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Enhancing Effect of Pokeweed Mitogen on the Proliferation and the Cytotoxicity of Lymphokine-activated Killer Cells

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In order to obtain more potent lymphokine-activated killer (LAK) cells for use in adoptive immunotherapy, pokeweed mitogen (PWM) was added to the culture medium for the initial 24–48 h of culturing. The proliferation rate of PWM-stimulated LAK cells reached about 1000-fold after 3-week culture. This rate was nearly the same as that of LAK cells stimulated by 10 ng/ml of OKT3, the mouse anti-CD3 monoclonal antibody. However, the cytotoxicity of PWM-stimulated LAK cells was significantly more potent than that of OKT3-stimulated LAK cells. Phenotypic analysis revealed that PWM-stimulated LAK cells were $CD3^+CD56^+$-dominant while OKT3-stimulated LAK cells were $CD3^+CD56^-$-dominant. About half of $CD3^+CD56^+$ PWM-stimulated LAK cells was $CD8^+$. These results suggest that more efficient adoptive immunotherapy is possible by using high-dose PWM-stimulated LAK cells with more potent cytotoxicity. Interleukin-1β and tumor necrosis factor α were significantly increased in the culture media after 24-h incubation with 1 μg/ml of PWM. Secretion of interferon-γ was not enhanced by this concentration of PWM within 24 h. Therefore, PWM is considered to activate monocytes or macrophages to produce these cytokines in advance, influencing the proliferation and the cytotoxicity of LAK cells.

Key words: LAK cell — Pokeweed mitogen

Adoptive immunotherapy using lymphokine-activated killer (LAK)[2] cells has been applied for the treatment of malignant diseases. Efficacy of adoptive immunotherapy using LAK cells and interleukin-2 (IL-2) has been reported on some malignancies such as malignant melanoma, renal cell carcinoma and non-Hodgkin's lymphoma.[1,2] However, one of the important problems of adoptive immunotherapy is how to obtain a number of potent LAK cells sufficient for frequent treatments to reduce the tumor burden. Rosenberg[3] recommended that more than 10^{10} LAK cells should be injected into one patient in one therapy.

On the other hand, mouse anti-CD3 monoclonal antibody enhanced the proliferation of LAK cells significantly.[4–10] Treatment of peripheral blood lymphocytes (PBL) with 10 ng/ml of OKT3 for the initial 48 h of culturing resulted in production of 1000-fold more LAK cells after 3 weeks' culture.[10] We also reported on the influence of OKT3 on the proliferation and the cytotoxicity of LAK cells.[11,12] With a static system, the LAK cells stimulated by 10 ng/ml of OKT3 for the initial 48 h proliferated 250-fold more after 3 weeks' incubation, and with a hollow fiber bioreactor system the number of LAK cells was increased 500-fold after 3 weeks' incubation. However, the cytotoxicity of these OKT3-stimulated LAK cells was significantly decreased, and this phenomenon might be explained by the decreased rate of $CD3^+CD56^+$ cells and the increase of $CD3^+CD56^-$ cells.[11] Then, in order to produce as potent and as many LAK cells as possible, we applied pokeweed mitogen (PWM),[13] which possesses a significant mitogenic activity on PBL, especially B cells, and induces immunoglobulin production from B cells. PWM could enhance both the proliferation and the cytotoxicity of LAK cells, and it made it possible to use more than 10^{10} LAK cells in one injection in adoptive immunotherapy.

In this paper, we report on the ability of PWM to produce almost the same proliferative rate of LAK cells as that of the LAK cells stimulated by OKT3, and also more potent cytotoxicity than that of the OKT3-stimulated LAK cells or LAK cells without any stimulation.

MATERIALS AND METHODS

Induction of LAK cells PBL were purified from heparinized blood of normal donors by using the Ficoll-Hypaque gradient sedimentation method. PBL were washed three times with phosphate-buffered saline (PBS), and incubated in RPMI 1640 containing 10^3 U/ml of recombinant IL-2 (TGP-3, Takeda Pharmaceutical Industries), 5 U/ml of heparin as an anticoagulant and

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[2] Abbreviations: LAK, lymphokine-activated killer; PWM, pokeweed mitogen; IL-2, interleukin 2; IL-1β, interleukin 1β; TNF, tumor necrosis factor; IFN-γ, interferon-γ; PBL, peripheral blood lymphocytes.

