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Additive effects of antitumor drugs and lymphokine-activated killer cell cytotoxic activity in tumor cell killing determined by lactate-dehydrogenase-release assay

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Summary. The effect of pretreatment with antitumor drugs on lymphokine-activated killer (LAK) cell cytotoxic activity, determined by lactate-dehydrogenase(LDH)-release assay, was investigated. LAK cells were induced by incubating peripheral blood lymphocytes of healthy donors in medium containing interleukin-2 (IL-2) and monoclonal anti-CD3 antibody for 6–7 days. A human lung squamous carcinoma cell line, SQ-5, was used as an adherent target. After 24 h exposure of the target cells to cisplatin, doxorubicin, or mitomycin C, the drugs were washed off and LAK cells were added at an E/T ratio of 5. During further incubation for 48 h, LDH release from cisplatin- or doxorubicin-pretreated target cells was markedly higher than that from non-pretreated target cells. The combination of cisplatin and LAK cells has an additive cytotoxic effect and that of mitomycin C and LAK cells does not; there may also be an additive effect late in the toxicity mechanism between doxorubicin and LAK cells.

Key words: Lactate dehydrogenase – LAK cell – Antitumor drugs

Introduction

Adoptive immunotherapy with lymphokine-activated killer (LAK) cells is emerging as a possible new treatment for cancer [5]. Clinically, however, LAK cell therapy requires a very large number of effector cells and has shown promise only for a few types of tumors [14, 15]. Various approaches to overcome this limitation of LAK therapy have been reported [16], one of the most promising being a combination of chemotherapy and LAK cell therapy. In tumor-bearing mice, combinations of doxorubicin or cyclophosphamide and LAK therapy were found to have synergistic effects on survival [10, 17]. In an in vitro study, Laroux et al. [9], found by ^{51}Cr-release assay that pretreatment with antitumor drugs enhanced the sensitivity of mouse tumor cells to the killing action of LAK cells. However, we do not know of any report on whether the combined effects of these treatments are merely additive or synergistic (supraadditive) on the target cells.

Although the conventional 4-h ^{51}Cr-release assay has been used for quantification of LAK activity in vitro, it has not yet been used for chemosensitivity testing extensively. Roper and Drewinko evaluated the 4-h ^{51}Cr-release assay as a method to measure drug-induced cell lethality, and they concluded the assay was insensitive in measuring the magnitude of injury caused by chemotherapeutic agents [13]. Therefore, the ^{51}Cr-release assay may not necessarily be suitable for evaluating the effect of chemotherapy in combination with LAK therapy.

We examined the possibility of using a lactate-dehydrogenase(LDH)-release assay, which is a simple way to quantify target tumor cells lysed by natural killer cells, cytotoxic T lymphocytes and macrophages, as described by Korzeniewski and Callewaert [8] and Decker and Matthes [4]. These previous investigators compared the LDH-release assay with the ^{51}Cr-release assay and concluded that results from the two assays correlated well.

Here we describe a modified LDH-release assay for simultaneous quantification of dead and surviving tumor cells to determine the cytocidal effect and cytostatic effect of drug treatment in combination with LAK cell co-culture. This method is attractive because it can easily be semi-automated by use of a computer-controlled laboratory processor.

Materials and methods

Target cells. The human lung squamous carcinoma cell line SQ-5 was used as adherent target tumor cells. The cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 in air at 37° C.

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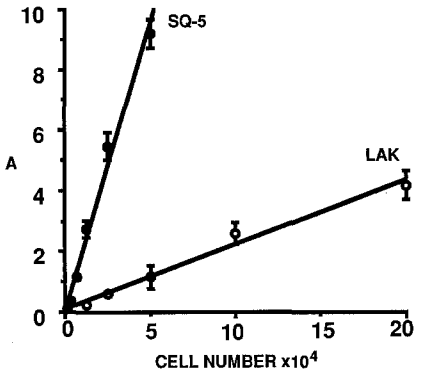

