Acta Medica et Biologica
Vol. 39, No. 2, 83-90, 1991

Induction of Cytotoxic T Lymphocytes against Allogeneic Leukemia Cell Line

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Received March 4, 1991

Summary. **In order to apply specific cytotoxic T lymphocytes (CTL) to human leukemia therapy, the induction of CTL against an allogeneic leukemia cell line from healthy volunteers and leukemia patients in remission was performed.**

Cytotoxicity was measured after mixed lymphocyte-tumor cell culture (MLTC) of peripheral blood lymphocytes (PBL) from healthy volunteers or patients and mitomycin C (MMC) treated YS-1 which is an allo-leukemia cell line. The results were as follows: 1) CTL were induced from PBL of both healthy volunteers and leukemia patients in remission; 2) in leukemia patients, CTL activity varied according to disease conditions; 3) a secondary culture induced significantly higher CTL activity than a primary culture.

These results suggest the feasibility of adoptive immunotherapy (AIT) for human leukemias using specific CTL.

INTRODUCTION

In recent years, adoptive immunotherapy (AIT) has been performed on malignant tumors. However, AIT uses mostly non-specific lymphokine activated killer (LAK) cells (LAK-AIT)[1-3]; more specific cytotoxic T lymphocytes (CTL) (CTL-AIT) are rarely used.[4]

In addition, for hematological malignancies, most of the LAK cells are still under *in vitro* investigation, and there are only a few studies on CTL.[5-16]

At present, LAK-AIT does not show results as good as those of *in vitro* studies. Furthermore, there are limitations to LAK-AIT partly because of the side effects of Interleukin-2 (IL-2). On the other hand, CTL-AIT may be more specific and physiological and therefore efficient. However, an adequate amount of the stimulator is often difficult to obtain. In this respect, leukemia may be a good indication for CTL-AIT because a large amount of the stimulator can be obtained relatively readily compared with many other solid tumors. To examine the possibility of applying CTL-AIT to human leukemia therapy, the author experimentally induced CTL against an allogeneic leukemia cell line from healthy volunteers and leukemia patients in remission.

MATERIALS AND METHODS

1. Induction of CTL

Fig. 1 shows the flow chart for the preparation.

1) *Stimulator cells*

YS-1,[17] an allogeneic leukemia cell line established from the peripheral blood of a patient with chronic myelogenous leukemia (CML) in blastic crisis, was treated with mitomycin C (MMC) and used as a stimulator. YS-1 is a B cell line positive for CD10, CD19, CD20, and CD21 and markedly expresses Class I and Class II antigens of the major histocompatibility complex (MHC).

2) *Responder cells*

Peripheral blood lymphocytes (PBL) were obtained from healthy volunteers in our department and leukemia patients in remission (chronic phase in CML) using Haemonetics V50, centrifuged by the density gradient method to remove erythrocytes, washed, and suspended in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) at 1×10^6/ml. Table 1 shows the profiles of the patients.