10% of human fresh plasma of the same blood type as that of the donors under a 5% CO_2 atmosphere at 37°C. With a static culture system the initial concentration of PBL was 2×10^6/ml.

PWM and OKT3 PWM (Sigma) was added at various concentrations from the start of the culture. PWM was washed out after 48-h incubation. Anti-CD3 monoclonal antibody OKT3 (Becton-Dickinson) was added to the culture for the initial 48 h at a concentration of 10 ng/ml as reported before. OKT3 was also washed out with PBS after 48-h incubation.

Cytotoxicity Three different target cells were used for the cytotoxicity assay. Cytotoxicity of LAK cells toward the natural killer-resistant cell lines Daudi and Raji was estimated by the 4-h ^{51}Cr-release assay. Cytotoxicity was calculated as follows: Cytotoxicity (%) = {(experimental release − spontaneous release) / (maximum release − spontaneous release)} × 100. On the other hand, the neutral red dye-uptake method was used for the cytotoxicity assay on such adhesive target cells as SW1116, human colon carcinoma cell line. Briefly, LAK cells and SW-1116 were incubated in a microtest plate at various effector-to-target ratios. After incubation for 48 h, when the control wells became confluent, LAK cells were washed out twice with PBS containing Ca^{2+} and Mg^{2+} (PBS+), and live adhesive target cells were allowed to take up neutral red during incubation for 1 h with neutral red-containing PBS+. Remaining neutral red was washed out and a mixture of 0.1 *N* HCl and 100% ethanol was added to destroy the target cells and to dissolve the neutral red. Absorbance was read by using a Titertek at the wavelength of 540 nm. Cytotoxicity was calculated as follows: Cytotoxicity (%) = $(1-A_{540}$ of experiment/A_{540} of control) × 100. Every cytotoxicity assay was carried out in triplicate.

Phenotype CD3, CD4, CD8, and CD56 were estimated by using monoclonal antibodies conjugated with fluorescein isothiocyanate or phycoerythrin. LAK cells were incubated in microtest plates with two different monoclonal antibodies for 1 h at 37°C. Cells were washed twice with PBS and two-color analysis was carried out by using a FACScan (Becton-Dickinson).

Cytokines Interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ) contained in the supernatant of LAK cells after 24-h incubation were estimated by enzyme-linked immunosorbant assay (ELISA) using monoclonal antibodies. ELISA kits were purchased from Otsuka Assay (IL-1β) and Genzyme (TNF-α, IFN-γ).

RESULTS

Optimal concentration of PWM PWM at various concentrations ranging from 0.1 μg/ml to 10 μg/ml was added during the initial 48 h of incubation, and the proliferation rate of LAK cells was estimated (Fig. 1). The incubation period was determined in preliminary experiments, and the concomitant presence of PWM and IL-2 for 24–48 h was sufficient to stimulate PBL. After 2 weeks' culture, LAK cells without PWM-stimulation had usually proliferated 2- to 5-fold. On the other hand, the proliferation rate of PWM-stimulated LAK cells was about 100-fold. There was no significant difference in the proliferation rate among the concentrations employed of PWM. After 3 weeks' culture the proliferation rate of PWM-stimulated LAK cells was increased about 1000-fold; it would be increased 2000- to 10,000-fold after another week or more of incubation. Higher concentrations of PWM stimulated PBL in 48 h, resulting in worse proliferation of LAK cells.