Fig. 1. Absorbance as a function of cell number using SQ-5 (●) and lymphokine-activated killer (LAK) cells (○). After cell plating, the cells were incubated with 0.65% dodecyldimethylaminoacetic acid betaine for 20 min. The samples were diluted 1/5 with phosphate-buffered saline just before measuring LDH activity. Absorbance was measured at 520 nm and the absorbance values (*A*) are shown as five times the observed values

LAK cells. Peripheral blood was obtained by venipuncture from healthy volunteers. Mononuclear cells were isolated by density-gradient sedimentation on a Leuco-PREP cushion (Beckton Dickinson Co., USA). LAK cells were induced by incubating peripheral blood lymphocytes at a density of 1×10^6/ml for 6–7 days in LAK medium consisting of a mixture of RPMI-1640 medium, Ham's F-12 medium, and MEMα at 3:1:1 by volume, supplemented with 5% plasma from the lymphocyte donor, 0.02 mg/l α-tocopherol, 0.002 mg/l sodium selenite, 0.004 mg/l linoleic acid, 0.2 mg/l cholesterol, 500 mg/l human albumin, 0.6 mg/l 2-mercaptoethanol, 0.6 mg/l ethanolamine, 5 mg/l insulin, 5 mg/l transferrin, and 20 μg/l monoclonal anti-CD3 antibody (Nichirei Co., Japan). Recombinant human interleukin-2 (Ajinomoto Co., Japan) was added at a concentration of 1000 Jurkat Unit/ml for the first 5–7 days of culture and then at 200 Jurkat Unit/ml.

Drug preparation. The antitumor drugs tested were cisplatin (*cis*-diaminedichloroplatinum), doxorubicin hydrochloride and mitomycin C, all obtained from commercial sources. Just before use they were diluted with MEM and added at final concentrations 1, 0.05, and 0.01 μg/ml respectively.

LDH-release assay. Target cells were seeded into 96-well microculture plates at a density of 1×10^3 cells/well with 100 μl MEM containing 10% FBS, and were precultured for 3 days at 37° C. Just before assay, the plates were washed once with MEM containing 5% FBS to remove LDH spontaneously released into the culture medium. Adherent target cells in six control wells were trypsinized for counting before LAK cell addition. Viable target cells were counted by a hematocytometer, then an appropriate number of LAK cells adjusted to the desired E/T ratios were added to each well in 100 μl LAK medium. The plates were then incubated at 37° C in an atmosphere of 5% CO_2 in air for an appropriate time. A sample of 100 μl phosphate-buffered saline (PBS) or 100 μl 0.65% dodecyldimethylaminoacetic acid betaine was added to each well. The plates were incubated further for 20 min to achieve complete solubilization of cells. The supernatant of wells with added PBS was named culture supernatant, and the supernatant of wells with added detergent was named cell lysate. After centrifugation at 140 × *g* for 3 min, 10 μl culture supernatant or cell lysate was transferred to corresponding wells of an optically clear microtiter plate. Spontaneous LDH release from tumor and LAK cells was determined by incubating target cells without LAK cells and LAK cells without target cells under the same conditions.

For determination of the augmenting effect of antitumor drugs, target cells were precultured for 3 days as mentioned above, washed with fresh culture medium, and incubated with antitumor drugs dissolved in 200 μl MEM containing 5% FBS for 24 h. Then they were washed once with MEM and incubated with LAK cells at an E/T ratio of 5 for 48 h. The culture supernatant and cell lysate were then obtained as described above.

Measurement of LDH activity. A commercially available enzymatic test kit for LDH (Kyokuto Pharmaceutical Industries, Co., Tokyo) was used. Samples of supernatant (50 μl) were transferred to the corresponding wells of an enzyme immunoassay/radioimmunoassay plate (Costar Inc.). Then 50 μl substrate and nitrozolium blue mixture (nitrozolium blue, NAD, diaphorase, DL-lithium lactate, and TRIS-buffer) was added to each well, and after 30 min incubation at 37° C, 100 μl 1 M HCl was added to each well to stop enzyme reaction. The absorbance at 520 nm was then determined. Using this assay system, the actual absorbance in wells was linear from 0.0 to 1.5. When necessary, the samples were diluted appropriately with PBS just before measuring LDH activity. The absorbance values shown in this paper were calculated as those before dilution.

