Nagoya J. Med. Sci. 44. 117 ~ 131, 1982

CRYOPRESERVATION OF HUMAN PERIPHERAL BLOOD LYMPHOID CELLS REACTIVE TO ALLOGENEIC ANTIGENS, VIRUS-TRANSFORMED AUTOLOGOUS CELLS AND AUTOLOGOUS LEUKEMIA CELLS IN *IN VITRO* SENSITIZATION

HIRONORI YAMADA*[1], YOSHIHISA KODERA*[1,2]
and KAZUMASA YAMADA*[1]

*[1] *First Department of Internal Medicine*
*[2] *Radioisotope Center, Nagoya University School of Medicine*

ABSTRACT

We asked if cryopreservation of human peripheral blood lymphoid cells (PBL) could maintain their function to generate cytotoxic activity against various antigens *in vitro*. Cells to be frozen were suspended in RPMI 1640 medium supplemented with 10% dimetylsulfoxide and 10% fetal calf serum at a concentration of 1×10^7 cells per ml. One and a half ml of this cell solution per polystyrene vial were frozen in an automated programmed freezer at a cooling rate of $-1°C$ per minute. When used for assays, cells were recovered by rapidly thawing the cell solution in the vials. The ability of thus cryopreserved PBL to generate lymphocyte cytotoxicity was compared to that of fresh PBL from the same individuals. The micromethod of *in vitro* sensitization was used to generate and measure lymphocyte cytotoxicity in a single 96-well plate. Reactivities to alloantigens were examined in the PBL from 6 individuals and reactivities to Epstein-Barr (E-B) virus-transformed autologous lymphocytes were tested in 4 independent experiments using the PBL from two individuals. In addition, PBL reactivity to autologous leukemia cells was more precisely analysed in one patient with acute myelogenous leukemia. The cytotoxic activities of human PBL directed against alloantigens, E-B virus-transformed autologous lymphocytes, and autologous leukemia cells were satisfactorily recovered after cryopreservation. This encourages the idea to apply this procedure in further analysis of cell-mediated immune reactions in various disease conditions. It may also raise the possibility of producing immune lymphocytes cytotoxic to autologous leukemia cells that could be therapeutically usable for leukemia patients.

INTRODUCTION

Study of the immunological responses of human lymphoid cells to alloantigens and various autologous antigens *in vitro* is becoming increasingly important in the fields of applied immunology for organ transplantation, viral infections, autoimmune diseases, and cancer. By utilizing freshly prepared peripheral blood lymphoid cells, it has been well established that these PBL are stimulated by allogeneic transplantation antigens (1), Epstein-Barr (E-B) virus-transformed cells (B-cell line) (2), and autologous leukemia cells (3), and subsequently generated cytotoxic cells selectively active toward the target cells syngeneic to the stimulator cells. This *in vitro* phenomenon, which is called *in vitro* sensitization, is considered to be a useful model for understanding the *in vitro* interactions between host immune apparatus and

山田博豊・小寺良尚・山田一正

Received for Publication February 24, 1982

variable antigens expressed on cell surfaces. One of the difficulties for these studies in the human situation is, nevertheless, the fact that the numbers of each subpopulation of lymphoid cells and their functions change from day to day according to numerous factors, such as "biolism" of normal individuals or administration of certain drugs to patients; thus, one can hardly repeat and analyse phenomena with reproducibility.

Since Ashwood-Smith reported the successful preservation of viable mouse lymphoid cells after slow freezing and rapid thawing in the presence of dimetylsulfoxide (DMSO) in 1964 (4), the cryopreservation technique has been expected to be a method to solve these problems and many investigators have provided examples of the ability of cryopreserved lymphocytes to maintain their functional properties. By using our micromethod of *in vitro* sensitization (5), which allows the serially dilution of the number of responding cells by relatively easy handling, we have studied the abilities of cryopreserved human peripheral blood lympoid cells to respond to E-B virus-transformed autologous cells and autologous leukemia cells as well as to allogeneic lymphoid cells.

MATERIALS AND METHODS

Collection of human peripheral blood lymphoid cells (PBL)

The buffy coat rich in platelets and lymphoid cells was collected from normal volunteers or patients in a plastic blood transfusion bag (Teruflex, Terumo Co. Ltd.) by a continuous blood cell separator (Hemonetics PEX, Hemonetics Co. Ltd.) with acid citrate dextrose (ACD) as an anticoaglant. The bag was then centrifuged at 200G. for 10 min. to separate lymphoid cells from the platelet rich plasma. The lymphoid cells were then transfered into test tubes, diluted with phosphate buffer saline without Ca^{++} and Mg^{++} (PBS (−)) and further purified by Ficoll-Conray density gradient centrifugation at 400G. for 30 min. (7). After centrifugation, the cells at the interphase were collected, washed three times with PBS (−), resuspended in RPMI 1640 medium supplemented with 20% fetal calf serum (FCS) (Centaurus, Lot. 649, Santa Anna, U. S. A.) and counted. Cell viability was assessed by the trypan blue dye exclusion test and the differentiation of mononuclear cells was performed by Türk staining. Both the viability and the percentage of mononuclear cells were more than 95% in all experiments.

