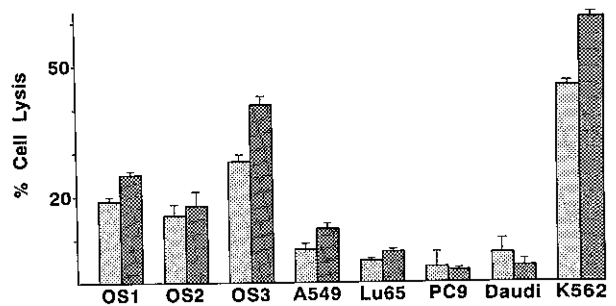

Fig. 1. PBL susceptibility of SCLC cell lines. ^{51}Cr-labeled SCLC cell lines (OS1, OS2 and OS3), non-SCLC cell lines (A549, Lu65 and PC9), or controls (Daudi and K562) were cultured with nylon wool-passed human PBL at the E/T ratio of 25:1 (▤) or 50:1 (▩). The % cell lysis was measured after 4 h incubation. The cytotoxicity at E/T=50 to OS1, OS2, OS3 and A549 was significantly different from that to Daudi ($P<0.001$, 0.02, 0.001 and 0.02, respectively).

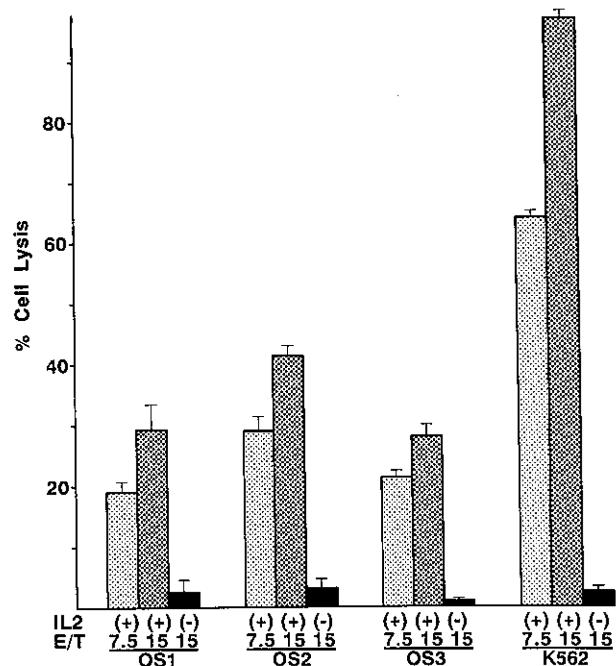

Fig. 2. LAK susceptibility of SCLC cell lines. Human PBL were activated with or without rIL-2. Effector cells (LAK) were harvested after 4-day culture, and the LAK sensitivity of SCLC cell lines (OS1, OS2 and OS3) or a control (K562) was measured by 4-h ^{51}Cr-release assay at the E/T ratio of 7.5:1 (▤) or 15:1 (▩, ■). The % cell lysis at E/T=15 of OS1(+), OS2(+), OS3(+) and K562(+) was significantly different from that of OS1(−), OS2(−), OS3(−) and K562(−), respectively ($P<0.01$, 0.001, 0.001 and 0.001, respectively).

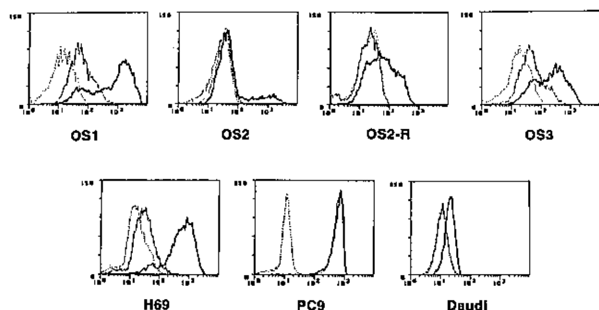

Fig. 3. HLA-class I antigen expression of SCLC cell lines treated with rIFN-γ. SCLC cell lines (OS1, OS2, OS2-R, OS3 and H69) or controls (PC9 and Daudi) were cultured with (——) or without (–·–·, ········) rIFN-γ. Cells were harvested after 4 days and HLA-class I antigen expression was examined by FACScan. The dotted line (----) shows the unstained control curve.