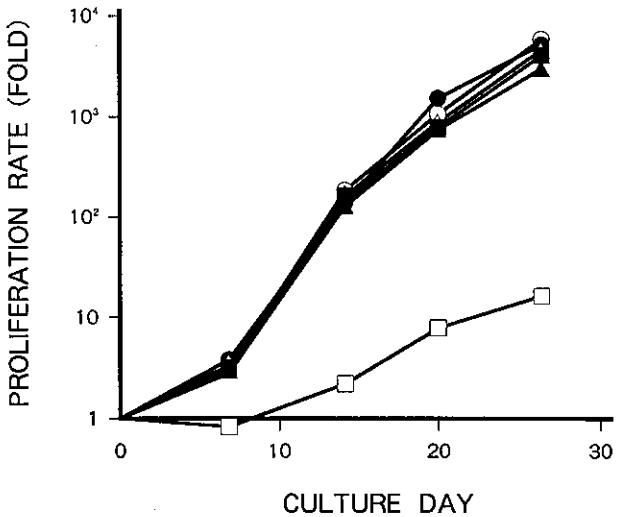

Fig. 1. Proliferation of LAK cells stimulated by various concentrations of PWM. PWM added: 0 μg/ml (□), 0.1 μg/ml (▲), 0.3 μg/ml (■), 1 μg/ml (△), 3 μg/ml (○) and 10 μg/ml (●).

Adherent cells were rarely observed in flasks when PBL was stimulated by PWM, while many cells, probably monocytes, were found to adhere to the bottom of flasks in ordinary culture without stimulant.

OKT3 at the concentration of 10 ng/ml could enhance the proliferation of LAK cells at almost the same rate as did PWM.

Cytotoxicity of PWM- or OKT3-stimulated LAK cells Cytotoxic activity toward SW1116 was estimated using PWM- or OKT3-stimulated LAK cells (Fig. 2). PBL was cultured with PWM at a concentration between 0.3 and 10 μg/ml for 48 h, or cultured with 10 ng/ml of OKT3 for 48 h. PWM or OKT3 was then removed and lymphocytes were incubated in IL-2-containing RPMI-

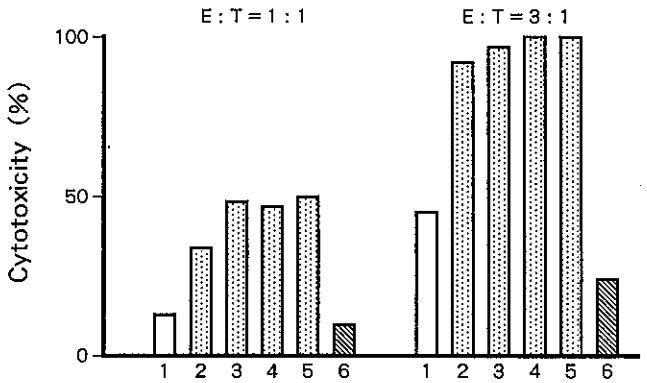

Fig. 2. Cytotoxicity of LAK cells stimulated by PWM or OKT3. 1: 0 μg/ml. 2: PWM 0.3 μg/ml. 3: PWM 1 μg/ml. 4: PWM 3 μg/ml. 5: PWM 10 μg/ml. 6: OKT3 10 ng/ml.

Table I. Effect of PWM on Cytotoxicity of LAK Cells

	Cytotoxicity against	
	Daudi	Raji
PWM (−)	60.6±7.6[a)]	24.9±8.1
PWM (+)	44.3±8.0	50.1±6.7

a) Mean±SE (%) was calculated from the results of four experiments.

1640. PWM-stimulated LAK cells showed more potent cytotoxicity than non-stimulated LAK cells. Any concentration of PWM between 0.3 and 10 μg/ml could enhance the cytotoxicity of LAK cells. However, OKT3-stimulated LAK cells usually killed a smaller number of target cells.

When Raji was used as target cells, 4-h ^{51}Cr-release assay revealed more potent cytotoxicity of PWM-stimulated LAK cells incubated in a gas-permeability bag for 2 weeks (Table I). As for Daudi, a natural killer-resistant but LAK-sensitive cell line, there was no significant difference in cytotoxicity between the non-stimulated and PWM-stimulated LAK cells.

The cytotoxicity of PWM-stimulated LAK cells was most potent after culture for 2 to 3 weeks.

Phenotypes Phenotypes of non-stimulated, PWM-stimulated and OKT3- stimulated LAK cells were studied by two-color analyses.

