Generation of a Small Cell Lung Cancer Variant Resistant to Lymphokine-activated Killer (LAK) Cells: Association with Resistance to a LAK Cell-derived, Cytostatic Factor

Isao Tachibana, Masatoshi Watanabe, Yoshiro Tanio, Seiji Hayashi, Shigeto Hosoe, Shin'ichi Saito, Machiko Matunashi, Tadashi Osaki, Yoshihisa Shigedo, Tomiya Masuno, and Ichiro Kawase

The Department of Internal Medicine III, Osaka University Medical School, 1-1-50, Fukushima, Fukushima-ku, Osaka-553, Japan

ABSTRACT

Cells of OS2-RA, a human small cell lung cancer line sensitive to lymphokine-activated killer (LAK) cells, were repeatedly cocultured with human LAK cells. Fourteen cycles of the coculture produced a variant, termed OS2-RA-R, capable of growing successfully in the presence of LAK cells. OS2-RA-R showed a moderate resistance to lysis by LAK cells in 4-h ⁵¹Cr release assays. OS2-RA-R acted positively as a cold target for lysis of OS2-RA by LAK cells, suggesting no loss of the binding site for LAK cells on the cell surface of the variant. On the other hand, LAK cells were shown to produce a factor capable of suppressing the proliferation of OS2-RA and certain other cell lines but not lymphocytes. Interestingly, OS2-RA-R exhibited a substantial resistance to the cytostatic activity of LAK cell supernatants. The cytotactic factor, eluted at the 57-kDa fraction in gel filtration, showed no activity of interleukin 1, γ-interferon, transforming growth factor β, or tumor necrosis factor. These results suggest that LAK cells exhibit antitumor activity through not only rapid cytosis but also slow-acting cytokine production, and the successful growth of OS2-RA-R in a coculture with LAK cells is the result of acquiring resistance to these two different LAK cell phenomena.

INTRODUCTION

SCLC is a highly aggressive malignant neoplasm with a rapid growth rate which frequently invades and metastasizes. The high rate of tumor recurrence and the frequent presence of subclinical metastases may make surgical cure impossible even at early stages. SCLC also relapses rapidly and develops resistance to chemotherapeutic agents, even though its initial response to chemotherapy may have been good. Thus, new treatment protocols are needed to improve the prognosis of patients with SCLC.

We have established several lines of SCLC from untreated primary tumors biopsied by diagnostic bronchofiberscopy. Recently, two SCLC lines were established from a patient with SCLC before chemoradiotherapy and after relapse. In vitro cytotoxicity tests revealed that cells of the relapsed SCLC line were highly sensitive to lymphocytes activated with human rIL 2, known as LAK cells (4), while the tumor cells showed an acquired resistance to chemotherapeutic agents and to irradiation. This suggests that adoptive immunotherapy with LAK cells and rIL 2, as proposed by Lafreniere and Rosenberg (5), may act as an adjuvant therapy for SCLC.

However, recent studies have revealed that, except for tumors of selected histological types such as melanoma, common human malignancies including lung cancer are hardly controlled with this treatment (6), whereas cells of a wide variety of cultured tumor lines are shown to be sensitive to lysis by LAK cells in vitro.

This study was undertaken to investigate the mechanisms regulating target cell sensitivity to LAK cells. We can report that (a) repeated coculture of cells of a LAK-sensitive cell line results in the generation of a variant capable of growing successfully in the presence of LAK cells and (b) the ability of the variant to proliferate in spite of long-term attacks from LAK cells is largely based on its resistance to a cytotactic factor produced by LAK cells.

