CRYOPRESERVATION OF HUMAN LYMPHOCYTES FOR USE IN IMMUNOLOGICAL TESTS

免疫学的検査に用いるヒトリンパ球の凍結保存法について

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RADIATION EFFECTS RESEARCH FOUNDATION
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SUMMARY

The following conclusions were obtained from experimentation on cryopreservation of human lymphocytes:

The optimum conditions for cryopreservation were freezing medium and recovery solution of pH 7.2, concentration of cells suspended in fetal bovine serum at the time of freezing of 5~7 × 10^6/ml, freezing rate of 2°C/min, and thawing temperature of 42°C. Under such conditions, viability after cryopreservation of 86.8% ± 3.9% was obtained.

It was demonstrated that under specific conditions cryopreserved lymphocytes are competent enough for lymphocyte blastogenesis and mixed lymphocyte culture tests and are not affected by cryopreservation within five months.

It was considered that T cells are more susceptible to damage by cryopreservation than B cells and the cell membrane more than the cytoplasm.

要　約

ヒトリンパ球の凍結を試み，次のような結論を得た.

凍結保存の至適条件は，凍結液と融解液のpH 7.2，凍結時のウシ胎児血清浮遊細胞濃度5～7×10^6／ml，凍結速度2℃／min，融解温度42℃であった．これらの条件下で，凍結後生存率86.8±3.9%が得られた．

一定条件の下では，凍結後リンパ球がリンパ球幼若化試験，リンパ球混合培養試験に十分耐え，5か月までは凍結の影響を受けないことが証明された．

凍結保存によって，B細胞よりT細胞が，細胞質より細胞膜がそれぞれ障害を受けやすいと考えられた．

INTRODUCTION

Collection and preservation of immunocompetent cells have become increasingly important in

緒　言

免疫学の研究において免疫担当細胞の採取，保存の

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immunological studies. For example, preservation of immunocompetent cells with their functions intact would increase their utility value, permitting further tests at any time and new immunological tests in future.

重要性が増してきた．例えば，免疫担当細胞がその機能を保持したまま保存できれば，随時取り出して検査でき，将来の新しい免疫検査にも対応できるので，その利用価値は大きい．

We have experimentally conducted cryopreservation of human lymphocytes using a programmed freezer according to the method of Stopford et al.[1] This is a report on our study of the preservability and functional changes of lymphocytes due to cryopreservation, using as indexes cell viability before and after preservation, blastogenesis in culture with mitogens [phytohemagglutinin-P (PHA-P), concanavalin A (Con A), and pokeweed mitogen (PWM)], mixed lymphocyte culture, E- and EAC-rosette formation methods, and nonspecific esterase staining.

我々は，Stopford ら[1]の方法に沿って，プログラムフリーザーを用いて，ヒトリンパ球の凍結保存を試み，凍結前後の細胞の生死，mitogen〔phytohemagglutinin-P (PHA-P)，concanavalin A (Con A) と pokeweed mitogen (PWM)〕による幼若化反応，リンパ球混合培養試験，E-，EAC-ロゼット形成試験，そして nonspecific esterase 染色を指標として，凍結保存によるリンパ球の保存性と機能変化を検討したので報告する．

MATERIALS AND METHODS

Lymphocyte Isolation. Lymphocytes were isolated from heparinized (20 units/ml) peripheral blood from healthy adults (aged 20-52) by Ficoll-Conray density centrifugation. These cells were used in experiments after determining their viability with trypan blue.

