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Inhibition of Colony Formation of Drug-resistant Human Tumor Cell Lines by Combinations of Interleukin-2-activated Killer Cells and Antitumor Drugs

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The cytotoxicity of interleukin-2-activated killer (LAK) cells with or without anticancer drugs against cell lines with acquired drug resistance was evaluated *in vitro* by colony assay. Human non-small cell lung cancer cell lines, PC-9 and PC-14, human leukemia cell line, K-562, and their sublines resistant to cisplatin (CDDP), PC-9/CDDP and PC-14/CDDP, and to adriamycin (ADM), K-562/ADM, were used as target cells. PC-9/CDDP demonstrated a marked increase in susceptibility to killing by both peripheral blood lymphocytes (PBL) and LAK cells, as compared to the parental cell line, PC-9. The cytotoxicity of PBL and LAK cells against PC-14/CDDP and K-562/ADM was similar to that against their parental cell lines. Moreover, the combination of LAK and CDDP had a synergistic effect on PC-14 and PC-14/CDDP.

Key words: Colony inhibition — Interleukin-2-activated killer cells — Human lung cancer — Drug-resistant cell

The development of drug resistance in tumor cells is a common and serious clinical problem which limits the therapeutic potential of chemotherapy. Recently, many investigators have reported various approaches to destroy the resistant cells remaining after chemotherapy.[1-3]

LAK[4] cells possess a novel cytotoxic function against tumor cells and have been used in the adoptive immunotherapy of neoplastic disease.[4] Yanovich *et al.* reported that a pleiotropic drug-resistant variant of K-562 showed a modest increase in resistance to natural killer (NK) cells.[5] In another study, however, human colon carcinoma cell lines with pleiotropic drug resistance established by continuous exposure to ADM were more efficiently killed by LAK cells than were the parental cells in some experiments.[6] On the other hand, Leroux *et al.* reported that treatment with some anticancer drugs enhances the sensitivity of murine lymphoma cells to the killing action of both LAK and cytotoxic T lymphocytes.[7]

In this study, we evaluated the cytotoxicity of LAK cells against CDDP-resistant human lung adenocarcinoma cell lines established in our laboratory and a human leukemia cell line with pleiotropic drug resistance. Additionally, we investigated the anti-tumor effects of LAK cells in combination with anticancer drugs the resistant cell lines to examine the feasibility of clinical application of the combination of LAK cells and anticancer drugs.

MATERIALS AND METHODS

Cell lines The human tumor cell lines PC-9 and PC-14 cells derived from human lung adenocarcinoma (kindly donated by Professor Y. Hayata, Tokyo Medical College) and their sublines resistant to CDDP (PC-9/CDDP and PC-14/CDDP) established in our laboratory were used as target cells. K-562 cells derived from human myelogenous leukemia, and its subline resistant to ADM, K-562/ADM (kindly provided by Dr. T. Tsuruo, Cancer Institute, Japanese Foundation for Cancer Research) were also used as target cells.

Establishment of these resistant cell lines was previously described.[8,9] Briefly, PC-9 and PC-14 were cultured in RPMI-FBS containing CDDP. When a large colony was observed in a well, the colony was isolated and propagated as a resistant subline. After 3–4 weeks of maintenance in medium containing CDDP, the cells were treated with gradually increasing concentrations of CDDP and the cloning procedure was continued to develop sublines resistant to a higher concentration of CDDP. The resistant cell lines used in this experiment were finally maintained at the concentrations of 0.5 μg/ml (PC-9/CDDP) and 1.5 μg/ml (PC-14/CDDP). We used these resistant sublines for experiments 2–3 weeks after culturing them in drug-free medium.