Preparation of E-B virus-transformed cells (B-cell line)

B-cell lines derived from two individuals were used for the 4 experiments presented in this paper. One line was spontaneously established from a patient's leukemia cell culture. The other was established by culturing PBL obtained from a healthy individual with a cell-free culture medium from the B 95-8 cell line. After being established, both cell lines were maintained with RPMI 1640 medium supplemented with 10% FCS, and it was confirmed that they possessed surface immunoglobulins and E-B associated nuclear antigens, and formed EAC rosetts. When used for experiments, the cells were harvested from culture flasks, washed three times with RPMI 1640 medium supplemented with 10% human AB male serum (test medium) and counted with trypan blue dye. Viability of the B-cells was usually more than 90%.

Collection of human leukemia cells

At the time of diagnosis, the patient's peripheral venous blood which was fully occupied with leukemia cells was drawn with heparin and subjected to Ficoll-Conray density gradient at 400G. for 30 min. After centrifugation, the leukemia cells at the interphase were collected, washed three times with PBS (−), then resuspended in RPMI 1640 with 20% FCS and counted.

Cell freezing

The PBL and leukemia cells to be frozen were suspended at 2×10^7/ml in RPMI 1640 medium containing 20% FCS and placed on ice to cool in 50 ml plastic tubes. When the cell solutions were cooled down to around 4°C, an equal amount of RPMI 1640 medium containing 20% DMSO was slowly added to them and gently mixed. The cell solutions were then aliquoted into pre-cooled 2-inch Nunc serum tubes (Nunc, Denmark) at 1.5 ml each and placed into the chamber of the Cryo-Med Liquid Nitrogen Controlled-rate freezer and allowed to equilibrate at 0°C for 10 min. A 30-gauge copper-constantan thermocouple was suspended through the cap of one of the samples to be frozen. A Leeds and Northrup Speedmax Type N/L recorder was used for continuous temperature recordings. The samples were cooled at a rate of −1°C/min. to −40°C and then −5°C/min. to −80°C and immediately transfered to liquid nitrogen for storage (−196°C).

Fresh PBL as unfrozen controls were set in round-bottomed 15 ml plastic test tubes with RPMI 1640 medium containing 20% FCS and kept at 4°C until use.

Thawing and dilution of frozen cell suspensions

Each frozen tube was thawed rapidly in a 40°C water bath until the ice was completly melted (90 sec.). As the last trace of ice melted, the cell solution was transfered into a 50 ml plastic test tube and then cold test medium was added dropwise to the tube while it was being gently swirled until the volume reached 15 ml. This cryopreserved cell suspension and the unfrozen control cell suspension were centrifuged at 200G. for 10 min.. After an additional washing for both samples, the cells were resuspended in test medium and counted. Viability of the cells was found in the range of 90-98% and cell recovery was in the range of 60-95%.

Micromethod of in vitro sensitization

The micromethod for *in vitro* sensitization of lymphoid cells was a modification of that used by Zarling and Bach (8). The cryopreserved or unfrozen, fresh responding PBL were adjusted to 2×10^6/ml with test medium and each of the samples was aliquoted into 6 wells in a line of a Nunc U-bottomed mircotestplate at 0.1 ml/well. Allogeneic fresh PBL or autologous leukemia cells, also adjusted to 2×10^6/ml, or autologous B-cell lines adjusted to 0.25×10^6/ml were then added as stimulating cells (irradiated 4,000 R) into the wells containing preseeded responding PBL. By using a Multi-Channel Pipette (Titertek), the responding and stimulating cells were then gently mixed and 0.1 ml of aliquot per well from 3 wells were transfered into the next 3 wells in a line prefilled with 0.1 ml of test medium. The same handling was repeated for the last 3 wells; thus, serially diluted, 4 different numbers of responding cells (triplicate in each) were set up, keeping a constant responder to stimulator cell ratio. As controls for the stimulating cells described above, 4,000 R irradiated autologous fresh PBL were used.

After the final adjustment of media volume to 0.2 ml/well with test medium, the plates were incubated at 37°C in a 5% CO_2 humid atmosphere for 7 days.

^{51}Cr release assay for cell-mediated cytotoxicity resulting from in vitro sensitization

For assaying the cytotoxicity directed against alloantigens, PHA stimulated PBL were used as target cells as well as stimulating cells for ^{51}Cr release assay. For assaying the cytotoxicity resulting from atuologous *in vitro* sensitization, the continuously cultured B-cell lines or the leukemia cells which had been cryopreserved and thawed by the methods described above were used as target cells. For the labeling of those cells with ^{51}Cr, they were washed and adjusted to 2×10^6/0.4 ml of test medium and 50 mCi/0.05 ml of ^{51}Cr($Na_2{}^{51}CrO_4$, New England Nuclear, S. A.: 300-400 mCi/mg) was added to each target cell solution. The mixtures were then incubated at 37°C incubator for 2 hours. After the incubation, the cells were washed 3 times with cold test medium and adjusted to 0.5×10^6/ml of test medium.

The microtestplates were then kept in the humidified incubator with 5% CO_2 for 7 days. Cells pelleted at the bottoms of the wells were gently dispersed by a Multi-Channel Pippete and then 0.02 ml of target cell solution was added into each well; thus, the cells generated to be cytotoxic effectors were co-cultured with 10^4 ^{51}Cr labeled target cells. To determine spontaneously and maximally releasable ^{51}Cr from the target cells during the experimental time period, the target cells were seeded in wells containing plain test medium and 0.5% N. P. −40 respectively. The plates were then incubated for an additional 8 hours. After the incubation, the supernatant was harvested by Supernatant Collection System (Titertek) and radioactivity of supernatant was counted in a well-type gamma counter. Assays were run in triplicate as described above and the mean and S. D. were calculated. The equation to obtain the percent cytotoxicity was as follows;

$$\text{percent cytotoxicity} = \left(\frac{\overline{X} \text{ of test release} - \overline{X} \text{ of spontaneous release}}{\overline{X} \text{ of maximum release} - \overline{X} \text{ of spontaneous release}} \right) \times 100$$

RESULTS

a) Responsiveness of cryopreserved PBL against alloantigens in in vitro sensitization.

