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EFFECT OF LYMPHOKINE-ACTIVATED KILLER CELLS ON HUMAN RETINOBLASTOMA CELLS (Y-79) IN VITRO : ENHANCEMENT OF THE ACTIVITY BY A POLYSACCHARIDE PREPARATION, KRESTIN

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SUMMARY: Peripheral blood mononuclear cells (PBMC) cultured in a medium containing interleukin 2 (IL 2) develop the ability to kill fresh tumor cells. This function has been termed lymphokine activated killing (LAK). Recently, cord LAK cell activity was demonstrated to be equally as cytotoxic against similar in vitro targets as adult (peripheral) LAK cells. We investigated the future therapeutic use of LAK adoptive immunotherapy by examining LAK in vitro cytotoxicity from both cord and peripheral blood mononuclear cells against pediatric malignant tumor cell lines Y-79 (retinoblastoma). Cord LAK cells show higher levels of cytotoxicity toward Y-79 targets than do adult LAK cells. Attempts to enhance the rIL 2-induced LAK activity by addition of rIFN-γ or PSK (krestin) were successful. Furthermore, we found that PSK has a function to enhance rIL 2-induced IFN-γ and TNF-α production. These findings suggest that combined administration of cord LAK cells and PSK may account for the improvement of advanced retinoblastoma in the neonatal period.

Human peripheral blood mononuclear cells (PBMC) are capable of killing certain established tumor lines such as K562 (1). Cells displaying this function have been designated natural killer (NK) cells. However, these cells that have NK activity are unable to kill tumor lines such as Raji and Dauji, or fresh tumor cells (all referred to as NK resistant). It was reported that short-term culture (3 to 5 days) of human PBMC with rIL 2 resulted in the development of cytotoxic activity against fresh tumor targets and NK-resistant targets, and termed this effector lymphokine-activated killer (LAK) cells (2-4). It is already known that

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the addition of rIFN-γ to rIL 2 cultures or pretreatment of PBMC with rIFN-γ results in the augmentation of LAK activity (5). Chin, et al (6) demonstrated that the classical neuroblastma cell line appears to be most susceptible to neonatal mononuclear LAK activity, compared to that of adult LAK activity. The response to retinoblastoma cell line, under the category of the same pediatric solid tumors as neuroblastoma cell line, still remains to be determined. In this study, we examined whether or not both adult and cord LAK cell activity towards Y-79. We found that cord LAK cells tend to show higher cytotoxicity than do adult LAK cells against Y-79 cells.

Recently, many workers have been reported that INF-β (7), γ (5) and TNF-α, β (8) augment LAK activity respectively. We also confirm that IFN-γ enhances rIL 2-induced LAK cytotoxicity against Y-79 cells. In addition, we examined whether or not PSK (krestin) can act as LAK activity enhancer as well as preceding cytokine. PSK is a biological response modifier (BRM) (9) and has been widely applied to cancer patients. A possible role of PSK is based on reports that PSK can increase IFN-γ level in the blood stream of tumor bearing mice(10). This information prompted us to conduct trials on PSK for LAK induction. In fact, we found that PSK augments rIL 2-induced LAK and increase both IFN-γ and TNF-α production. These results suggest that PSK may be able to develop the method of administration of LAK for the treatment of cancer patients of all types.

MATERIALS AND METHODS

Cells : The established human retinoblastoma cell line Y-79, human T leukemic cell line MOLT-4, and Burkitt lymphoma cell line Raji were used in the LAK sensitivity study. They were routinely maintained at 37 ℃ in a 95% air-5% CO_2 humidified atmosphere in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and antibiotics.

LAK cell generation : Cord blood mononuclear cells (CBMC) derived from a healthy individual were isolated by centrifugation on a Ficoll-Conray gradient (sp. gr. 1.077, Immunology and Biology Research Laboratories, Takatsuki, Japan) at 400 ×g for 20 min. Peripheral blood mononuclear cells (PBMC) were drawn from the same individuals as specimens from CBMC. Mononuclear cells of both origins were incubated in RPMI 1640 medium supplemented with 10% heat-inactivated self serum, antibiotics and 10 ng/ml of rIL 2 for 3 to 5 days.