Freezing Procedure. The isolated peripheral lymphocytes were suspended in fetal bovine serum (FBS), and the number of cells was adjusted to 5～7 × 10^6/ml. Eagle's minimum effective medium (MEM) as test medium [containing 100 units/ml of penicillin, 0.5 mg/ml of streptomycin, 0.02 mg/ml of fungizon, 0.3 mg/ml of 1-glutamine, and 25 mM of N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)] with 20% glucose and dimethyl sulfoxide (DMSO) were mixed at a volume ratio of 3:2 and adjusted to pH 7.2 using 1 N NaOH. This freezing medium was slowly dripped into the lymphocyte suspension under ice-cold condition, until the final volume ratio of the latter to the former was 3:1. One milliliter each of this mixture was dispensed into nunc vials and frozen using a programmed freezer (Cryo-Med Co., USA) under the following conditions:

材料及び方法

リンパ球分離． 健康成人（年齢20－52歳）から得たヘパリン添加末梢血（20単位／ml）中のリンパ球を，Ficoll-Conray 比重遠沈法で分離した．これらの細胞を，trypan blue で生存率を決定した後実験に用いた．

凍結保存法． 分離した末梢血リンパ球をウシ胎児血清（FBS）に浮遊させ，細胞数を 5～7 ×10^6／ml に調製した．20%グルコースを含むイーグル最低有効培養液（MEM）〔ペニシリン100単位／ml，ストレプトマイシン0.5mg／ml，ファンギゾン0.02mg／ml，l-グルタミン0.3mg／ml，N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid（HEPES）25mM を含むもので，以下 test medium と呼ぶ〕と dimethyl sulfoxide（DMSO）を容量比3：2に混和し，1N NaOH を用いて pH 7.2に調製した．氷冷条件下で，この凍結液をリンパ球浮遊液にゆっくり滴加し，最終的にリンパ球浮遊液と凍結液の容量比を3：1にした．この混合液を1ml ずつ nunc vialに分注し，プログラムフリーザー（米国 Cryo-Med 社製）を用いて凍結した．プログラムフリーザーの条件は次のとおりである．

Starting temperature
開始時温度 . . . . 0°C

Liquid phase: Freezing rate
液相 凍結速度 . . . . 2°C/min

Phase change: 相変更	Start phase change 相変更開始. . .	−5°C
	Temperature drop 温度降下	−80°C
	End phase change 相変更終了. . .	−20°C
Solid phase: 固体相	Freezing rate 凍結速度.	3°C/min
	End temperature 最終温度	−80°C

The frozen lymphocytes were preserved in liquid nitrogen.

凍結したリンパ球は，液体窒素中に保存した.

Thawing of Lymphocytes. The frozen lymphocyte suspension was thawed in a 42°C constant temperature water bath and transferred to a test tube kept in ice, into which 1 ml of 4°C FBS was slowly dripped. Following this, 4 ml of test medium containing 20% glucose maintained at 4° C was slowly added and mixed. After mixing, lymphocytes were collected by centrifugation at 1,800 rpm for 10 minutes. After the cells were washed twice with test medium containing 20% FBS and their viability was determined with trypan blue, they were used in experiments.

リンパ球の融解法. 凍結リンパ球浮遊液を42℃恒温温浴中で融解し，氷中のスピッツに移し，4℃ FBS 1 mlをゆっくり滴加した．次いで4℃，20%グルコースを含むtest medium 4 mlを徐々に加え，混和した．混和後，1,800rpm，10分間の遠沈で集められたリンパ球を，20% FBSを含むtest mediumで2回洗浄し，trypan blueで生存率を決定した後，実験に用いた.

Lymphocyte Blastogenesis in Culture with PHA-P, PWM, and Con A. Lymphocytes suspended in MEM containing 10% FBS were dispensed into the wells of microtest plate-II (Falcon Co.) so that each well would contain 2.5×10^5 cells. Then, mitogens PHA-P (Wellcome Co.), Con A (Sigma Co.), and PWM (Gibco Co.) were added at the rate of 10 μl/ml, 20 μg/ml, and 5 μl/ml, respectively, and the cells were cultured in a 5% CO_2 incubator at 37°C. Culture period was four days with PHA-P and Con A, and five days with PWM. Sixteen hours before completion of these cultures, 0.4 μCi of ^{3}H-thymidine (^{3}H-TdR, Radiochemical Centre, Amersham, England, 5 Ci/m mol) was added. The cells were harvested with a micro-cell-harvester (Otto-Hiller Co.), and radioactivity was determined with a liquid scintillation counter.