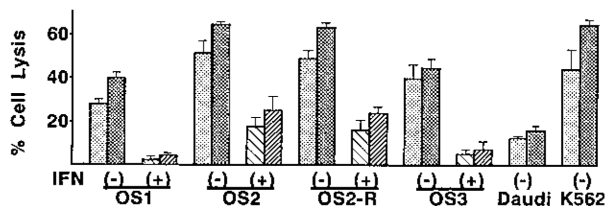

Fig. 4. PBL susceptibility of SCLC cell lines treated with rIFN-γ. SCLC cell lines (OS1, OS2, OS2-R and OS3) were cultured with or without rIFN-γ. Cells were harvested after 4 days and the PBL sensitivity was examined by 4-h ^{51}Cr-release assay at the ratio of 50:1 (□, ▧) or 100:1 (▩, ▨). The % cell lysis at E/T=100 of OS1(−), OS2(−), OS2-R(−) and OS3(−) was significantly differently from that of OS1(+), OS2(+), OS2-R(+) and OS3(+), respectively (P<0.001, 0.01, 0.001 and 0.01, respectively).

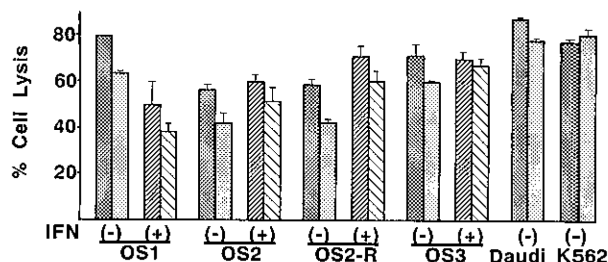

Fig. 5. LAK susceptibility of SCLC cell lines treated with rIFN-γ. A 4-h ^{51}Cr-release assay was performed at the E/T ratio of 3.75:1 (□, ▧) or 15:1 (▩, ▨). The % cell lysis at E/T=3.75 of OS1(−) and OS2-R(−) was significantly different from that of OS1(+) and OS2-R(+), respectively (P<0.01 and 0.02, respectively).

toxicity against SCLC (Fig. 2). The effector cells seemed to be NK cells. On the other hand, PBL activated with rIL-2 lysed all SCLC cell lines even at an effector:target (E/T) ratio of 7.5 as shown in Fig. 2. These results showed that the chemo-radiosensitivity of SCLC could not predict its sensitivity to PBL or LAK cells.

HLA-class I and class II, and ITK-2 antigen expression of SCLC cells Expression of cell surface antigens of the SCLC cell lines was analyzed by an indirect immunofluorescence technique. HLA-class II antigen expression was negative and ITK-2 antigen expression was positive in all SCLC cell lines (data not shown). On the other hand, HLA-class I antigen expression of SCLC cell lines was lower than that of non-SCLC cell lines or PC9, whereas 4-day culture of the SCLC cell lines with rIFN-γ (100 U/ml) changed their expression from low to high levels (Fig. 3). However, neither HLA-class II nor ITK-2 antigen expression was changed under the same experimental conditions (data not shown).

Susceptibility of rIFN-γ-treated SCLC cells to PBL and LAK We next questioned if rIFN-γ could modulate biological characteristics of SCLC cell lines other than HLA-class I antigen expression. Under the condition that SCLC cells were pretreated with 100 U/ml of rIFN-γ for 4 days, the cell viability was not changed and there seemed to be almost no changes of *in vitro* growth rate and *in vivo* tumorigenicity in rIFN-γ-treated SCLC cells (data not shown). All 4 SCLC cell lines were sensitive to whole PBL but pretreatment with rIFN-γ significantly decreased the sensitivity almost to the level of Daudi (Fig. 4). The effect of rIFN-γ on LAK sensitivity, however, was variable among SCLC cell lines, although untreated cell lines were similarly sensitive. As shown in Fig. 5, there was a slight reduction of sensitivity after the rIFN-γ treatment in OS1 cells but no reduction in OS3 cells. On the other hand, both IFN-treated OS2 and OS2-R were more sensitive than untreated cell lines; this was confirmed by several other experiments (data not shown). The above-mentioned results were obtained from allogeneic systems. Since NK cells might directly participate in allograft rejection,[9] the inverse correlation should be confirmed by examining autologous SCLC cells and PBL. OS6 and OS7 were established while the patients were alive in hospital. HLA-class I antigen expression of both OS6 and OS7 was low, but was increased by rIFN-γ treatment (Figs. 6A, 7A). In parallel with this, both OS6 and OS7 became more resistant to not only allogeneic but also autologous PBL after rIFN-γ treatment (Figs. 6B, 7B). Their LAK sensitivities were similarly high, although the effect of rIFN-γ was variable (data not shown).