Cytotoxicity assay : Target cells (Y-79, MOLT-4, Raji) were labeled with ^{51}Cr and adjusted to a cell concentration of 1×10^5 cells/ml. Aliquots of 0.1 ml were then placed in a 96-well round-bottom microtiter plate, and effector mononuclear cells were then added to each well at an effector-to-target cell ratio of 5:1, 10:1, 20:1, 40:1 respectively. After a 4 hr incubation, aliquot samples were counted

in a scintillation counter. Percentage specific lysis was calculated by the following formula:

$$\text{Specific Cytotoxicity} = \frac{\text{Experimental mean cpm} - \text{Spontaneous mean cpm}}{\text{Maximal mean cpm} - \text{Spontaneous mean cpm}} \times 100$$

where maximal cpm was determined in medium containing 2% hydrochloric acid. Experiments in which the spontaneous release was more than 30% were not included in the results.

IFN, IFN assay : Recombinant human gamma interferon (rIFN-γ) was provided by Kyowa Hakko, Tokyo, Japan, in a frozen-dry condition. The chemical was dissolved immediately before the experiments and diluted with the complete medium. To see the effect of IFN on rIL 2-induced LAK activity, it was added to a culture medium on day 0 for a final concentration of 10, 100 U/ml. After 5 days incubation, LAK activity against Y-79 was counted as described above.

IFN-γ activity of the IFN-γ preparation and culture fluids was assayed by inhibition of the cytopathic effect of Sindbis virus in human FL fibroblasts in 96-well tissue culture plates. The reciprocal of the dilution factor of a sample that protected approximately 50% of the cells from lysis by Sindbis virus was defined as the titer. The titer were compared with an IFN-γ standard of the National Institute of Health, Japan.

TNF-α Assay : A double antibody radioimmunoassay system (TNF-α IRMA : Medgenix) was used to measure TNF-α levels in supernatant fluids of CBMC, which were incubated with 10 ng/ml of rIL2 in the presence or absence of 0.5 mg/ml PSK for 3 days. In this system, several antibodies directed against distinct epitopes of TNF-α had been used. These were attached to the lower and inner surface of the plastic tube. The second antibody was labelled with ^{125}I. After washing, the remaining radio-activity bound to the tube reflecting the antigen concentration. In brief, 200μl supernatants of each tube were vortexed, 50μl of tracer antiTNF-α antibody was dispensed into each tube and they were incubated for 18 hrs at room temparature. Complete contents of each tube were aspirated, and then tubes were washed with washing solution. Contents were aspirated. The activity was counted in a gamma counter for 60 seconds.

Drug : PSK (Kureha Chemical Ind., Tokyo, Japan) was dissolved in sterile physiological saline, sterilized at 100℃ for 15 min and stored at 4 ℃ as 5 mg/ml solution until use. To determine whether PSK can enhance LAK activity, fresh isolated CBMC was incubated with 10 ng/ml rIL 2 and various concentrations of PSK for 5 days. Induced LAK cells were washed twice, and then tested for their LAK activity toward Y-79.

RESULTS

Effect of LAK activity against MOLT-4 and Raji : LAK activity toward standard NK-sensitive and -resistant cell lines is demonstrated in Figure 1 as the activity toward the MOLT-4 (NK sensitive) and Raji (NK resistant but LAK sensitive) cell line. Cord cell cytotoxicity against MOLT-4 was greater than the comparative values for adult cells. A similar tendency was also observed with the Raji targets.