Figure 1 shows an experiment of the comparative study of the cytotoxicity generated from fresh or cryopreserved PBL after 7 days *in vitro* sensitization against alloantigens. Maximal cytotoxicity was obtained at the equivalent level in both cryopreserved PBL and fresh PBL groups in well with 10×10^4 cells.

Table 1 shows the summary of the experiments of the comparative studies of the cytotoxicities generated from fresh or cryopreserved PBL after the allogeneic *in vitro* sensitization. In 5 out of 6 experiments, the cytotoxicity generated from cryopreserved PBL reached their maximum levels equivalent to those from fresh PBL at the same or one-fold different numbers of responding cells. In one case, the cytotoxicity from the cryopreserved PBL was somewhat higher than that from the fresh PBL.

b) Responsiveness of cryopreserved PBL against E-B virus-transformed autologous cells in in vitro sensitization.

Figure 2 shows data obtained in the comparative study of the cytotoxicity generated from fresh or cryopreserved PBL after they were sensitized with the E-B virus-transformed autologous cells *in vitro*. As shown in the figure, the maximum cytotoxicity generated from the cryopreserved PBL was equivalent to that from fresh cells at the same numbers (20×10^4) of responding cells in a well, and the cytotoxicity diminished in parallel fashion in accordance with the dilution of the responding cells in both cases.

Table 2 shows the summary of the comparative studies on the cytotoxicity generated from fresh or cryopreserved PBL after *in vitro* sensitizations against E-B virus-transformed autologous cells. In 3 out of 4 experiments, the maximum cytotoxicity induced from cryopreserved PBL was not significantly different from that induced from fresh PBL. In the other case, the cytotoxicity generated from the cryopreserved PBL was somewhat higher than that from fresh PBL.

c) Responsiveness of cryopreserved PBL against autologous leukemia cells.

Among our routine experiments for studing the antigenecities of human leukemia cells and the host immune responses to those antigens, it was found by using the method of *in vitro* sensitization that the PBL of patient T. H., whose profile was summarized in Table 3, generated cytotoxic cells when they wer co-cultured with his own leukemia cells in the

presence of Nocardia rubra cell wall skeleton (N-CWS). Thus, we drew 4×10^8 of his PBL by a blood cell separator, aliquoted it into 30 vials, and cryopreserved it for further investigations.

Table 1. Comparison of the responsiveness between fresh and cryopreserved PBL against allogeneic antigens in the micromethod of *in vitro* sensitization

Exp. No.	Fresh or cryo.	Mean of percent cytotoxicity ± S. D. generated from the responding cell numbers of				Difference[2] of maximum cytotoxicity between fresh & cryo.
		20[1]	10[1]	5[1]	2.5[1]	
1	F	45.3 ± 7.0	40.0 ± 6.8	39.8 ± 2.6	9.9 ± 1.5	N. S.[3]
	C	31.4 ± 11.5	51.3 ± 8.2	31.4 ± 10.9	13.9 ± 5.5	
2	F	59.7 ± 17.3	74.9 ± 12.9	50.2 ± 2.4	18.8 ± 4.8	N. S.
	C	45.8 ± 7.9	79.9 ± 6.0	54.0 ± 5.0	21.3 ± 10.3	
3	F	32.6 ± 12.6	38.4 ± 9.9	23.9 ± 11.8	9.0 ± 5.8	N. S.
	C	34.0 ± 3.6	27.3 ± 4.8	18.9 ± 7.3	4.6 ± 2.6	
4	F	59.6 ± 9.9	61.7 ± 6.6	31.9 ± 5.9	10.0 ± 3.4	N. S.
	C	46.3 ± 3.5	64.9 ± 11.6	23.8 ± 7.8	0	
5	F	35.4 ± 12.4	54.5 ± 16.3	43.4 ± 10.8	13.5 ± 5.0	Sig.[4]
	C	97.5 ± 0.7	52.8 ± 18.7	51.6 ± 10.3	40.8 ± 12.9	
6	F	33.0 ± 8.6	48.7 ± 4.0	72.1 ± 20.3	13.0 ± 1.9	N. S.
	C	68.3 ± 1.5	70.7 ± 3.3	62.7 ± 5.5	19.6 ± 2.2	

Table 1. Comparison of the responsiveness between fresh and cryopreserved PBL against allogeneic antigens in the micromethod of *in vitro* sensitization

1: Responding cell numbers $\times 10^{-4}$ in a well of Nunc-U plate

2: by student t-test

3: not significant

4. significant

Table 2. Comparison of the responsiveness between fresh and cryopreserved PBL against autologous E-B virus-transformed cells in the micromethod of *in vitro* sensitization