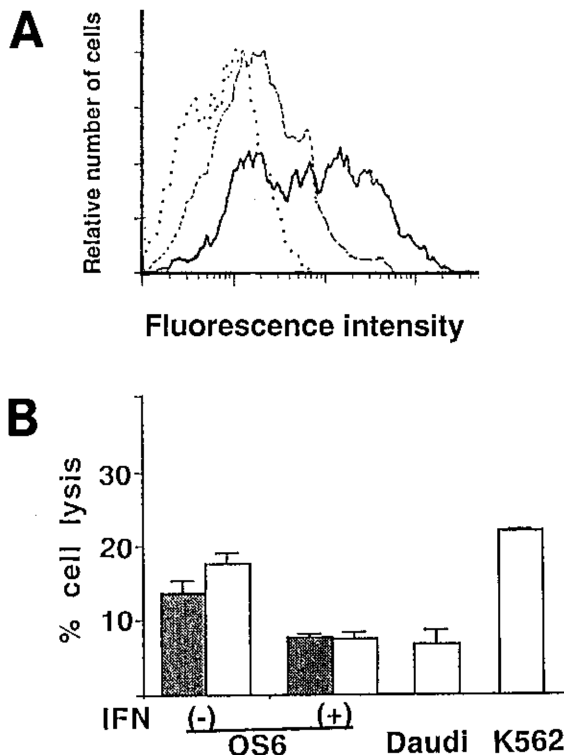

Fig. 6. HLA-class I antigen expression and autologous PBL susceptibility of OS6 treated with rIFN-γ. Both autologous (■) and allogeneic (□) PBL were prepared as a source of NK cells. OS6 cells were pretreated with (+) or without (−) rIFN-γ for 4 days; A) their HLA-class I antigen expression was examined by FACScan: (——) IFN-treated OS6, (········) non-treated OS6, (- - - -) unstained control; B) A 4-h ^{51}Cr-release assay was carried out at the E/T ratio of 50:1. The % cell lysis of OS6(−) was significantly different from that of OS6(+) against both autologous and allogeneic PBL (P<0.05 and 0.01, respectively).