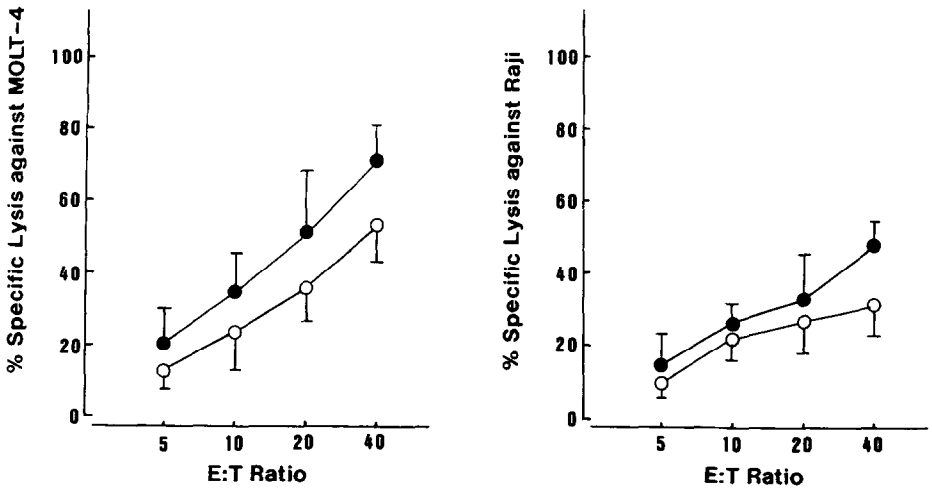

Figure 1. Effect of LAK activity against MOLT-4 and Raji. Cord(●) and adult (○) mononuclear cells were incubated with rIL 2 (10 ng/ml), and then incubated with ^{51}Cr-labeled MOLT-4 and Raji target cell line for 4 hr in an effector target ratio of 5:1, 10:1, 20:1, 40:1, respectively. Percentage specific lysis was determined as described in "Materials and Methods". Bars, SE (n=10).

Effect of LAK activity against the Y-79 retinoblastoma cell line : There was a significant increase in cord LAK activity demonstrated toward Y-79 compared with adult LAK activity in accordance with increase of E:T ratio (Figure 2). There was only 5% or less specific cell lysis shown in the cytotoxic assay in both cord and peripheral blood mononuclear cells cultured without rIL 2 against MOLT-4, Raji (data not shown) and Y-79 cell lines (Figure 2).

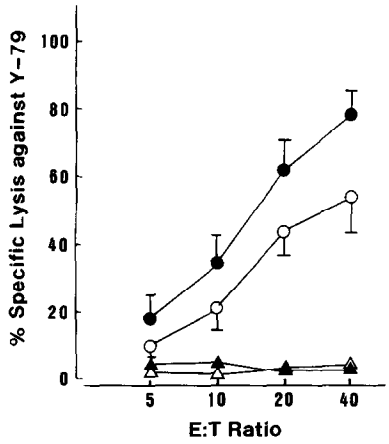

Figure 2. Effect of LAK activity against the Y-79 retinoblastoma cell line. Cord (●) and adult (○) mononuclear cells were incubated with rIL 2 (10 ng/ml), and then incubated with ^{51}Cr-labeled Y-79 target cell line for 4 hr in an effect or target ratio of 5:1, 10:1, 20:1, 40:1, respectively. Also shown is the spontaneous NK activity in cord (▲) and adult (△) mononuclear cells . Percentage specific lysis was determined as described in "Materials and Methods". Bars, SE (n=10).

Effect of rIFN-γ on rIL 2-induced LAK activity : It has been reported that the endogenous IFN-γ in rIL 2 treated cultures and the addition of rIFN-γ to rIL 2 treated cultures play an important role in the IL 2-initiated developement of LAK activity (5). We also examined the effect of exogenous rIFN-γ on rIL 2-induced LAK activity. The LAK activity generated by rIFN-γ treatment of CBMC was increased as compared to the level achieved by CBMC cultured with rIL 2 alone to some extent (Table 1). The addition of rIFN-γ alone did not induce LAK cytotoxicity (data not shown).

Effect of PSK on rIL 2-induced LAK activity : Recently it has been reported that PSK induces the endogenous TNF-α , IL-1 and IFN-γ in PBMC culture system in vitro. Moreover, it is known that PSK augments the natural killer (NK) cell activity (10). However, the potential function against LAK activity has not yet been demonstrated. We analyzed the effect of PSK treatment on LAK cytotoxicity against Y-79. As shown in Table 1, addition of PSK to CBMC at concentration of 0.5mg/ml for 5 days caused a dramatic enhancement of LAK activity to Y-79, as compared with control cultures.