MATERIALS AND METHODS

Cell Lines. SCLC line OS2-RA was established in our laboratory from a patient with SCLC relapsed after chemoradiotherapy and SCLC line OS3 from another, untreated case of SCLC. Other SCLC lines, H69 and N231, were a gift from Dr. Y. Shimozato, National Cancer Research Institute, Tokyo, Japan. PC9, a lung adenocarcinoma line, was donated by Dr. Y. Hayata, Tokyo Medical College, Tokyo, Japan. The cell lines of squamous cell carcinoma of the lung, QG56, adenocarcinoma of the lung, A549, erythroleukemia, K562, and Burkitt's lymphoma, Daudi, and a murine fibroblast line, L929, were all obtained from the American Type Culture Collection, Rockville, MD. All cell lines except OS2-RA were serially passaged in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated FBS (General Scientific Laboratories, Los Angeles, CA), 2 mm l-glutamine (Flow Laboratories, North Lyde, Australia), 100 units/ml penicillin (Meiji Seika, Tokyo, Japan), and 100 µg/ml streptomycin (Meiji Seika). This medium was designated the complete medium. OS2-RA was maintained in the complete medium supplemented with 10 nm hydrocortisone (Sigma Chemical Co., St. Louis, MO), 5 µg/ml insulin (Shionogi Pharmaceutical Co., Osaka, Japan), 10 µg/ml transferrin, 10 nm 17β-estradiol, and 30 nm sodium selenite (all from Sigma). This medium was designated the HITES medium (3). All of these cell lines were shown to be free of Mycoplasma infection when tested with Bacto pleuropneumonia-like organism agar (Difco, Detroit, MI).

Antibodies. The derivation of ITK-2, an IgG1 MoAb recognizing the cluster 1 antigen of SCLC, was described previously (7). MOC-1 (8) and NKH-1 (9), both cluster 1 antigen-recognizing MoAbs, were purchased from Bio-Science Products, Emmenbruecken, Switzerland, and Coulter Immunology, Hialeah, FL, respectively. IOT2 (anti-human class I MHC) and IOT2a (anti-human class II MHC) MoAbs were both purchased from Immunotech, S. A., Marseilles, France.

Preparation of PBMCs. PBMCs from normal volunteers were isolated on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), washed, and suspended in an appropriate medium.

Induction of LAK Cells. PBMCs were suspended at a cell density of...
10⁶ cells/ml in RPMI 1640 medium supplemented with 5% heat-inactivated normal human AB serum, 5 × 10⁻⁵ M 2-mercaptoethanol (Wako Pure Chemical Co., Osaka, Japan), 2 mM l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were then activated for 4 days with 300 units/ml human rIL-2 (Takeda Pharmaceutical Co., Osaka, Japan). After activation with rIL-2, the cells were washed twice and resuspended in an appropriate medium.

Preparation of Target Cells. OS2-RA, OS3, and N231 were enzymatically digested with 0.01% DNase (Sigma) and 0.05% collagenase S-1 (Nitta Gelatin, Osaka, Japan) for 1 h, washed, and then cultured again in the HITES medium overnight. After this brief culture, single-cell suspensions of these cell lines were prepared by gentle pipetting. Single-cell suspensions of A549 and QG56 were obtained through brief digestion with 0.25% trypsin (Gibco, Grand Island, NY).

Coculture of OS2-RA with LAK Cells. Ten thousand OS2-RA cells were mixed with 10⁴-10⁵ LAK cells in 200 µl of HITES-2ME medium in a well of a 96-well microculture plate (No. 3596; Costar, Cambridge, MA). Five days after the culture, 100 µl of the culture supernatant was replaced with the same volume of the HITES-2ME medium every other day until the tumor cells had proliferated enough to form visible clusters. The clusters were digested enzymatically with DNase and collagenase S-1 as described above, and the resulting tumor cells were cultured again with LAK cells at E/T ratios ranging from 1/1 to 10/1.

Cytolysis Assay. Ten thousand ²⁵¹Cr-labeled target cells were mixed with 2.5–100 × 10⁵ effector cells in 200 µl of the complete medium in a well of a microculture plate (No. 76–013–05; Linbro, McLean, VA). The plate was centrifuged at 800 rpm for 1 min and cultured for 4 h. After incubation, 100 µl of the supernatant was harvested and its radioactivity counted with a gamma-counter. Cytolytic activity in triplicate cultures was calculated as follows:

\[
\text{% specific cytosis} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Total cpm} - \text{Spontaneous cpm}} \times 100
\]

Spontaneous cpm, measured by incubating labeled target cells alone, was <10% of total cpm.