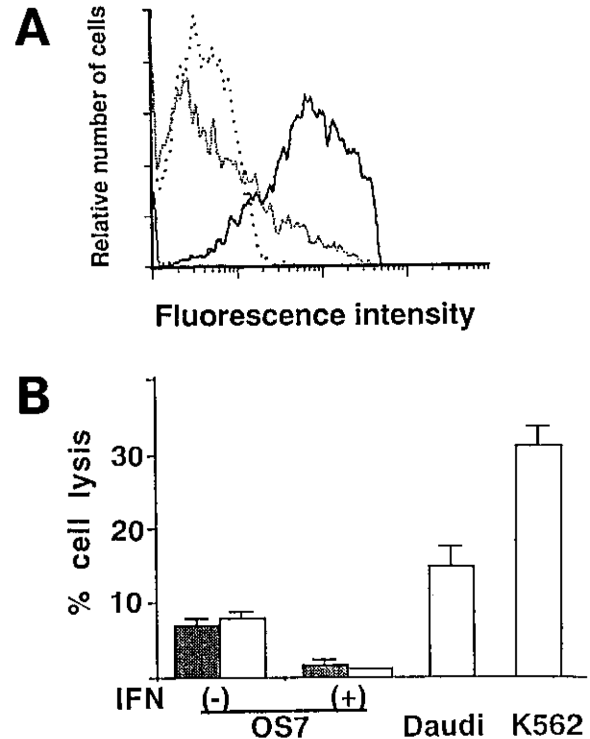

Fig. 7. HLA-class I antigen expression and autologous PBL susceptibility of OS7 treated with rIFN-γ. Both autologous (■) and allogeneic (□) PBLs were prepared as a source of NK cells. OS7 cells were pretreated with (+) or without (−) rIFN-γ for 4 days; A) their HLA-class I antigen expression was examined by FACScan: (——) IFN-treated OS7, (········) non-treated OS7, (- - - -) unstained control; B) A 4-h ^{51}Cr-release assay was carried out at the E/T ratio of 50:1. The % cell lysis of OS7(−) was significantly different from that of OS7(+) against both autologous and allogeneic PBL (P<0.02 and 0.01, respectively).

Dose-dependent effect of rIFN-γ To analyze further the effect of rIFN-γ on sensitivity to PBL and HLA-class I expression of SCLC cell lines, we set up 2 identical sets of target cells (OS1) and changed the dosage of rIFN-γ from 0.1 to 1,000 U/ml. As shown in Fig. 8A, OS1 cells gradually became resistant to PBL as the dose of rIFN-γ was increased. This was inversely correlated with HLA-class I antigen expression of OS1 cells (Fig. 8B). For clarity, data at 0.1 and 1.0 U/ml in FACS analysis were omitted, and the results are representative values of 2 experiments.

Kinetics of sensitivity to PBL and HLA-class I antigen expression after rIFN-γ treatment In preliminary experiments, 100 U/ml of rIFN-γ raised HLA-class I antigen expression of SCLC cells to the maximum level after 4 days. Therefore after 4-day treatment with rIFN-γ, OS1 cells were washed to remove rIFN-γ and cultivation was continued for 2 to 7 days. The PBL sensitivity of OS1 cells increased from the lowest level to the constitutional level after removal of rIFN-γ (Fig. 9A). HLA-class I antigen expression of OS1 cells decreased during 7 days in rIFN-γ-free medium and the peak of fluorescence intensity shifted from the right side to the constitutional level (Fig. 9B). This also indicated that the effect of rIFN-γ lasted for more than 7 days after 4-day treatment.

Induction of allo-specific killer cells against SCLC cell lines (Table II) To test the function of HLA-class I antigens elevated by rIFN-γ, we carried out MLTC 4 to 6 times. Human PBL were stimulated with rIFN-γ-treated SCLC cells once a week. To maintain cell proliferation for 1 to 2 weeks, rIL-2 was added to the medium 3 days after MLTC once a week. At one week

