Original Article

Study on Lymphokine Activated Killer (LAK) Cells

I. Improved LAK cell induction in vitro

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Abstract: The optimum incubation period, serum or plasma concentration, recombinant interleukin 2 (rIL-2) concentration, cell density and kind of culture bottles for inducing lymphokine activated killer (LAK) cells in vitro from normal peripheral blood lymphocytes (PBL) were examined. Culture of PBL, at a high cell density of 2×10^6/ml with 9 U/ml of rIL-2 (TGP-8) and 2% of serum (or plasma) for 3 days in a flat bottle, was most effective, although the cell density of 1×10^6/ml and 8 U/ml of rIL-2 has been used so far. ABO matched allogeneic frozen plasma was shown to be as effective to induce LAK cells as AB sera. This modification could reduce the volume of medium, number of culture bottles and CO₂ incubator space, and cost required in mass culture for practical use.

Key words: LAK, IL-2, Adoptive immunotherapy

INTRODUCTION

Modern biotechnology has enabled us to proliferate just the useful cells selectively in vitro, indefinitely, by using suitable growth and/or differentiation factor(s). Owing to gene technology, such factors are now mostly available in recombinant form, which ensures quality and quantity.

One of such factors, interleukin 2 (IL-2) has already been known to proliferate activated T cells, natural killer (NK) cells, and cytotoxic T cells. IL-2 was at first expected to be useful for treating patients with malignant tumors, by inducing cytotoxic cells. However, its effect was found to be rather limited. One of the reasons was that the half life of IL-2 was within several minutes in vivo, so an enormous amount was required to maintain an effective concentration which then produced severe side effects.

On the other hand, Rosenberg et al. proposed “adoptive immunotherapy”, which meant that instead of inducing cytotoxic cells in vivo by injecting large amounts of IL-2, cytotoxic cells were induced in vitro in the presence of a suitable concentration

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of IL-2 and then cytotoxic cells were injected back to the same patient with a malignant tumor from whom peripheral blood lymphocytes (PBL) had been collected for induction.

They called such cytotoxic cells as lymphokine activated killer (LAK) cells\(^2,3\) and they could kill even NK resistant solid tumor cells rather in a broad spectrum without MHC restriction. However, they did not kill normal cells due to some unknown recognition mechanism.

In their clinical trial\(^9,11\) such LAK cell therapy was effective against drug resistant and radiation resistant malignant tumor in advanced stage.

Here, we present some basic data on LAK cell induction in vitro before we attempt clinical application against malignant tumors.

**Materials and Methods**

*Effector cells.* Lymphocytes were collected from heparinized peripheral blood of normal donor by using a Ficoll-Paque (Pharmacia, Sweden) gradient centrifugation method. Throughout the experiment the blood donor was the same normal individual.

*Medium.* RPMI-1640 (Gibco, USA) powder was dissolved with ultra pure water made by a Milli Q system (Millipore, USA) and sterilized through a filter with a pore size of 0.22 \(\mu\) (Millipore, USA).

Complete medium was made by addition of 100 U/ml penicillin G (Meiji Pharmaceutical Co., Japan), 100 \(\mu\)g/ml streptomycin (Meiji Pharmaceutical Co., Japan), and 2 mM glutamine (Nakarai Chemical, Japan) to the RPMI-1640 medium.

Serum or plasma was also added in various concentrations as indicated after inactivation by heating at 56°C for 30 minutes.

*Target Cells.* Daudi, a NK resistant human Burkitt’s lymphoma cell line, or K562, a NK-sensitive human erythroleukemia cell line was used for the cytotoxic assay. Normal PBL were also used as a control.

*Recombinant IL-2.* TGP-3 was supplied by Takeda Pharmaceutical Co Ltd., Japan.

*Induction of LAK cells.* After confirming the viability over 95% by dye exclusion test with 0.1% nigrosin B, 1−3×10^6/ml of PBL were cultured in complete media with various concentrations of serum or plasma as indicated, with various concentrations of rIL-2 for 2 days to 6 weeks at 37°C under 5% \(\text{CO}_2\) in a \(\text{CO}_2\) incubator (Tabai Espec, Japan). Culture was done either left standing or with rotation on a roller bottle apparatus, (Bellco, USA) at 1 rpm.

*Cytotoxicity Assay.* \(^{51}\text{Cr}\) (ICN, USA) was labeled to target cells by incubating 100–150 \(\mu\)Ci of \(^{51}\text{Cr}\) with 1×10^6 target cells in 0.2 ml of complete medium for one hour in a \(\text{CO}_2\) incubator. After washing cells with complete medium 3 times, viability was checked by the dye exclusion test and usually it was over 95%.