Procedures in the assay, such as addition of PBS, detergent, or drugs, transfer of the supernatant and determination of LDH activity were performed automatically with a laboratory processor (Biomek 1000, Beckman Instruments, Inc., USA).

Calculations. Since LDH activities in the culture supernatant and the cell lysate were proportional to the amounts of dead and surviving cells, specific cytotoxicity of LAK cells and surviving target cells were calculated according to following formulae:

$$\text{specific cytotoxicity} = ET_s - E_s - T_s$$

where ET_s is the LDH activity of the culture supernatant from wells containing effector and target cells, E_s is the LDH activity spontaneously released from effector LAK cells in the absence of target cells, and T_s is the spontaneous release of LDH from target cells in the absence of effector cells. A measure of target cell survival is given by

$$(ET_c - ET_s) - (E_c - E_s)$$

where ET_c is the LDH activity of the cell lysate, and E_c and E_s are the LDH activities of the cell lysate and culture supernatant from effector cells in the absence of target cells.

Statistical analysis. All values are expressed as means ± SD, for four or more samples. Statistical analyses were performed by Student's *t*-test.

Results

Modified LDH-release assay

As described in Material and methods, we modified the LDH-release assay to determine killed target tumor cells and surviving target tumor cells simultaneously, by measuring LDH activity released into the culture supernatant and cell lysate respectively. A laboratory automated processor was used in the assay of LDH activity. The relationship between the cell number and the LDH activity is shown in Fig. 1. One target SQ-5 cell showed an absorbance of 1.91×10^{-4}/cell, while one LAK cell showed a value of 0.21×10^{-4}/cell.

Figure 2 shows the time course of change in LDH activity in the culture supernatant at an E/T ratio of 5. Spontaneous release of LDH from the target and the LAK cells under identical conditions was very low. The LDH activity in the culture supernatant did not increase until 12 h after addition of LAK cells at this E/T ratio, but elevation of the activity was obvious after incubation for 24 h. Figure 3 shows the specific cytotoxicity and surviving target cells after co-culture with LAK cells at E/T ratios of 1.25–20 for 24 h and 48 h. We named these two co-culture periods the 24-h assay and the 48-h assay respectively.

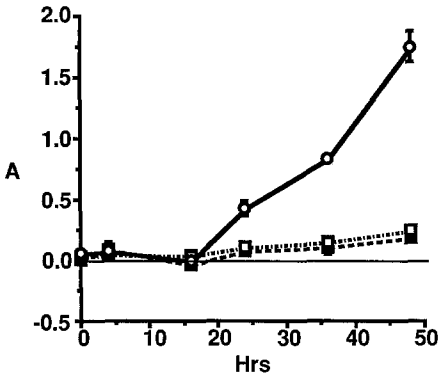

Fig. 2. Time course of release of lactate dehydrogenase (LDH) after adding LAK cells at an E/T ratio of 5. LDH activity (-○-) of the supernatant from wells containing effector and target cells, and spontaneous LDH release in wells containing only tumor cells (- · - □ - · -) and only effector cells (– – ■ – –) were measured. *Points* are means from four replicate samples; *bar*, SD

In the 24-h assay, the specific cytotoxicity increased linearly with E/T ratios of 1.25–10 and then leveled off at an E/T ratio of 20. In the 48-h assay, the level was optimal at an E/T ratio of 10, and then decreased. In contrast, surviving target cells decreased with increase in the E/T ratio in both assays. In the 48-h assay, spontaneous LDH release from LAK cells was not negligible at an E/T ratio of 20. This increase in spontaneous release resulted in a decrease in specific cytotoxicity at this E/T ratio. Thus, this assay should be performed at an E/T ratio less than 20.