Initial induction of resistance against K-562 was achieved by continuous exposure of K-562 cells to vincristin over 1 month. The marginally resistant cells

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[4] The abbreviations used are: LAK, interleukin-2-activated killer cells; CDDP, cisplatin (*cis*-diamminedichloroplatinum); ADM, adriamycin; PBL, peripheral blood lymphocytes; NK, natural killer cells; RPMI-FBS, RPMI-1640 medium with fetal bovine serum; MEM, Eagle's minimum essential medium; E/T, effector-to-target ratio.

obtained (2- to 3-fold resistant to ADM) were further treated with gradually increasing concentrations of ADM *in vitro* (increases every 2–3 weeks) until the concentration finally reached 450 n*M* ADM, and then kept for 4 months at 500 n*M*. Then the cells were cloned and stored in liquid nitrogen until use in an experiment. K-562/ADM expressed pleiotropic drug resistance related to P-glycoprotein.[10] Characteristics of the tumor cell lines used in this study are shown in Table I.

Preparation of freshly obtained PBL Venous blood was obtained by venipuncture from healthy volunteers. The blood was diluted with Eagle's minimum essential medium (MEM), and the mononuclear cells were isolated by density gradient sedimentation on a Ficoll-Conray cushion (1080*g*) according to Boyum's method.[11] Cells accumulating at the interface were washed twice with MEM and once with RPMI-1640 medium with fetal bovine serum (RPMI-FBS). The mononuclear cells in RPMI-FBS were incubated in a Falcon 3003 plastic dish (Falcon Plastic Co., USA) in a humidified atmosphere of 5% CO_2, 95% air, at 37°C for 1 h. Later, nonadherent cells were collected by repeated extensive washing with RPMI-FBS. More than 95% of these nonadherent cells were lymphocytes. The number of cells was adjusted to three different effector-to-target (E/T) ratios, 12.5:1, 25:1, 50:1, before the cytotoxicity assay.

Preparation of LAK cells For the preparation of LAK cells, non-adherent lymphocytes suspended in RPMI-FBS (2×10^6/ml) were incubated with 100 U/ml of rIL-2 (Shionogi Pharmaceutical Co., Osaka) for 3 days. Before the experiment, the number of LAK cells was adjusted to three different E/T ratios, 12.5:1, 25:1 and 50:1.

Cytotoxicity assay The cytotoxicity assay used in this study was a modification of the double agar method developed by Hamburger and Salmon.[12] The bottom layer was 0.5% agar in enriched McCoy's 5A medium, which consisted of 400 ml of McCoy's 5A medium (Gibco) with 10% heat-inactivated FBS, 5% heat-inactivated horse serum, 4 ml of 2.2% Na-pyruvate, 4 ml of 200 m*M* glutamine, 0.8 ml of 2.1% serine and antibiotics. The top layer contained 0.3% agar in RPMI-FBS.

For the determination of cytotoxicity of PBL and LAK cells, 3×10^4 (final plating number, 1×10^4) tumor cells were mixed with lymphocytes at E/T ratios of 12.5:1, 25:1, and 50:1 and the cells were incubated for 5 h at 37°C in 2.7 ml of top medium without agar. The antitumor activity of LAK cells with anticancer drugs was also determined by two different experiments to see whether they had a synergistic effect. In the first experiment, 3×10^4 tumor cells were exposed to 1.0 μg/ml of CDDP or 0.3 μg/ml of ADM for 1 h and washed with RPMI-FBS, followed by incubation with LAK cells (E/T ratio=50:1) for 5 h in 2.7 ml of RPMI-FBS. In the second experiment, 3×10^4 tumor cells were exposed to CDDP or ADM with LAK cells simultaneously at the same concentrations and E/T ratio as above for 5 h in 3.0 ml of RPMI-FBS, then washed with RPMI-FBS and 2.7 ml of RPMI-FBS was added. After these treatments, 0.3 ml of 3% agar was added to each tube. Aliquots (1 ml each) of the resultant mixture were plated over the bottom layer of 6-well plate dishes (Linbro) as described above. The colony formation of tumor cells without any lymphocytes nor anticancer drugs was considered to be the control. The dishes were incubated in a humidified atmosphere of 5% CO_2 at 37°C for 5–8 days depending on the growth characteristics of tumor cells. Colonies at least 60 μm in diameter were counted with a colony analyzer (CP 2000, Shiraimatsu Instrument Ltd., Osaka). The actual number in each control plate ranged from 1356 to 2151. Each experiment was performed in triplicate and repeated more than three times. The repeated experiments gave consistent results. The percent inhibition of colony formation was determined by the following formula;

$$100-\frac{\text{number of colonies from different treatment}}{\text{number of colonies per control plate}}\times100.$$

Statistical analysis Differences between groups were tested for significance by means of Student's two-tailed *t*-test, and *P* values were calculated.