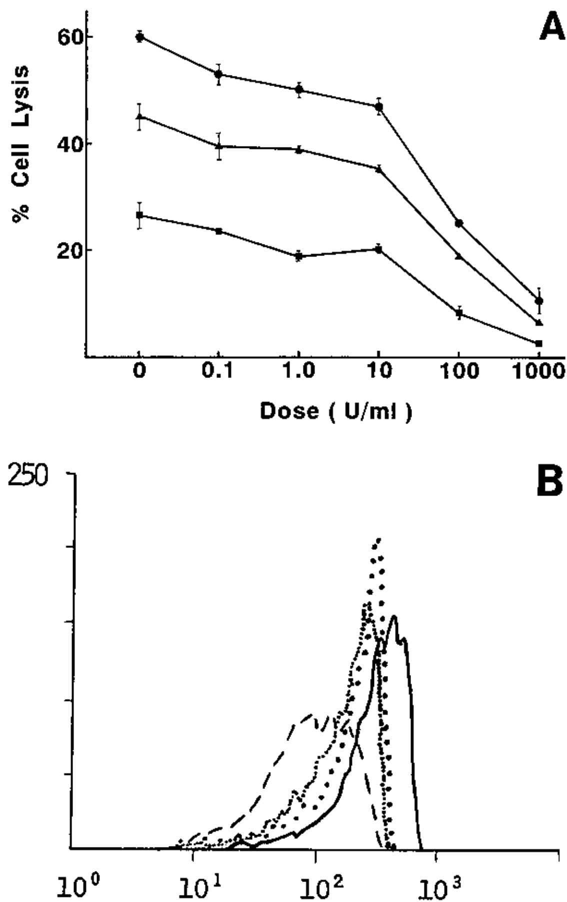

Fig. 8. Dose-dependent effect of rIFN-γ on: A) PBL susceptibility of OS1, B) HLA-class I antigen expression of OS1. Two identical sets of OS1 cells were cultured with graded doses of rIFN-γ: 1,000 μg/ml (——), 100 μg/ml (······), 10 μg/ml (·········), and 0 μg/ml (----). Cells were harvested after 4-day culture and one set was used for ^{51}Cr-release assay at the E/T ratio of 100:1 (●), 50:1 (▲) or 15:1 (■), and the other set was examined for HLA-class I antigen expression. The % cell lysis at E/T=100 of OS1 treated with IFN at the dose of 0.1, 1.0, 10, 100 and 1,000 μg/ml was significantly different from that of the control (P<0.05, 0.01, 0.01, 0.001 and 0.001, respectively).

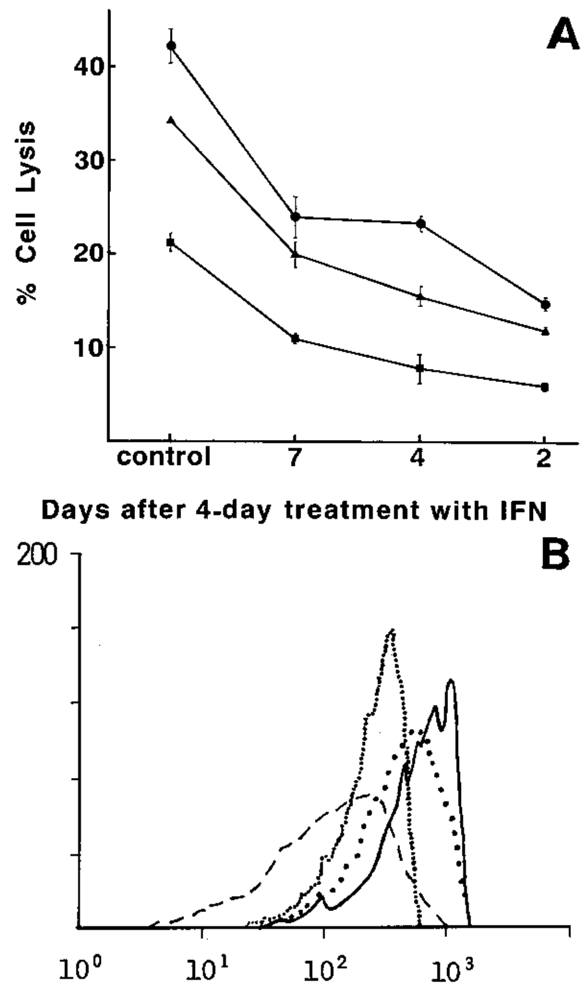

Fig. 9. Kinetics of rIFN-γ effect on: A) PBL susceptibility of OS1, B) HLA-class I antigen expression of OS1. Two identical sets of OS1 cells were cultured with rIFN-γ (100 μg/ml) for 4 days. After washing out of the rIFN-γ, cells were cultured again for 2 days (——), 4 days (······) and 7 days (·········). The control (----) was cultured without rIFN-γ for 11 days. One set was used for ^{51}Cr release assay at the E/T ratio of 100:1 (●), 50:1 (▲) or 15:1 (■), and the other set was examined for HLA-class I antigen expression. The % cell lysis at E/T=100 of the 7-day group, 4-day group and 2-day group was significantly different from that of the control (P<0.01, 0.001 and 0.001, respectively).

after the 4th MLTC, ^{51}Cr-release assay was performed. As compared to LAK cells, which were activated only by adding rIL-2 to the medium once a week, responder cells in MLTC lysed the respective SCLC cells more strongly and more specifically. Furthermore, rIFN-γ-treated target cells were more susceptible to allo-killer cells than non-treated target cells. Although representative data using OS3 are shown, OS1 and OS2-R were also used as stimulators and similar results were obtained. In experiment 1, LAK activity was still detected since Daudi and K562 were considerably lysed. Therefore MLTC was performed 6 times against OS3 and the killing activity was again assayed. Then more specific effector cells were induced against OS3 and lysed even non-treated OS3, although to a lesser extent than with IFN-treated OS3. These experiments revealed that enhanced HLA-class I antigens of SCLC could function as allo-antigens against T cells.