Exp. No.	Fresh or cryo.	Mean of percent cytotoxicity ± S. D. generated from the responding cell numbers of				Difference[2] of maximum cytotoxicity between fresh & cryo.
		20^1	10^1	5^1	2.5^1	
1	F	13.0 ± 5.7	40.3 ± 10.9	37.7 ± 12.1	26.7 ± 21.3	N. S.[3]
	C	11.1 ± 7.6	21.9 ± 2.3	25.0 ± 8.0	16.1 ± 3.5	
2	F	13.2 ± 279	35.0 ± 3.9	24.5 ± 4.3	13.0 ± 6.3	Sig.[4]
	C	56.6 ± 9.6	53.3 ± 11.2	40.7 ± 12.7	24.1 ± 5.5	
3	F	30.0 ± 5.4	27.6 ± 1.6	12.6 ± 0.9	8.3 ± 1.6	N. S.
	C	26.7 ± 2.1	22.6 ± 1.4	17.4 ± 3.3	7.9 ± 1.9	
4	F	49.3 ± 7.6	59.1 ± 3.3	68.8 ± 2.5	71.0 ± 8.6	N. S.
	C	42.7 ± 3.4	52.2 ± 13.1	50.0 ± 16.0	55.7 ± 17.9	

Table 2. Comparison of the responsiveness between fresh and cryopreserved PBL against autologous E-B virus infected cells in the micromethod of *in vitro* sensitization

1: Responding cell numbers $\times 10^{-4}$ in a well of Nunc-U plate

2: by student t-test

3: not significant

4. significant

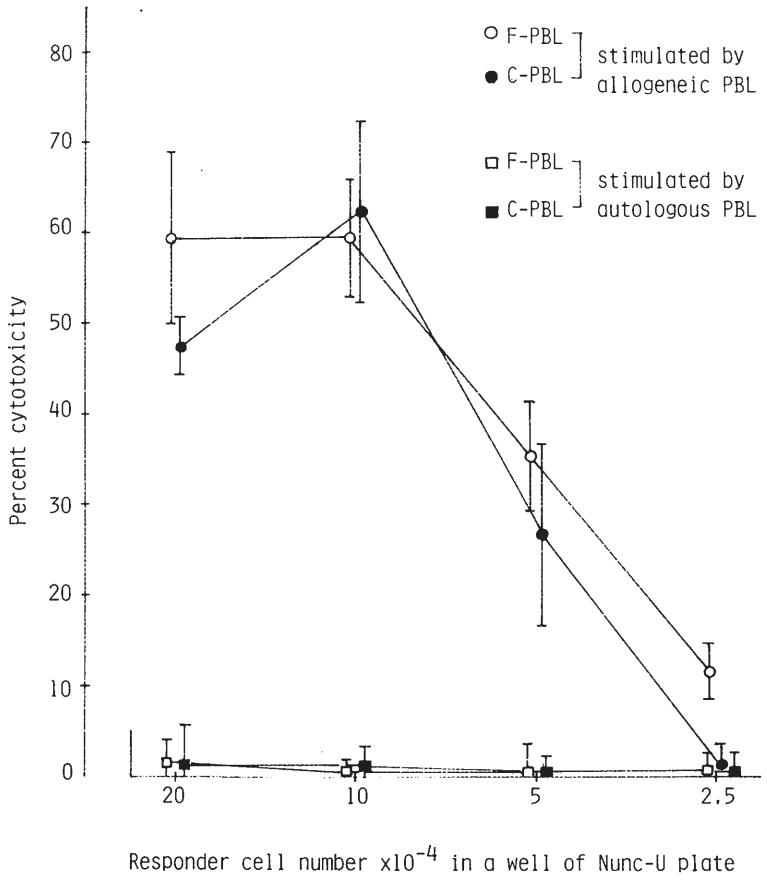


Fig. 1 Comparison of the responsiveness of fresh (F) and cryopreserved (C) PBL against allogeneic antigens in the micromethod of *in vitro* sensitization

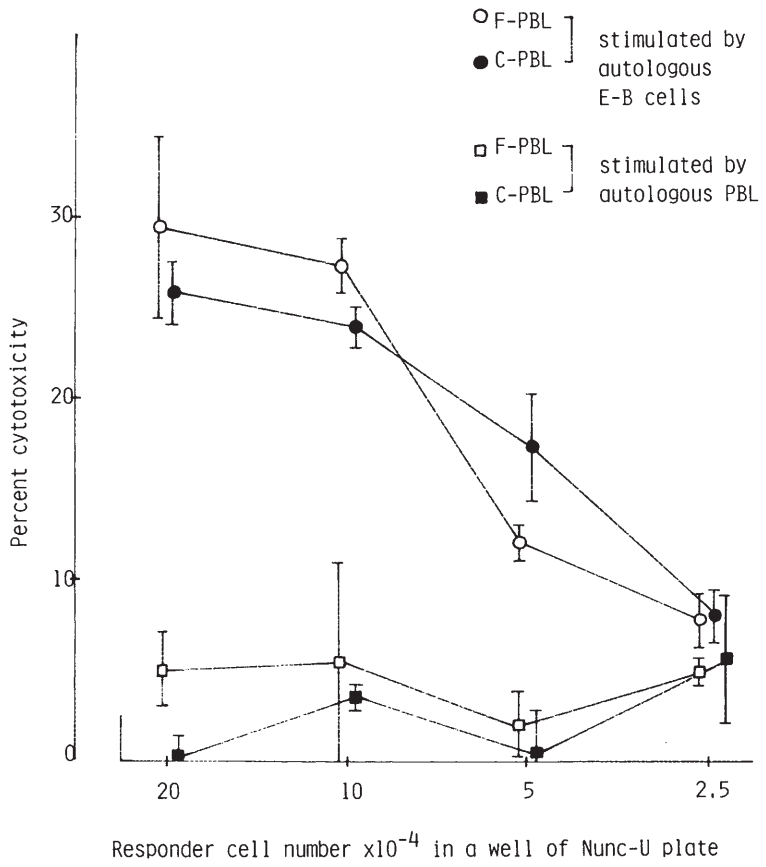


Fig. 2 Comparison of the responsiveness of fresh (F) and cryopreserved (C) PBL against autologous E-B virus-transformed cells in the micromethod of *in vitro* sensitization