PHA-P，PWM，Con Aに対するリンパ球幼若化反応. リンパ球を10% FBSを含むMEMに浮遊させ，細胞数を2.5×10^5／wellになるようmicrotest plate-Ⅱ(Falcon社製)の各孔に分注し，PHA-P (Wellcome社製) 10 μl／ml，Con A (Sigma社製) 20 μg／ml，PWM (Gibco社製) 5 μl／mlとなるように各mitogenを加え，37℃，5% CO_2恒温器中で培養した．培養期間は，PHA-PとCon Aについては4日間，PWMでは5日間とした．培養終了の16時間前に，0.4 μCiの^{3}H-thymidine (^{3}H-TdR，英国Amersham，Radiochemical Centre，5 Ci／m mol)を加え，micro-cell-harvester (Otto-Hiller社製)で細胞をharvestし，液体シンチレーションカウンターにより放射能活性を測定した.

Mixed Lymphocyte Culture (MLC). Fresh lymphocytes obtained from the same person were irradiated with 1,250 rad of cobalt using RT-100S (Shimazu Co.) and used as stimulator cells; lymphocytes of before and after freezing were used as responder cells. Both cells were adjusted to 2.5×10^6/ml and suspended in MEM containing 10% FBS, and then dispensed into

リンパ球混合培養試験. Stimulator cellは島津社製RT-100Sで，1,250radのコバルト照射を施された同一人新鮮リンパ球を用い，responder cellとしては凍結前後のリンパ球を用いた．Stimulator cell，responder cellともに2.5×10^6／mlに調製し，10% FBSを含むMEMに浮遊させ，microtest plate-Ⅱの

wells of the microtest plate-II in aliquots of 0.1 ml each and cultured for seven days in a 5% CO_2 incubator at 37°C; 0.4 μCi of ^{3}H-TdR was added 16 hours before completion of culture, and radioactivity was determined.[2] Every test was conducted three times and the mean value was used. T and B lymphocyte counting by E- and EAC-rosette formation methods and nonspecific esterase staining are described elsewhere.[3]

各孔に各々0.1mlずつ分注し，37℃，5％CO_2恒温器で7日間培養した．培養終了の16時間前に，0.4μCiの^{3}H-TdRを加え，放射能活性を測定した．[2] なお，すべての培養実験は3回行い，その平均値をとった．E-ロゼット，EAC-ロゼット形成試験とnonspecific esterase染色によるTリンパ球，Bリンパ球の算定については，別に述べる．[3]

RESULTS

The optimum conditions of cryopreservation of lymphocytes were studied in eight experiments concerning viability of frozen lymphocytes.

The effects of pH of freezing medium and recovery solution are shown in Figure 1. Viability of 70% or more was maintained with pH in the range of 7.0-7.5, and it decreased remarkably whether pH was higher or lower than this range. The range of optimum pH for freezing was small, being pH 7.2, at which viability was 85% ± 5%.

The effect of the number of cells suspended in FBS at the time of freezing is shown in Table 1. Sufficient viability was maintained when the number of lymphocytes was 5×10^6/ml or more. However, when this number was 1×10^7/ml or more, clots increased after freezing. That is, $5\sim7 \times 10^6$/ml was the suitable concentration of frozen lymphocytes.

The effect of freezing rate on viability after freezing is shown in Table 2. There was no difference in viability between freezing rates of 1°C/min and 2°C/min in the liquid phase.

Thawing time differed by the diameter of the tube used for freezing. Using nunc vials, viability decreased at thawing temperatures of 37°C and 52°C (Table 3). The optimum thawing temperature was 42°C.

When lymphocytes were frozen under these optimum conditions in 15 experiments, the viability after freezing was 86.8% ± 3.9% as against 99.7% ± 0.7% before freezing. As shown in Table 4 viability and PHA-P, Con A, and PWM responses were maintained up to five months (n=3).

