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CRYOPRESERVATION OF HUMAN LYMPHOCYTES FOR USE IN IMMUNOLOGIC TESTS: REPORT 2

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: 第 2 報

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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: 第2報

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SUMMARY

As the number of atomic bomb survivors has been decreasing each year, it would be advisable to preserve their valuable cells for future studies. Thus, cryopreservation of cells has become increasingly important.

The authors conducted experiments on cryopreservation of human lymphocytes and have previously reported the effects of cryopreservation on mitogen response and mixed lymphocyte culture. Having established a method of cryopreservation and evaluated its effects on lymphocyte subpopulation, immunoglobulin production, and cytotoxicity, the following conclusions were obtained.

1) The optimal conditions for cryopreservation were: the use of freezing and thawing media of pH 7.2, concentration of dimethyl sulfoxide of 10%, concentration of cells suspended in fetal calf serum at the time of freezing of $5 \sim 15 \times 10^6$/ml, freezing rate of $-1 \sim -2$°C/min, and thawing temperature at 37°C. Under such conditions, the average viability and recovery rate after cryopreservation were 90% and 80%, respectively.

要 約

原爆被爆者は，年々減少しており，貴重な細胞を保存することは後世の調査にも役立ち，細胞の凍結保存の重要性は増大している．

著者らはヒトリンパ球の凍結保存を試み，凍結保存が mitogen 反応性，mixed lymphocyte culture に及ぼす影響を以前報告した．今回，更に凍結保存技術を確立し，凍結保存が，リンパ球 subpopulation，免疫グロブリン産生能，細胞障害活性に及ぼす影響を検討し，以下の結論を得た．

1) 凍結保存の至適条件としては，凍結液，融解液の pH 7.2，dimethyl sulfoxide 濃度10%，凍結時のウシ胎仔血清浮遊細胞濃度 $5 \sim 15 \times 10^6$/ml，凍結速度 $-1 \sim -2$°C/min，融解温度 37°C であり，これらの条件下で凍結後，平均生存率90%，回収率80%を得た．

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2) Neither E receptor nor Fc receptor of the cell surface marker was affected by cryopreservation. The proportion of T cells, helper-inducer T cells, cytotoxic-suppressor T cells, B cells, monocytes, and natural killer (NK) cells was determined by monoclonal antibodies and was found to be stable following cryopreservation.

3) The ability of B lymphocytes to produce immunoglobulin was not significantly altered after cryopreservation.

4) Antibody-dependent cell-mediated cytotoxicity (ADCC) was not affected by cryopreservation when determined by a plaque assay using sheep red blood cells as target cells, but there was an average 35% reduction in ADCC after cryopreservation when examined by ^{3}H-proline cytotoxicity assay with T-24 target cells.

5) NK cell activity showed an average of 40%-60% decrease immediately after thawing, but a recovery of NK cell activity was observed after preincubation for 18 hours. No change was observed in either lymphocyte subpopulations or viability after preincubation following cryopreservation, but as an increase was observed in the ratio of target cells binding to K-562, it was considered that preincubation may be a cause for the recovery of NK cell activity.

6) Freezing damages are considered to depend on the freezing process rather than on the length of cryopreservation period. This was confirmed by the fact that mitogen responses, E- and EAC-rosette formation, and NK cell activity could be kept intact for 12 months, 14 months, and 14 months, respectively.

INTRODUCTION

With the development of basic and clinical immunology, a great deal of progress has been made in the study of lymphocytes, so that preservation of lymphocytes has become increasingly necessary. Cryopreservation of lymphocytes is especially useful scientifically, because it enables immunologic studies under a fixed condition, comparison of results of each sample, and retrospective study of a large number of cells obtained from one person or many people successively under optimal conditions. It is especially important to establish a technique for cryopreservation of cells which

2) 細胞表面マーカーのEレセプター，Fc レセプターは，凍結保存の影響を受けなかった．また，モノクローナル抗体を用いたリンパ球 subpopulation の検出で，T 細胞，helper-inducer T 細胞，cytotoxic-suppressor T 細胞，B細胞，単球，natural killer (NK)細胞の割合を保持して，凍結保存が可能であった．

3) B細胞の機能を示す免疫グロブリン産生能は凍結保存後もその機能は保持されていた．

4) 抗体依存性細胞障害(ADCC)は，ヒツジ赤血球を標的細胞とするプラーク法では凍結保存による影響を受けなかったが，T-24 を標的細胞とする ^{3}H-proline を用いた ADCC 試験では，凍結保存後，平均35%の機能低下を認めた．

5) NK 細胞活性は，凍結融解直後平均40%～60%の機能低下を示したが，18時間 preincubation によって，細胞活性の回復を認めた．凍結保存後の preincubation でリンパ球 subpopulation の割合，生存率に変化は認められなかったが，K-562 に対する target-binding cell の割合の増加を認め，preincubation が NK 細胞活性の回復の一因となると考えられた．

6) 凍結障害は，凍結期間よりも，凍結の際の障害に依存すると考えられ，これは mitogen 反応性は12か月，E, EAC ロゼット形成能は14か月，NK 細胞活性は14か月まで保存可能であることから確認された．

緒　言

基礎免疫学，臨床免疫学の進歩とともに，リンパ球を用いる研究が進み，リンパ球の凍結保存の必要性が増してきた．リンパ球の凍結保存は，同一人あるいは多数の人から大量の細胞を継次的に最適な条件で保存することによって，一定の条件の下でその免疫学的検討をすることが可能となり，成績を各サンプルについて比較すること，また，retrospective にも検討することも可能となり，研究面での有用性は大きい．特に対象者数が減少していく原爆被爆者の免疫能を調査する場合，後世の研究調査にも利用

enables the use of cells in future for the immunologic studies of A-bomb survivors who have been decreasing in number.

Further, preservation of useful cell sources including sperm, blood cell components, and bone marrow cells has become increasingly important in both basic and clinical medicine. In Japan, too, the establishment of cell banks and gene banks is being planned and the cryopreservation technique has become one of the basic techniques necessary not only for medical science but also for all biological sciences. Here the authors report the establishment of a technique for cryopreservation of lymphocytes and its effects on immunologic studies.

MATERIALS AND METHODS

Isolation of Peripheral Mononuclear Cells. Peripheral blood obtained from healthy adults (aged 20-62) was defibrinated using glass beads, and mononuclear cells were isolated by Ficoll-Hypaque density centrifugation. These mononuclear cells were used in immunologic experiments after determining their viability with trypan blue. The remaining cells were suspended in fetal calf serum (FCS), adjusted to $5\sim15\times10^6$ cells/ml, and cryopreserved by the following method.

Freezing Procedure. The freezing procedure[1] described previously was improved and used. Eagle minimum essential medium (MEM) containing 40% dimethyl sulfoxide (DMSO), 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1% nonessential amino acid (NEAA), and 1% essential amino acid was prepared as freezing medium and adjusted to pH7.2 with NaOH before use.

To three parts of cell suspension was added one part of the freezing medium and the two were thoroughly mixed by gently shaking the tube, and aliquots of 1 ml were dispensed into cryopreservation vials (Nunc). They were placed in a programmed freezer (Cryo Med), and frozen to −80°C at freezing rates of −1°C/min in liquid phase and −2°C/min in solid phase (Figure 1), and immediately transferred to a liquid nitrogen tank for preservation.

可能であり，細胞の凍結保存法の確立の意義は大きい．

更に，有用な細胞資源の保存は，研究分野のみでなく，精子，血球成分，骨髄細胞など基礎医学及び臨床医学の両分野でも，ますますその重要性が増大してきている．我が国でも細胞銀行，遺伝子銀行の設立が考えられている現在，凍結保存技術は，医学のみでなくあらゆる生命科学に必要な基礎的技術の一画を占めつつある．そこで著者らは，リンパ球の凍結保存技術の確立と，免疫学的検査への影響について報告する．

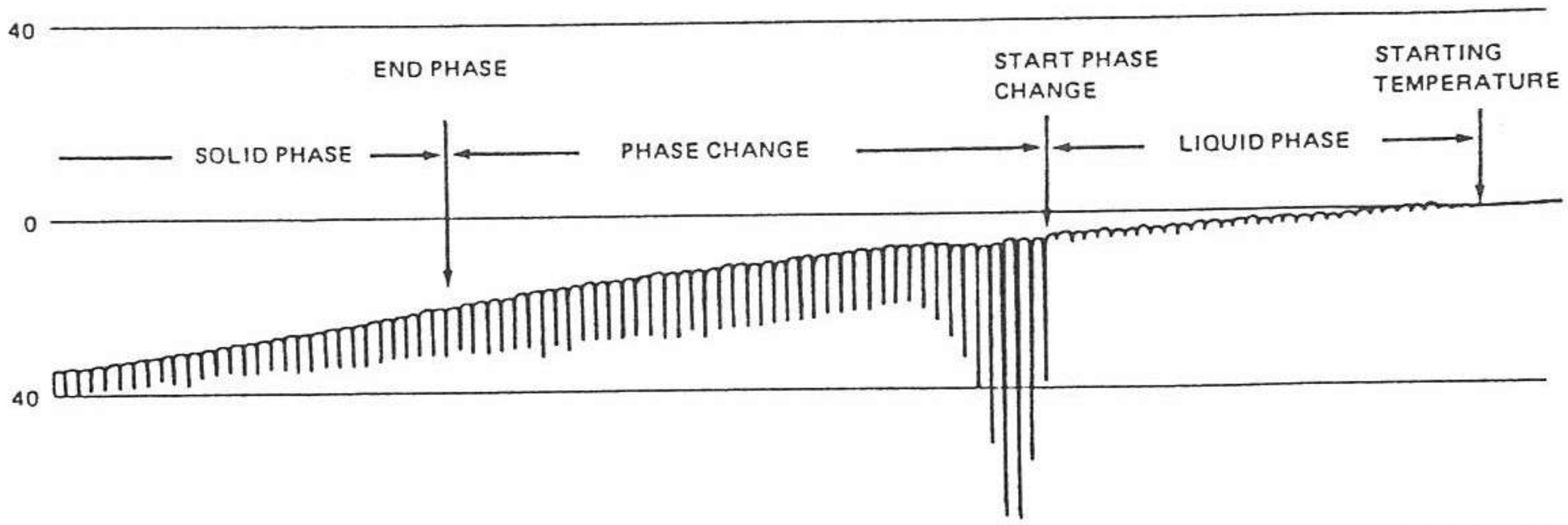
材料及び方法

末梢血単核球の分離． 健康成人(年齢20～62歳)から採取した末梢血をガラスビーズを用いて脱フィブリン化し，Ficoll-Hypaque 比重遠沈法で単核球を分離した．得られた単核球は trypan blue により生存率を決定した後，免疫学的実験に使用した．残りの細胞は，細胞数 $5\sim15\times10^6$/ml になるようウシ胎仔血清(FCS)に浮遊して，次の方法で凍結保存した．