Cold Target Inhibition in Cytolysis. Unlabeled tumor cells were mixed with 10⁴ ²⁵¹Cr-labeled target cells in 100 µl of the complete medium at cold/hot ratios ranging from 1.25/1 to 10/1 and then added to a well containing 2.5 × 10⁴ LAK cells in 100 µl of the complete medium. A 4-h ²⁵¹Cr release assay was carried out as described above.

Clonogenic Assay in Soft Agar. Ten thousand tumor cells suspended in 0.3 ml HITES medium containing 0.3% Noble agar (Difco) were plated on a support layer of 0.5 ml HITES medium containing 0.5% agar in a well of a 24-well culture plate (No. 3424; Costar), as described by Hamburger and Salmon (10). Colonies containing >40 cells were counted 2 weeks after plating. CFE in triplicate cultures was calculated as follows:

\[
\text{CFE} = \frac{\text{No. of colonies}}{\text{No. of cells plated}} \times 100
\]

Tumor Inoculation in Nude Mice. Five million tumor cells, obtained by enzymatic digestion as described above, were suspended in 50 µl of the complete medium and inoculated s.c. into the back of BALB/c nu/nu mice (Shizuoka Agricultural Cooperative for Experimental Research, Shizuoka, Japan).

Cytostasis Assay. One thousand or 10,000 tumor cells suspended in 100 µl HITES medium or in the complete medium were added to a well containing 100 µl test samples in a 96-well microculture plate (No. 3596; Costar) and cultured for 7 days. The concentrations of FBS, l-glutamine, antibiotics, hydrocortisone, insulin, transferrin, 17β-estradiol, and sodium selenite were adjusted to produce the HITES medium for OS2-RA, OS3, and N231 and the complete medium for the other cell lines. Tumor cultures with medium alone served as controls. Tumor growth was assessed by calculating the number of viable tumor cells with trypsin blue dye exclusion tests, following enzymatic digestion if necessary, or by microculture tetrazolium assays as described below.

Cytostatic activity in triplicate cultures was calculated as follows:

\[
\frac{\text{% growth inhibition}}{A} = \frac{(A - B)}{A} \times 100
\]

A refers to the data shown in control experiments and B to those in experiments using test samples.

MTT Assay. The colorimetric assay was performed according to the method of Alley et al. (11). Briefly, 50 µl of RPMI 1640 medium containing 50 µg of MTT (Sigma) was added to a well containing tumor cells and cultured for 5 h. Wells containing culture medium alone but no tumor cells served as reference. The culture supernatant was removed by centrifugation, and 150 µl of dimethyl sulfoxide (Wako Pure Chemicals Co.) was added to each well. After the reaction mixtures were triturated with a pipet, the absorbance at 540 nm was measured with a microculture reader.

Sensitivity to TNF and IFN-γ. nTNF-α and recombinant IFN-γ were donated by, respectively, Dr. S. Sone, Tokushima University, Tokushima, Japan, and Shionogi Pharmaceutical Co., Osaka, Japan. Ten thousand OS2-RA cells were suspended in 200 µl of the HITES medium containing nTNF-α or IFN-γ at various concentrations, and cultured in a well of a microculture plate (No. 3596; Costar) for 2–7 days. Cytotoxicity in triplicate cultures was determined by microculture tetrazolium assay.

Cytostasis Assay in Diffusion Chambers. Five million LAK cells suspended in 1.2 ml HITES-2ME medium were added to a lower compartment of a 24-well double chamber culture plate (Transwell, No. 3413; Costar). Ten thousand OS2-RA cells suspended in 0.3 ml of the same medium were added to an upper compartment with a microporous membrane (pore size, 0.4 µm) at the bottom. The upper compartment was placed in the lower compartment so that its lower half was steeped in 1.2 ml of the cell suspension, and the plate was cultured for 7 days. After the culture, the number of viable tumor cells in the upper compartment was determined by trypsin blue dye exclusion test following enzymatic digestion. Tumor cultures without LAK cells served as controls.

