Urol Res (1989) 17:259–264

Urological Research

Assessment of in vitro lymphokine activated killer (LAK) cell activity against renal cancer cell lines and its suppression by serum factor using crystal violet assay

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Accepted: October 7, 1988

Summary. Lymphokine activated killer (LAK) cell activity against renal cancer cell lines was assessed in vitro using a crystal violet assay. A standard 4-h ^{51}chromium release assay and a 48-h crystal violet assay showed that both natural killer-susceptible (NC65) and -resistant (ACHN) renal cancer cell lines were sensitive to LAK cells which had been generated by a 3-day incubation of peripheral blood mononuclear cells (PBMC) with recombinant interleukin 2 (rIL-2). Optimal LAK activity was generated by a 5-day culture of PBMC with 1 U rIL-2/ml. LAK activity was enhanced by the presence of IL-2 in the crystal violet assay system, while it was suppressed by fresh autologous serum. The suppressive effect was found in serum from both normal donors and patients with metastatic renal cell carcinoma, suggesting that non-specific suppressive factor(s) affecting LAK cell activity were present in human sera.

Key words: LAK cells – Renal cancer – Serum suppressive factor – Crystal violet assay

Introduction

Lymphokine activated killer (LAK) cells were originally described by Grimm et al. [5] as interleukin 2 (IL-2)-activated effector cells. Natural killer (NK)-resistant as well as NK-susceptible target cells were lysed by LAK cells, and LAK activity was demonstrated against freshly isolated autologous and allogeneic tumor cells as well as tumor cell lines.

Recent results of clinical trials by Rosenberg et al., in which in vitro generated autologous LAK cells were infused with recombinant (r) IL-2 into patients with advanced cancer, demonstrated a promising new approach to the immunotherapy of patients with metastatic cancer [14, 15]. Among the various malignancies treated in their study, renal cancer seems to have been one of the most sensitive targets. However, the precise antitumor mechanism of adoptively transferred LAK cells and IL-2 is not understood yet, and several different inhibitory responses against LAK activity by the host have been reported [3, 17]. Therefore, we developed a simple non-isotopic method using crystal violet staining to assess the in vitro LAK cell activity against renal cancer cell lines, and then utilized this experimental model for analyzing factors which modulate LAK cell activity both at the induction and effector phases.

Materials and methods

Target cells

Target cell lines used in the present study were the K562 myeloid leukemia cell line, the NC65 renal cell carcinoma cell line from Dr. F. H. Schroeder, Department of Urology, Erasmus University Rotterdam [6] and the ACHN renal cell carcinoma cell line from the American Type Culture Collection. All cell lines were maintained in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS: Flow Laboratories, Inc., McLean, Va.), hereafter referred to as complete medium (CM).

IL-2

Purified human rIL-2 was obtained from Takeda Chemical Industries Inc. (Osaka, Japan). Its specific activity was 3.5×10^4 units/mg protein as determined by the ability to maintain the NKC3 IL-2 dependent murine cell line, as described previously [8], and was equivalent to 1.2×10^7 standard units/mg of rIL-2 provided by the Biological Response Modifier Program, NCI.

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of healthy donors and from patients with

Table 1. Antitumor activities of fresh PBMC and LAK cells in 4-h ^{51}Cr release assay[a]

Target cells	% specific lysis at following E/T ratios			
	Fresh PBMC		LAK cells	
	40:1	10:1	40:1	10:1
K562	59 ± 2[b]	37 ± 3	83 ± 8	68 ± 7
NC65	23 ± 9	12 ± 8	70 ± 10	60 ± 1
ACHN	−4 ± 3	−3 ± 2	58 ± 7	30 ± 3

[a] Antitumor activities of PBMC cultured with 1 U rIL-2/ml for 3 days and fresh PBMC from five normal donors were assessed in a 4-h ^{51}Cr release assay

[b] Mean ± SD

metastatic renal cell carcinoma by Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient centrifugation and were suspended in CM at a concentration of 1×10^6/ml as previously described [2]. Nonadherent cells obtained by a 1-h incubation of PBMC in a plastic dish (25,020, Corning Glass Works, Corning, NY) were referred as peripheral blood lymphocytes (PBL).