凍結保存法． 凍結保存法は，前回報告[1]の方法を改良して用いた．凍結保存液として，40% dimethyl sulfoxide (DMSO)，25mM N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid (HEPES)，1% nonessential amino acid (NEAA)，1%essential amino acid を含む Eagle minimum essential medium (MEM)を作製し，NaOH で pH 7.2に調整した溶液を用いた．

氷冷条件下で，単核球浮遊液に，凍結保存液を3：1の割合になるように，ゆっくり滴下し，1ml ずつ vial(Nunc)に分注した．分注後，vial をプログラムフリーザー(Cryo Med)に入れ，液相の凍結速度 −1°C/min 固相の凍結速度 −2°C/min で−80°C まで凍結し(図1)，直ちに液体窒素タンクに移し保存した．

FIGURE 1 FREEZING CONDITION
図 1　凍結条件



STARTING TEMPERATURE		0°C
LIQUID PHASE:	FREEZING RATE	−1 ~ −2°C/min
PHASE CHANGE:	START PHASE CHANGE	−5°C
	TEMPERATURE DROP	−80°C
	END PHASE CHANGE	−20°C
SOLID PHASE:	FREEZING RATE	−2 ~ −3°C/min
	END TEMPERATURE	−80°C

LYMPHOCYTES WERE FROZEN WITH A PROGRAMMED FREEZER.
リンパ球はプログラムフリーザーで凍結した.

Thawing Procedure. Eagle MEM containing 20% FCS, 25 mM HEPES, 1% NEAA, and 1% amino acid mixture was adjusted to pH 7.2, cooled to 4°C, and used as thawing medium.

Nunc vials were removed from the liquid nitrogen tank and were rapidly thawed with gentle agitation at 37°C in a water bath. Immediately after thawing, they were kept in ice-cold water. Then, the contents were slowly transferred into a centrifuge tube containing 1 ml of FCS kept at 4°C and, after adding 1 ml of FCS and 4 ml of thawing medium twice, this suspension was washed by centrifugation at 4°C and 240 ×g for 10 minutes. After the cells were washed twice by centrifugation with MEM containing 10% FCS, their viability and recovery rate were determined by trypan blue staining for use in experiments.

E-rosette-Forming Test (T Lymphocytes Counting Test). For the counting of T cells, 0.1 ml of sheep red blood cell (SRBC) solution (2%) treated with 140 M aminoethylisothiouronium bromide hydrobromide (AET, pH9.0), and 0.1 ml

融解法. 融解液として，20% FCS, 25mM HEPES, 1% NEAA, 1% amino acid mixture を含む Eagle MEM を pH 7.2に調整し，4°C に冷却して用いた.

液体窒素タンクから出した vial は直ちに 37°C 恒温温浴中で，ゆっくり振盪しながら急速に融解した. 融解後，直ちに氷水中に入れて冷却し，内容物は，あらかじめ 4°C, 1 ml の FCS を入れておいた遠沈管にゆっくり滴下し，次いで 1 ml FCS, 4 ml 融解液を2回加え，4°C, 240 ×g で10分間遠沈して単核球を集めた. 10% FCS を含む MEM で2回遠沈洗浄後，trypan blue 染色を用いて，細胞の生存率と回収率を求め実験に用いた.

Eロゼット形成試験（Tリンパ球算出試験）. T細胞の算定には，0.1ml リンパ球浮遊液（4×10^6/ml）と 140 M aminoethylisothiouronium bromide hydrobromide (AET, pH 9.0) で処理したヒツジ赤血球 (SRBC)

of lymphocyte suspension (4×10^6/ml) were mixed. Following this, they were centrifuged at 120 × g for five minutes and incubated in ice-cold water for two hours. Then, they were resuspended and examined microscopically on a hemocytometer. A total of 200 lymphocytes and the number of rosette-forming cells, lymphocytes with three or more SRBC attached, were counted to determine the percentage of T cells:

$$\frac{\text{Number of rosette-forming cells (ロゼット形成細胞数)}}{\text{Total number of lymphocytes} \times \text{viability (総リンパ球数×生存率)}} \times 100\,(\%)$$

EA-rosette-Forming Test (Test for counting all the cells possessing IgG-Fc receptor) and Double Rosette-Forming Test (Test for counting Tγ cells: T lymphocytes possessing IgG-Fc receptor). In EA- and double-rosette-forming tests, a silica suspension (Japan Antibody Research Institute) was added to the blood in a volume of 1/10 of the blood in order to remove monocytes, and the blood was kept at 37°C for 60 minutes with occasional shaking. Then, the whole blood was overlaid on Ficoll-Hypaque and lymphocytes were isolated.

In the EA-rosette-forming test, 0.1 ml of lymphocyte suspension (2×10^6/ml) was mixed with 0.1 ml of 1% IgG antibody-sensitized chicken erythrocytes (CK-EA_{IgG}, Japan Antibody Research Institute), and kept in ice-cold water for two hours. Then, the rosette-forming cells in which one or more CK-EA_{IgG} were attached were counted to obtain the percentage of EA-rosettes.

In the double rosette-forming test, 0.1 ml of SRBC treated with AET (1%) was added to 0.1 ml of EA-rosette suspension and, after centrifugation at 50 × g for five minutes, the mixture was kept in ice-cold water for two hours. Then, microscopic examination was conducted to determine the proportion of double rosette (Tγ cells) combined with one or more CK-EA_{IgG} and three or more SRBCs.

Identification of Lymphocyte Subpopulation Using Monoclonal Antibodies. Using anti-Leu-1, anti-Leu-2a, anti-Leu-3a, anti-HLA-DR, anti-Leu-7 (Becton Dickinson), Mo2, and B1 (Coulter Clone)

溶液(2%)0.1ml を混和した．その後，120×g で5分間遠沈し，氷水中で2時間反応させた．次いで再浮遊させ，血球計算盤上で鏡検した．リンパ球を200個数え，3個以上の SRBC が付着したリンパ球をロゼット形成細胞として，その割合を求めた．

EA ロゼット形成試験(IgG-Fc レセプター保有全細胞算出試験)，ダブルロゼット形成試験(IgG-Fc レセプター保有Tリンパ球＝Tγ細胞の算出試験)． EA 及びダブルロゼット形成試験では，単球除去のため，血液の 1/10 量のシリカ懸濁液(日本抗体研究所)を加え，時々かくはんしながら 37°C で60分間処理し，全血のまま Ficoll-Hypaque に重層しリンパ球を分離した．

EA ロゼット形成試験は，0.1ml リンパ球浮遊液(2×10^6/ml)と 1% IgG 抗体感作ニワトリ赤血球(CK-EA_{IgG}，日本抗体研究所)0.1ml を混和後，氷水中で2時間静置し，1個以上の CK-EA_{IgG} とロゼットを形成した細胞をロゼット形成細胞として，その割合を求めた．

ダブルロゼット形成試験は EA ロゼット形成後，SRBC を加え反応させた．すなわち EA ロゼット浮遊液 0.1ml に AET 処理 SRBC(1%) 0.1ml を加え，50×g で5分間遠沈後，氷水中で2時間静置して鏡検し，CK-EA_{IgG} 1個以上，SRBC 3個以上と結合したダブルロゼット(Tγ細胞)の割合を求めた．

モノクローナル抗体を用いたリンパ球 subpopulation の同定． モノクローナル抗体は，anti-Leu-1, anti-Leu-2a, anti-Leu-3a, anti-HLA-DR, anti-Leu-7 (Becton Dickinson)，Mo2，B1 (Coulter Clone) を

as monoclonal antibodies, the proportions of T cells, cytotoxic-suppressor T cells, helper-inducer T cells, B cells, monocytes, and NK cells were obtained by the same method as described in TR 4-86.[2]

Capacity of IgG Production. The number of mononuclear cells suspended in MEM containing 10% FCS (hereinafter referred to as "test medium") was adjusted to 5×10^6/ml, 2.5×10^6/ml, and 1.25×10^6/ml. Then, 0.1 ml of this cell suspension was dispensed into the wells of microtest plate II (Falcon) prefilled with 0.1 ml of test medium. After adding 1 μl of pokeweed mitogen (PWM) as B lymphocyte stimulant, the cells were incubated for seven days. After incubation, they were centrifuged at 400 × g for 10 minutes, and 0.1 ml of supernatant fluid was obtained. IgG in the supernatant fluid was measured by radioimmunoassay.[3]

Measurement of Antibody-dependent Cell-mediated Cytotoxicity. ADCC activity of lymphocytes was measured by plaque assay and ^{3}H-proline cytotoxicity assay.