Figure 3 shows the results of the comparative study of the cytotoxicity generated from fresh or cryopreserved PBL by *in vitro* sensitization with autologous leukemia cells. After the 7-day co-culture of the patient's fresh or cryopreserved PBL and his autologous leukemia cells in the presence of N-CWS, the generated cytotoxicity was tested on the autologous leukemia cells as targets. As shown in the figure, the cytotoxicity generated from 10×10^4 of the cryopreserved PBL in a well was equivalent to the maximum cytotoxicity generated from the same numbers of the fresh responding cells. Although the data were not shown in this paper, we repeatedly tested the same batch of this patient's PBL and confirmed that these cryopreserved PBL also exhibited their maximum reactivity when the numbers of responding cells were 10×10^4 in a well.

d) Studies of the antigenecities of human leukemia cells and the cellular immune responses to these antigens in a leukemia patient, T. H., by using his cryopreserved PBL.

As it was demonstrated in the preceeding section that patient T. H.'s cryopreserved PBL were equally responsive as fresh ones, we tried to study the antigenecities of human leukemia cells and cellular immune responses to these antigens in the case of T. H. by using his cryopreserved PBL.

Figure 4 shows the augmenting effect of N-CWS in *in vitro* sensitization of patient T. H.'s PBL against his autologous leukemia cells. In 2 out of 5 previous experiments of *in vitro* sensitization, his PBL reacted to his own leukemia cells without the addition of any immune modulator *in vitro* (in table 3). Such reactivity was lost at the time when the PBL were cryopreserved. However, when the PBL were co-incubated with his autologous leukemia cells in the presence of N-CWS for 7 days, cytotoxicity on autologous leukemia target cells reappeared.

Figure 5 shows the influence of the removal of the medium containing N-CWS on the assay day. As the target leukemia cells were directly seeded into the wells of a microtestplate where the patient's PBL, his autologous leukemia cells and N-CWS were co-incubated for 7 days by the micromethod of *in vitro* sensitization, the cytotoxicity may be induced from the direct effect of N-CWS on the target cells. To examine this possibility, we removed the medium containing cultured cells and N-CWS on day-7 (the assay day) from each well, spun them down, washed the cells twice, resuspended the cells in the test medium and reseeded them to a new microtestplate with ^{51}Cr-labeled target leukemia cells. As seen in the figure, such removal of N-CWS from the medium did not influence the cytotoxicity, revealing that the cytotoxicity was a cell-mediated one, not a direct effect of N-CWS itself.

As shown in the figure 6, the cytotoxicity was decreased when the autologous leukemia cells were more diluted, showing that the presence of leukemia cells was a prerequisite for the generation of cytotoxic activity in this system.

Table 3. Profile of patient T. H.

Age 36 male acute myelogenous leukemia

1) At the time of diagnosis, his leukemia cells, which almost fully occupied his peripheral blood and bone marrow, were collected and cryopreserved.

2) He was induced into complete remission by chemotherapy.

3) Two out of 5 *in vitro* sensitization tests showed positive results when the experiments were performed with freshly prepared PBL during the complete remission. PBL were collected by a blood cell separator and cryopreserved.

The cytotoxicity generated in this way was then tested on patient T. H.'s target cells to see whether this cytotoxic action was a selective one. PBL obtained from an unrelated individual were cultured with T H.'s leukemia cells to generate the cytotoxicity reactive to both target cells. As shown in figure 7, the cytotoxicity induced from the clutures of the patient's PBL and his own leukemia cells in the presence of N-CWS was selectively active against his leukemia cells. The generated cytotoxicity of PBL obtained from an unrelated individual against T. H.'s alloantigens was exhibited on both target cells, suggesting that the biological susceptibility of them to any cytotoxicity would be equivalent.