Review of Effect of Freezing on Blastogenesis in Culture with Mitogen. Lymphocytes

結果

リンパ球凍結保存の至適条件を凍結後リンパ球生存率について8例で検討した．

凍結液，融解液のpHが与える影響は図1のとおりで，pH 7.0から7.5では70％以上の生存率を保持できたが，pHがそれより低くても高くても生存率は著しく低下し，凍結至適pHの範囲は狭く，pH 7.2が最適で，生存率は85％±5％であった．

凍結時FBS浮遊細胞数の効果は表1に示した．細胞数5×10^6／ml以上のリンパ球を用いると十分な生存率が保持できた．しかし1×10^7／ml以上になると，凍結後clotが多くなる．すなわち，5～7×10^6／mlが適当な凍結リンパ球濃度であった．

凍結速度が凍結後生存率に与える影響は表2のとおりで，液相の凍結速度1℃／minと2℃／minの間では差がなかった．

凍結に用いるチューブの直径によって融解時間は異なってくるが，nunc vialを用いると37℃，52℃で生存率が低下した（表3）．至適融解温度は42℃であった．

これらの至適条件で15例に凍結を行うと，凍結前の生存率が99.7％±0.7％であるのに対し，凍結後は86.8％±3.9％であった．表4に示したように，生存率及びPHA-P，Con A，PWMの反応性は5か月まで保たれた（n＝3）．

リンパ球のmitogenに対する幼若化反応への凍結による影響の検討．2時間凍結保存した後，融解した

FIGURE 1 EFFECT OF pH IN FREEZING MEDIUM AND RECOVERY SOLUTION ON CELL VIABILITY
図1 凍結液と融解液の pH が細胞の生存率に及ぼす影響

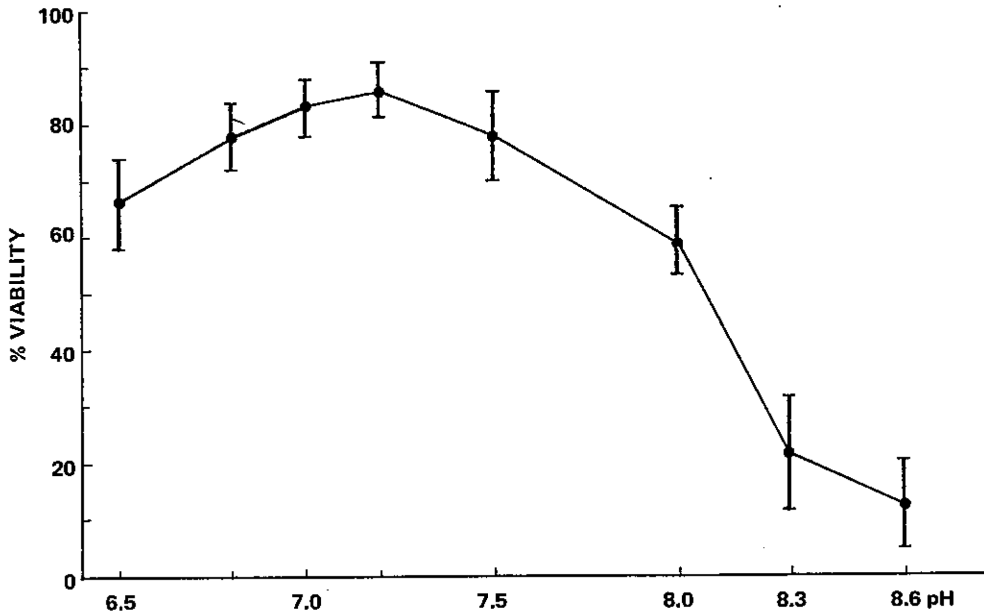

TABLE 1 EFFECT OF CELL NUMBERS IN FREEZING MEDIUM (FBS) ON CELL VIABILITY
表1 凍結液（FBS）の細胞数が細胞の生存率に及ぼす影響

Cells/ml	% viability
2×10^6	36 ± 6
4×10^6	73 ±10
5×10^6	87 ± 5
6×10^6	85 ± 5
7×10^6	85 ±11
10×10^6	83 ±10