Plaque Assay. Ten microliters of 5% SRBC was added to the wells of microtest plates treated with poly-L-lysine (Sigma). Then, the plate was centrifuged at 400 × g for five minutes so that SRBCs would attach to the bottom of the wells. In order to remove monocytes, mononuclear cells were incubated in a T-60 plastic flask (Corning) containing test medium for an hour at 37°C. Lymphocytes were suspended at 2.5×10^6/ml in test medium. Then, the same volume of anti-SRBC-rabbit serum IgG fraction (0.5 mg/100 ml, Japan Antibody Research Institute) was added, and 2μl of this mixture was added onto each monolayer of SRBC prepared as above and incubated at 37°C for three hours. After incubation, staining was conducted using the mixture of 0.25% glutaraldehyde (Wako) and 0.0003% brilliant creysyl blue (Merck) containing 1% agarose (Difco), and the percentage of plaque-forming cells among the added lymphocytes was obtained microscopically to determine the ADCC activity:

用いて TR 4-86[2] に準じた方法で，T細胞，cytotoxic-suppressor T細胞，helper-inducer T細胞，B細胞，単球及び NK 細胞の割合を求めた．

IgG 産生能． 10% FCS を含む MEM（以下 test medium）に浮遊した単核球を，細胞数 5×10^6/ml，2.5×10^6/ml，1.25×10^6/ml に調整した．この細胞浮遊液 0.1ml を，既に test medium 0.1ml を分注しておいた microtest plate II（Falcon）の各孔に分注した．次いでBリンパ球刺激物質として pokeweed mitogen（PWM）を 1 μl 添加し，細胞を 7 日間培養した．培養終了後，400×g で10分間遠沈し，培養上清 0.1ml を採取した．培養上清中の IgG の測定は radioimmunoassay で行った．[3]

抗体依存性細胞障害の測定． リンパ球の ADCC 活性はプラーク法と，^{3}H-proline cytotoxicity assay を用いて測定した．

プラーク法． 5% SRBC 10μl を，poly-L-lysine（Sigma）で処理し，microtest plate の各孔に加えた．次いで plate を 400×g で 5 分間遠沈し，SRBC を plate の孔底面に付着させた．一方，単球除去のために，test medium に単核球を浮遊し，T-60 プラスチックフラスコ（Corning）中で 1 時間 37°C で培養した．単球除去したリンパ球を 2.5×10^6/ml となるよう test medium に浮遊し，等量の抗 SRBC-ウサギ血清 IgG 分画（0.5mg/100ml，日本抗体研究所）を加え，この 2μl ずつを，上記のとおり作製された SRBC の単層上に添加，37°C で 3 時間培養した．判定は incubation 終了後，1% agarose（Difco）を含む 0.25% glutaraldehyde（和光）と 0.0003% brilliant creysyl blue（Merck）の混合液で固定染色し，顕微鏡下で添加リンパ球数に対するプラーク形成細胞の割合を求め ADCC 活性とした．

$$\frac{\text{Number of plaque-forming cells}\ \text{プラーク形成細胞数}}{\text{Number of lymphocytes} \times \text{viability}\ \text{リンパ球数}\times\text{生存率}} \times 100\ (\%)$$

^{3}H-proline Cytotoxicity Assay. According to ^{3}H-proline cytotoxicity assay of Bean et al,[4] T-24 cells[5] (human cell line originating from urinary bladder cancer patient) were used as target cells. The T-24 cells which had been incubated in a plastic flask (Corning) were labeled with 2 ml of ^{3}H-proline (50μCi/ml) for 18 hours. As the proline was incorporated into target cells in this labeling technique, NEAA was not added to culture medium. After labeling, the cells were trypsinized (0.05%) and washed twice with the test medium. These target cells were adjusted to 10^5/ml and dispensed in aliquots of 10μl into the wells of microtest plates prefilled with 0.1 ml of test medium and incubated for three hours before the addition of effector cells.

Lymphocyte suspension (2.5×10^6/ml) from which monocytes were removed using plastic flasks was dispensed in aliquots of 0.1 ml into the wells of the above-mentioned microtest plate containing T-24 cells. The ratio of effector cells to target cells (E:T ratio) was 250:1. Anti-T-24 rabbit serum (1:10,000) was added to the respective wells and, after incubation in a 5% CO_2 incubator at 37°C for 24 hours, the plate was washed and dried. After drying, bottoms of the wells were punched and the radioactivity of ^{3}H-proline incorporated in the surviving target cells was measured with a liquid scintillation counter to determine activity of lymphocytes which can damage target cells in the presence of antibodies. Thus, ADCC activity was calculated using the following expression.

^{3}H-proline cytotoxicity assay. Bean ら[4] の ^{3}H-proline cytotoxicity assay に準じ，標的細胞として T-24 細胞[5]（膀胱癌患者由来ヒト細胞株）を用いた．プラスチックフラスコ(Corning)中で培養された T-24 細胞に，2 ml ^{3}H-proline (50μCi/ml) を加え，18時間培養し標識を行った．この場合，プロリンの標的細胞内への取り込みを利用した標識法であるため，培養液には NEAA を加えなかった．標識後プラスチックフラスコの底に付着している標的細胞を 0.05% トリプシン処理により剝離し，test medium で 2 回洗浄した．この標的細胞を 10^5/ml に調整し，あらかじめ 0.1ml test medium を入れておいた microtest plate に 10μl ずつ分注後，エフェクター細胞を加えるまでに 3 時間培養した．

プラスチックフラスコを用いた吸着法により単球除去したリンパ球浮遊液 (2.5×10^6/ml) 0.1ml ずつを，T-24 細胞を含む上記 microtest plate の各孔に分注した．エフェクター細胞と標的細胞の比 (E:T) は250対 1 とした．抗 T-24 ウサギ血清 (1 対10,000) を各孔に加え，37°C, 5 % CO_2 インキュベーター中で24時間反応させた後，plate を洗浄，乾燥させた．乾燥後，各孔の底を打ち抜き，生き残った標的細胞に取り込まれている ^{3}H-proline の放射能活性を，液体シンチレーションカウンターで測定することにより，抗体存在下で標的細胞を障害し得るリンパ球の活性を求めた．すなわち，ADCC 活性は次式より算定した．

$$\left(1-\frac{\text{Target cell count remaining after incubation with anti-T-24 serum}\ \text{抗 T-24 血清添加培養後の生存標的細胞数}}{\text{Target cell count remaining after incubation without anti-T-24 serum}\ \text{抗 T-24 血清未添加培養後の生存標的細胞数}}\right)\times 100\,(\%)$$

NK Cell Activity. For the measurement of NK cell activity, a ^{51}Cr release assay was conducted using K-562 (originating from leukemia cells)[6] as target cells. To $5\sim10\times10^6$ K-562, 100μCi of $Na^{51}CrO_4$ (New England Nuclear, specific activity; 200 mCi/mg or more) were added, and incubated in a 5% CO_2 incubator at 37°C for an hour. Following the labeling, the cells were washed three times by centrifugation with test medium and, after the number of cells were adjusted to 5×10^4/ml, were dispensed into the wells of a U-shaped microtest plate (Nunc) in

NK 細胞活性． NK 細胞活性の測定には，K-562（白血病細胞由来）[6] を標的細胞として，^{51}Cr release assay を用いた． $5\sim10\times10^6$ 個の K-562 に，$Na^{51}CrO_4$ (New England Nuclear, specific activity 200mCi/mg 以上) 100μCi を加え，37°C, 5 % CO_2 インキュベーター中で 1 時間標識を行った．標識後，test medium で 3 回遠沈洗浄し，細胞数を 5×10^4/ml に調整し，U型 microtest plate (Nunc) に 0.1ml ずつ

aliquots of 0.1 ml. Lymphocytes from which monocytes were removed using silica suspension were adjusted to 4×10^6/ml as effector cells, and 0.1 ml each was added to the plate so that the E:T ratios would be 80:1, 40:1, 20:1, and 10:1. Also, by adding test medium only and by adding 1% triton X (Nakarai), radioactivity of spontaneous release and maximum release was measured. After centrifugation at 200×g for two minutes, the cells in this plate were incubated in a 5% CO_2 incubator at 37°C for four hours. Following incubation, they were centrifuged at 200×g for two minutes, and the released radioactivity in 0.1 ml of the supernatant was measured with a γ-counter. Thus, NK cell activity was calculated by the following expression:

分注した．シリカ懸濁液により単球を除去したリンパ球をエフェクター細胞として 4×10^6/ml に調整し，E:T 比が80対1，40対1，20対1，10対1になるように0.1ml ずつplate に加えた．なお，エフェクター細胞を加えず test medium のみ，及び1% triton X (半井)を加えたものの，自然放出及び最大放出の放射能活性を測定した．このplate を 200×g で2分間遠沈後，37°C，5% CO_2 インキュベーター中で4時間反応させた．反応終了後，200×g で2分間遠沈し，0.1ml の上清中に含まれる放出放射能活性を，γカウンターで測定した．更に NK 細胞活性を以下の計算式により算出した．

$$\frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100\ (\%)$$

$$\frac{\text{放出量(実験値)－自然放出量}}{\text{最大放出量－自然放出量}} \times 100\ (\%)$$

Target-binding Cell Assay Against K-562. To determine binding capacity of lymphocytes against K-562, a target-binding cell assay[7] was conducted. Lymphocytes were suspended in test medium, and 0.1 ml of this suspension (2×10^6/ml) and 0.1 ml of K-562 suspension (1×10^6/ml) were mixed in small tubes, centrifuged at 200×g for five minutes at room temperature, and incubated in ice-cold water for 30 minutes. After reaction, they were resuspended and 500 lymphocytes were counted microscopically to determine the proportion of lymphocytes bound to K-562.