Effect of PSK on rIL 2-induced IFN-γ and TNF-α production in CBMC : It is known that IFN-γ production was induced by the addition of rIL 2 on PBMC (5). We next examined whether or not PSK can induce endogeneous IFN-γ production on rIL 2-stimulated CBMC. CBMC were cultured with 10 ng/ml of rIL 2 in the presence or absence of 0.5 mg/ml PSK for 3 days, and IFN-γ activity of their culture supernatants was assayed. As shown in Table 2, PSK augmented IFN-γ production

Table 1
Effect of rIFN-γ or PSK on rIL 2-induced LAK activity*
against Y-79 cells

% Specific Lysis	rIFN-γ (U/ml)			PSK (mg/ml)		
	0	10	100	0	0.1	0.5
E:T=10:1	35±2.5	40±3.1	60±4.4	32±2.5	35±1.7	55±4.4
E:T=20:1	55±4.1	62±6.2	74±5.3	52±3.0	50±3.6	71±3.2
E:T=40:1	70±6.1	72±4.6	90±6.9	68±4.4	72±3.7	88±4.8

*CBMC were incubated with 10 ng/ml of rIL 2 in the presence or absence of various concentrations of compounds (rIFN-γ, PSK) for 5 days. After washing twice, the cells were tested for their LAK activity toward Y-79.

Table 2
Effect of PSK on rIL 2-induced IFN-γ and TNF-α production against CBMC

Cultured with	IFN-γ Activity (U/ml)	TNF-α Activity (pg/ml)
rIL 2 alone (10ng/ml)	47.5 ±12.8	1359 ±301
rIL 2 + PSK(0.5mg/ml)	83.8 ±24.6	2815 ±194

CBMC from 10 donors were incubated for 3 days in RPMI medium supplemented with 10% heat inactivated self serum in the presence of 10ng/ml of rIL 2 and 0.5mg/ml of PSK, and were then assayed. The values represent the means ±SD of 10 donors. The mesurement of IFN-γ and TNF-α activity was performed as shown in "Materials and Methods".

from rIL 2-stimulated CBMC. IFN-γ could not be detected in cultures with PSK alone in the absence of rIL 2 (data not shown).

Furthermore, under the same conditions , we analyzed the effect of PSK on TNF-α production from CBMC. As shown in Table 2, PSK augmented TNF-α production in the culture fluid of these cells.

DISCUSSION

Retinoblastoma, along with leukemia and neuroblastoma, is one of the most common childhood malignancies and is the most common intraocular neoplasm. Though several different chemotherapeutic drugs (11-13) have been attempted, the survival rate is very poor once the tumor becomes clinically advanced (metastatic). Therefore, it is necessary to develop a new approach to the treatment of retinoblastoma. Recently it has been reported that the systemic administration of LAK cells and rIL 2 is effective to the patients with some metastatic cancers (14). Although the effect of LAK therapy has been studied extensively in adults with cancer, it has not been well documented and studied in younger population with retinoblastoma. In this study, we demonstrate that Y-79 appears to be more susceptible to cord LAK activity, compared to that of adult LAK activity.

The differential cytotoxicity between cord and adult LAK cells may be due to the difference of spontaneous proliferative ability. In fact, although NK activity is less in cord blood, its spontaneous proliferative ability is greater than PBMC (15, 16). This may reflect a greater cell population with IL 2 receptor in

cord blood than adult blood consequently showing higher LAK cytotoxicity in cord blood. It is well known that IL 2 can enhance NK and LAK cell activity. It has been suggested that IL 2 may indirectly amplify these immune responses through the induction of IFN-γ production, which then regulates the differentiation of cytotoxic effector cells (17). In addition, IFN-γ may, under certain conditions, increase the numbers of receptors for IL 2 on lymphocytes (18), facilitating NK and LAK cells to interact with IL 2. Our results with the treatment of IFN-γ are consistent with those of Ito et al. (5), who demonstrated the ability of an exogenous IFN-γ to enhance LAK activity.