Effect of pretreatment with antitumor drug on LAK cell cytotoxic activity

To determine whether there is any synergistic effect of antitumor drugs and LAK cells, we separated treatments with the drugs and with LAK cells, because exposure of LAK cells to antitumor drugs usually reduces LAK cell viability and consequently their activity. The target tumor cells were cultured in medium containing antitumor drugs (1 μg/ml cisplatin, 0.05 μg/ml doxorubicin, or 0.01 μg/ml mitomycin C) for 24 h, and then washed once with fresh medium. At this point, there was no significant difference in the fraction surviving between target cells treated with each drug and control target cells (Fig. 5a, b, c at 0 h). After the washing, we added LAK cells to the target cells and co-cultured them for 24 h or 48 h. We then measured LDH activity in the culture supernatant and in cell lysate

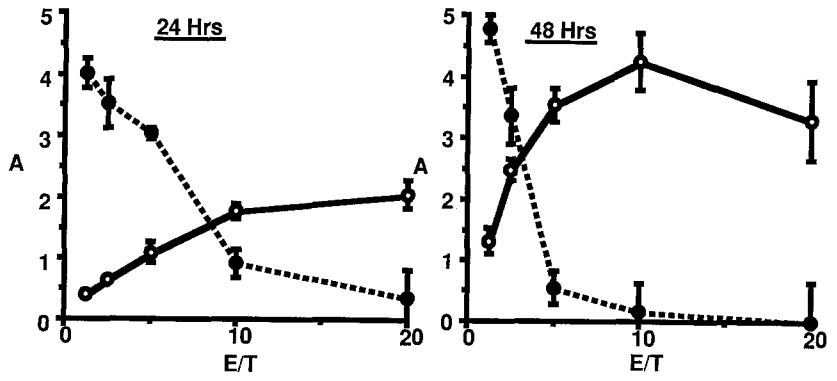

Fig. 3. Cytotoxicity of LAK cells to SQ-5 at E/T ratios of 1.25–20. Specific cytotoxicity (–○–) and survival of target cells (– – ● – –) were calculated as described in Materials and methods. *Points* are means from four replicate samples; *bar*, SD

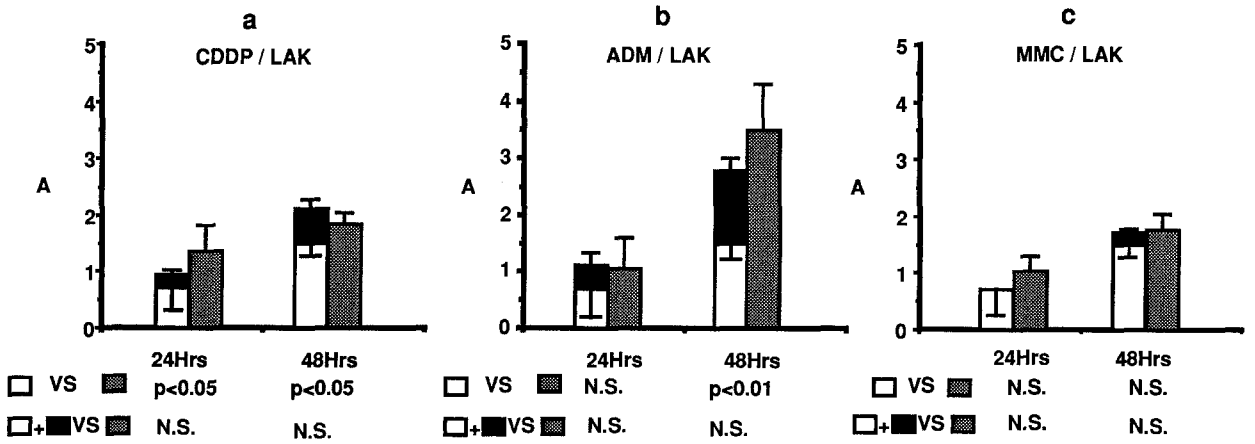

Fig. 4. Effect of pretreatment of target cells with antitumor drugs on LAK cell cytotoxicity detected as specific cytotoxicity. Target tumor cells are precultured in medium containing cisplatin (*CDDP*; 1 μg/ml), doxorubicin (*ADM*; 0.05 μg/ml) or mitomycin C (*MMC*; 0.01 μg/ml) for 24 h. The specific cytotoxicities of LAK cells combined with antitumor drugs (*shaded columns*), LAK cells without antitumor drugs (*white columns*), and antitumor drugs without LAK cells (*black columns*) are shown. *Columns* are means for eight determinations; *bar*, SD; *N. S.*, not significant