K-562 に対する target-binding cell assay. K-562 に対するリンパ球の結合能を調べるために target-binding cell assay[7] を行った．Test medium に浮遊したリンパ球浮遊液 (2×10^6/ml) 0.1ml と，K-562 浮遊液 (1×10^6/ml) 0.1ml を小試験管中で混和し，室温で 200×g，5分間遠沈後，氷水中で30分間反応させた．反応後，再浮遊し，顕微鏡下で，リンパ球500個を数え，K-562 と結合しているリンパ球の割合を求めた．

Statistical Procedure. All experimental values obtained for lymphocytes before and after cryopreservation were compared using paired t-test.

統計方法． 凍結保存前，後のリンパ球を用いて得られた値は，paired t-test を用いて比較した．

RESULTS

結　果

Effect of Cryopreservation on Lymphocyte Subpopulations. Binding capacity of lymphocytes and SRBCs was used as an index to determine T lymphocytes in peripheral blood. In the previous study, as a result of using untreated SRBCs, the proportion of E-rosette decreased to 57.0%±16.2% after cryopreservation.[1] However, in the present experiment in which SRBCs treated with AET were used, the proportion before freezing was 71.9%±6.4%,

凍結保存がリンパ球 subpopulation に与える影響．末梢血中のTリンパ球を求めるために，リンパ球とSRBC との結合能を指標とした．以前の実験では無処理 SRBC を用いた結果，Eロゼットの割合は，凍結保存後 57.0%±16.2% に低下した．[1] しかし，今回の実験で，AET 処理 SRBC を用いると，凍結前 71.9%±6.4%，凍結保存後 69.9%±7.0%で差は

which was not significantly different from 69.9%±7.0% after freezing. Thus, no effects of cryopreservation was observed (Table 1).

なく，凍結保存による影響を認めなかった(表1)．

TABLE 1 EFFECT OF CRYOPRESERVATION ON E- AND EA-ROSETTE FORMATION
表1 Eロゼット，EAロゼット形成能における凍結保存の影響

	No. of subjects	Fresh (%)	Frozen (%)	*
E-rosette				
(SRBC treated with AET)	17	71.9±6.4	69.9± 7.0	NS
(SRBC without treatment)	5	71.2±6.5	57.0±16.2	$P<0.05$
EA-rosette	12	18.4±4.1	17.5± 4.1	NS
Double rosette (T_γ cell)	12	13.5±3.2	12.8± 4.0	NS

E: Sheep red blood cells (SRBC) ヒツジ赤血球(SRBC)
EA: Chicken erythorcytes coated with anti-chicken erythrocyte antibody (IgG)
抗ニワトリ赤血球抗体(IgG)付着ニワトリ赤血球
Mean±SD 平均±標準偏差
*Student t-test; NS-not significant 有意でない

As the index to detect all the cells possessing IgG-Fc receptors, the rosette-forming (EA-rosette) test was conducted using the cells and chicken erythrocytes sensitized with antichicken erythrocytes rabbit serum (IgG fraction). The proportion of EA-rosettes before freezing was 18.4%±4.1%, not significantly different from 17.5%±4.1% after freezing. Thus, no effect of cryopreservation was observed in the cells with IgG-Fc (Fc_γ) receptor detected in this EA-rosette-forming test (Table 1).

IgG-Fc レセプターを保有するすべての細胞を検出する指標として，細胞と抗ニワトリ赤血球ウサギ血清(IgG 分画)で感作したニワトリ赤血球とのロゼット形成(EA ロゼット)試験を用いた．EA ロゼットの割合は凍結前 18.4%±4.1%，凍結保存後 17.5%±4.1%で，凍結保存前後で差は認められず，このEA ロゼット形成試験で検出される IgG-Fc (Fc_γ) レセプターをもつ細胞群には，凍結保存による影響はなかった(表1)．

To detect T cells (T_γ cells) among cells possessing Fc_γ receptor, the double rosette-forming test was conducted. The values before and after freezing were 13.5%±3.2% and 12.8%±4.0%, respectively. Thus, no change was observed in the proportion of T_γ cells (Table 1).

$Fc\gamma$ レセプターをもつ細胞群中のT細胞(T_γ細胞)の検出には，ダブルロゼット形成試験を行った．凍結保存前後の値はそれぞれ 13.5%±3.2%，12.8%±4.0%で，T_γ細胞の割合は不変であった(表1)．

Monoclonal antibodies which are more specific to cell membrane antigens than the above-mentioned rosette-forming test were used to determine the proportion of lymphocyte subpopulations.

上記のロゼット形成試験に比べて，細胞膜抗原にもっと特異性を示すモノクローナル抗体を用いて，リンパ球 subpopulation の割合を検索した．

Anti-Leu-1 is a monoclonal antibody against all T cells, anti-Leu-2a against suppressor-cytotoxic T cells, anti-Leu-3a against helper-inducer T cells, anti-HLA-DR against B cells and monocytes, anti-Leu-7 against NK cells, Mo2 against monocytes, and B1 against B cells.

モノクローナル抗体の anti-Leu-1 は総T細胞に，anti-Leu-2a は suppressor-cytotoxic T 細胞に，anti-Leu-3a は helper-inducer T 細胞に，anti-HLA-DR はB細胞及び単球に，anti-Leu-7 は NK 細胞に，Mo2 は単球に，B1 はB細胞に対する抗体である．

As shown in Table 2, the proportion of various subpopulations of lymphocytes determined by monoclonal antibodies was not affected by cryopreservation. Also, no difference was observed in the degree and nature of fluorescent staining of cell membrane before and after cryopreservation.

表2に示すように，モノクローナル抗体を用いたリンパ球の各種subpopulationの割合は，凍結保存により影響を受けなかった．また，細胞膜の蛍光染色の度合，性状にも，凍結保存前後で差はなかった．

TABLE 2 MONOCLONAL ANTIBODY-DETERMINED SUBPOPULATIONS OF FRESH AND FROZEN LYMPHOCYTES

表2 モノクローナル抗体を用いた新鮮，凍結リンパ球のsubpopulation

	No. of subjects	Fresh (%)	Frozen (%)
Viability	34	99.9 ± 0.1	89.2 ± 4.7
Leu-1 (total T cell)	34	67.2 ± 6.8	67.6 ± 8.3
Leu-2a (suppressor-cytotoxic T cell)	34	27.2 ± 6.8	27.7 ± 7.3
Leu-3a (helper-inducer T cell)	34	43.4 ± 8.6	44.1 ± 7.9
HLA-DR (B cell, monocyte)	33	21.4 ± 9.8	20.9 ± 8.0
Leu-7 (NK cell)	16	13.6 ± 3.5	13.6 ± 5.0
Mo2 (monocyte)	10	3.2 ± 2.7	2.0 ± 1.0
B1 (B cell)	16	16.5 ± 7.2	14.8 ± 6.1

Mean ± SD 平均±標準偏差

The proportion of peripheral blood lymphocyte subpopulations was determined under a fluorescence microscope by the indirect immunofluorescence antibody method using monoclonal antibodies.

末梢血リンパ球subpopulationの割合は，モノクローナル抗体を使用して間接免疫蛍光抗体法によって蛍光顕微鏡下で測定した．

Next, the effect on length of cryopreservation on lymphocyte subpopulations was observed on five subjects up to a maximum period of 12 months after freezing, but as shown in Table 3, no differences were observed in the proportion of subpopulations by cryopreservation period. Presently, further observations are being made by extending the cryopreservation period.

次に，凍結保存期間のリンパ球subpopulationに及ぼす影響を検討した．5例の対象者において，凍結保存後12か月まで検討したが，表3に示すように凍結保存期間に関係はなく，subpopulationの割合に変化はみられなかった．現在，更に凍結保存期間を延長して検討中である．

TABLE 3 LYMPHOCYTE SUBPOPULATIONS AFTER DIFFERENT PERIODS OF CRYOPRESERVATION

表3 凍結保存期間別リンパ球subpopulation

	Cryopreservation period				
	Fresh	2 weeks	1 month	2 months	12 months
Viability	99.8 ± 0.2	85.8 ± 4.7	89.7 ± 3.7	83.5 ± 7.0	86.9 ± 4.0
Leu-1	66.7 ± 5.8	63.8 ± 9.9	67.6 ± 5.4	65.0 ± 6.4	67.9 ± 2.1
Leu-2a	26.1 ± 6.3	25.1 ± 4.2	27.2 ± 5.8	29.7 ± 6.8	28.7 ± 6.1
Leu-3a	44.1 ± 8.8	45.0 ± 5.8	39.9 ± 7.5	49.1 ± 4.2	47.0 ± 2.3
HLA-DR	16.5 ± 6.7	16.6 ± 3.2	15.8 ± 5.3	16.7 ± 2.4	18.8 ± 4.7
Leu-7	17.9 ± 4.2	18.1 ± 4.0	20.2 ± 4.6	–	18.8 ± 4.2

Mean % ± SD of 5 experiments. 5測定値の平均(%)±標準偏差

Effect of Cryopreservation on Various Examination of Lymphocyte Functions.