Furthermore, IFN-γ is known to be closely correlated with TNF-α. For example, Nedwin et al. (19) have shown that rIL 2 induces TNF-α and TNF-β production in PBMC, and that this production can be further enhanced by IFN-γ. Scheurich et al. (20) have shown that TNF is effective as a costimulator of rIL 2-dependent IFN-γ production in T-cells. Finally, TNF-α may promote enhanced rIL 2 responsiveness as well as IFN-γ. It is likely that TNF-α and IFN-γ stimulate activities with each other.

Several trials of IFN-γ in the clinical therapy of various diseases have been carried out. One must also be reminded of the evidence that in killing tumor cells IFN-γ exert its effects adversely on immune responses of the hosts. We have therefore been looking for some substances which prevent or reduce such adverse effects against the hosts. Thus, we used here an immunomodulator PSK instead of IFN-γ. Earlier studies showed that PSK augments NK activity and IFN-γ level in blood. However, the effect of PSK against LAK activity in vitro has not been mentioned. Our results suggest that PSK stimulates CBMC to produce endogenous IFN-γ and TNF-α, which in turn acts as a differentiation signal that may be involved in the IL 2-initiated LAK generation. In a sense, PSK may act just like IL 2 or increase the sensitivity of IL 2 toward effector cells. PSK has a slight growth inhibitory effect against Y-79 in vitro (unpublished data). Therefore, the combined administration of cord LAK cells and PSK could be of benefit for the treatment of advanced retinoblastoma patients. It is important to recognize, however, that only Y-79 was examined in this study and that studies using more cell lines and fresh isolated tumor tissue will strengthen these initial observations. Further studies are necessary to elucidate these problems.

the possibile use of recombinant apoaequorin as an intracellular probe for monitoring rapid changes in $[Ca^{2+}]_i$ when coelenterazine is introduced into intact yeast cells and aequorin is regenerated *in vivo*.

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REFERENCES

1. Shimomura,O., Johnson,F,H., & Saiga,Y. (1962) J. Cell. Comp. Physiol. **59**, 223-239.
2. Shimomura,O., Johnson,F,H., & Saiga,Y. (1963) J. Cell. Comp. Physiol. **62**, 1-8.
3. Shimomura,O., Johnson,F.H., & Saiga,Y. (1976) Symp. Soc. Exp. Biol. **30**, 41-54.
4. Cormier,M.J., Lee,J., & Wampler,J.E. (1975) Annu. Rev. Biochem. **44**, 255-272.
5. Shimomura,O. & Johnson,F.H. (1978) Proc. Natl. Acad. Sci. USA **75**, 2611-2615.
6. Shimomura,O. & Johnson,F.H. (1978) Methods Enzymol. **57**, 271-291.
7. Shimomura,O. & Johnson,F.H. (1975) Nature (London) **256**, 236-238.
8. Inouye,S., Noguchi,M., Sakaki,Y., Takagi,Y., Miyata,T., Iwanaga,S., Miyata,T., & Tsuji,F.I. (1985) Proc. Natl. Acad. Sci. USA **82**, 3154-3158.
9. Charbonneau,H., Walsh,K.A., McCann,R.O., Prendergast,F.G., Cormier,M.J., & Vanaman,T.C. (1985) Biochemistry **24**, 6762-6771.
10. Ridgway,E.B. & Ashley,C.C. (1967) Biochem. Biophys. Res. Commun. **29**, 229-234.
11. Blinks,J.R., Mattingly,P.H., Jewell,B.R., Van Leeuwen,M., Harrer,G.C., & Allen,D.G. (1978) Methods Enzymol. **57**, 292-328.
12. Borle,A.B. & Snowdowne,K.W. (1982) Science **217**, 252-254.
13. McNeil,P.L.& Taylor,D.L. (1985) Cell Calcium **6**, 83-93.
14. Borle,A.B., Freudenrich,C.C., & Snowdowne,K.W. (1986) Amer. J. Physiol. **251**, C323-C326.