Gel Filtration. Five million LAK cells washed three times were suspended in 2 ml RPMI 1640 medium supplemented with 5 × 10⁻⁵ M 2-mercaptoethanol alone and cultured for 2 days in a well of a 24-well culture plate (No. 3424; Costar). One hundred ml of the culture supernatant was concentrated 20-fold, dialyzed, and applied on a Sephacryl S-200 column (1.6 x 98 cm; Pharmacia) as described previously (12). After dialysis against RPMI 1640 medium, each fraction (3 ml) was assayed for its cytostatic effect on OS2-RA cells as described above.

Assay for TNF, IFN, TGF-β and IL 1. Two assay methods for human TNF activity were used, one a biological L-cell assay and the other an ELISA using a MoAb to human TNF-α (13). The sensitivity of the ELISA was 20 pg/ml. Human IFN activity was titrated for its ability to inhibit the cytopathic effect of the sindbis virus on a monolayer of Mv1Lu cells (15). This assay system was capable of detecting 0.1 ng/ml of TGF-β. IL 1 activity was assayed with an ELISA, using MoAbs to human IL 1-α and IL 1-β (16), that was capable of detecting 10 pg/ml of IL 1-α and IL 1-β.

Flow Cytometry. Ten thousand cells were stained with FITC-2 or NKH-1 at a concentration of 5 µg/ml or with MOC-1 at a dilution of 1:10, washed twice, and then labeled with FITC-conjugated rabbit antimouse immunoglobulin (Zymed Labortatories, South San Francisco, CA) at a dilution of 1:20. A normal mouse IgG (Serotec, Kidlington, England) was used as a control antibody at a concentration of 5 µg/ml. Cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

Lysis and Trypsin Treatment. Test materials were dialyzed against 0.1 M glycine-HCl buffer at pH 2.0 for 12 h at 4°C, and the pH was restored to neutrality by dialysis against RPMI 1640 medium for 12 h. The susceptibility to trypsin was examined as described elsewhere (17). Briefly, trypsin (Sigma) was added to an aliquot of a test sample at a
RESULTS

Induction of LAK Resistance on OS2-RA Cells. When OS2-RA cells were cocultured with LAK cells at E/T ratios ranging from 1/1 to 10/1, most of the tumor cells were destroyed in 1–2 days even at an E/T ratio of 1/1. One or 2 weeks after the initiation of the coculture, a few OS2-RA cells which escaped from the attack of LAK cells started to proliferate, forming one or two colonies per well. One month later, cells obtained by coculture with LAK cells at an E/T ratio of 5/1, termed OS2-RA-R, were compared with the parental line with respect to cell surface phenotype, proliferative activity, and LAK sensitivity.

Statistical Analysis. The mean difference between control and experimental groups was evaluated statistically by Student’s t test. Statistical significance of the difference in the number of mice bearing tumors between the two groups was determined by χ² test.

Fig. 1. Cold target inhibition test in 4-h ⁵¹Cr release assay. Increasing number of unlabeled (cold) tumor cells, OS2-RA (C), OS2-RA-R (B), and Daudi (A), were mixed with 10⁵ labeled (hot) tumor targets, OS2-RA (A) and Daudi (B) at cold/hot (C/H) ratios ranging from 2.5/1 to 10/1. Cytotoxicity was assessed by 4-h ⁵¹Cr release assay at an E/T ratio of 2.5/1. The percentage specific cytolyis to OS2-RA and Daudi determined without cold targets was 61.7 ± 4.2 and 81.7 ± 3.2%, respectively. Points, mean percentage of the relevant controls; bars, ±SE.
TUMOR RESISTANCE TO A LAK-DERIVED, CYTOSTATIC FACTOR

Table 4 Production of cytostatic factor by LAK cells and difference in the sensitivity to the factor between OS2-RA and OS2-RA-R

Ten thousand tumor cells and 5 × 10^5 LAK cells were added to the upper and lower compartment of diffusion chambers, respectively, and cultured for 1 week. After the culture, the number of viable tumor cells in the upper compartment was determined by trypan blue dye exclusion test following enzymatic digestion.