With CD3 and CD56 antigens (Fig.3), which are expressed on mature T cells and NK cells, respectively, $CD3^-CD56^+$ cells increased to reach about 50% of all non-stimulated LAK cells after 2 weeks' incubation. On the contrary, $CD3^-CD56^+$ cells accounted for less than 20% of PWM-stimulated LAK cells after 3 weeks' incubation. OKT3-stimulated LAK cells also gave a lower percentage of $CD3^-CD56^+$.

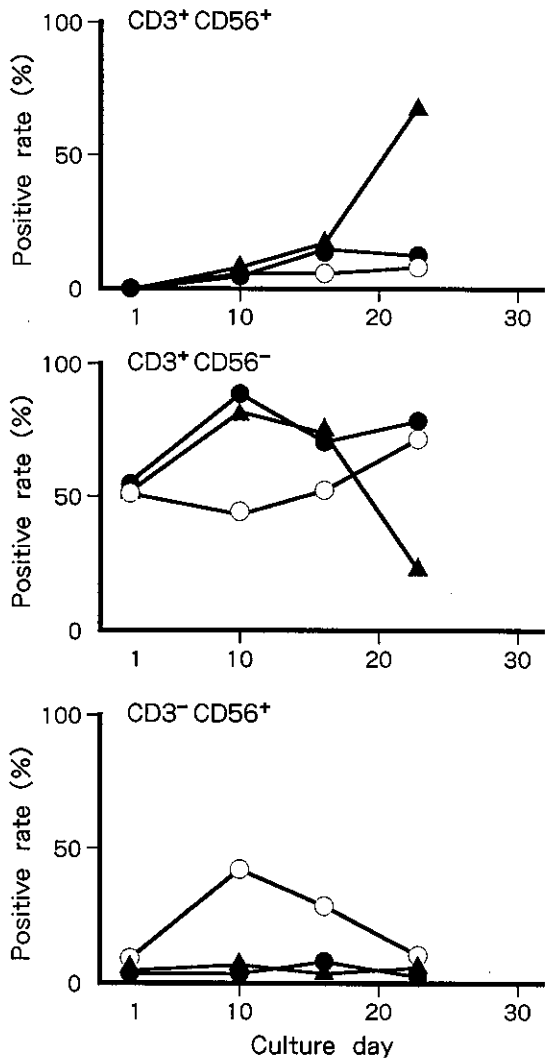

Fig. 3. Change of positive rates of $CD3^+CD56^+$, $CD3^+CD56^-$ and $CD3^-CD56^+$ LAK cells without stimulation (○) or stimulated by 0.3 μg/ml of PWM (▲) or 10 ng/ml of OKT3 (●).

On the other hand, $CD3^+CD56^+$ double-positive cells increased during incubation and these cells became dominant, amounting to about 30–50% of all PWM-stimulated LAK cells after 3 weeks' incubation. These double-positive cells continued to proliferate and accounted for 50% of PWM-stimulated LAK cells. Usually $CD3^+CD56^+$ cells were less than 10% of non-stimulated LAK cells and OKT3-stimulated LAK cells.

$CD3^+CD56^-$ cells, which accounted for about 60% of PWM-stimulated LAK cells just after stimulation by