Mean percentages ± SD of eight experiments.
8例の平均百分率±標準偏差値

TABLE 2 EFFECT OF FREEZING RATE ON CELL VIABILITY
表2 凍結速度が細胞の生存率に及ぼす影響

Freezing rate (°C/min)	% viability
1	85 ±2
2	85 ±2

Mean percentages ± SD of eight experiments.
8例の平均百分率±標準偏差値

TABLE 3 EFFECT OF THAWING TEMPERATURE ON CELL VIABILITY
表3 融解温度が細胞の生存率に及ぼす影響

Temperature	% viability
37°C	76 ±3
42	83 ±3
52	67 ±3

Mean percentages ± SD of eight experiments.
8例の平均百分率±標準偏差値

TABLE 4 VIABILITY AND MITOGEN RESPONSE AFTER DIFFERENT STORAGE PERIODS
表4　異なる保存期間内の生存率と MITOGEN 反応

	Fresh	2 hours	3 months	5 months
Viability (%)	98.2 ± 0.3	87.7 ± 2.1	89.7 ± 1.6	89.0 ± 1.0
Control* (cpm)	1642 ± 796	1877 ± 999	1271 ± 762	1587 ± 788
PHA-P	38684 ± 18150	38917 ± 19474	36573 ± 11007	38008 ± 10683
ConA (cpm)	35200 ± 12336	32975 ± 7242	34582 ± 14656	33742 ± 8272
Control** (cpm)	1151 ± 489	1261 ± 508	1101 ± 311	1386 ± 504
PWM (cpm)	26606 ± 7715	21920 ± 7247	29215 ± 8076	25573 ± 9944

*Culture for four days 4日間の培養
**Culture for five days, mean ± SD of three experiments 5日間の培養，3例の平均値±標準偏差値
cpm = counts per minute 毎分値

TABLE 5 RESPONSE OF FROZEN-THAWED CELLS TO MITOGEN
表5　MITOGEN に対する凍結融解細胞の反応

Responder cells/well	Control 4-day culture		PHA-P		ConA		Control 5-day culture		PWM	
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
2.5×10^5	1834	2099	41245	42593	34139	35364	1942	1896	31847	29148
(n = 15)	±1006	±1559	±14655	±12962	± 8757	±12169	±1245	±1478	± 9902	±11133
T-test	NS		NS		NS		NS		NS	
1.8×10^5	1815	1454	33615	15450	25697	14692	1684	1437	24616	16128
(n = 5)	± 441	± 284	± 3201	± 3044	± 4110	± 2486	± 189	± 462	± 3500	± 3570
T-test	NS		$P<0.005$		$P<0.005$		NS		$P<0.025$	
1.25×10^5	1259	1237	25261	14029	22306	3754	1358	1024	22100	9792
(n = 5)	± 51	± 128	±12574	± 6354	±10410	± 3438	± 434	± 128	±10557	± 7154
T-test	NS		$P<0.05$		$P<0.05$		NS		$P<0.05$	

Mean count per minute ±SD of triplicate studies in each group.
各グループで3回行った実験の平均毎分値±標準偏差値

cryopreserved for two hours and thawed were compared with fresh cells cultured under identical conditions. As shown in Table 5, under the condition of 2.5×10^5 cultured lymphocytes/well there was no significant difference between before and after freezing (i.e., no effect of freezing, n=15). Under the conditions of 1.25×10^5 cells/well and 1.8×10^5 cells/well, incorporation of ^{3}H-TdR in the controls was not significantly different between before and after freezing, but mitogen response decreased after freezing (n=5).

Regarding the relationship of mitogen response to viability after freezing, no significant difference in mitogen response was observed when viability was 80% or over (n=15), but the response decreased when viability was less than 80% (n=4). Viability of 80% or over after freezing was necessary (Table 6).