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>Upper compartment</th>
<th>Lower compartment</th>
<th>% growth inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS2-RA</td>
<td>-</td>
<td>42.3 ± 3.6</td>
<td>91.9 ± 0.5</td>
</tr>
<tr>
<td>OS2-RA-R</td>
<td>LAK cells</td>
<td>3.4 ± 0.2</td>
<td>41.9 ± 6.6</td>
</tr>
<tr>
<td></td>
<td>LAK cells</td>
<td>12.4 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

<math>\% \text{ growth inhibition} = \left(\frac{\text{No. of viable tumor cells in the upper compartment}}{\text{No. of viable tumor cells in the lower compartment}}\right) \times 100</math>

<math>\text{Mean} \pm \text{SE. The difference between } c \text{ and } d \text{ was statistically significant (P < 0.01).}</math>

Table 5 Sensitivity of OS2-RA and OS2-RA-R to the cytostatic activity of LAK cell culture supernatant

Five million LAK cells, washed 3 times, were resuspended in 2 ml of the complete medium supplemented with 5 × 10^{-5} M 2-mercaptoethanol and cultured for 2 days. The supernatant was harvested and diluted 1- to 4-fold with RPMI 1640 medium. One hundred μl of diluted LAK cell culture supernatant was added to a well containing 10^4 tumor cells in 100 μl of appropriate medium. Several reagents were added to make up the HITES medium as described in "Materials and Methods." Tumor cultures with the HITES medium alone served as controls.

<table>
<thead>
<tr>
<th>Dilution of LAK cell culture supernatant (fold)</th>
<th>Cell no. calculation</th>
<th>MTT assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS2-RA</td>
<td>2</td>
<td>88.1 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>84.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>65.6 ± 3.9</td>
</tr>
<tr>
<td>OS2-RA-R</td>
<td>2</td>
<td>34.1 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>32.3 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>30.7 ± 6.5</td>
</tr>
</tbody>
</table>

<math>\% \text{ growth inhibition} = \left(\frac{\text{No. of viable tumor cells in the upper compartment with LAK cells in the lower compartment}}{\text{No. of viable tumor cells in the upper compartment without LAK cells in the lower compartment}}\right) \times 100</math>

<math>\text{Mean} \pm \text{SE. There was no statistical significance between OS2-RA and OS2-RA-R.}</math>

Table 6 Sensitivity of OS2-RA and OS2-RA-R cells to TNF and IFN-γ

Ten thousand tumor cells were cultured in the presence of nTNF-α or IFN-γ for 2 or 7 days, respectively, and then subjected to an MTT assay. Tumor cells cultured alone served as controls.

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>5 × 10^2</th>
<th>10</th>
<th>5 × 10^3</th>
<th>IFN-γ (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L929</td>
<td>53.3 ± 3.7</td>
<td>NT</td>
<td>50.9 ± 0.6</td>
<td>NT</td>
</tr>
<tr>
<td>OS2-RA</td>
<td>0.0 ± 0.0</td>
<td>27.9 ± 4.5</td>
<td>117.7 ± 4.8</td>
<td>NT</td>
</tr>
<tr>
<td>OS2-RA-R</td>
<td>2.2 ± 2.9</td>
<td>20.8 ± 3.5</td>
<td>23.6 ± 5.0</td>
<td>NT</td>
</tr>
</tbody>
</table>

<math>\text{Mean} \pm \text{SE. There was no statistical significance between OS2-RA and OS2-RA-R.}</math>