The interaction of LAK cells and anticancer drugs was determined by using the multiplicative model.[13] When the product of the surviving fractions of cells treated with LAK cells or anticancer drugs was higher than the observed surviving fraction of cells given the combined treatment, the interaction was considered to be synergistic.

RESULTS

Characteristics of the tumor cell lines Table I shows the characteristics of all the parental and resistant cell lines used in this study. The plating efficiency of PC-9/CDDP was slightly lower than that of the parental cell line, but other resistant cell lines showed almost the same plating efficiency as the parental cells. Since the growth of each tumor cell line was different, we initially determined the optimal day for counting colony numbers. Relative resistance was calculated as the ratio of 50% inhibitory concentrations (IC_{50}s) in resistant cells and IC_{50}s in parental cells for CDDP (PC-9/CDDP and PC-14/CDDP) and those of IC_{30}s for ADM (K-562/ADM), with 5-h exposure to each drug, as shown in Table I.

Cytotoxicity of PBL and LAK cells to the resistant cells Cytotoxicity of freshly obtained PBL and LAK cells to

the tumor cell lines at the E/T ratio of 50:1 was examined by colony assay following a 5-h incubation of the cells with PBL or LAK cells (Table II).

Table I. Characteristics of the Tumor Cell Lines Used in This Study

Cell line	Plating efficiency[a] (%)	Incubation period[b] (days)	Relative resistance[c]
PC-9	20.5	7	1.0
PC-9/CDDP	17.0	8	6.4
PC-14	20.2	7	1.0
PC-14/CDDP	19.7	8	2.8
K-562	18.6	5	1.0
K-562/ADM	18.8	6	159

a) The plating efficiency (%) was calculated by means of the following formula;

(number of colonies/number of cells plated) × 100.

b) The incubation time was set for each cell line as two days less than the time when the colony number reached maximum.

c) Relative resistance was calculated as the ratio of IC_{50}s in resistant cells and of IC_{50}s in parental cells for CDDP (PC-9/CDDP, 14/CDDP) and those of IC_{30}s for ADM (K-562/ADM).

Table II. Cytotoxicity of PBL and LAK Cells against Resistant Sublines[a]

Cell line	PBL	LAK cells
PC-9	2.6±3.7 ⎤[b]	8.9±4.4 ⎤[b]
PC-9/CDDP	43.2±4.8 ⎦	65.5±7.0 ⎦
PC-14	10.0±4.9	41.1±9.2
PC-14/CDDP	15.7±5.2	52.2±11.4
K-562	21.7±9.2	77.7±5.8
K-562/ADM	38.8±3.5	66.8±9.6

a) Data are expressed as a percentage of colony inhibition ± SE in the colony assay. The tumor cells were incubated for 5 h with PBL or LAK cells at the E/T ratio of 50:1.

b) The difference was statistically significant ($P<0.01$).

Incubation of PBL with 100 U/ml rIL-2 augmented the cytotoxicity against all cell lines. PC-9/CDDP showed a marked and significant ($P<0.01$) increase in their susceptibility to killing by both PBL and LAK cells, as compared with the parental cell line, PC-9. However, no significant difference between PC-14 and PC-14/CDDP, and between K-562 and K-562/ADM was observed in their susceptibility to either PBL or LAK cells.

Figure 1 shows the cytotoxicity of PBL and LAK cells against PC-9 and PC-9/CDDP at three different E/T ratios. A marked increase in the cytotoxicity of PBL and LAK cells against PC-9/CDDP as compared to the parental cells was observed at all E/T ratios. Cytotoxicity of LAK cells against PC-9/CDDP was higher than that of PBL at all E/T ratios, but the difference showed no statistical significance.