リンパ球と新鮮リンパ球とを同一条件で培養し，比較した．表5に示したように，培養リンパ球2.5×10^5／wellの条件では凍結前後に有意差はみられず，凍結による影響はみられなかった(n =15)．細胞数1.25×10^5／well，1.8×10^5／wellでは，コントロールの^{3}H-TdR の取り込みに凍結前後での有意差はなかったが，mitogen による反応性は凍結後低下した(n = 5)．

凍結後の生存率と mitogen 反応性の関係をみると，生存率80%以上では凍結前後で有意の差はみられなかったが(n =15)，80%未満では mitogen 反応性が低下し(n = 4)，凍結後80%以上の生存率が必要であった(表6)．

TABLE 6 EFFECT OF VIABILITY AFTER FREEZING ON MITOGEN RESPONSE
表6 凍結後の生存率が MITOGEN 反応に及ぼす影響

	Viability			
	Fresh	Frozen		
		90% over	80-89%	70-79%
Subjects	15	3	12	4
Control* (cpm)	1834 ± 1006	1947 ± 1052	1713 ± 1514	2189 ± 867
PHA-P (cpm)	41246 ± 14655	43031 ± 16538	41846 ± 12108	29132 ± 20932
ConA (cpm)	34139 ± 8757	33502 ± 18470	36409 ± 11548	16962 ± 14222
Control** (cpm)	1942 ± 1245	1814 ± 509	1943 ± 1690	2168 ± 1444
PWM (cpm)	31847 ± 9902	30463 ± 15001	28627 ± 9937	16086 ± 12207
T-test		NS	NS	P<0.005

*Culture for four days 4日間の培養
**Culture for five days, data present mean of triplicate studies ±SD
5日間の培養，3回の実験の平均値±標準偏差値

TABLE 7 COMPARISON OF FRESH AND FROZEN LYMPHOCYTES IN MIXED LYMPHOCYTE CULTURE (MLC)
表7 リンパ球混合培養における，新鮮リンパ球と凍結リンパ球の比較

	Control		MLC	
	Fresh	Frozen	Fresh	Frozen
Thymidine incorporation (cpm)	2543 ± 2027	2224 ± 1826	13405 ± 3956	12140 ± 4289
T-test	NS		NS	

Mean counts per minute ±SD of 11 experiments 11例の平均毎分値±標準偏差値

Review of Effect of Freezing on Mixed Lymphocyte Culture. As with mitogen response, lymphocytes were cryopreserved for two hours and thawed, then cultured under identical conditions prior to freezing, and MLC conducted before and after freezing were compared as responder cells in 11 experiments. As shown in Table 7, there was no significant difference between before and after freezing (i.e., no effect of freezing).

Review of Effect of Freezing on Lymphocyte Subpopulation. Review was made of five experiments using E- and EAC-rosette formation methods and nonspecific esterase staining. As shown in Table 8, EAC-rosette formation showed no significant difference. On the other hand, E-rosette formation showed decrease. No significant difference was observed in nonspecific esterase staining. Cryopreservation caused no change in the ratio of T and B cells in nonspecific esterase staining.

リンパ球混合培養試験への凍結による影響の検討. mitogen による反応性と同様に，リンパ球を2時間凍結保存した後融解し，凍結前のリンパ球と同一条件で培養し，responder cell として凍結前後の MLC を11例で比較した．表7のとおり，凍結前後に有意の差はみられなかった．すなわち，凍結による影響はなかった．

凍結によるリンパ球 subpopulation への影響の検討. E-ロゼット，EAC-ロゼット形成試験，nonspecific esterase 染色を用いて5例を検討した．表8のように，EAC-ロゼットに有意差はなかったが，E-ロゼットには低下が見られた．nonspecific esterase 染色には有意差がなかった．nonspecific esterase 染色を用いたT細胞，B細胞の割合には，凍結による変化がなかった．

TABLE 8 EFFECT OF CRYOPRESERVATION ON E- AND EAC-ROSETTE FORMATION AND ON α-NAPHTHYL ACETATE ESTERASE (ANAE) ACTIVITY
表8 凍結保存が E-並びに EAC-ロゼット形成と，α-naphthyl acetate esterase（ANAE）活性に及ぼす影響