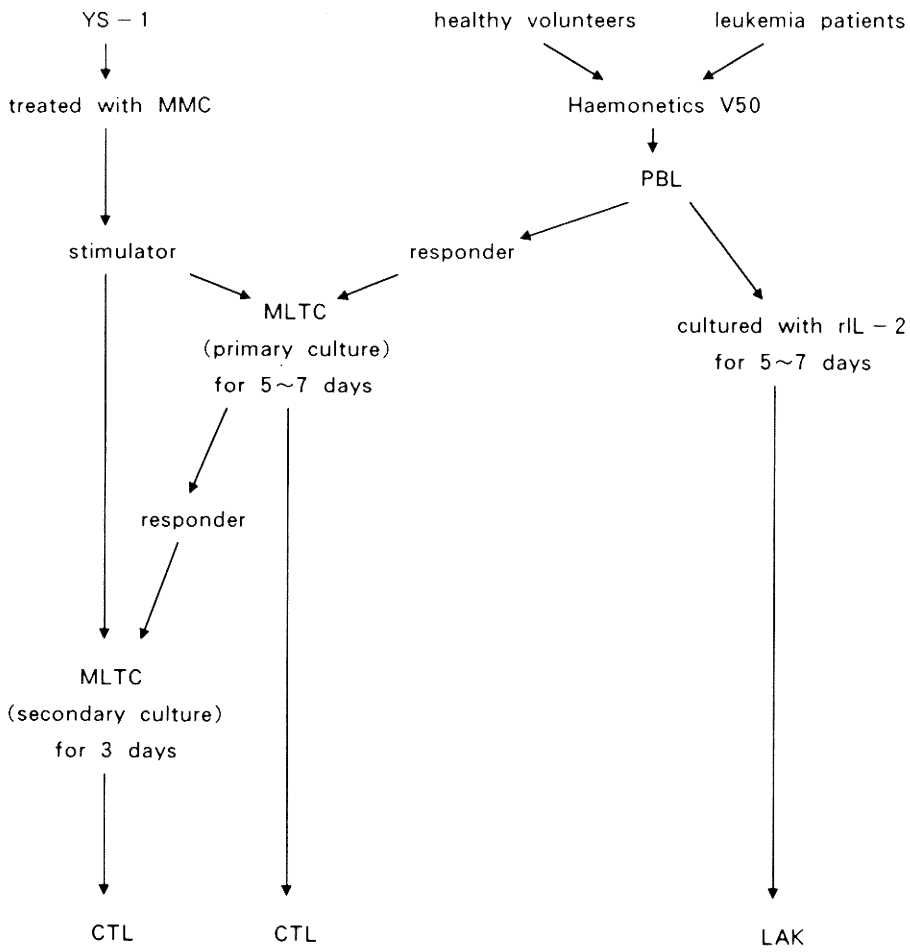

Fig. 1. Induction of CTL and LAK. MMC: mytomicin C, PBL: peripheral blood lymphocyte, MLTC: mixed lymphocyte-tumor cell culture, rIL-2: recombinant interleukin 2 CTL: cytotoxic T lymphocyte, LAK: lymphokine activated killer.

Table 1 Patients Studied

Case	Age	Sex	Dx
1	21	M	AML (M2) 1CR
2	17	M	CML 1CP
3	24	M	ALL (L2) 1CR
4	33	F	AML (M2) 1CR
5	24	F	ALL (L2) 1CR
6	51	F	CML 1CP
7	27	F	ALL (L2) 1CR
8	39	M	ALL (L2) 1CR

M: male, F: female, AML: acute myelogenous leukemia, CML: chronic myelogenous leukemia, ALL: acute lymphoblastic leukemia, ( ): FAB classification, CR: complete remission, CP: chronic phase

3) *Mixed lymphocyte-tumor cell culture (MLTC): primary culture*

The stimulator and responder cells were mixed at various ratios and cultured at 37℃ in 5% CO_2 incubator for 5-7 days.

4) *Secondary culture*

In some experiments, the count of the responder cells was adjusted to 1×10^6/ml after the primary culture, and the responder cells were incubated with newly added stimulator cells for 3 days.

2. Induction of LAK cells

Fig. 1 shows the flow chart for the preparation.

Recombinant IL-2 (rIL-2) (TGP-3, supplied by Takeda Pharmaceutical Co.) was added to the responder cells obtained in 1-2) to a final concentration of

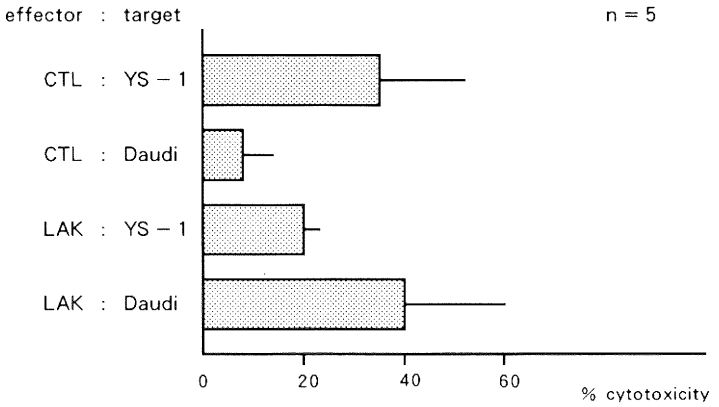

Fig. 2. CTL and LAK activities in healthy volunteers. The CTL activity against YS-1 was higher than the LAK activity against YS-1. This CTL was not significantly cytotoxic to Daudi.