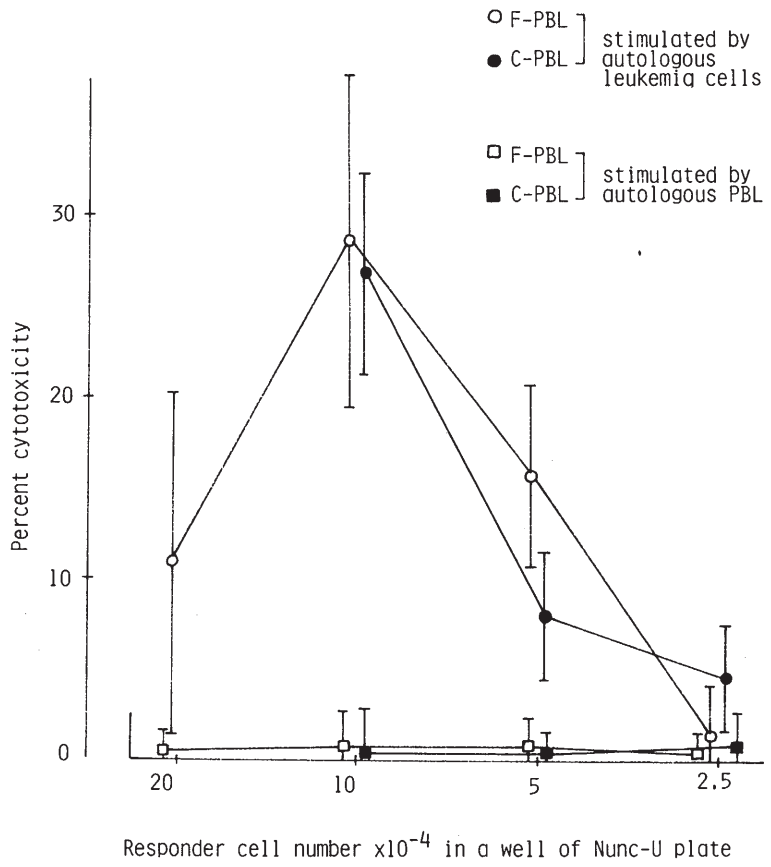
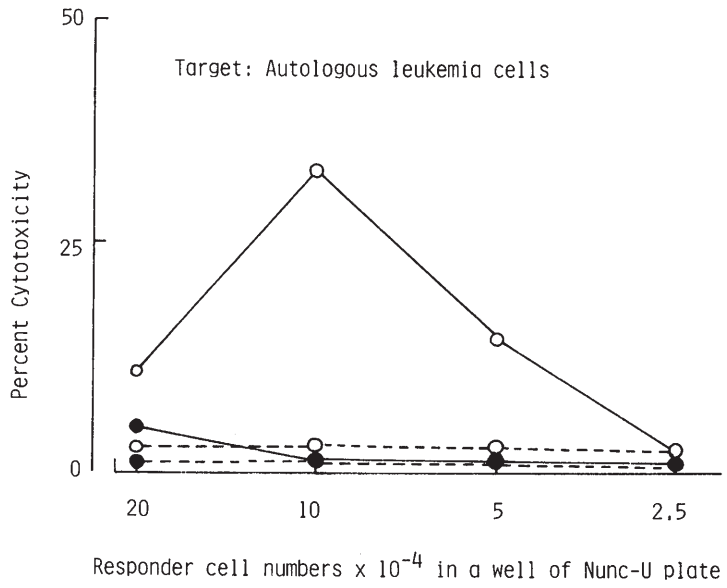
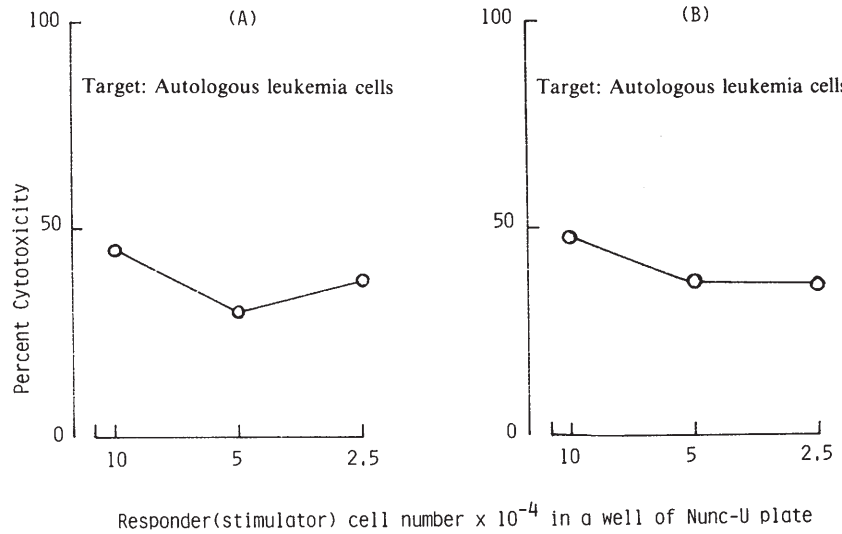


Fig. 3 Comparison of the responsiveness of fresh (F) and cryopreserved (C) PBL against autologous leukemia cells in the micromethod of *in vitro* sensitization



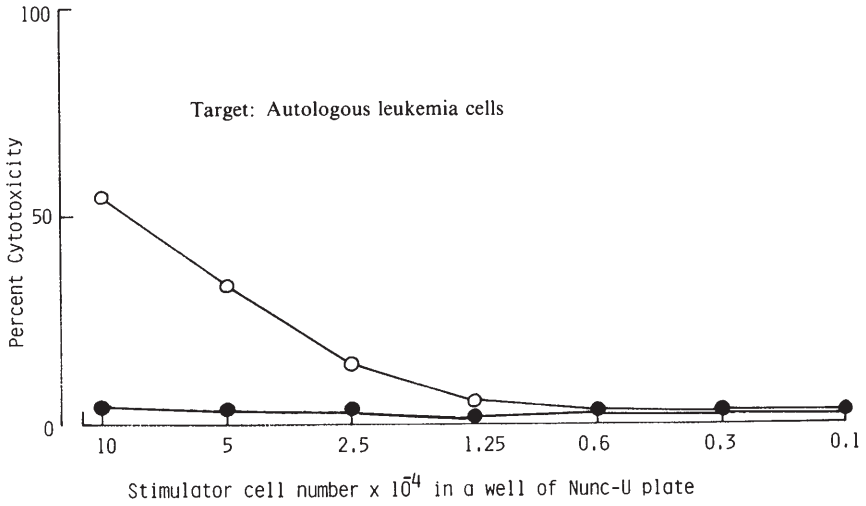
T. H.'s PBL were stimulated by O——O; autologous leukemia cells and N-CWS (10μg/ml), O- - -O; autologous leukemia cells alone, ●——●; N-CWS alone, ●- - - -●; no stimulator.