Induction of lymphokine activated killer cells

PBMC at 1×10^6/ml in CM were cultured with various concentrations of rIL-2 for 1 to 7 days at 37°C in a humidified atmosphere of 5% CO_2 in air in 25 cm^2 flasks (25,100: Corning).

Human sera

Peripheral blood was drawn from healthy donors and patients with metastatic renal cell carcinoma. After the blood had been allowed to clot and retract, the serum was removed by centrifugation and used as fresh autologous human serum (AHS). Human AB serum (Flow) was stored at −20°C, and was used as control human serum (CHS). AHS and CHS were used without heat-inactivation unless otherwise stated.

^{51}Cr-release assay

A 4-h ^{51}Cr release assay was performed as described previously [20]. Briefly, 100 µl labeled target cells (5×10^3) and 100 µl effector cells in different numbers were added to wells of round-bottomed microtiter plates (Linbro Chemical, Hamden, CT). After a 4-h incubation the supernatant was collected and the radioactivity was counted in a gamma counter. The percent lysis was calculated by the formula: [(experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)] × 100. Spontaneous cpm was the amount of ^{51}Cr released from the target cells in the absence of effectors. Maximum cpm was the amount of ^{51}Cr released from target cells lysed by the addition of 8% Detergent 7X (Flow).

Crystal violet assay

Target cells (2×10^4) in 1 ml of CM were added to 24-well plates (25,820: Corning). After 20 h of incubation, effector cells at different numbers were added, and the plates were incubated at 37°C in 5% CO_2 for 48 h. The plates were then washed to remove effector cells and lysed target cells, and were fixed and stained with 0.5% crystal violet in 20% methanol. The dye was eluted with Sorenson's buffer (6.1 ml of 0.1 M disodium citrate, 3.9 ml of 0.1N HCl, and 10 ml of 95% ethanol), and the eluted material's absorbance at 570 nm was determined using a spectrophotometer (Shimazu Corporation, Kyoto, Japan). The cytostatic and cytolytic activities were expressed as the percent growth inhibition, which was calculated as follows: [(control optical density(OD) − experimental OD)/control OD] × 100. Control OD was determined from wells in which target cells were incubated alone.

Statistical analysis

All determination were made in triplicate, and statistical evaluation of the data was performed using the Student's t test.

Results

Susceptibility of renal cell carcinoma cell lines to NK cells and LAK cells

ACHN and NC65 were tested for susceptibility to fresh NK cells and LAK cells (Tables 1 and 2). PBMC or PBL expressed considerable antitumor activity against NC65 cells in a 4 h ^{51}Cr release assay and in a 48 h crystal violet assay. Their antitumor activity was enhanced by culture of effector cells with IL-2. On the other hand, ACHN cells were neither lysed nor growth inhibited by PBMC or PBL, while they were susceptible to LAK cells in both assays.

Optimal conditions for LAK induction

PBMC were cultured for 3 days with different concentrations of rIL-2 and tested against NK-resistant ACHN cells in a 48-h crystal violet assay (Fig. 1). Growth inhibitory activity was detectable when PBMC were cultured with 0.1 U rIL-2/ml, reaching maximum activity at 1–10 U/ml. Then, PBMC were cultured with 1 U rIL-2/ml for time intervals varying from 1 to 7 days. An antitumor effect was evident on day 3, and peaked on day 5 (Fig. 2).

Enhancing effects of IL-2 at the effetor phase of LAK

The effects of rIL-2 on LAK activity at the effector phase were examined using a 48-h crystal violet assay. Growth inhibitory effects of optimally induced LAK

Table 2. Antitumor activities of fresh PBL and LAK cells in 48-h crystal violet assay[a]

Target cells	% growth inhibition at following E/T ratios					
	Fresh PBL			LAK cells		
	20:1	5:1	1.25:1	20:1	5:1	1.25:1
NC65	37 ± 10[b]	15 ± 5	2 ± 1	96 ± 2	76 ± 9	39 ± 8
ACHN	0.3 ± 0.3	0.3 ± 0.5	0.1 ± 0.1	91 ± 3	57 ± 8	10 ± 6