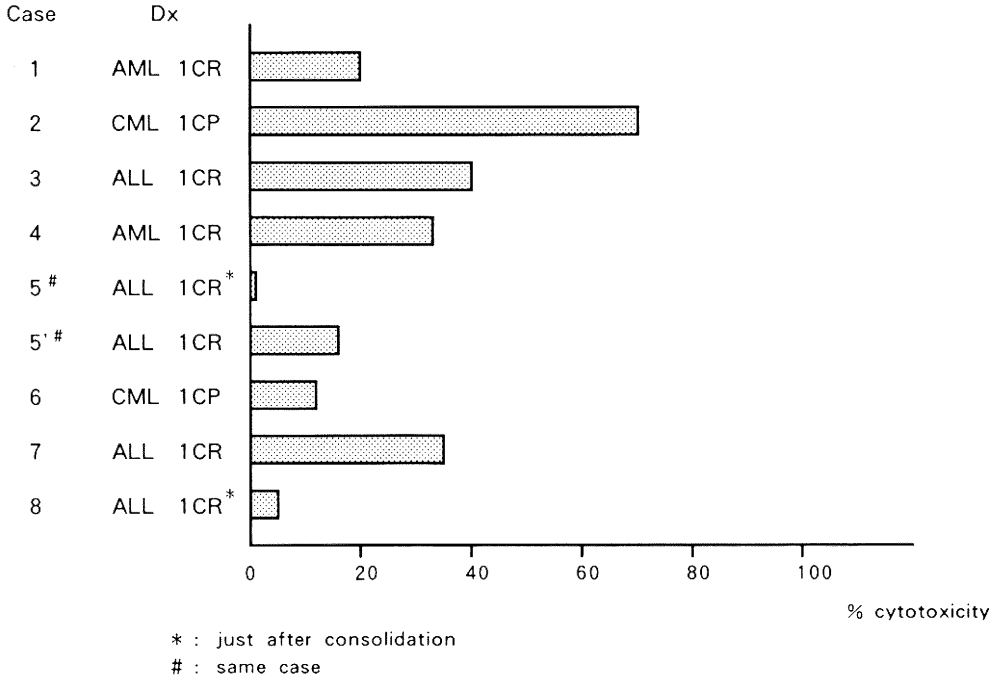

Fig. 3. CTL activity in leukemia patients. Significant CTL activity (≧20%) was induced in 5 cases. In cases just after consolidation, no CTL activity (Case 5) or only negligible activity (5.2%, Case 8) was induced.

2U/ml, and 1×10^6/ml cells were cultured for 5-7 days.

3. Cytotoxicity assays

YS-1 and Daudi, a natural killer (NK)-insensitive cell line, were labeled with ^{51}Cr and used as targets. CTL and LAK were used as effectors after density gradient centrifugation to remove dead cells. Four h-^{51}Cr release assay was performed at E:T=40:1, and % cytotoxicity was calculated as follows:

$$\%\ \text{cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

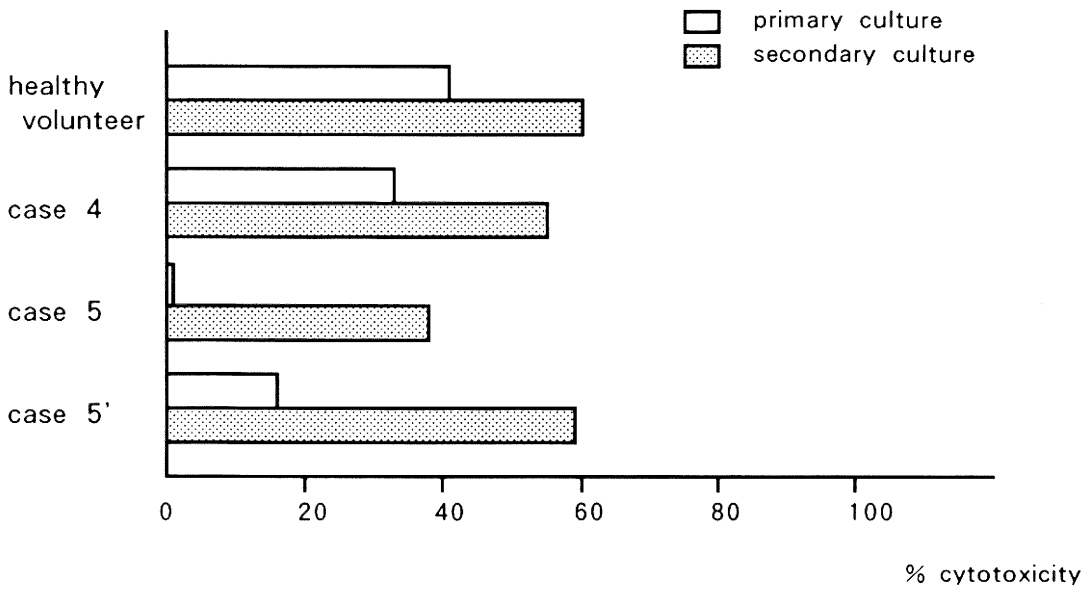

Fig. 4. Comparison between primary and secondary cultures. A secondary culture induced higher CTL activity in all cases. In Case 5, no CTL activity was observed after the primary culture, but a significant CTL activity (38.1%) was observed after the secondary culture.