Into each well of a 96-well U type microtiter plate (Nunc, Denmark) we added 1×10^4 radiolabeled target cells and effector cells at the ratio of 50:1, 25:1, 12:1, or 6:1 against the target cells. Total volume was 220 \(\mu\)l/well. The plate was incubated at 37°C in 5% \(\text{CO}_2\) for 4 hours, and then centrifuged at ×200 g, at 4°C for 5 minutes. The radioactivity in 100 \(\mu\)l of each supernatant was counted by a \(\gamma\)-counter (Aloka, Japan). Spontaneous release was counted from the well without the addition of effector cells. Maximum release was counted from the well where the same number of target cells were incubated with 0.05% NP-40 for 4 hours.
Percent killing was calculated according to the following formula and expressed as mean value±SD of triplicate experiments.

\[
\% \text{Killing} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100
\]

Statistical significance was examined by Student’s t-test.

**Results**

**Concentration of rIL-2**

PBL 1x10^6/ml were cultured for 6 days with 2% autologous serum and concentrations of rIL-2 at 0.5 U/ml, 1 U/ml, or 3 U/ml and cytotoxicity was examined against Daudi or K562 cells at different E/T ratios. As shown in Table 1, in both target cells the highest cytotoxicity of LAK was observed at a concentration of 3 U/ml of rIL-2 at most E/T ratios.

**Concentration of serum**

PBL 1x10^6/ml were cultured for 6 days with 3 U/ml of rIL-2 with 0.1, 0.5, 1, 2 or 5% of autologous serum and cytotoxicity was compared against Daudi or K562 cells. As shown in Table 2, no significant difference was observed between 1% to 5% against either target cells. However, at 0.1% and 0.5% it was too difficult to harvest enough cells to do the cytotoxic assay, and at 1%, the number of harvested cells was less than half of that at 2%.

**Comparison between autologous fresh plasma and allogeneic frozen plasma and allogeneic AB serum**

From a practical point of view for future application, cytotoxicity was compared among PBL cultured with 2% autologous fresh plasma, 2% allogeneic frozen plasma (ABO blood type matched) or 2% allo-

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**Table 1. Effect of concentration of rIL-2 on induction of LAK cells.**

<table>
<thead>
<tr>
<th>Target cells</th>
<th>conc. of rIL-2 (U/ml)</th>
<th>25/1</th>
<th>12/2</th>
<th>6/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daudi</td>
<td>0.5</td>
<td>61.1±6.2(^a)</td>
<td>@</td>
<td>44.3±6.0★</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>73.7±3.5(^★)</td>
<td>51.2±4.5</td>
<td>47.9±1.5</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>95.3±13.0(^★)</td>
<td>@</td>
<td>58.0±4.5★</td>
</tr>
<tr>
<td>K562</td>
<td>0.5</td>
<td>78.1±9.5</td>
<td>85.4±5.2</td>
<td>73.0±2.6(^★)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>69.8±8.5(^★)</td>
<td>83.0±2.3</td>
<td>51.5±7.3(^★)</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>90.7±3.6(^★)</td>
<td>78.5±8.1</td>
<td>69.9±8.4(^★)</td>
</tr>
</tbody>
</table>

* \(^a\) ★☆\(\odot\) p<0.05  ★ p<0.01

**Table 2. Effect of concentration of autologous serum on induction of LAK cells.**

<table>
<thead>
<tr>
<th>Target cells</th>
<th>conc. of serum</th>
<th>25/1</th>
<th>12/1</th>
<th>6/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daudi</td>
<td>1%</td>
<td>79.5±6.3</td>
<td>46.7±5.0</td>
<td>60.4±5.2</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>77.0±9.6</td>
<td>56.8±11.2</td>
<td>60.8±6.2</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>80.7±2.3</td>
<td>50.6±4.1</td>
<td>52.9±6.3</td>
</tr>
<tr>
<td>K562</td>
<td>1%</td>
<td>87.6±2.4(^★)</td>
<td>61.3±6.6</td>
<td>45.1±8.7</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>78.8±5.3</td>
<td>53.7±9.0</td>
<td>57.8±3.1</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>67.7±8.5(^★)</td>
<td>57.7±7.9</td>
<td>48.0±6.4</td>
</tr>
</tbody>
</table>

* \(^★\) p<0.05

Analysis among groups with different rIL-2 concentration

Analysis among groups with different serum concentration
geneic AB serum for 6 days. As shown in Table 3, significantly higher activity in autologous fresh plasma was observed at 50/1 and 25/1 E/T ratios compared with allogeneic plasma. However, no significant difference was observed in AB sera compared with autologous or allogeneic plasma.