<math>\text{NT, not tested.}</math>

to be different from that on Daudi, because neither of the two cell lines competed with the other in cytolyis by LAK cells. Resistance of OS2-RA to Cytostatic Activity of LAK Cells. Preliminary experiments showed that LAK cell activity of rIL 2-stimulated PBMCs dramatically declined within 4–5 days after removal of rIL 2 (data not shown). When cultured with LAK cells, however, OS2-RA cells which had escaped from lysis by LAK cells did not show any proliferative activity of their own, even after most of the LAK cells had expired, until the culture supernatant was replaced with fresh medium. This suggests that LAK cells may produce factors capable of suppressing the growth of OS2-RA. To investigate this possibility, OS2-RA and LAK cells, washed twice, were cultured separately in diffusion chambers for 7 days, and the number of tumor cells was calculated. As shown in Table 4, the growth of OS2-RA in the upper compartments was almost completely suppressed by the addition of LAK cells to the lower compartment, indicating that soluble factors produced by LAK cells could suppress the growth of OS2-RA. Interestingly, the separate cultures of OS2-RA-R and LAK cells resulted in only a partial suppression of the growth of OS2-RA-R. To confirm these findings, supernatants of LAK cells cultured alone were further tested for cytostatic activity with both cell-counting and colorimetric assays (MTT assay). LAK cells, washed twice, were cultured in the complete medium supplemented with 5 × 10^{-5} M 2-mercaptoethanol for 2 days, and the culture supernatant was added to a 7-day culture of the parent and the variant lines. Table 5 clearly indicates that the 2-day culture supernatant of LAK cells strongly suppressed the growth of OS2-RA, while its variant line exhibited a substantial resistance to the cytostatic activity. There was no significant difference between these two assay methods in sensitivity for assessing cytostasis.

Sensitivity to TNF and IFN-γ. The 2-day culture supernatant of LAK cells was shown to contain small amounts of both TNF (62 pg/ml) and IFN-γ (107 IU/ml). To investigate the possibility that TNF and IFN-γ might play a role in the LAK cell-derived cytostasis, the sensitivities of the cell lines to these cytokines were tested. As shown in Table 6, both the parent and the variant lines exhibited a high resistance to TNF. On the other hand, these two cell lines were both weakly sensitive to IFN-γ, without any significant difference in their sensitivity. These results suggest that the cytostatic factor to which the two cell lines show different sensitivities is distinct from TNF and IFN-γ.

Semipurification and Characterization of the Cytostatic Factor. LAK cells, washed twice, were further cultured for 2 days in RPMI 1640 medium supplemented with 5 × 10^{-5} M 2-mercaptoethanol. The FBS-free culture supernatant was concentrated and subjected to gel filtration using a Sephacryl S-200 column. As shown in Fig. 2, the activity suppressing the growth of OS2-RA was eluted at the 57-kDa fraction. On the other hand, a small peak of TNF activity was found at the 17-kDa fraction. The fractions from the peak at 57-kDa were pooled, concentrated 2-fold, and further analyzed for its physicochemical properties and cytokine activities.

As summarized in Table 7, the cytostatic factor semipurified by gel filtration was heat labile and trypsin sensitive, and its activity was drastically reduced by treatment at pH 2.0. Assays for various cytokine activities revealed that the semipurified sample was free of TNF-α, TGF-β, and IL 1 activities and exhibited only a trace activity of IFN (Table 8).

Target Specificity of the Cytostatic Factor. The cytostatic factor semipurified by gel filtration was further analyzed for its...
target specificity by testing the inhibitory activity on 7-day growth of tumor cells, with the results summarized in Table 9. This factor effectively suppressed the growth of a variety of lung cancer cell lines. However, cells of certain tumor lines, including SCLC and non-SCLC lines, could proliferate, showing a natural resistance to the cytostatic activity. Furthermore, hematopoietic tumor lines such as K562 and Daudi were completely resistant to the factor. In addition, this factor showed no inhibitory effect on the mitogen response of PBMC to Con A. OS2-RA-R also showed a substantial resistance to the cytostatic activity of the semipurified LAK cell supernatant. The substantial differences in sensitivity to the cytostatic factor did not correlate with those in the doubling time.