Capacity of IgG Production (Table 4). Mononuclear cells at concentrations of 1.25×10^5/well, 2.5×10^5/well, and 5×10^5/well were stimulated by PWM, but their capacity to produce IgG was not affected by cryopreservation. However, antibody production of controls (unstimulated cells) decreased after cryopreservation when the number of cells was adjusted to 5×10^5/well. Antibody production also decreased after preservation in the other two suspensions, but the change was not statistically significant. It was considered that the appropriate number of cultured cells should be 2.5×10^5/well for the determination of IgG production.

凍結保存が各種リンパ球機能検査に与える影響.

IgG 産生能(表4). 1.25×10^5/well, 2.5×10^5/well, 5×10^5/well の単核球を PWM で刺激した場合の IgG 産生能は，凍結保存による影響を受けなかった．しかし，コントロール(無刺激細胞群)は，細胞数を 5×10^5/well に調整した場合，凍結保存後抗体産生の低下を認めた．細胞数 2.5×10^5/well, 1.25×10^5/well でも，凍結保存後抗体産生が低下したが，その変化は統計的に有意ではなかった．したがって凍結保存リンパ球を IgG 産生能の検査に使用する場合は，培養細胞数 2.5×10^5/well が適当と思われた．

TABLE 4 IgG PRODUCTION BY FRESH AND FROZEN LYMPHOCYTES

表4 新鮮，凍結リンパ球による IgG 産生能

Cell number /well	No. of subjects	IgG concentration (ng/ml)			
		Control		PWM-induced IgG production	
		Fresh	Frozen	Fresh	Frozen
5×10^5	13	993 ± 701	617 ± 516	3254 ± 1295	2923 ± 1158
*		P<0.05		NS	
2.5×10^5	12	438 ± 499	345 ± 313	1882 ± 1311	1852 ± 1111
*		NS		NS	
1.25×10^5	14	327 ± 428	146 ± 118	872 ± 604	753 ± 532
*		NS		NS	

Mean ± SD 平均±標準偏差

*Student's t-test; NS—not significant. NS，有意でない

Mononuclear cells were stimulated by PWM for 7 days. Concentration of IgG in the supernatant was measured by radioimmunoassay.

単核細胞は7日間 PWM で刺激した．上清中の IgG の濃度は radioimmunoassay によって測定した．

Antibody-dependent Cell-mediated Cytotoxicity. As shown in Table 5, in the plaque assay using SRBCs as target cells, ADCC activity before freezing was 6.9% ± 2.5% as against 7.0% ± 2.7% after freezing, indicating no effect of cryopreservation. When ^{3}H-proline cytotoxicity assay was used, ADCC activity against T-24 cells decreased by 6% or less in 3 of the 15 cases studied, but by 10%-30% in 6 cases, and 30% or more in another 6 cases (Figure 2). The average value of ADCC activity was 77.5% ± 13.6% before freezing which was highly different ($P<0.001$) from 50.1% ± 20.0% after freezing, thus a decrease in ADCC activity was clearly evident after cryopreservation.

抗体依存性細胞障害． 標的細胞として SRBC を用いたプラーク法では，表5に示すように，ADCC 活性は凍結前 6.9% ± 2.5%，凍結保存後 7.0% ± 2.7% で，凍結保存による変動は認められなかった．^{3}H-proline cytotoxicity assay を用いて，T-24 細胞に対する ADCC 活性を測定した場合，凍結保存リンパ球の ADCC 活性は，15例の検査対象者中3例は6%以下の低下に留まったが，6例は10%～30%，及びほかの6例は30%以上の低下を示した(図2)．凍結前の ADCC 活性の平均値 77.5% ± 13.6% に対し，凍結保存後は 50.1% ± 20.0% で，明らかに ADCC 活性の低下を認めた．

TABLE 5 EFFECT OF CRYOPRESERVATION ON THE PERCENTAGE OF KILLER CELLS

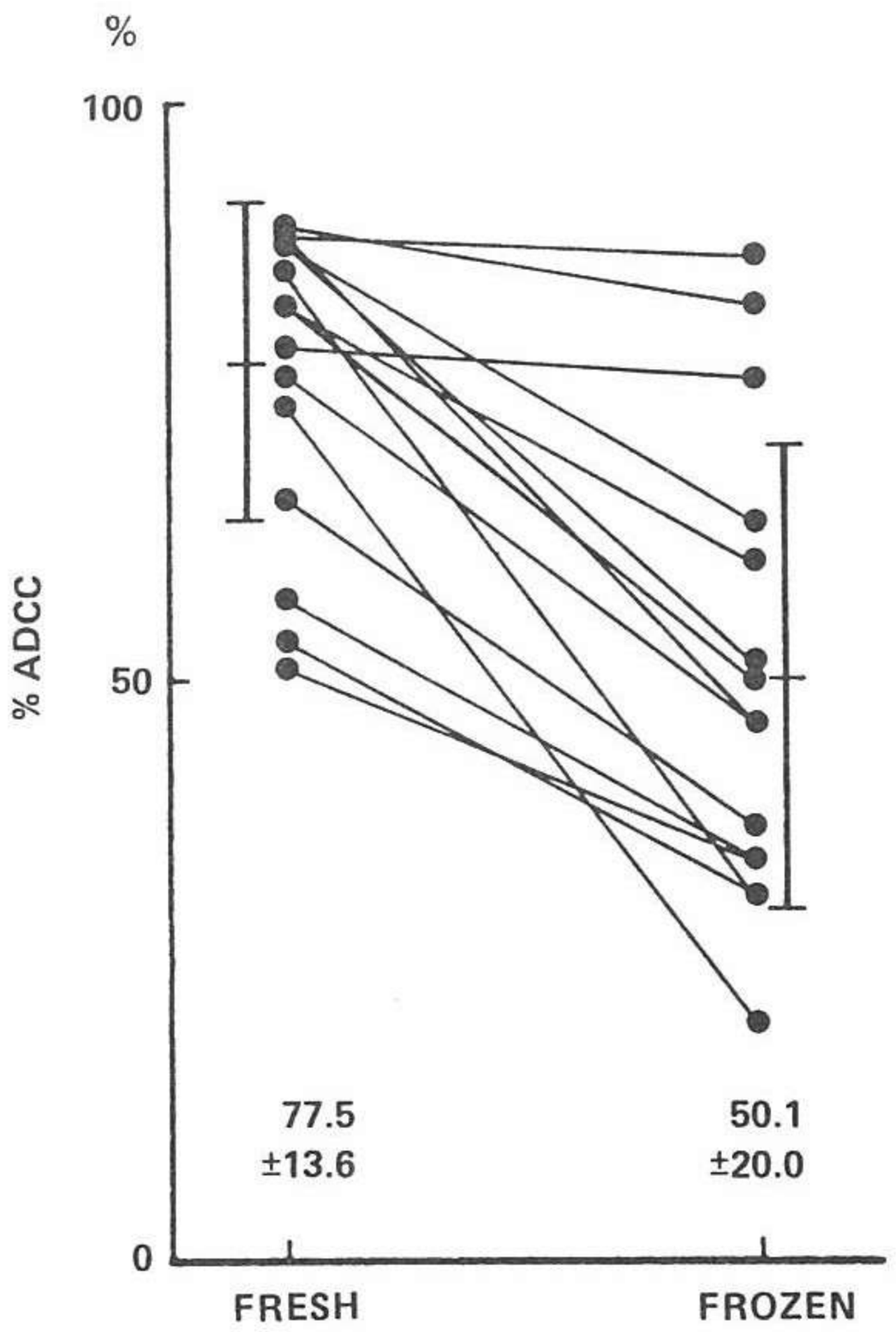
表5 キラー細胞の割合に対する凍結保存の影響

	Fresh (%)	Frozen (%)	*
Recovery	–	65.5 ± 21.9	
Viability	99.7 ± 0.3	84.9 ± 6.3	
Killer cells	6.9 ± 2.5	7.0 ± 2.7	NS

Mean ± SD of 16 experiments. 16測定値の平均±標準偏差
*Students t-test; NS-not significant. NS 有意でない
Killer cells lysed antibody-coated SRBC to form plaques.
キラー細胞は抗体付着 SRBC を溶解しプラークを形成した.

FIGURE 2 COMPARISON OF ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY (ADCC) OF FRESH AND FROZEN CELLS

図2 新鮮，凍結細胞における抗体依存性細胞障害の比較



EFFECTOR LYMPHOCYTES AND T-24 TARGET CELLS LABELED WITH ^{3}H-PROLINE (250:1) WERE INCUBATED IN THE PRESENCE OF ANTI-T-24 ANTIBODY FOR 24 HOURS, AFTER WHICH THE AMOUNT OF RADIOACTIVITY IN VIABLE TARGET CELLS WAS DETERMINED.

エフェクターリンパ球と ^{3}H-proline (250：1) 標識 T-24 標的細胞は，抗 T-24 抗体とともに24時間反応させた後生存標的細胞の放射能活性を測定した.

NK Cell Activity. It is generally considered difficult to keep NK cell activity intact under the heretofore used conditions. Thus, the authors studied two important items, i.e., freezing medium and freezing rate, in order to improve preserving conditions.

First, using pooled human serum and FCS as the serum component of freezing medium, NK cell activities after freezing were compared. As shown in Table 6, after freezing NK cell activity decreased equally in both cases, so that we employed FCS as freezing medium in subsequent experiments.