Table II. Induction of Allo-specific Cytotoxic Lymphocytes against SCLC Cell Lines

Stimulator[b]	E/T ratio	% Cell lysis[a]							
		OS1-IFN	OS1	OS2-IFN	OS2	OS3-IFN	OS3	K562	Daudi
Experiment 1: 4 stimulations									
Control	7.5	8.6±2.1[c]	19.8±1.5	27.1±5.1	11.2±5.6	7.8±1.7	14.3±1.5	29.0±1.9	29.1±2.7
	15.0	11.5±2.4	30.6±2.7	36.7±4.0	17.7±4.5	16.1±0.3	21.2±1.1	58.1±4.6	44.0±1.1
OS3-IFN	7.5	10.1±2.3	NT[d]	22.7±5.4	NT	26.2*±1.1	19.3±0.1	29.3±2.2	16.1±0.4
	15.0	16.5±2.0	NT	27.8±1.9	NT	34.4±1.4	35.1±3.5	40.4±2.3	27.3±2.4
Experiment 2: 6 stimulations									
OS3-IFN	7.5	1.6±0.2	NT	0.3±0.3	NT	16.4**±1.5	9.0±0.9	1.4±0.4	0.4±0.3
	15.0	2.4±0.3	NT	0.0±0.0	NT	29.9***±0.4	13.4±0.2	4.1±0.3	0.1±0.1

a) Target cells were pretreated with or without rIFN-γ for 4 days and 4-h ^{51}Cr-release assay was performed.
b) Human PBL were stimulated with or without (control) rIFN-γ-treated SCLC cells in MLTC once a week and after 3 days' culture with rIL-2.
c) Mean±SE of triplicate wells. *: $P<0.01$, OS3-IFN vs. OS3, OS1-IFN or Daudi. ** : $P<0.02$, OS3-IFN vs. OS3, $P<0.001$, OS3-IFN vs. OS1-IFN, OS2-IFN, K562 or Daudi. *** : $P<0.001$, OS3-IFN vs. OS3, OS1-IFN, OS2-IFN, K562 or Daudi.
d) Not tested.

DISCUSSION

SCLC comprises about 16 to 21% of primary lung cancer cases autopsied in Japan and the ratio is tending to increase.[12, 13] Although recent progress in chemotherapy has made some malignant tumors, such as testicular carcinomas and Hodgkin's lymphomas, curable cancers, the 3-year survival rate of SCLC is still only about 10%. One of the main reasons for its poor prognosis is early metastasis to not only regional lymph nodes but also distant organs such as brain and bone marrow. Why does SCLC metastasize so quickly to lymph nodes and later to neuroendocrine organs? Host immune surveillance may be an important factor here.