DISCUSSION

Tumor variants resistant to lysis by LAK cells have been successfully induced from LAK-sensitive tumor lines by culturing cells with LAK cells repeatedly (18, 19). Rivoltini et al. (18) have shown that only three cycles of a coculture of a LAK-sensitive melanoma line with LAK cells results in the generation of variants exhibiting a >8-fold reduction in the susceptibility to LAK lysis. Similarly, melanoma variants acquiring a significantly low lysability to CTL have been established by a coculture with CTL repeated only four times (19). In our study, however, >10 cycles of a coculture with LAK cells were required to establish a variant, termed OS2-RA-R, capable of growing successfully in a coculture with LAK cells from a LAK-sensitive SCLC line, OS2-RA. Furthermore, 14 cycles of a coculture of the SCLC line with LAK cells resulted in a reduction to only half its sensitivity to lysis by LAK cells. No further reduction in its sensitivity to LAK lysis was achieved by an additional 4 cycles of a coculture of OS2-RA-R with LAK cells (data not shown). On the other hand, the moderate resistance of OS2-RA-R to LAK lysis was stable for only 1 month and then decreased by 30%/month when the variant was cultured alone. The decreased resistance was restored by repeated reexposures of the cells to LAK cells. Therefore, OS2-RA-R was maintained in vitro through intermittent coculture with LAK cells.

The establishment of OS2-RA-R by repeated cocultures with LAK cells seems to be the result of a selection of a subpopulation with a moderate resistance to LAK lysis from the parent SCLC line with heterogenous LAK sensitivity. In fact, among a variety of clones obtained by limiting dilution of the parent cell line, OS2-RA, successful cocultures with LAK cells resulted in a reduction to only 30% of the parental cells' sensitivity to lysis by LAK cells. This factor effectively suppressed the growth of a variety of tumor cells, with the results summarized in Table 9.
SCLC line, no clone exhibited a higher resistance to LAK lysis than did OS2-RA-R. Furthermore, the decrease and the restoration of the resistance of OS2-RA-R to LAK lysis, caused by removal and readdition of LAK cells to the tumor cell culture, were accompanied by an inverse change in its growth rate. This suggests that OS2-RA-R is still heterogeneous, i.e., a major subpopulation with both a low LAK sensitivity and a slow-growing ability may be gradually replaced by a small but rapidly-growing subpopulation with a high LAK sensitivity when the variant is cultured without LAK cells. Therefore, such a significant difference in ability to induce resistance to lysis by cytotoxic effectors between melanoma and SCLC lines may depend on a different degree of heterogeneity of these cell lines in their susceptibility to cytolysis.

Topalian et al. (19) demonstrated that the reduction in the sensitivity of melanoma cells to lysis by CTL, induced by repeated cocultures with CTL, might be due to the loss of the cell surface-binding sites for CTL, as suggested by data of cold target inhibition studies. On the other hand, OS2-RA-R could well act as a cold target for lysis of OS2-RA by LAK cells, suggesting no loss of the binding site for LAK cells. Furthermore, the reduction in LAK sensitivity of the SCLC line by repeated cocultures with LAK cells was accompanied by a similar decrease in the sensitivity to lysis by NK cells (data not shown). Thus, it seems possible that a reduced sensitivity to cytotoxic substances including perforin plays a role in the decreased lysability to LAK cells in the SCLC variant, although the present study lacks data suggesting this possibility.

The selected OS2-RA-R showed a significant decrease in both in vitro proliferation and in vivo growth. This is in agreement with the report of Rivoltini et al. (18), which suggested that a LAK-resistant subpopulation with a retarded growth rate had undergone terminal differentiation and thus had been lost from the tumor culture and had been replaced by cells similar to the parent ones.

Cell surface phenotyping showed a certain increase in the expression of the class I MHC antigen in OS2-RA-R, while there was no difference in either N-CAM or the class II MHC antigen expression between the parental and the variant lines. This increase in the class I MHC antigen expression seems to be due to IFN-γ produced by LAK cells when cultured in the presence of FBS. Incubation of tumor cells with IFN-γ has been reported to reduce their sensitivity to lysis by LAK cells (20, 21). However, a brief incubation of OS2-RA in the presence of 100 IU/ml IFN-γ, resulting in an augmented expression of the class I MHC antigen, did not reduce the sensitivity to LAK lysis, although a substantial decrease in NK susceptibility was observed. Therefore, it is unlikely that the reduced lysability of OS2-RA-R to LAK cells, achieved by repeated coculture with LAK cells, is due to IFN-γ produced by LAK cells.