Standing or rolling culture

PBL (1×10⁶/ml) were cultured with 2% autologous serum and 3 U/ml of rIL-2 for 6 days, in a flat flask (Falcon, USA) for standing culture or in a roller bottle (Falcon, USA) for rolling at 1 rpm on an apparatus (Bellco, USA) and cytotoxicity was compared.

As shown in Table 4, no significant difference was observed between standing culture and rolling culture. There was no difference in growth rate (data not shown).

Cytotoxicity of LAK cell against normal cells

To examine the cytotoxic effect of LAK cells against normal cells, normal PBL were used as the target. PBL (1×10⁶/ml) were cultured for 6 days with 3 U/ml rIL-2 and 2% autologous serum in standing or rolling culture and the cytotoxic test was performed against normal PBL. As shown in Table 5, almost no cytotoxicity was observed in either standing or rolling culture at any E/T ratio. A simultaneous experiment showed that these LAK cells remained highly cytotoxic when the target was tumor cells (Table 4).

Time for LAK cell induction

PBL were cultured at 1×10⁶/ml with 3 U/ml of rIL-2 and 2% autologous serum for 2~42 days, and cytotoxicity against Daudi cells was compared at different E/T ratios. Medium was changed every week in continued cultivation. As shown in Figure 1, LAK activity appeared on day 2, reached a peak on day 3 and continued until day 7. Thereafter, the activity decreased for six weeks.

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**Table 3.** Effect of autologous fresh plasma, allogeneic ABO matched frozen plasma or allogeneic heterologous frozen AB serum on induction of LAK cells.

<table>
<thead>
<tr>
<th>Plasma or serum</th>
<th>concentration</th>
<th>E/Ta</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50/1</td>
<td>25/1</td>
<td>12/1</td>
</tr>
<tr>
<td>autologous fresh plasma</td>
<td>2%</td>
<td>94.8±1.1*</td>
<td>78.0±2.7@</td>
<td>59.0±5.4</td>
</tr>
<tr>
<td>allogeneic frozen plasma</td>
<td>2%</td>
<td>80.2±3.2*</td>
<td>59.5±6.8@</td>
<td>49.4±4.8</td>
</tr>
<tr>
<td>AB serum</td>
<td>2%</td>
<td>93.1±10.6</td>
<td>70.2±8.2</td>
<td>55.3±7.7</td>
</tr>
</tbody>
</table>

* Target cells, Daudi analysis among groups with different plasma or serum
@ p<0.05  * p<0.01

**Table 4.** Effect of standing or rolling culture of PBL with rIL-2 on induction of LAK cells.

<table>
<thead>
<tr>
<th>Target cells</th>
<th>Cultivation</th>
<th>E/T</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>25/1</td>
<td>12/1</td>
<td>6/1</td>
</tr>
<tr>
<td>Daudi</td>
<td>Rolling</td>
<td>88.0±1.4</td>
<td>59.6±4.1</td>
<td>44.8±8.0</td>
</tr>
<tr>
<td></td>
<td>Standing</td>
<td>87.5±10.7</td>
<td>47.5±0.0</td>
<td>38.9±1.9</td>
</tr>
<tr>
<td>K562</td>
<td>Rolling</td>
<td>55.8±1.6</td>
<td>35.4±4.2</td>
<td>28.7±5.3*</td>
</tr>
<tr>
<td></td>
<td>Standing</td>
<td>61.0±4.1</td>
<td>53.5±7.3</td>
<td>48.5±4.8*</td>
</tr>
</tbody>
</table>

* p<0.05 analysis among groups with different culture condition
Table 5. Cytotoxicity of LAK cell against normal PBL.

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>E/T 25/1</th>
<th>E/T 12/1</th>
<th>E/T 6/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standing</td>
<td>1.4±2.2</td>
<td>4.5±1.0</td>
<td>2.1±2.5</td>
</tr>
<tr>
<td>Rolling</td>
<td>3.2±4.2</td>
<td>2.6±2.8</td>
<td>3.2±1.2</td>
</tr>
</tbody>
</table>

Table 6. Effect of cell density on induction of LAK cells.

<table>
<thead>
<tr>
<th>Cell density (×10^6/ml)</th>
<th>E/Ta 25/1</th>
<th>E/Ta 12/1</th>
<th>E/Ta 6/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1×10^6</td>
<td>70.4±5.1</td>
<td>59.9±4.4</td>
<td>57.7±8.0</td>
</tr>
<tr>
<td>2×10^6</td>
<td>88.0±2.7</td>
<td>76.8±2.9</td>
<td></td>
</tr>
<tr>
<td>3×10^6</td>
<td>67.6±5.3</td>
<td>63.8±1.2</td>
<td>56.3±9.1</td>
</tr>
</tbody>
</table>

*a Target cells, Daudi analysis among groups with different cell densities
@ p<0.05 # p<0.01 $ p<0.001

Table 7. Effect of rIL-2 concentration on induction of LAK cells at a high cell density.