All assays were done in triplicate, with 20% or more cytotoxicity considered significant.

4. Cell surface marker analysis

Cell surface markers were examined by direct and indirect immunofluorescence methods using monoclonal antibodies (MoAb). For analysis, FACScan (Becton Dickinson) was used.

RESULTS

1. Induction of CTL from healthy volunteers

CTL was induced at responder (R) to stimulator (S) ratio of 1:1, 2:1, 10:1 (data not shown). At an R/S ratio of 2:1, CTL was induced most stably and efficiently. Therefore, the following experiments were done at this ratio. CTL was judged to be induced because the cytotoxic effector cells were considered T lymphocytes positive for CD3 but negative for NK markers (as described below).

2. CTL and LAK activities in healthy volunteers

Fig. 2 shows the results. In 5 subjects whose CTL activity and LAK activity were simultaneously measured, the CTL activity was $34.5 \pm 17.2\%$ using YS-1 as the target and $7.7 \pm 5.8\%$ using Daudi. The LAK activity using YS-1 and Daudi as the target was $20.1 \pm 3.3\%$ and $40.1 \pm 20.1\%$, respectively.

3. Induction of CTL from leukemia patients

Fig. 3 shows the results. The CTL activity measured 9 times in 8 patients varied from 0.0 to 70.4% ($25.7 \pm 20.4\%$); 20% or more CTL activity was observed in 5 patients. Just after consolidation, no CTL activity was induced in Case 5, but after 2 months, the activity was 15.6%. In Case 8, CTL activity was only 5.2% just after consolidation.

4. Comparison between primary and secondary cultures

Fig. 4 shows the results in 1 healthy volunteer and 2 patients. The secondary culture induced a higher CTL activity than the primary culture. In Case 5, no CTL activity was observed after the primary culture, but 38.1% CTL activity was observed after the secondary culture. In Case 5', the CTL activity was 15.6% after the primary culture but 49.4% after the secondary culture.

5. Surface markers of CTL effector

Fig. 5 shows the results of 2-color analysis in 1 healthy volunteer. Two cell groups were detected, one positive for CD4 and the other positive for CD8. These cells were negative for CD16 and CD57 which are NK-related markers. The CD8-positive cells were CD11-negative; they therefore had cytotoxic T cell markers. The effector was CD3-positive (not shown