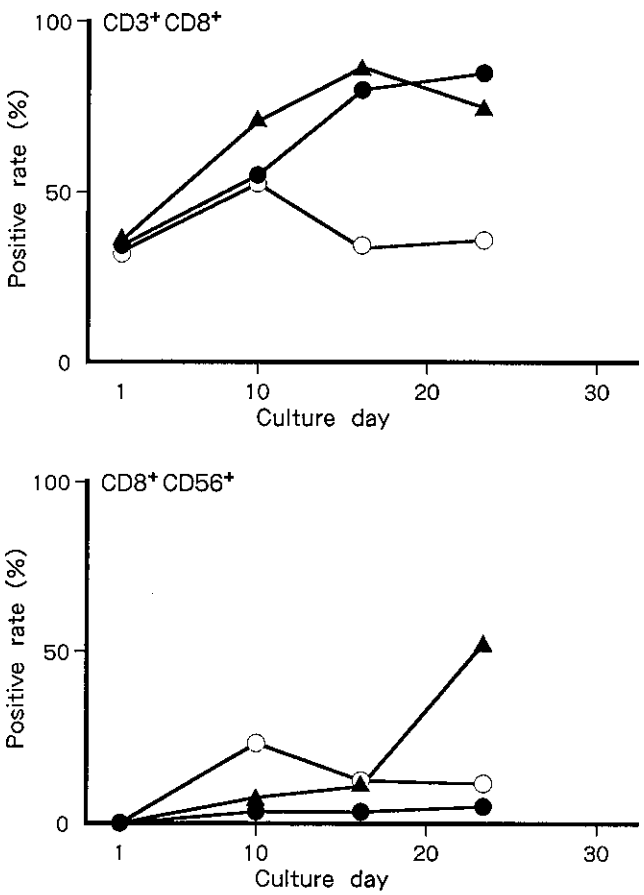

Fig. 4. Change of positive rates of $CD3^+CD8^+$ and $CD8^+$-$CD56^+$ LAK cells without stimulation (○) or stimulated by 0.3 μg/ml of PWM (▲) or 10 ng/ml of OKT3 (●).

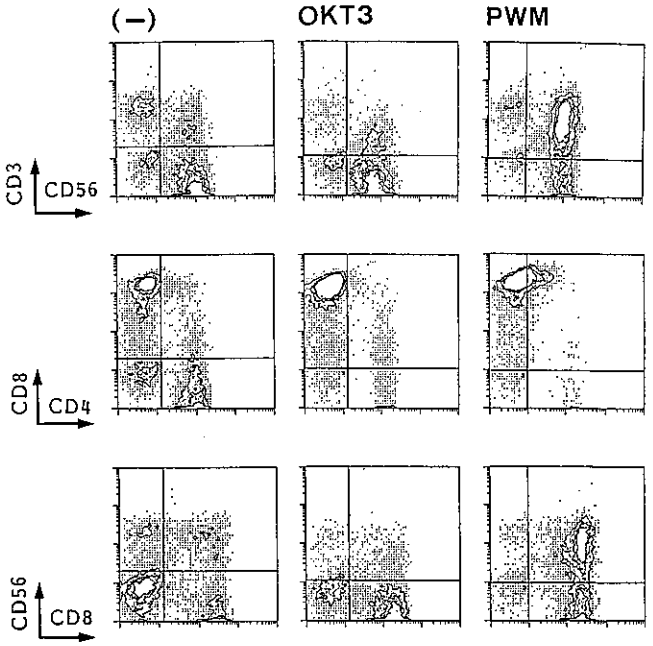

Fig. 5. Phenotypic analysis of LAK cells on day 23 after initial stimulation by 10 ng/ml of OKT3 or 0.3 μg/ml of PWM.

PWM, increased to about 80% after 7 to 10 days' incubation. Then, $CD3^+CD56^-$ cells decreased continuously during a further 2-week incubation. OKT3-stimulated LAK cells maintained a $CD3^+CD56^-$ rate of about 70 to 95% throughout the incubation period. Non-stimulated LAK cells showed a gradually increasing rate of $CD3^+$-$CD56^-$ cells, reaching about 70–90% after 3 weeks' incubation.

The $CD8^+$ cells, corresponding to about 30% of lymphocytes after PWM-stimulation, continuously increased during culture to reach 80–90% of all PWM-stimulated LAK cells. Their fluorescence was not dim but was light, meaning that they are strongly CD8 positive cells (Fig. 4). About half of these $CD8^+$ cells became $CD56^+$ after 3 weeks' incubation, and their level depended on the rate of $CD3^+CD56^+$ cells (Fig. 4). These cells were $CD3^+$-$CD8^+CD56^+$ triple-positive cells. The OKT3-stimulated $CD8^+$ LAK cells were $CD3^+$ but not $CD56^+$ (Fig. 4).

Representative results of FACS analyses are shown in Fig 5.