	Fresh	Frozen	T-test
E-rosettes (%)	71.2 ±6.5	56.4 ±17.5	0.01<P<0.05
EAC-rosettes (%)	21.3 ±1.6	20.0 ± 3.5	NS
ANAE positive (%)	72.5 ±4.7	72.0 ± 3.6	NS
ANAE negative (%)	21.3 ±3.9	17.8 ± 4.3	NS

Mean percentages ±SD of units tested from five experiments
5例の平均百分率±標準偏差値

DISCUSSION

Using a programmed freezer and the Stopford et al[1] method, we observed no effect of cryopreservation in mitogen response and MLC when the optimum number of cultured lymphocytes was employed. Nor did we observe any effect in the ratio of lymphocyte subpopulation in nonspecific esterase staining. We were able to cryopreserve lymphocytes with their function, though short of intact, maintained.

Miller et al[4] reported that antibody-dependent cell-mediated cytotoxicity tests made on a large number of human lymphocytes showed no difference before and after cryopreservation. Adkison and Coggin[5] reported that in their study of tumor development in vivo mixing tumor cells with murine peritoneal exudate cells (PEC), no difference was observed in inhibition of tumorigenesis before and after cryopreservation when the ratio of PEC was high.

In our experiments, good results were obtained in mitogen response and MLC when the number of cultured cells was of a specific level or larger. Probable reasons are: 1) lymphocytes have been damaged to some extent by cryopreservation, 2) macrophages have been damaged by cryopreservation, and 3) DMSO has had inhibitory effect during culture.

As for cell damage by cryopreservation, it is considered that the lipoprotein membrane is most susceptible to damage at the eutectic temperature.[6] Reportedly, in mitogen response, mitogen combines with the lymphocyte membrane receptor, which is followed by a series of phenomena to lymphocyte proliferation.[7]

By our experiments, intracellular metabolic activity toward DNA synthesis was considered to be maintained because there was no difference

考察

Stopford ら[1]の方法に準じて，プログラムフリーザーを用いて，培養リンパ球を至適細胞数にしたとき，mitogen による幼若化反応と MLC に凍結保存による影響は認められなかった．nonspecific esterase 染色によるリンパ球 subpopulation の割合にも影響が認められなかった．完全とは言えないまでも，リンパ球機能を保持したままでリンパ球を凍結保存することができた．

Miller ら[4]は，細胞数を多くしてヒトリンパ球の antibody-dependent cell-mediated cytotoxicity を実施したところ，凍結前後に差がなかったことを報告した．Adkison と Coggin[5]は，murine peritoneal exudate cell（PEC）と腫瘍細胞を混合し in vivo で腫瘍発育をみたところ，PEC の割合を大にすると凍結前後の腫瘍増殖の抑制に差がなかったことを報告している．

我々の実験でも，mitogen による幼若化反応，MLC では一定培養細胞数以上について好成績が得られたが，その理由としては，1) 凍結保存によりリンパ球がある程度細胞障害を受けている，2) 凍結保存によりマクロファージが障害を受けている，3) 培養中に DMSO が抑制作用を示すことなどが挙げられる．

凍結による細胞障害は，共融温度でリポ蛋白膜が最も影響を受けやすいと考えられている．[6] mitogen による幼若化反応では，mitogen がリンパ球膜レセプターに結合し，リンパ球増殖に至る一連の現象に続くとされている．[7]

我々の実験では，培養細胞数が少ないときでもコントロールの^{3}H-TdR 取り込みにおいて凍結前後の細胞に

between fresh and frozen-thawed cells in the uptake of ^{3}H-TdR by controls even when the number of cultured cells was small, but the mitogen-bound membrane receptor was considered to have sustained damage.

It is well known that macrophages are involved in mitogen response.[8] Sears et al[9] reported a decrease in the number of macrophages after freezing. Hem and Munthe-Kaas[10] reported decreased ability to adhere to glass and decreased number of membrane receptors. The optimum conditions for freezing can naturally be considered to differ between macrophages and lymphocytes, and macrophage damage by cryopreservation is probably one of the reasons for the decreased mitogen response. However, the number of necessary macrophages is very small, and it is unknown to what extent they are involved in decreasing the mitogen response after cryopreservation. This will have to be studied.