NK 細胞活性．一般に，NK 細胞活性を十分に保ったまま凍結保存することは従来の条件では困難とされている．そこで著者らは従来の条件を更に改良するために，次の重要な2点について検討を行った．

まず，凍結保存液の血清成分にヒト保存血清とFCS を用いて，凍結保存後の NK 細胞活性を比較した．表6に示すように，いずれの血清を凍結保存液に使用しても，凍結保存後 NK 細胞活性が低下していた．したがって，以後の実験には FCS を凍結保存液に用いた．

TABLE 6 EFFECT OF SERUM IN FREEZING MEDIUM ON NK CELL ACTIVITY

表 6 NK 細胞活性に対する凍結溶液における血清の影響

E:T ratio	NK cell activity (%)		
	Fresh	Frozen	
		FCS	PHS
40:1	49.8 ± 13.4	28.8 ± 7.6	27.1 ± 7.2
20:1	38.2 ± 12.8	18.2 ± 3.4	18.1 ± 3.8
10:1	29.1 ± 11.2	9.9 ± 3.3	10.6 ± 2.3

Mean ± SD of 9 experiments. 9 測定値の平均±標準偏差

FCS: Fetal calf serum; PHS: Pooled human serum.
ウシ胎仔血清 ヒト保存血清

Lymphocytes were frozen at a rate of −1°C/min.
リンパ球は凍結速度 −1°C/min で凍結した．

E:T ratio, effector cell to target cell ratio. エフェクター細胞と標的細胞の比

Table 7 shows the effect of freezing rate (−1°C/min, −2°C/min) of the liquid phase on NK cell activity after cryopreservation. When the freezing rate was −1°C/min, NK cell activity was slightly higher, but not significantly, than when the freezing rate was −2°C/min.

Although these conditions mentioned above were applied, it was still difficult to preserve NK cell activity in the same state as prior to freezing. Therefore, various attempts as below were made to improve the recoverability of NK cell activity after cryopreservation.

表7に，液相の凍結速度（−1°C/min, −2°C/min）が，凍結保存後の NK 細胞活性に及ぼす影響を示した．凍結速度 −1°C/min のときは，−2°C/min のときと比べ NK 細胞活性が，わずかに高値を示したが，有意の差は認めなかった．

これらの条件で実験をしても，NK 細胞活性を，凍結前の状態で保存することは困難であった．したがって，次に凍結後の NK 細胞活性の回復能を改良するために種々の検討を試みた．

TABLE 7 EFFECT OF FREEZING RATE ON NK CELL ACTIVITY
表 7 NK 細胞活性に対する凍結速度の影響

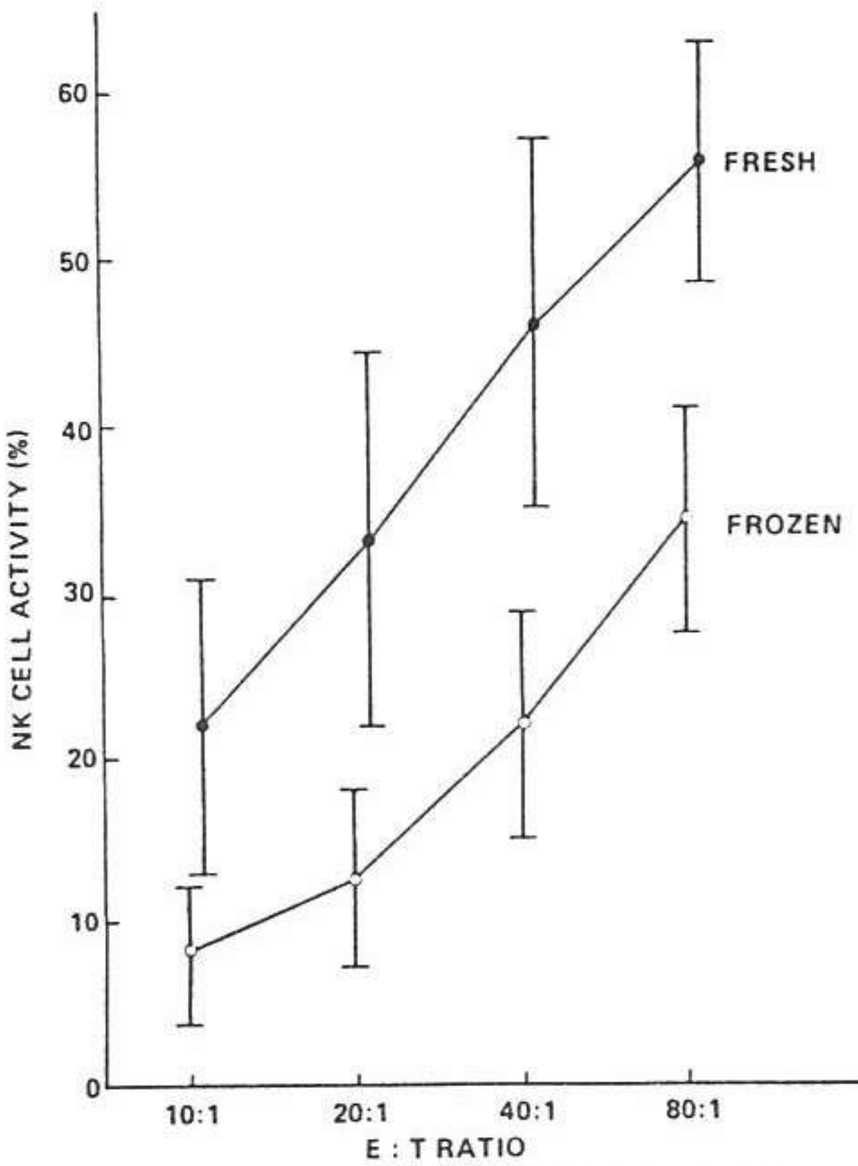
E:T ratio	NK cell activity (%)		
	Fresh	Frozen	
		−1°C/min	−2°C/min
80:1	56.2 ± 7.4	37.0 ± 12.6	33.8 ± 11.6
40:1	43.8 ± 6.2	22.0 ± 6.1	19.3 ± 5.5
20:1	29.4 ± 7.3	11.0 ± 4.6	9.5 ± 4.3
10:1	18.8 ± 3.2	5.1 ± 2.0	3.8 ± 1.8

Mean ± SD of 5 experiments. 5 測定値の平均±標準偏差
Lymphocyte were frozen in freezing medium with fetal calf serum.
リンパ球はウシ胎仔血清を含む凍結保存溶液中で凍結した.

Effect of E:T ratio. As shown in Figure 3, the NK cell activity of cryopreserved lymphocytes was depressed, but thier functional recovery rate [(NK cell activity after freezing/NK cell activity before freezing) × 100] increased as the E:T ratio was greater (Table 8), e.g., when the E:T ratio was 80:1, the recovery rate was 60.6% ± 14.8%.

E:T 比の影響. 図3に示すように，凍結保存リンパ球の NK 細胞活性は低いものの，その機能的回復率〔(凍結後 NK 細胞活性/凍結前 NK 細胞活性) ×100〕は，E:T 比が大きくなるほど，上昇している. E:T 比 80対1の条件で，その回復率は 60.6% ± 14.8% であった(表8).

FIGURE 3 EFFECT OF E:T RATIO ON NK CELL ACTIVITY
図 3 NK 細胞活性に対する E:T 比の影響



E:T RATIO = EFFECTOR CELL TO TARGET CELL RATIO.
エフェクター細胞と標的細胞の比

LYMPHOCYTES WERE CRYOPRESERVED AND MEASURED FOR NK CELL ACTIVITY IMMEDIATELY AFTER THAWING.
リンパ球は凍結保存して，融解直後に NK 細胞活性を測定した.

TABLE 8 RELATIONSHIP OF PREINCUBATION TIME AND NK CELL ACTIVITY AFTER CRYOPRESERVATION

表 8　凍結保存後の pre incubation 時間と NK 細胞活性の関係

	Fresh (%)	Frozen (%)			
		Immediate	Preincubation		
			3 hrs.	18 hrs.	43 hrs.
No. of subjects	20	11	8	20	3
E:T ratio					
80:1	55.5 ± 7.3	34.1 ± 10.1** (60.6 ± 14.8)	50.4 ± 9.0* (86.6 ± 15.2)	54.7 ± 8.4† (99.3 ± 13.9)	43.2 ± 11.3† (86.1 ± 24.1)
40:1	45.0 ± 9.7	22.0 ± 6.6** (47.6 ± 8.3)	35.2 ± 8.7** (59.0 ± 11.6)	40.0 ± 12.0* (88.8 ± 20.0)	29.9 ± 13.0† (81.8 ± 33.2)
20:1	33.8 ± 9.6	12.5 ± 5.4** (39.9 ± 15.9)	23.9 ± 5.8** (50.4 ± 16.7)	27.4 ± 10.4** (80.2 ± 19.2)	18.4 ± 9.6† (64.5 ± 29.7)
10:1	23.1 ± 7.5	8.2 ± 4.7** (38.9 ± 16.4)	–	18.1 ± 7.8** (79.4 ± 24.9)	–

( ): Functional recovery = 機能的回復率 $\frac{\text{NK cell activity of frozen cells 凍結細胞の NK 細胞活性}}{\text{NK cell activity of fresh cells 新鮮細胞の NK 細胞活性}} \times 100\ (\%)$

The data after cryopreservation was compared with the data before cryopreservation using Student t-test.

凍結保存後のデータは t-test を使用して凍結保存前のデータと比較した.

† Not significant 有意でない

* $P<0.05$

** $P<0.001$

Comparison of NK cell activities before and after freezing showed a correlation (Figure 4): the correlation coefficient (r) was 0.625 ($P<0.05$), 0.801 ($P<0.01$), 0.494 ($P<0.2$), and 0.708 ($P<0.02$) when E:T ratio was 80:1, 40:1, 20:1, and 10:1, respectively. Therefore, it was considered that the change of NK cell activity could be estimated by comparing samples after freezing.