IL-1β, TNF-α and IFN-γ PBL at the concentration of 10^6/ml was cultured in RPMI1640 containing 5% autologous plasma and 1 μg/ml of PWM. No IL-2 was added in this experiment. PBL was separated from blood of 10 normal donors and 7 malignant patients. After 24-h incubation, supernatants were collected and IL-1β, TNF-α and IFN-γ were measured (Fig. 6). Significant increases of IL-1β and TNF-α were induced in the presence of PWM, while secretion of IFN-γ was not affected by PWM. The mean concentration of IL-1β was increased by PWM from 355.6 pg/ml to 534.7 pg/ml. The concentration of TNF-α also changed from 670.3 pg/ml to 1167.6 pg/ml. On the other hand, the mean concentrations of IFN-γ were 156.5 pg/ml in the absence of PWM and 156.2 pg/ml in the presence of PWM. There was no significant difference in the concentration of these three cytokines between normal donors and malignant patients. Down-regulation of IL-1β and TNF-α by PWM observed in some donors was related to poor proliferation of PWM-stimulated LAK cells.

DISCUSSION

The influence of PWM on the proliferation and the cytotoxicity of LAK cells was investigated and compared

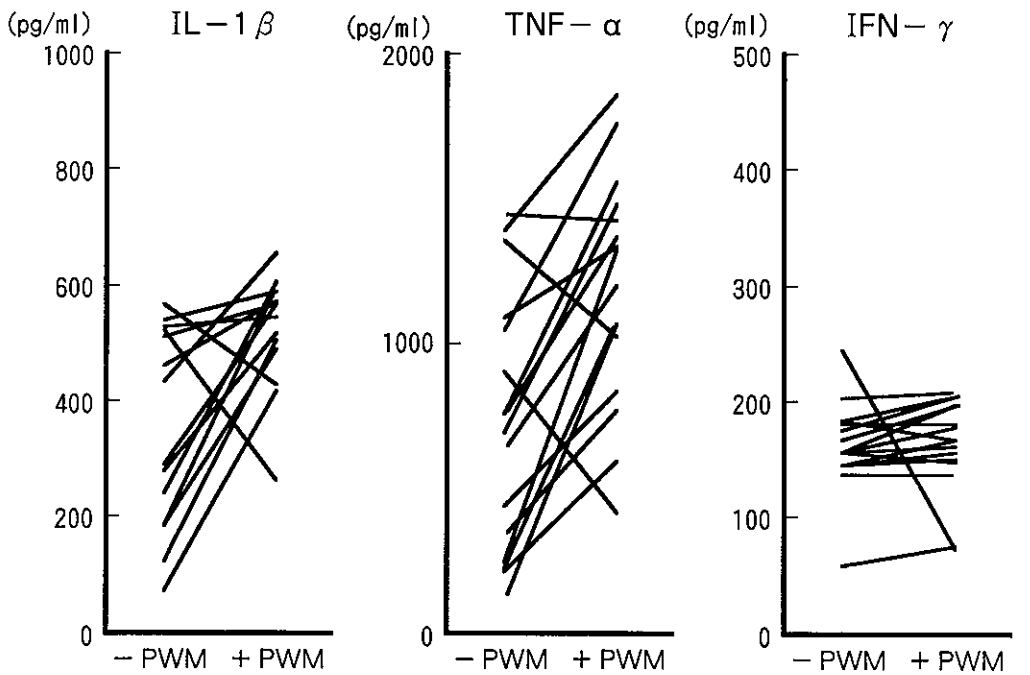

Fig. 6. Concentrations of IL-1β, TNF-α and IFN-γ in supernatants of PBL 24 h after incubation with or without PWM.

to the effect of OKT3. Optimal timing and concentration of PWM to be added to the culture of PBL were the initial 48 h and between 0.3 and 3 μg/ml, respectively. PWM-stimulated LAK cells showed a huge proliferation rate of about 1000-fold in 3 weeks' culture and 2000- to 10,000-fold in 4 weeks' culture. These proliferation rates were almost the same as those found in OKT3-stimulated LAK cell culture. However, the cytotoxicity of PWM-stimulated LAK cells, which reached the maximum at about 2 weeks' culture was more potent than that of non-stimulated LAK cells or OKT3-stimulated LAK cells. OKT3-stimulated LAK cells usually showed less potent cytotoxicity toward SW1116 than that of non-stimulated ordinary LAK cells.