Cytotoxicity of LAK cells with CDDP to CDDP-resistant sublines To evaluate the combination therapy of LAK cells and CDDP compared with each therapy alone, the antitumor effect of LAK cells following an initial 1-h exposure of CDDP and that of LAK cells incubated simultaneously with CDDP for 5 h were determined in human non-small cell lung cancer cell lines and their resistant sublines.

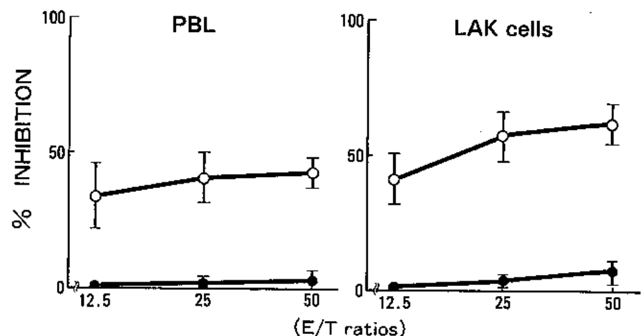

Fig. 1. Cytotoxicity of PBL and LAK cells to PC-9 (●) and PC-9/CDDP (○) at three different E/T ratios.

Table III. Cytotoxicity of LAK Cells (E/T ratio=50:1) with CDDP (1.0 μg/ml) against Human Lung Cancer Cell Lines[a]

	PC-9	PC-9/CDDP	PC-14	PC-14/CDDP
LAK (5 h)	8.9±4.4	65.5±7.0	41.1±9.2 ⎤[b]	52.2±11.4 ⎤[b]
CDDP (1 h)	32.2±2.8	13.5±6.8	11.2±5.6 ⎥	13.1±1.1 ⎥
CDDP (1 h) → LAK (5 h)	39.7±8.5	56.8±11.4	55.2±6.2 ⎥	66.1±7.7 ⎥
CDDP (5 h)	75.1±3.2	28.2±3.7	30.0±6.5 ⎥	13.3±6.8 ⎥
CDDP+LAK (5 h)	79.0±2.5	65.3±3.2	79.9±1.9 ⎦	79.9±5.5 ⎦

a) Data are expressed as a percentage of colony inhibition ± SE in the colony assay.

b) The difference was statistically significant ($P<0.05$).

Table IV. Cytotoxicity of LAK Cells (E/T ratio=50:1) with ADM (3.0 μg/ml) against K-562 and K-562/ADM[a)]

	K-562	K-562/ADM
LAK (5 h)	77.7±5.8	66.8±9.6
ADM (1 h)	79.5±8.7	2.5±2.1
ADM (1 h) → LAK (5 h)	86.1±3.9	63.8±10.7
ADM (5 h)	88.0±1.8	1.6±1.1
ADM+LAK (5 h)	84.2±3.8	62.9±14.6

a) Data are expressed as a percentage of colony inhibition±SE in the colony assay.

The results with LAK cells at the E/T ratio of 50:1 and 1.0 μg/ml of CDDP are shown in Table III. A significant increase ($P<0.05$) in cytotoxicity of the combined treatment with a 5-h exposure to LAK cells and CDDP simultaneously as compared to the cytotoxicity of LAK cells or CDDP alone was observed with PC-14 and PC-14/CDDP. These combined treatments had a synergistic effect on both cell lines. There was no synergistic effect in the cytotoxicity of LAK cells with CDDP against PC-9 and PC-9/CDDP cells.

Cytotoxicity of LAK cells with ADM to K-562/ADM Table IV shows the cytotoxicity of ADM with or without LAK cells against K-562 and K-562/ADM. The procedure was the same as that for CDDP with LAK cells at the E/T ratio of 50:1 and 0.3 μg/ml of ADM. No statistically significant difference in cytotoxicity was observed between the combination of LAK cells with ADM and either treatment alone.