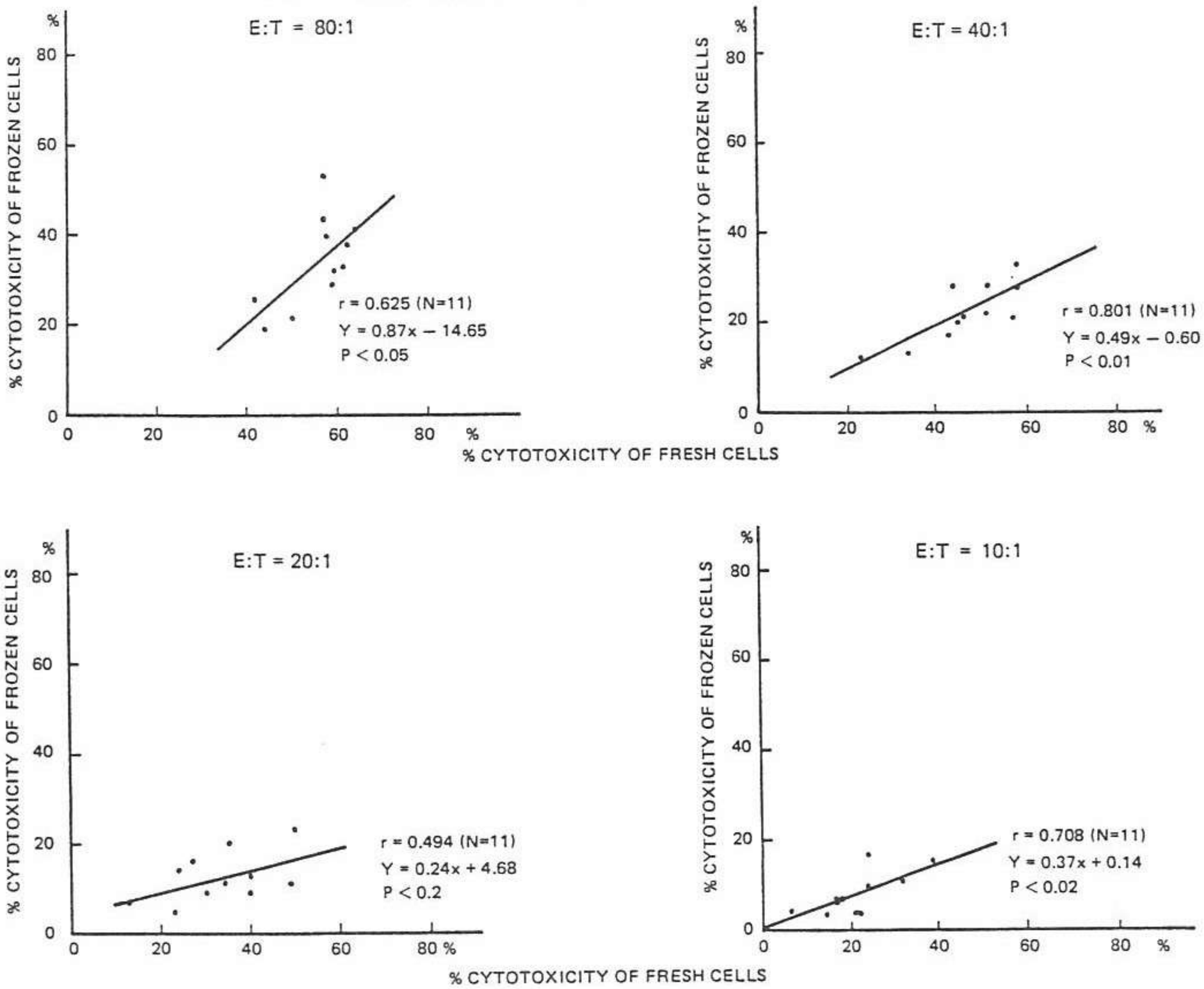
Effect of Preincubations. As the insufficient recovery of cell membrane and cytoplasmic metabolism was considered to be one of the causes of lowered NK cell activity of cryopreserved cells, the cells were incubated at 37°C in the presence of 5% CO_2 for various periods after thawing to examine any changes in NK cell activity.

凍結前と，凍結保存後の NK 細胞活性を比較すると，図 4 に示すように，相関係数 (r) は E:T 比80対1の条件で 0.625 ($P<0.05$)，40対1で 0.801 ($P<0.01$)，20対1で 0.494 ($P<0.2$)，10対1で 0.708 ($P<0.02$) となり，凍結保存前後の NK 細胞活性は，相関関係を認めた．したがって，凍結保存後の各サンプルを比較検討することで，NK 細胞活性の動態が推測できると考えられた．

Preincubation の影響． 凍結保存細胞の NK 細胞活性の低下には，細胞膜や細胞形質代謝の回復が十分でないことも一因と考えられるので，細胞を凍結保存融解後，一定期間 37°C，5 % CO_2 存在下で incubation を行い，NK 細胞活性の変動を検討した．

FIGURE 4 COMPARISON OF NK CELL ACTIVITY OF FRESH AND FROZEN LYMPHOCYTES

図 4　新鮮，凍結リンパ球における NK 細胞活性の比較



THE NK CELL ACTIVITY OF FROZEN LYMPHOCYTES WAS MEASURED IMMEDIATELY AFTER THAWING.

凍結リンパ球の NK 細胞活性は融解直後に測定した.

First, using fresh lymphocytes, effect of preincubation on NK cell activity was determined on four subjects. The average percentages of NK cell activity of fresh lymphocytes were 51.6%±10.3%, 35.1%±0.3%, and 24.3%±8.1% for E:T ratios of 80:1, 40:1, and 20:1, respectively, and after preincubation for 18 hours, they were 49.8%±4.1%, 38.9%±6.1%, and 29.1%±6.3%, respectively; no significant differences were noted between the above findings.

Next, lymphocytes after cryopreservation were preincubated as described above for 3, 18, and 43 hours, and NK cell activity was measured. To minimize errors which could be caused by measurement conditions, NK cell activity was measured immediately after cryopreserved cells were thawed, and then after 3, 18, and 43 hours of preincubation.

まず，新鮮リンパ球を用いて，NK 細胞活性に及ぼす preincubation の影響を，4 例の対象者について検討した．E:T 比が80対 1，40対 1，20対 1 の条件で，新鮮リンパ球の NK 細胞活性の平均値は，51.6%±10.3%，35.1%±10.3%，24.3%±8.1%であり，18時間 preincubation 後は，それぞれ 49.8%±4.1%，38.9%±6.1%，29.1%±6.3%で，新鮮リンパ球の NK 細胞活性に，preincubation による変化は認められなかった．

次に，凍結保存後のリンパ球を，3，18，43時間同様に preincubation を行った後，NK 細胞活性を測定した．NK 細胞活性の測定条件による誤差をなるべく最小にとどめるために，融解直後と凍結保存細胞を測定の3，18，43時間前に融解し preincubation した後の NK 細胞活性の測定を実施した．

As shown in Table 8, when the E:T ratio was 80:1, recovery rate of NK cell activity immediately after thawing was 60.6%±14.8% as against 86.6%±15.2% after preincubation for 3 hours, 99.3%±13.9% for 18 hours, and 86.1%±24.1% for 43 hours, and it was found that preincubation for 18 hours would lead to the recovery of almost 100%. When E:T ratios were 40:1, 20:1, and 10:1, NK cell activity decreased after freezing compared to before freezing even after preincubation but, after preincubation for 18 hours, the recovery rate of NK cells were 88.8%±20.0%, 80.2±19.2%, and 79.4%±24.9% for the E:T ratios of 40:1, 20:1, and 10:1, respectively.

To study the mechanism involved in the recovery of NK cell activity by preincubation 1) viability of cells, 2) recovery rate of cells, 3) proportion of lymphocyte subpopulations, and 4) proportion of target-binding cells were compared (Table 9).

Viability of cells immediately after thawing was 92.9%±3.2%, which was not significantly different from 92.5%±3.5% after preincubation for 18 hours.

表8に示すように，E:T比80対1の条件で，凍結保存融解直後のNK細胞活性の回復率は60.6%±14.8%であるのに対し，3時間preincubation後は86.6%±15.2%，18時間preincubation後は99.3%±13.9%，43時間preincubation後は86.1%±24.1%で，18時間のpreincubationを行うことにより，ほぼ100%凍結前の値に回復することがわかった．Preincubationを行ってもE:T比が40対1，20対1，10対1の条件では，凍結前に比べ凍結後NK細胞活性の低下が認められたが，18時間preincubation後のNK細胞の回復率は，E:T比40対1で88.8%±20.0%，20対1で80.2%±19.2%，10対1で79.4%±24.9%であった．

一方，preincubationによる凍結保存リンパ球のNK細胞活性の回復における機序を検討するために以下の点について調べた．すなわち，1) 細胞の生存率，2) 細胞の回収率，3) リンパ球subpopulationの割合，4) target-binding cellの割合について検討を加えた（表9）．

細胞の生存率は，凍結保存融解直後92.9%±3.2%，18時間preincubation後92.5%±3.5%で，変化はみられなかった．

TABLE 9 EFFECT OF PREINCUBATION AFTER CRYOPRESERVATION ON FROZEN LYMPHOCYTES

表9 凍結リンパ球に対する凍結保存後のpreincubationの影響

	No. of subjects	Immediate	18 hrs preincubation
Viability	20	92.9 ± 3.2	92.5 ± 3.5
Recovery of cell number	20	81.7 ±23.7	69.9 ±25.5
Lymphocyte subpopulation	14		
Leu-1		67.0 ± 9.2	67.5 ± 9.0
Leu-2a		26.1 ± 5.3	25.8 ± 5.1
Leu-3a		40.9 ± 5.9	41.6 ± 6.8
HLA-DR		15.9 ± 7.1	14.1 ± 3.8
Leu-7		21.4 ± 5.6	21.3 ± 5.6
% target-binding cells	21	8.2 ± 2.6	13.0 ± 5.1

Mean % ± SD 平均%±標準偏差

One portion of frozen lymphocytes was used to measure each immunologic parameter immediately after thawing and the remaining cells were incubated for 18 hours and then each parameter was measured.