This significant difference in the cytotoxicity of PWM-stimulated LAK cells and OKT3-stimulated LAK cells may be explained by the difference of phenotypes of these LAK cells. $CD3^+CD56^+$ double-positive cells, which are considered to possess potent cytotoxicity,[14] proliferated dominantly in PWM-stimulated LAK cells after 2 or 3 weeks' incubation, while almost all OKT3-stimulated LAK cells were $CD3^+CD56^-$ cells. The CD56 antigen molecule is expressed on mature natural killer cells which possess non HLA-restricted cytotoxicity without any pre-sensitization.[15, 16] However, Lanier *et al.*[17] reported that CD56 antigen was expressed on HLA class I-restricted cytotoxic T cell clone or $CD4^+$ and $CD8^+$ T cell clones, and that none of the latter clones showed cytotoxicity. Moreover, a comparative study of the cytotoxicity of $CD3^+$ LAK cells and $CD56^+$ LAK cells after sorting by FACScan revealed that the potency of LAK cells depended on $CD3^-CD56^+$ cells. This difference was in part due to target cells. However, the facts that the maximum cytotoxicity of PWM-stimulated LAK cells could be obtained after 2 weeks' culture and maintained for a week, and that in this period not $CD3^-CD56^+$ but $CD3^+CD56^+$ LAK cells corresponded to about 50–60% of the total suggested that $CD3^+CD56^+$ LAK cells also possess potent cytotoxicity. This may also account for the less potent cytotoxicity of OKT3-stimulated LAK cells, which did not express CD56 molecules.

The $CD3^+CD56^+$ PWM-stimulated LAK cells also expressed CD8 antigen, being $CD3^+CD8^+CD56^+$ triple-positive. Lanier *et al.*[17] reported on triple-positive cytotoxic T clones. However, it appears that the expression of CD56 antigen would not have direct relationship to cytotoxic activity as reported by Lanier *et al.*, because the positive rates of $CD3^-CD56^+$ cells and $CD3^+CD56^+$ cells did not correspond to the potency of the cytotoxicity and despite the $CD8^+$ status, the cytotoxicity was MHC-nonrestricted.

In fact, the relationship between the CD56 molecule and cytotoxicity has not been clarified yet. We also carried out one experiment to examine whether anti-

CD56 monoclonal antibody could inhibit the cytotoxicity of $CD56^+$ LAK cells against SW1116. The result that none or little inhibition of cytotoxicity was observed suggested that the CD56 molecule alone does not have a direct relationship to cytotoxicity (data not shown).

PWM has usually been used for induction of immunoglobulin from B cells. As for T lymphocytes, the cytotoxic activity of T cells was significantly enhanced by contact with PWM for as short a period as 1 h. Other lectins did not show such an enhancing effect on the cytotoxicity of lymphocytes with only 1-h contact. These facts suggested that use of PWM for stimulating LAK cells might improve adoptive immunotherapy, for which more potent and significantly larger amounts of LAK cells might be useful. It should be noted in addition that PWM is less expensive than OKT3, and is much more stable in the medium than the monoclonal antibody.

We have already reported adoptive immunotherapy using LAK cells.[18–20] Recently PWM-stimulated LAK cells were applied in our allogeneic LAK therapy, and no side effects have been experienced. PWM was thoroughly washed out after 24- or 48-h incubation. So PWM, should it remain in the culture medium, would be present only at a very low concentration while LAK cells were proliferating. Therefore, PWM should not show any toxic effect on patients who received PWM-stimulated LAK cells. More than 10^{10} allogeneic LAK cells could be injected frequently, and such an amount of the cells can be obtained from PBL of 20 ml of normal fresh blood.

IL-1β is a multipotent cytokine secreted mainly by macrophages and monocytes. In the initial 24 h of the incubation, PWM is thought to stimulate monocytes especially to induce production of IL-1β and TNF-α. Both of these cytokines might stimulate the further proliferation of LAK cells, which would produce more IFN-γ and TNF-α later. IFN-γ and TNF-α were reported to have potent cytotoxic effects on tumor cell lines,[21, 22] and they were considered to be the most important cytokines contributing to the cytotoxic activity of LAK cells. The relationship between the amounts of cytokines, especially TNF-α and IFN-γ, secreted by LAK cells and the potency of cytotoxicity of these LAK cells is now under investigation.

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