Fig. 4 The effect of the addition of N-C W S against autologous leukemia cells in the micromethod of *in vitro* sensitization



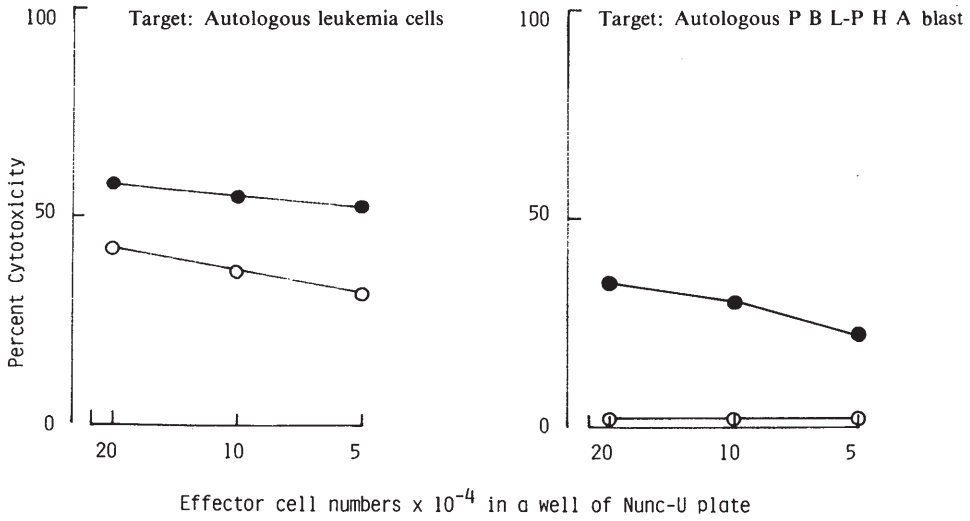
(A) Culture medium was not replaced.
(B) Culture medium was replaced by new test medium before ^{51}Cr release assay.

Fig. 5 The effect of the removal of N-CWS containing culture media on the cytotoxic activity against autologous leukemia cells in the micromethod of *in vitro* sensitization.



T. H.'s P B L were stimulated by O——O; variable numbers of autologous leukemia cells and N-C W S (10μg/ml) ●——●; N-C W S alone.

Fig. 6 Titration of leukemia cells as stimulators in the micromethod of *in vitro* sensitization



T. H.'s PBL were stimulated by autologous leukemia cells and N-CWS (10μg/ml); O——O
PBL obtained from an unrelated individual were stimulated by T. H.'s leukemia cells and N-CWS (10μg/ml); ●——●

Fig. 7 Selectivity of the cytotoxicity

DISCUSSION

Cryopreservation of human peripheral lymphoid cells is usually conducted in the medium with 10% DMSO and 10% FCS containing 10^7/ml of cells, using an automated programmed freezer at a cooling rate of $-1°C$/min.. The frozen cells are kept in liquid nitrogen and are thawed rapidly at 40°C upon use. It has been well established that the above procedure is sufficient to preserve the function of lymphoid cells in terms of response to mitogen (9), to certain soluble antigens like purified protein derivatives (PPD) (10), and to allogeneic antigens (9) and that of cytotoxic lymphocytes (11) and antibody dependent cell-mediated cytotoxicity (ADCC) effector cells (12). Since each lymphoid subpopulation might have different susceptibility to a cooling rate (13), we have postulated that the cells committed for the generation of cytotoxic cells in *in vitro* sensitization might not be totally recovered after cryopreservation, especially when cells with weaker antigens are used as stimulators.

Although the recovered cryopreserved lymphoid cells showed 90-98% viability when tested by the trypan blue dye exclusion test, it must be taken into consideration whether or not the viable cells defined by the dye exclusion test were truely viable enough to sustain their own functions (14). The cryopreserved PBL might die soon after they are seeded into cluture plates, and the cryopreserved PBL might show less function than fresh PBL even when the same number of "viable" cells are cultured. Therefore, in order to compare the fucntion of fresh and cryopreserved PBL, one should evaluate them not at a fixed number of testing cells but at various numbers of cells by serially diluting testing cells. We applied this serially dilution method for the present study and evaluated the recovery of the function of cryopreserved PBL in terms of the generation of cytotoxic cells by *in vitro* sensitization. Using the micromethod of *in vitro* sensitization which we devised previously (5), we could more easily dilute the number of responding cells or that of stimulating cells at the start of *in vitro* sensitization culture.

As shown in table 1 and figure 1, the maximum cytotoxic activity of fresh PBL, which were sensitized *in vitro* against allogeneic PBL, was observed at the cell concentration of 2.5 to 20×10^4 per well of a microplate. When cryopreserved PBL from the same donors were used in the same experiments, the equivalent levels of maximum cytotoxicity were observed at the same or one-fold different numbers of seeded cells as those of fresh PBL. This result indicated that cryopreservation could preserve a subset of human PBL which become allo-reactive cytotoxic cells in *in vitro* sensitization.