<table>
<thead>
<tr>
<th>rIL-2 conc. (U/ml)</th>
<th>E/Ta 25/1</th>
<th>E/Ta 12/1</th>
<th>E/Ta 6/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>88.0±10.0</td>
<td>82.0±2.7</td>
<td>76.8±2.5</td>
</tr>
<tr>
<td>6</td>
<td>98.1±4.7</td>
<td>89.8±5.0</td>
<td>78.7±8.1</td>
</tr>
<tr>
<td>9</td>
<td>103.3±3.5</td>
<td>98.5±1.0</td>
<td>85.6±1.2</td>
</tr>
</tbody>
</table>

*a Target cells, Daudi analysis among groups with different rIL-2 concentrations
@ p<0.05 # p<0.01 $ p<0.001

Fig. 1. Days for LAK cell induction
1×10^6/ml of PBL were cultured with 3 U/ml of rIL-2 and 2% autologous serum in standing culture for 6 weeks and cytotoxicity against Daudi cell was examined at the E/T ratio of 25/1 (○), 12/1 (●) and 6/1 (▲).

Cell density

Since, the culture showed maximum cytotoxicity on day 3, different cell densities of 1×10^6/ml, 2×10^6/ml or 3×10^6/ml were compared on day 3. As shown in Table 6, cell density of 2×10^6/ml gave the highest cytotoxicity when PBL were cultured with 3 U/ml of rIL-2. Then, a much higher concentration of rIL-2 than that shown in Table 1 was tested for such a high cell density to obtain a higher cytotoxicity.

As shown in Table 7, when cytotoxicity was compared among different rIL-2 concentrations such as, 3, 6 or 9 U/ml at a cell density of 2×10^6/ml, 9 U/ml of rIL-2 could induce the highest cytotoxicity, especially at a low E/T ratio.

Discussion

Before applying adoptive immunotherapy clinically, we examined the optimum conditions for inducing LAK cells using our tissue culture facilities from the practical point of view.

LAK cell killing was shown to be so effective in vitro, that they could kill about 90% of the target cells even at a ratio of 6/1 after 3 days of culture (Table 6), although they did not kill PBL which were used as a representative of normal cells. (Table 5)

PBL, cultured at the initial cell density of 2×10^6/ml with 9 U/ml of rIL-2 and 2% plasma for 3 days by standing culture showed the most effective cytotoxicity. The cell density of 1×10^6/ml and 3 U/ml of
rIL-2 are usually used. The LAK activity decreased after culture over 1 week, as shown in Fig. 1. However, the time to change the medium was also a question.

One group reported\(^\text{11}\) that they had injected up to \(1 \times 10^{10} - 10^{11}\) LAK cells to patients, which meant that they used ten to hundred liters of medium, because \(1 \times 10^6 / \text{ml}\) of PBL is usually used. Therefore, from a practical point of view, it is necessary to minimize the volume of medium. Our results showed that by increasing the concentration of rIL-2 to 9 U/ml it was possible to culture PBL at such a high density as \(2 \times 10^6 / \text{ml}\) to induce high LAK activity. This modification could reduce the volume of medium, number of culture bottles and size of incubator space required into half.

In our study, rolling culture which has been recommended by others\(^\text{11}\) did not produce a higher cytotoxicity than standing culture for some unknown reasons even though the rolling system was in 5% CO\(_2\).

Since no discrepant cytotoxicity was observed between NK sensitive and NK resistant target cells, NK cytotoxicity was not considered to be involved in the present assay.

Our data also showed that ABO-matched allogeneic frozen plasma was almost as efficient as AB sera for inducing LAK cells although less efficient than autologous fresh plasma. It would be better to use allogeneic normal plasma than autologous plasma, because the patient’s plasma may contain a suppressive factor (manuscript in preparation). Considering the difficulty of obtaining as much AB sera as Rosenberg et al\(^\text{11}\) used, the present improvement to use frozen allogeneic plasma would help further clinical application and economy.

We have started to treat patients with renal cell carcinoma, hepatoma, colon carcinoma or glioblastoma using such improvements. (manuscript in preparation) Further improvements are expected to improve the effectiveness and prevent the side effects of this LAK therapy.

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References


Induction of LAK Cells