DISCUSSION

Drug resistance is one of the major obstacles in cancer chemotherapy. Recently, various approaches to overcome acquired resistances *in vitro* have been reported.[1-3] In the present study we examined the possibility of an immunological approach using LAK cells to circumvent drug resistance.

We have previously reported that LAK activity can be detected by colony assay because of a positive correlation between percent inhibition of colony formation in colony assay and percent cytolysis obtained in ^{51}Cr-release assay.[14] These findings suggested the possibility of determining the cytotoxicity of LAK cells in combination with anticancer drugs by colony assay. By using this method, we have found that some of the sublines resistant to CDDP are more sensitive than others to recombinant human tumor necrotizing factor.[15]

Our results in this study showed that one of the sublines resistant to CDDP, PC-9/CDDP, was much more susceptible to killing by both PBL and LAK cells, than was the parental cell, PC-9. Allavena *et al.* reported that LAK cells efficiently kill colon carcinoma cell lines with pleiotropic drug resistance,[6] though Yanovich *et al.* found that a pleiotropic drug-resistant variant of K-562 demonstrated a modest increase in resistance to NK cells.[5] In the present study, we obtained results similar to those of Allavena *et al.* However, there were some differences in that we used CDDP-resistant cells and observed a more marked increase in cytotoxicity of LAK cells against PC-9/CDDP as compared to the parental cell. Furthermore, other resistant cell lines (PC-14/CDDP and K-562/ADM) showed a degree of susceptibility to killing by LAK cells similar to that of the parental cells, suggesting that LAK cells could have a similar degree of cytotoxicity even if the tumor cells acquired resistance anticancer drugs. Similar results have recently been reported by Passerini *et al.*, indicating chemo-surviving human melanoma or small cell lung cancer cells after a short treatment with mafosfamide were not less sensitive to lysis by rIL-2 activated PBL than their untreated counterparts, and in some cases the drug treatment rendered the neoplastic cells even more sensitive to lymphocyte killing *in vitro*.[16] The reasons why the present results on a pleiotropic drug-resistant K-562 cell line differ from those of Yanovich *et al.* on a resistant K-562 cell line may be partly the difference in the detection method of cytotoxicity and partly the difference in the degree of resistance to ADM. These reports, including our results, suggest that LAK therapy may be a useful therapy for drug-resistant tumors. Further examination with various drug-resistant cells should be done to evaluate the efficacy of LAK therapy.

It has also been found that the fatty acid composition of the cell membranes and its modification by certain drugs including ADM play an important role in the efficiency of the "lethal hit" in cell destruction by specific antibody and complement.[17] On the other hand, ADM has been shown to modify the expression of some cell surface receptors,[18] which might also influence the expression of putative LAK cell receptors. Additionally, Leroux *et al.* reported that pretreatment with mitomycin C or ADM enhanced the sensitivity of murine lymphoma cells to the killing action of LAK cells.[7] Our results with PC-9/CDDP suggest that CDDP may cause such a modification on the surface of tumor cells and enhance their sensitivity to LAK cells. The basis for the observed differences in LAK susceptibility of the drug-resistant tumor cell line (PC-9/CDDP) compared with the parental cell line, PC-9, can in part be approached by examination of target structure expression in assays of effector: target conjugate number and cold target inhibition. Experiments from this standpoint are in progress in our laboratory.

The second purpose of our study was to evaluate the availability of LAK therapy in combination with anticancer drugs. In this study, a synergistic effect between LAK cells and CDDP was observed on PC-14 and PC-14/CDDP, although no synergism of LAK cells and anticancer drugs was observed on PC-9 and PC-9/CDDP or K-562 and K-562/ADM.

In conclusion, we found a marked increase in sensitivity to LAK cells in one of the CDDP-resistant cell lines (PC-9/CDDP) as compared to the parental cell line, and a synergistic effect between LAK cells and CDDP in PC-14 and PC-14/CDDP. These results suggest that LAK cells may have some value in the therapy of tumors with acquired drug resistance. Investigation of the *in vivo* susceptibility of these drug-resistant tumor cell lines to NK and LAK killing would be an interesting extension of these *in vitro* studies.

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