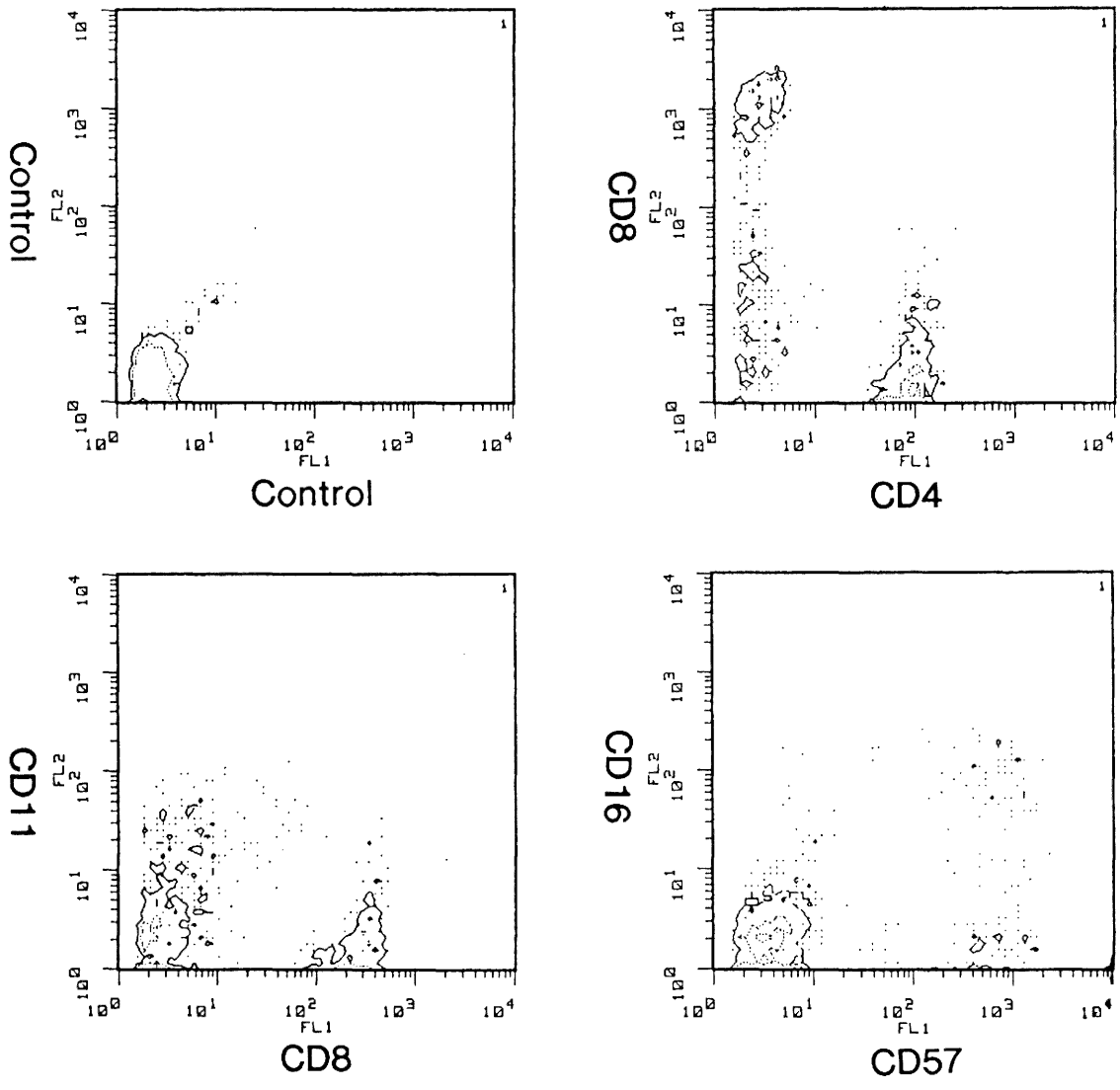

Fig. 5. Surface markers of CTL effector. Dual staining in 1 healthy volunteer. Horizontal axis: fluorescein (FITC), vertical axis: phycoerythrin (PE). There are 2 cell groups; one was CD4-positive, and the other, CD8-positive (right upper). These cells were negative for CD16 and CD57 (right lower). The CD8-positive cells were CD11-negative (left lower).

in the figure). Similar results were obtained in all cases examined.

DISCUSSION

As a preliminary study in order to apply CTL to leukemia therapy, CTL against an allogeneic leukemia cell line, YS-1, from healthy volunteers and leukemia patients in remission was induced.

In the healthy volunteers, the activity of induced CTL was higher than that of simultaneously induced LAK. This CTL did not show any cytotoxic activity against Daudi. Therefore, it was possibly specific to YS-1. In the leukemia patients in remission, CTL activity comparable to that in healthy volunteers was induced. Significant CTL activity was observed in 5 of the 8 patients. However, negligible activity was noted in Case 8 and no activity in Case 5 at the time of the induction just after consolidation although in Case 5, 15.6% CTL activity could be induced 2 months later. In these cases, anti-leukemic drugs on CTL precursor cells might have affected the activity. However, these results suggest differences in CTL

activity among leukemia patients according to disease conditions.

In AIT, induction of the effector with high cytotoxic activity is necessary. In this respect, the usefulness of the secondary culture was evaluated. In cases showing no significant CTL activity (0.0%, 15.6%) after a primary culture, significant CTL activity (38.1%, 49.1%) was observed after a secondary culture. The secondary culture induced higher CTL activity than the primary culture in all cases examined. Therefore, a secondary culture can be a useful method to obtain effector cells with higher cytotoxic activity. In particular, the secondary culture induced CTL activity in cases showing no CTL activity after the primary culture. Such a finding has significance for clinical application.