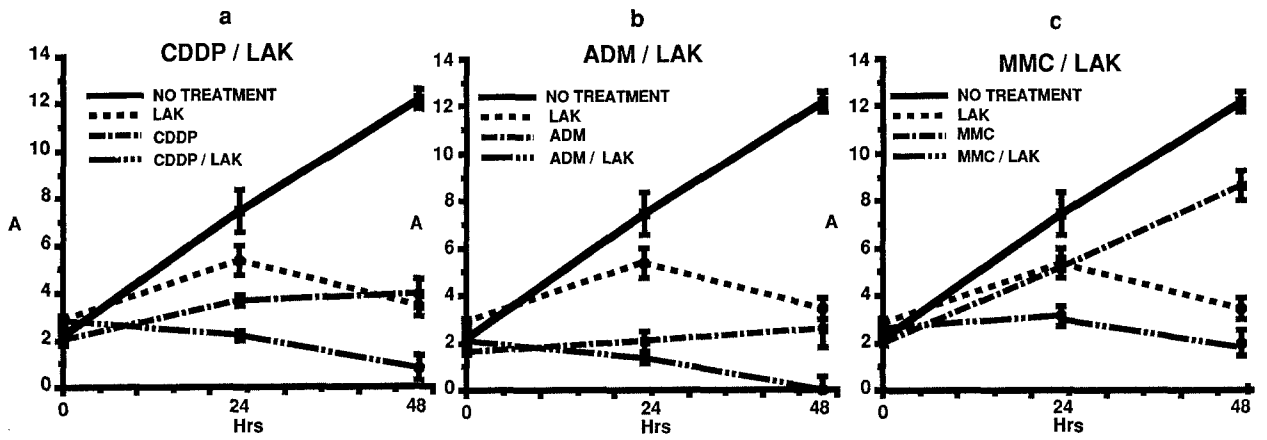

Fig. 5. Effect of pretreatment with antitumor drugs on LAK cell cytotoxicity detected as survival of target cells. Target tumor cells are precultured in medium containing cisplatin (*CDDP*; 1 μg/ml), doxorubicin (*ADM*; 0.05 μg/ml) or mitomycin C (*MMC*; 0.01 μg/ml) for 24 h. Survival of target cells without treatment (———), after treatment with LAK cells only (– – – –), after treatment with antitumor drugs only (–●–), and after treated with LAK cells and antitumor drugs (–●●–) are shown. Survival of target cells was calculated as described in Materials and methods. *Points* are means for eight determinations; *bar*, SD

and determined the specific cytotoxicity and target cell survival as defined in Materials and methods. The results are shown in Figs. 4 and 5 respectively. In Fig. 4, comparison is made between target cells 24 h and 48 h after the LAK cell addition with (shaded columns) or without (white columns) pretreatment of a drug. In the case of cisplatin (Fig. 4a), pretreatment of the target tumor cells resulted in significant (P <0.05) increase in release of LDH activity into the culture supernatant both in the 24-h assay and in the 48-h assay. Doxorubicin augmentated the LAK cell cytotoxicity only in the 48-h assay (Fig. 4b, P <0.01). No significant augmentation of LDH release from target cells was observed on treatment with a combination of mitomycin C at 0.01 μg/ml and LAK cells in either the 24-h assay or the 48-h assay (Fig. 4c).

Target cells that were pretreated with an antitumor drug for 24 h and then incubated for a further 24 h or 48 h without co-culturing with LAK cells released various amount of LDH activity into the culture supernatant (Fig. 4, black columns). The sum of this amount and the LDH activity released from the target cells without pretreatment of a drug (white columns in Fig. 4) was not significantly different from the LDH activity released from target cells treated with combinations of any one of the three drugs and LAK cells (shaded colums).