A tumor variant acquiring a resistance to TNF has been shown to become resistant to lysis by NK cells (22). More recently, Lichtenstein et al. (23) demonstrated good correlation between resistance to TNF and to LAK lysis and between these two types of resistance and c-erbB-2 gene expression in human ovarian cancer cells (23). These findings suggest a role of TNF in the LAK cell phenomenon. As shown in our study, however, the parent SCLC line was completely resistant to TNF.

The 2-fold difference in the lysability to LAK cells between the parent and the variant SCLC lines cannot explain the different ability of these two lines to proliferate in a coculture with LAK cells. The present study clearly indicates that LAK cells produce a cytostatic factor capable of suppressing the growth of OS2-RA and several other tumor cell lines. Interestingly, OS2-RA-R exhibited a substantial resistance to the factor. The cytostatic activity, eluted at the 57-kDa fraction in gel filtration, was heat labile and trypsin sensitive and became inactivated at low pH. Candidates for cytostatic cytokines, produced by rIL 2-stimulated PBMCs, are TNF (24), IFN-γ (24), IL 1 (25), and probably TGF-β (26). Furthermore, it has been reported that IL 1 exhibits a synergistic antiproliferative effect with TNF (27). In this study, however, the cytostatic factor semipurified by gel filtration was shown to be free of TNF-α, TGF-β, and IL 1 activities and exhibited only a trace activity of IFN. Furthermore, the semipurified sample showed no activity of either IL 2 or IL 6 (data not shown). These results suggest that the LAK cell-derived, cytostatic factor is probably distinct from these cytokines. This possibility may be further confirmed by testing the effect of antibodies to these cytokines on the cytostatic activity of the semipurified factor. The characteristics of the factor will be clarified through further purification processes.

Hersh et al. (28) have demonstrated a similar finding, namely, that IL 2-stimulated human PBMCs produce a cytotoxic factor that has a molecular size larger than 50 kDa and is probably distinct from TNF, IFN, and IL 1. In their report, the factor was shown to be able to suppress the growth of certain tumor cell lines but not lymphoblasts or hematopoietic tumor lines, including K562 and Daudi, when subjected to cell culture for 3–7 days. Consistent with their finding, considerable differences in the sensitivity to the cytostatic factor were observed among cell lines tested in our present study. As shown in our study, the sensitivity to the cytostatic factor did not correlate with either the proliferative potential or the lysability to LAK cells. The cytostatic factor reported by Hersh et al. was also shown to have a cytolytic effect on tumor cells. In our study, however, no apparent decrease, but a reduction in the increase, in the number of tumor cells was observed, suggesting that our factor exerts no cytolytic activity. This difference may be due partly to a difference in the production of the LAK cell-derived factor. For their study, Hersh et al. obtained the LAK cell culture supernatant 5 days after the initiation of the PBMC culture with IL 2, and they used it as a source of the factor without removing IL 2. On the other hand, washed LAK cells were further cultured in serum-free medium alone for another 2 days to produce the factor used in our study.

In conclusion, LAK resistance of OS2-RA-R was shown to be based on the reduction in the sensitivity not only to cytolysis but also to cytostasis. Although the mechanism involved in the cytostatic activity of LAK cell supernatant remains unclear, this activity may be an important component of the LAK cell phenomenon. Therefore, OS2-RA and OS2-RA-R may be useful entities for investigating the characteristics of the cytostatic factor and its effector mechanism. Further study is now under way to delineate the scope of this mechanism, using a broader panel of cultured cell lines from different cell types.

ACKNOWLEDGMENTS

The authors wish to thank Dr. H. Fujiwara of the Biomedical Research Center of Osaka University Medical School for his assay of TGF-β activity. We are also grateful to Otsuka Assay Laboratories, Tokushima, Japan, for assays of IL 1-α, IL 1-β, and IL 6 activities, and to Biochemical Medical Laboratories Co. Ltd., Tokyo, Japan, for assays of IL 2, IFN, and TNF-α (ELISA) activities.

REFERENCES

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Isao Tachibana, Masatoshi Watanabe, Yashiro Tanio, et al.


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