[a] Antitumor activities of PBMC cultured with 1 U rIL-2/ml for 3 days and fresh PBL from five normal donors were assessed in a 48-h crystal violet assay
[b] Mean ± SD

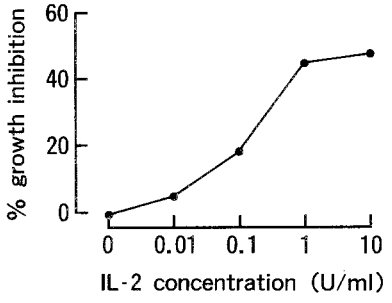

Fig. 1. PBMC from a normal donor were cultured with different concentrations of rIL-2 for 3 days, and growth inhibitory activities were assessed against ACHN in a 48-h crystal violet assay at 10:1 effector/target(E/T) ratio. The standard deviations did not exceed 10%. Similar results were obtained in 4 different donors

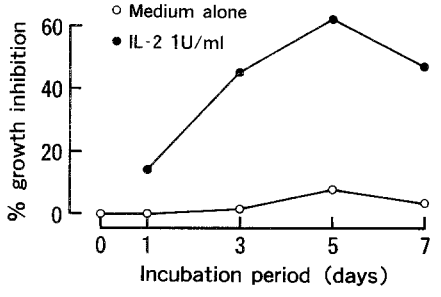

Fig. 2. PBMC from a normal donor were cultured alone (○) or with 1 U rIL-2/ml (●) for various time intervals, and assessed for the antitumor activity against ACHN in a 48-h crystal violet assay at 10:1 E/T ratio. The standard deviations did not exceed 10%. Similar results were obtained in 3 different donors

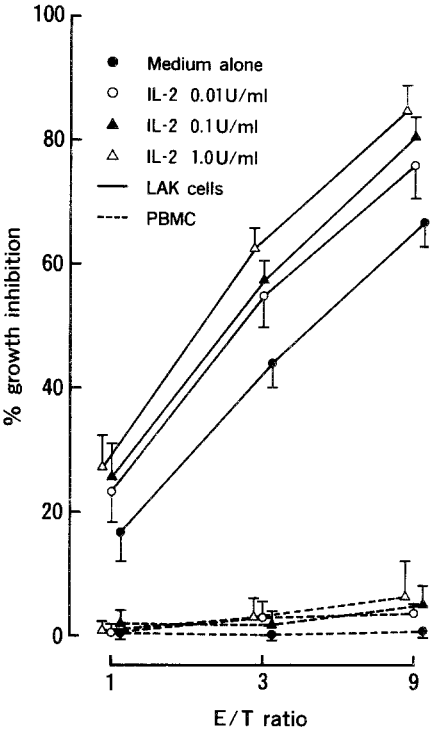

Fig. 3. PBMC from 5 normal donors were cultured alone or with 1 U rIL-2/ml for 5 days, and the antitumor activities were assessed against ACHN in the presence of different concentrations of rIL-2 in the assay. The addition of rIL-2 significantly enhanced the LAK activity at all E/T ratios tested ($P < 0.01$). Each point represents mean ± SD of 5 separate experiments

cells (5-day incubation with 1 U rIL-2/ml) against ACHN cells were enhanced when rIL-2 was added to the assay system (Fig. 3). This enhancement was dependent on rIL-2 doses and seen at all effector/target (E/T) ratios tested. By contrast, PBMC cultured alone for 5 days showed no antitumor activity even with the addition of IL-2 at the effector phase.