Figure 5 shows the growth patterns of target cells treated with cisplatin, doxorubicin and mitomycin C. Cell growth was monitored by LDH activity remaining in the target cell lysate after culture with or without LAK cells. The target SQ-5 tumor cells grew linearly for 48 h when not treated with drugs or LAK cells. However, their growth was apparently reduced on pretreatment with cisplatin, doxorubicin, or mitomycin C (Fig. 5a, b, c respectively). Co-culture of the target cells with LAK cells, without the pretreatment of drugs, also inhibited growth of the target cells. In the 48-h assay, although the target cells had grown considerably by 24-h of co-culture, LAK cells reduced the number of target cells to the level at the start of co-culture. Combination treatment of the target cells with each of the three drugs and LAK cells reduced the target cell number, the combination of doxorubicin and LAK cells causing complete target cell killing in the 48-h assay, as shown in Fig. 5b. After 48 h (Fig. 5a) the mean value of the cisplatin/LAK group was significantly different (P <0.01) from those of the groups treated with cisplatin alone or LAK alone. These significant differences were also observed after 48 h in the doxorubicin/LAK and the mitomycin C/LAK groups (Fig. 5b, c).

Discussion

The results of studies of human cells and animal models have suggested that adoptive immunotherapy should be effective in combination with chemotherapy for the treatment of tumors. In vitro studies have shown that human tumor cell lines displaying multi-drug resistance that is either intrinsic or the result of in vitro drug exposure retain their sensitivity to LAK cells [2, 6, 12]. In animal studies, Salup and Wiltrout [17] demonstrated that doxorubicin and LAK cells act synergistically in increasing the survival rate of animals with an established murine renal carcinoma. Cyclophosphamide and LAK therapy were also reported to have synergistic effects on the survival of mice bearing an established tumor [10].

In the present study we found that target tumor cells pretreated with cisplatin or doxorubicin released a significantly greater amount of LDH activity into the culture supernatant than did control target cells without pretreatment with drugs (Fig. 4a and b). Leroux et al. [9] have reported similar results: using a ^{51}Cr-release assay, they found that pretreatment with mitomycin C or doxorubicin enhanced the sensitivity of murine lymphoma cells to the killing action of LAK cells. However, we found that, when LDH release induced by pretreatment with antitumor drugs was taken into account, the effects of LAK cells and the drugs were not synergistic but merely additive, suggesting that the cytocidal effects of cisplatin or doxorubicin and LAK cells were additive.

We determined the cytostatic effect of LAK cells on the target cells by measuring LDH activity in the cell lysate. Results indicated that LAK cells exerted cytostatic effects against drug-pretreated cells as well as against untreated control target cells. This observation is consistent with a report by Passerini et al. [11]. Furthermore, with a suitable combination of treatments, such as doxorubicin and LAK cells, 100% killing of tumor cells could be achieved in the 48-h assay.

Recently, assay methods that do not require isotope-labeled targets, such as tetrazolium-based compound (MTT) assay and clonogenic assay, have been proposed as alternatives to ^{51}Cr-release assay [7, 18]. Both the MTT and clonogenic assays have been used extensively for assessment of the chemosensitivity of tumor cells, and thus may also be available for evaluating the effects of combinations of antitumor drugs and LAK cells. However, they cannot be used to differentiate cytocidal and cytostatic effects on cells as discussed by Carmicael et al. [3]. The LDH-release assay was first described in 1983 by Korzeniewski and Callewaert as a sensitive method for measuring natural cytotoxicity. Later, this method was applied to cytotoxicity assays for macrophages, natural killer cells, cytotoxic T cells and a cytokine, tumor necrosis factor, and also to assess drug toxicity [1, 4]. In the present study, we modified this technique and used it for assessing the effects of combinations of antitumor drugs and LAK cells in vitro.

The main advantage of this method is that it can be used to detect both cytocidal and growth-inhibitory effects of treatments simultaneously. In addition, this enzyme-release assay is easily adjustable to a laboratory automated processor. The disadvantage of our modified LDH-release assay is that spontaneous LDH release from LAK cells is not negligible at an E/T ratio of 20. Thus the assay should be performed at E/T ratios of less than 20. However, the LDH assay is an inexpensive and quantitative method for measurement of cytotoxicity at low E/T ratios, and is suitable for use in examining the combined effects of chemotherapy and LAK cell therapy in a model system.

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