Recent developments in molecular biology have advanced our knowledge of the biology of SCLC. However, little immunological work on SCLC has been reported since Doyle's paper.[1] We have established SCLC cell lines derived from untreated primary tumors and analyzed the relationship between HLA-class I antigens of SCLC cell lines and host immune-competent cells. It should be noted that our SCLC cell lines have representative characteristics of chemo-radiosensitivity which correlated with the clinical courses of their hosts.[10] The sensitivities to anti-cancer drugs and radiation were quite different among 3 cell lines but their immunological characteristics were almost the same. As shown in Fig. 1, these 3 SCLC cell lines were similarly sensitive to PBL as compared to non-SCLC cell lines and an NK-sensitive cell line, K562. All SCLC cell lines analyzed above were equally sensitive to LAK cells (Fig. 2). On the other hand, cell surface markers of SCLC cell lines were analyzed by FACS and as a result HLA-class I antigens were found to be similarly low but inducible to higher levels by rIFN-γ treatment (Fig. 3). This rIFN-γ treatment of SCLC cell lines conversely reduced their PBL sensitivity. This was demonstrated in all 6 SCLC cell lines, independently of whether a cell line is chemo-radiosensitive or not. Furthermore, this inverse relationship between HLA-class I and PBL sensitivity was confirmed by both dose-response and time-course experiments (Figs. 8 and 9), and also with autologous systems (Figs. 6 and 7). Recently Harker *et al.*[14] reported that chemoresistance of renal cell carcinoma lines did not predict resistance to NK or LAK cells because drug-resistant cell lines were variably resistant to NK-mediated lysis and equally sensitive to LAK cells. In the present paper, we used SCLC cell lines with natural and acquired resistance as well as lines sensitive to chemo-radiotherapy and found they had similar sensitivity to PBL and LAK cells. Furthermore, the inverse relationship between PBL sensitivity and HLA-class I antigen expression was found in all SCLC cell lines used in our experiments.

On the other hand, the effect of rIFN-γ on LAK sensitivity was not constant. It seemed to depend on which cell line was used as a target. This was in contrast to the IFN effect on PBL sensitivity, which may suggest NK and LAK cells recognize different target molecules on SCLC cell lines, or the triggering and activation mechanisms of NK and LAK could be different as Powell *et al.*[15] suggested. However, it was shown that rIFN-γ treatment protected several human tumor cell lines from LAK-mediated lysis by de Fries and Golub.[16] On the other hand, Lagadec *et al.*[17] reported that pretreatment with rIFN-γ increased the LAK sensitivity of

7 out of 9 SCLC cell lines. Since LAK cells consist of 2 major populations, NK and T cells,[8] the above discrepancy may depend on which population is predominant.

There are many papers dealing with the relationship between HLA-class I antigens and PBL sensitivity, but the results remain controversial. Stam *et al.*[18] reported that the transfection of an SCLC cell line with HLA-A2 or HLA-B27 did not influence PBL susceptibility, and they concluded that there was no correlation between HLA-class I antigens and NK susceptibility. However, Storkus *et al.*[19] examined the role of carbohydrate in the HLA-class I protective effect by using HLA-A2, A3, B7 and BW58, and found that only HLA-A2 was unable to give protection. Therefore, they suggested that the $\alpha 1/\alpha 2$ domains of class I molecules may confer resistance to NK cells. Recently, Liao *et al.*[20] used mutant mice which were deficient in MHC-class I expression because of a mutant β2-microglobulin gene, and found that normal T cell blasts from the mice were NK-sensitive and that NK cells from the mice did not lyse class I-deficient target cells. Thus, they concluded that class I molecules may participate in the positive selection or tolerance induction of NK cells. Our present data may be explained by such mechanisms.

Although SCLC cell lines were quite sensitive to PBL or LAK cells *in vitro*, most patients die of progressive SCLC even if initial therapy is successful. SCLC metastasizes preferentially to lymph nodes, where NK activity is lower than in peripheral blood,[9] and finally blood-borne metastasis to neuroendocrine organs occurs when host defense mechanisms are suppressed. Therefore, we tried to enhance the sensitivity of SCLC cells to specific killer cells by administering rIFN-γ. Table II showed that rIFN-γ enhanced the HLA-class I antigen expression of SCLC and such SCLC cells became sensitive to allo-killer cells in spite of the reduction of sensitivity to PBL. Furthermore, MLTC with rIFN-γ-treated SCLC was capable of inducing specific killer cells. Recently we successfully induced autologous killer T cells against SCLC using both IL-2 and IL-6 in the MLTC system, although their cytotoxic activity was lower than LAK activity (data not shown). Further investigation will be necessary to make such immunotherapy effective.

In conclusion, most SCLCs are sensitive to PBL or LAK cells but not to killer T cells because of low-level expression of class I antigens, which may explain their preferential metastasis to lymph nodes, where NK activity is quite low. Sophisticated use of cytokines may be helpful to prevent the metastasis of SCLC.

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