Surface marker analysis showed that the effector cells induced in this experiment were CTL positive for CD3 but negative for NK-related marker CD16 and CD57. There were two cell groups, one positive for CD4, and the other positive for CD8. The CD8-positive cells were CD11-negative; that is, they had cytotoxic T cell markers. Many reports on CTL and tumor infiltrating lymphocytes (TIL) have shown that the effector cells are CD8-positive. It is speculated that the CD8-positive effectors recognize MHC Class I antigen on as the target and exert cytotoxic action. Though there are only a few reports on CD4-positive CTL, their restriction by MHC Class II antigen has been stated. Since YS-1 used in the present study is positive for both MHC Class I and Class II, the two effector groups positive for CD4 or CD8 may have been induced. Tanaka[18] reported a correlation between an expression of Class I antigen on tumor cells and the induction of CD8-positive TIL. Fujimoto[19] found that Class I-positive targets were recognized by CTL type LAK and Class I-negative targets by NK type LAK, but Class II antigen did not affect LAK activity. Therefore, in AIT, attention should be paid to the state of expression of MHC antigens on tumor cells.

In this study, an allogeneic cell line was used as for basic research, but no system for direct clinical application has been established. For more effective AIT, high anti-tumor activity and large amounts of the effector cells are necessary. MLTC, which requires a stimulator, are limited in quantity. In this experiment, MLTC was performed at R:S = 2:1. For clinical application, effective effector cells should be obtained at the highest possible R/S ratio. A secondary culture may be useful for inducing effector cells with higher anti-tumor activity but requires additional amounts of the stimulator. Therefore, the balance between the quality and quantity of the effector should be considered more carefully in CTL-AIT than in LAK-AIT.

Though NK activity in leukemia patients is decreased, it is often normalized in remission, only to decrease again at recurrence.[20-22] Similar changes have been reported in LAK activity.[10,11] These findings suggest differences in the activity of cytotoxic cells among leukemia patients according to the state and stage of the disease. In this experiment, CTL activity was compared between two different points of time in only 1 patient, and its differences seem to have been associated with the effects of anti-leukemic drugs on CTL precursor cells. Fujimoto[23] suggested the importance of suppressor T cells for negative immunity in tumor immunity and administered cyclophosphamide (Cy), considering that the inactivation of suppressor T cells is necessary to enhance the effects of CTL-AIT.[4,24] However, cytotoxic drugs such as Cy and other anti-leukemic drugs may act on both CTL precursor cells and suppressor T cells (or their precursor cells). When cytotoxic drugs are used, the balance of their effects on both types of cells should be considered. Further studies are needed on these drugs, including the possibility of preserving CTL precursor cells as much as possible while inactivating suppressor T cells.

AIT is very rarely performed on hematological malignancies. According to *in vitro* studies, AIT is expected to show good results in the remission stage when the tumor burden is decreased. AIT in this stage aims to further reduce the remaining leukemic cells, obtain a remission of higher quality, and prolong the remission period. Since the evaluation of residual leukemic cell masses is difficult, the length of the remission period should be taken into consideration in evaluating the effects of AIT, and thus, a long-term protocol is needed. However, this therapy may be more useful in the remission stage than in the terminal stage, improving the prognosis of leukemia.

From the same point of view, the application of AIT to bone marrow transplantation (BMT) is worth studying. It can be applied to purging in auto BMT[25] and can be performed for the same purpose as that in patients in remission when adequate recovery is observed after take. In allo BMT, more effective grafts versus leukemia (GVL)-like effects might be obtained. However, the possible induction of graft versus host disease (GVHD) is a very difficult problem and requires further investigation. If this problem could be solved, effector cells could also be obtained from the donor. Since various impairments of lymphocyte function are observed after BMT,[26]

effective effector cells may not be induced from the recipient depending on the stage. However, this problem would be solved if induction was from the donor. The effectiveness of CTL-AIT can be enhanced by administering Cy to the recipient in doses sufficient to inactivate suppressor T cells and by inducing CTL from the donor not affected by the Cy administration.

CONCLUSION

We induced CTL against an allo-leukemia cell line, YS-1, from healthy volunteers and leukemia patients in remission.

1) CTL against YS-1 was induced from PBL of both healthy volunteers and leukemia patients in remission.

2) In leukemia patients, CTL activity varied according to disease conditions.

3) Higher CTL activity was obtained by a secondary culture.

Acknowledgements. The author would like to express his thanks to Prof. Akira Shibata, First Department of Internal Medicine, Niigata University School of Medicine, and Associate Prof. Shoji Shinada, Blood Transfusion Division, Niigata University Hospital, for their helpful suggestions. Drs. Sadao Aoki, Koichi Nagai, and Nobuhiko Nomoto, and Ms. Izumi Tanaka and Ms. Minako Kimura are also extended many thanks for their cooperation and assistance.

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