Suppressive effects of fresh autologous serum at the effector phase of LAK

To determine the effects of human serum at the effector phase of LAK cells, complete medium was either supplemented with 10% fresh autologous serum or with 10% control human AB serum. The LAK activity

Table 3. Suppressive effects of fresh autologous serum on LAK activity at the effector phase[a]

LAK cells	% growth inhibition at 5:1 E/T ratio	
	Control serum	Autologous serum
Normal donor		
1	56 ± 5	33 ± 5
2	34 ± 2	22 ± 3
3	45 ± 7	23 ± 3
4	50 ± 5	31 ± 4
5	34 ± 1	23 ± 3
Mean ± SD	44 ± 10	26 ± 5[b]
RCC patient		
1	55 ± 7	27 ± 4
2	46 ± 5	23 ± 4
3	24 ± 1	13 ± 2
4	51 ± 5	34 ± 4
5	54 ± 1	45 ± 2
Mean ± SD	46 ± 12	28 ± 12[c]

[a] PBMC from 5 healthy donors and 5 patients with metastatic renal cell carcinoma were cultured with 1 U rIL-2/ml for 5 days, and the antitumor activities were tested against ACHN in a 48-h crystal violet assay in two different media: 1) CM + 10% control serum; 2) CM + 10% fresh autologous serum

[b,c] $P < 0.01$ compared with control serum

was significantly reduced when 10% fresh autologous serum was added to the crystal violet assay as compared to CM supplemented with 10% control human AB serum (Table 3). The effector phase suppression was demonstrable using serum from both normal donors and patients with metastatic renal cell carcinoma. There were no difference in numbers of ACHN cells cultured for 2 days with fresh autologous serum and control human AB serum in the absence of effector cells. The suppressive activity of fresh serum was not abolished by heating at 56°C for 30 min (Fig. 4).

The effect of fresh autologous serum at the effector phase was also tested in a 4-h ^{51}Cr release assay (Fig. 5). However, the LAK activity was not reduced by the addition of fresh autologous serum in a short term ^{51}Cr release assay.

Discussion

A short term ^{51}Cr release assay has been used to assess the cytotoxicity of NK and LAK cells against freshly isolated solid tumor cells and established tumor cell lines. When cultured tumor cell lines of parenchymal origin are used as targets, they are usually adherent cell lines which require trypsinization for harvesting and a post-trypsinization incubation (> 4 h) for them to

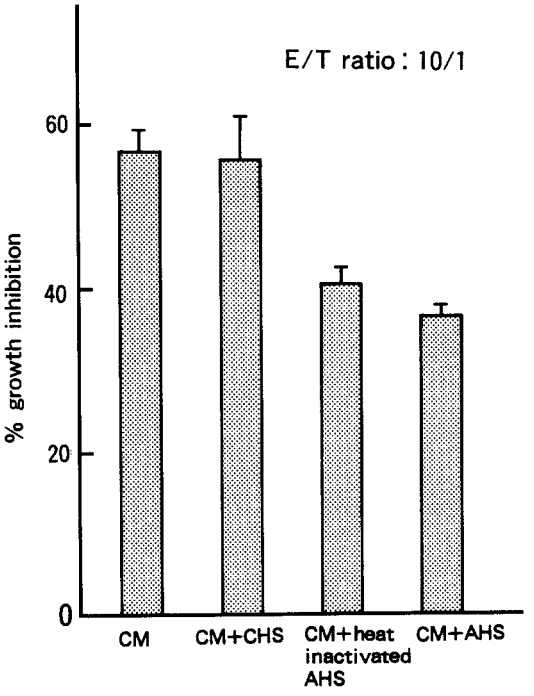

Fig. 4. PBMC from a normal donor were incubated with 1 U rIL-2/ml for 5 days, and the influence of heat-inactivation on the suppressive activity of fresh autologous serum was tested. The addition of 10% heat-inactivated AHS as well as fresh 10% AHS significantly reduced the LAK activity against ACHN as compared to CM + 10% CHS ($P < 0.02$) or CM alone ($P < 0.01$) at 10:1 E/T ratio. The data are from one of 4 representative experiments. Columns = mean of triplicate samples; bars = SD