Mangi and Mardiney[11] reported that residual DMSO after freezing inhibited lymphocyte blastogenesis. Hence, mitogen response can be considered to be affected by DMSO in culture. Effects of polymorphonuclear leukocytes and dead cells can also be considered. In any case, it is believed that the effects of such agents on cells after cryopreservation can be compensated for by increasing the number of cells to that necessary for response.

On the other hand, there is a question whether all kinds of lymphocytes are affected by cryopreservation or a specific lymphocyte population is affected selectively. In the present study rosette formation methods and nonspecific esterase staining were employed to identify the ratio of T and B cells. EAC-rosette formation method and nonspecific esterase staining showed no difference before and after freezing, and the ratio of E-rosettes was decreased after freezing. Membrane receptors were used in rosette formation methods. Nonspecific esterase staining, in which α-naphthyl acetate esterase (ANAE) positive cells were identified as T cells and negative cells as B cells,[12,13] can be said to be a zymochemical identification method. It is considered that esterase activity is localized in the vesicle-like organelles in the cytoplasm.[14] From our finding, it is assumed that the cytoplasm is less susceptible to damage by freezing than the cell membrane and that the membrane of B cells is less susceptible than the membrane of T cells.

差がなかったことから，DNA合成への細胞内代謝活性は保たれているが，mitogen結合膜レセプターは障害を受けていると考えられた．

マクロファージがmitogenによるリンパ球幼若化反応に関与していることはよく知られている．[8] Searsら[9]は，凍結後マクロファージの減少を報告している．HemとMunthe-Kaas[10]は，ガラス面への付着能力の低下と細胞膜レセプターの減少を報告している．凍結至適条件はマクロファージとリンパ球とでは当然違うと考えられ，凍結によるマクロファージの障害は，おそらくmitogen反応性の低下の原因の一つであろう．しかし，マクロファージの必要数は微量であり，それが凍結後のmitogen反応性の低下にどの程度関与しているのかは不明で，今後検討されなければならない．

MangiとMardiney[11]は，凍結後のDMSO残量がリンパ球幼若化反応を抑制すると報告している．したがって，mitogen反応性への培養中DMSOの影響も考えられる．このほかにも，多核白血球と死細胞の影響も考えられるが，いずれにしても凍結後にこれらの因子が細胞に与える影響は，細胞数を多くすることによって反応に必要な細胞を補えると思われる．

一方，凍結保存することによってあらゆる種類のリンパ球が影響を受けるのか，ある一定のリンパ球subpopulationが選択的に影響を受けるのかは問題である．我々の研究では，ロゼット形成試験とnonspecific esterase染色を行ってT，B細胞の割合を同定した．EAC-ロゼット形成試験，nonspecific esterase染色で凍結前後に差はなく，E-ロゼットの割合は凍結後低下していた．ロゼット形成試験には膜レセプターを利用した．Nonspecific esterase染色は，α-naphthyl acetate esterase（ANAE）陽性細胞をT細胞，陰性細胞をB細胞とした．[12,13] これは酵素化学的な同定法と言える．Esterase活性は，細胞内のvesicle-like organellesに局在していると考えられている．[14] 我々の所見では，凍結による細胞障害は，膜よりも細胞質内の方が受けにくく，T細胞の膜面よりもB細胞の膜面の方が障害を受けにくいと推測される．

Notwithstanding the efforts of many researchers, no method has yet been established to cryopreserve lymphocytes with all of their functions intact. The preservation method must be improved by studying the individual functions of lymphocytes which are gradually being elucidated and using a larger number of parameters. Also, the morphological, biochemical, and biological changes caused by freezing and thawing must be clarified.

多くの研究者の努力にもかかわらず，リンパ球の全機能を保ったまま凍結保存する方法はまだ確立されていない．徐々に明らかにされつつあるリンパ球機能を個々に検索し，より多くのパラメーターを駆使して保存法を改良してゆかなければならない．同時に，凍結融解によって形態的，生化学的，生物学的にどのような変化がもたらされるのかも解明されなければならない．

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