As shown in tables 2 and 3 and figures 2 and 3, when the E-B virus-transformed autologous PBL or autologous leukemia cells wer used, almost the same results were obtained, although the quantity and the quality of the antigens on these autologous cells might have been considerably different from those on allogeneic cells.

As an example of the usefulness of such cryopreserved PBL, we presented the data on the antigenecities of human acute leukemia cells and the host immune responses to those antigens by the method of *in vitro* sensitization. In the past, we studied 25 cases of acute leukemia in 47 *in vitro* sensitization experiments where freshly prepared patients' PBL were used, and 5 out of 25 cases (6 out of 47 experiments) showed the PBL to become cytotoxic cells *in vitro* against their autologous leukemia cells (15). However, in most cases, we could not repeatedly demonstrate the generation of such cytotoxicity in those patients presumably because of the administration of anti-leukemic agents including prednisolone, which are known to produce

a lymphocidal effect. Therefore, tried to cryopreserve enough of the patients' PBL during their "drug free" periods to confirm the reproducibility of the once observed results and to analyse the mechanisms involved.

The data obtained from the *in vitro* sensitization study of patient T. H. are summarized as follows; 1) T. H.'s leukemia cells possessed certain cell-surface antigens which were not present on his normal lymphoid cells. 2) T. H.'s PBL recognized these antigens and generated cytotoxic cells *in vitro*. 3) *In vitro* addition of an immunological adjuvant, N-CWS augmented the generation of cytotoxic cells which were reactive against autologous leukemia cells.

We believe that the present result on the successful cryopreservation of autoreactive lymphoid cell subsets encourages us to apply this technique for adoptive transfer of autologous lymphoid cells clinically as well as for further *in vitro* analysis of human cellular immune reactions with high reproducibility which is an essential prerequisite for science.

ACKNOWLEDGMENTS

We are grateful to Prof. Itsuro Sobue for his long-term support to our work and also for his advice. We thank to Profs. Nobuo Kato and Izumi Nakashima and Drs. Ryuzo Ohno and Hiroshi Shiku for critical reading of the manuscript and their advices. We also wish to thank Mss. Sayoko Sugiura and Atsuko Nagata for their expert technical assistance.

REFERENCES

1) Engers, H. D., Brunner, K. T., Cerottini, J. C.: The generation of cytolytic T lymphocytes *in vitro* using mixed lymphocyte cultures, in *In vitro methods in cell-mediated and tumor immunity*, pp. 737—740, edited by B. R. Bloom and J. R. David, Academic Press, New York, 1976.
2) Tanaka Y., Sugimura K., Hinuma Y. *et al.*: Memory of Epstein-Barr virus specific cytotoxic T cells in normal seropositive adults as revealed by an *in vitro* restimulation method. *J. Immunol.*, **125**, 1426—1431, 1980.
3) Zarling, J. M., Raich, P. C., Mackeough, M. *et al.*: Generation of cytotoxic lymphocytes *in vitro* against autologous human leukemia cells. *Nature* **262**, p. 196, 1976.
4 Ashwood-smith, M. J.: Low temperature preservation of mouse lymphocytes, with dimetyl-sulfoxide. *Blood,* **23**, 494—501, 1964
5) Kodera, Y. and Ezaki, K.: Studies of leukemia cell surface antigens and their solubirized form by the method of *in vitro* sensitization, *the Japanese Journal of Clinical Hematology,* **21**, 1291—1295, 1980 (in Japanese)
6) Knight S. C. and Farrant, J.: The problem of assaying the recovery of lymphocytes and other blood cells after freezind and thawing. *Cryobiology,* **15**, 230—231, 1978.
7) Böyum, J. M. and Bach, F. H.: Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Lab. Invest.*, **21**, Suppl. 97, 77—89, 1968.
8) Zarling, J. M. and Bach, F. H.: A sensitive micromethod for generating and assaying specifically cytotoxic human lymphocytes, in *In vitro methods in cell-mediated and tumor immunity,* pp. 741—748, Edited by B. R. Bloom and J. R. Devid, Academic Press, New York, 1976.
9) Mangi, R. J. and Mardiney, M. R.: The *in vitro* transformation of frozen-stored lymphocyte in the mixed lymphocyte reaction and in culture with phytohemagglutinin and specific antigens. *J. Exp. Med.*, **132,** 401—416, 1970.
10) Factor, M. A. Strong, D. M., Miller, J. L. *et al.*: Cell-mediated lymphocytotoxicity following *in vitro* sensitization of frozen stored human mononuclear cells in heterologous serum. *Cryobiology,* **12**, 521—529, 1975.
11) Golub, S. H., Sulit, H. L., Morton, D. L.: The use of viable frozen lymphocytes for studies in human tumor immunology. *Transplantation,* **19**, 195—202, 1975.
12) Miller, R. A., Bean, M. A., Kodera, Y. *et al.*: Cryopreservation of human effector cells active in antibody-dependent cell-mediated cytotoxicity. *Transplantation,* **21**, 517—519, 1976.
13) Knight, S. C., Farrant, J., Morris, G. L.: Separation of populations of human lymphocytes by freezing and thawing. *Nature New Biology,* **239**, 88—89, 1972.
14) Harada, M. and Hattori, K.: Does the dye exclusion test indicate true viability? *Cryobiology,* **15**, p. 681, 1978.
15) Kodera, Y., Tanimoto, M. Yamada, H. *et al.*: *In vitro* sensitization against autologous leukemia cells: Search for the reproducibility. *Proceeding of the Japanese Cancer Association,* the 39th Annual Meeting, November 1980.