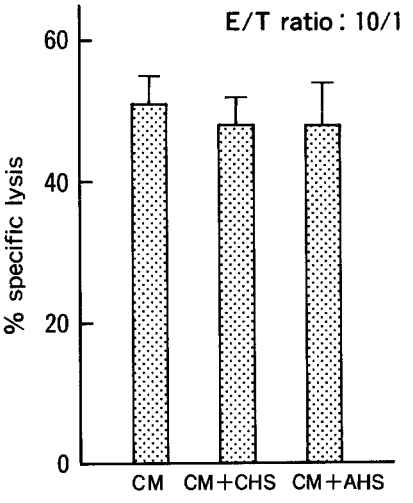

Fig. 5. PBMC from a normal donor was incubated with 1 U rIL-2/ml for 5 days, and the effect of fresh autologous serum at the effector phase was tested in a 4-h ^{51}Cr release assay. The LAK activity against ACHN was not significantly reduced in the presencve of 10% AHS as compared to CM + 10% CHS or CM alone at 10:1 E/T ratio. The data are from one of three representative experiments. Columns = mean of triplicate samples; bars = SD

recover from antigen modification induced by the enzymatic treatment [19]. However, such recovery period cannot occur in a standard ^{51}Cr release assay because of the rapid increase of spontaneous ^{51}Cr release from cells of adherent lines. In the present study, we applied a non-isotopic method employing crystal violet staining [9, 18], a technique already used to investigate the antiproliferative effects of cytokines, in order to assess the cytostatic and cytolytic activities of PBL and LAK cells against adherent tumor cell lines of renal cell carcinoma origin. Thus, a possible trypsinization-induced antigen modification of the target cells could be excluded in our 48-h assay. Culture of PBMC from healthy donors with IL-2 resulted in an enhancement, and induction, of antitumor activity against NK-sensitive and -resistant renal cell carcinoma cell lines, respectively. These results were obtained in both a standard 4-h ^{51}Cr release assay and a 48-h crystal violet assay. The optimum condition required for LAK induction obtained by the use of a 48-h assay in the present study was similar to that done by using a short term ^{51}Cr release assay in previous reports [11, 12].

The present study demonstrated that rIL-2 could enhance in vitro LAK cell activity at the effector phase. This finding is of importance, since it has been shown in animal models that combined treatment with LAK cells and rIL-2 had a greater therapeutic effect than either treatment alone [10]. The exact role of rIL-2 during such adoptive transfer of LAK cells in vivo, however, has not yet been understood. There are two possible explanations. One is that the activity of adoptively transferred LAK cells is enhanced, and the other is that LAK precursor cells are activated in vivo to become LAK effector cells[4]. Our in vitro results, together with recent results by others [13] that IL-2 promotes conjugation of effector cells with tumor target cells, could support the former possibility, although the latter can not be ruled out.

In the present study, fresh autologous serum suppressed LAK activity at the effector phase in a 48-h crystal violet assay. Furthermore, not only serum from advanced renal cancer patients but also that from normal donors, although tested at only one serum concentration, inhibited the antitumor function of LAK cells. In contrast, it has been reported that the serum of melanoma patients suppressed the induction of LAK activity, with no effect on the effector phase of LAK cells using a 4-h ^{51}Cr release assay. The presence of their serum-borne suppressive factor correlated strongly with the stage of the disease, while the serum from normal donors did not show any suppressive activity [1, 7]. These differences may be due to different assay systems, since the effector phase suppression by autologous serum was not observed in our study when a short term ^{51}Cr release assay was used. Therefore, the serum suppressive effect shown in a 48-h assay seems to be associated with less rapid mechanisms of cytolysis or cytostasis mediated by LAK cells rather than the rapidly occuring cytotoxic events which can be detected by a 4-h assay.

The serum suppressive factor observed in the present study may also be different from the "blocking factor" described by Hellström et al., since that is believed to consist of either tumor-associated antigen-antibody complexes or free antigens [16]. Several other mechanisms, such as prostaglandin-mediated immune suppression, anti-LAK antibody or circulating IL-2 receptors, have been reported to inhibit the generation and the activity of LAK cells [3, 21]. However, the possible role of these mechanisms in the effector phase suppression has not been explored in the present study, and the identity of the serum suppressive factor(s) is not known. The present results indicate that a non-specific suppressive factor present in fresh human serum could affect in vivo the antitumor activity of passively transferred LAK cells.

Acknowledgements: The authors are grateful to T. Yamashita for technical assistance and to Dr. A. Uchida, Department of Radiation System Biology, Radiation Biology Center, Kyoto University, for comments on the manuscript.

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