凍結リンパ球の一部は融解直後に各免疫パラメーターを測定するために使用し，残りの細胞は18時間インキュベーション後に各パラメーターを測定した．

Recovery rate of number of cells immediately after thawing was 81.7%±23.7% as against 69.9%±25.5% after preincubation for 18 hours, which indicated that preincubation had caused decrease of number of cells by about 10%.

細胞の回収率は，凍結保存融解直後81.7%±23.7%に対し，18時間 preincubation 後は 69.9%±25.5%になり，preincubation 操作により，約10%の細胞数の減少を示した．

Proportion of lymphocyte subpopulations was studied using monoclonal antibodies. There was no difference in proportion of NK cells (Leu-7 positive cells) between 21.4%±5.6% immediately after thawing and 21.3%±5.6% after preincubation. Also, the differences between proportion of lymphocyte subpopulations immediately after thawing and those after preincubation were not statistically significant in the proportion of T cells, cytotoxic-suppressor T cells, helper-inducer T cells, B cells, and monocytes.

リンパ球 subpopulation の割合は，モノクローナル抗体を用いて検討した．NK 細胞(Leu-7 陽性細胞)の割合は，凍結保存融解直後 21.4%±5.6%，pre-incubation 後 21.3%±5.6%で差はなかった．また，T 細胞，cytotoxic-suppressor T 細胞，helper-inducer T 細胞，B 細胞及び単球の割合も融解直後と preincubation 後では変動はなかった．

Proportion of target-binding cells immediately after thawing was 8.2%±2.6% as against an evidently increased value of 13.0%±5.1% after preincubation, which showed significant difference ($P<0.01$), and it was elucidated that cells binding with K-562 increased.

Target-binding cell の割合は，凍結保存融解直後 8.2%±2.6%に対し，preincubation 後は 13.0%±5.1%になり明らかに増加し($P<0.01$で有意)，K-562 に結合する細胞が増加することが判明した．

The above-mentioned results show that, though neither viability of cryopreserved lymphocytes nor percentage of NK cells was affected by preincubation, the number of cells binding to K-562 increased. This suggests that recovery of the target cell-binding capacity of cryopreserved cells is the cause of recovery of NK cell activity.

以上の結果から，preincubation によって，凍結保存リンパ球の生存率，NK 細胞の割合は変化がなかったが，K-562 に結合する細胞の増加を認め，凍結保存細胞の標的細胞結合能の回復が，NK 細胞活性の回復の一因となっていることを示唆している．

Effect of Length of Cryopreservation Period. Cells cryopreserved for various periods from two weeks to 14 months were thawed and, after preincubation for 18 hours, NK cell activity was measured. As shown in Figure 5, when E:T ratios were 80:1 and 40:1, NK cell activity was not different between before and after freezing up to 14 months of storage. When E:T ratios were 20:1 and 10:1, after two weeks of preservation period, the activity decreased compared to before freezing, but no further decrease was observed when cryopreservation period was extended to 14 months.

凍結保存期間の影響． 2 週間から14か月間まで，種々の期間凍結保存した細胞を融解し，18時間 preincubation 後に，NK 細胞活性を測定した．図 5 から，E:T 比が80対 1，40対 1 の条件では，凍結保存期間が14か月までは，凍結前後の NK 細胞活性に差を認めなかった．E:T 比が20対 1，10対 1 では，保存期間 2 週間で，凍結前に比し，明らかに細胞活性が低下したが，14か月まで凍結保存期間を延長しても，それ以上の低下はみられなかった．

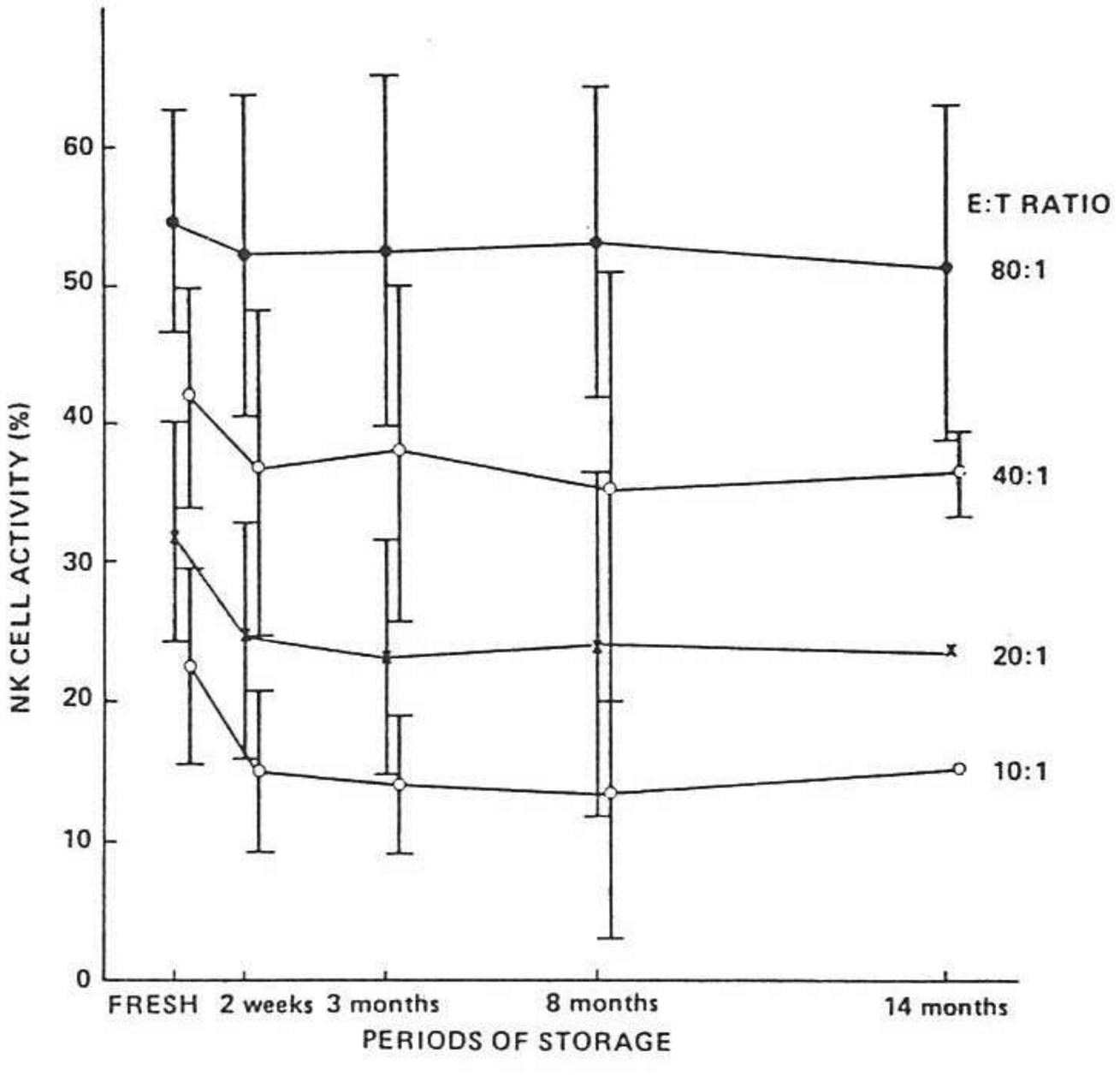
DISCUSSION

Technical difficulties of cryopreservation rest in the fact that low temperature damages and destroys various functions of cells and tissues while maintaining their viability for a long time,

考　察

低温は，細胞や組織の生存能を長期間維持する作用と，それらの種々な機能を障害，破壊する作用が

FIGURE 5 NK CELL ACTIVITY AFTER DIFFERENT PERIODS OF STORAGE
図 5 保存期間別 NK 細胞活性



and a perfect cryopreservation technique has not yet been established despite the desire to use it in not only medical but also in other fields of science where the efforts of many researchers have thus far made. The authors reviewed problems in cryopreservation of human lymphocytes and possibilities for its application to immunology and clinical medicine, using various immunologic parameters as indexes.

In freezing cells, there is a difference in freezing mechanism between inside and outside of a cell, separated by the cell membrane as the border. It will be most important to determine the optimal conditions for cryopreservation preventing or minimizing the effect of freezing damage on the cell membrane.

Lymphocytes have a large number of antigens as well as various receptors on their membrane. Thus, effects of cryopreservation on lymphocyte membrane have been studied with respect to surface markers, such as the E-rosette receptor, complement receptor, Fc receptor, cell surface immunoglobulin, etc. In general, there have been many reports[8-11] indicating that these

表裏一体の関係にあり，そこに凍結保存の技術的な難しさがあり，医学のみでなく広い分野からその活用が望まれ，現在まで多くの研究者の努力にもかかわらず，完璧な凍結保存法が確立されていないゆえんである．著者らは，種々の免疫学的パラメーターを指標にして，ヒトリンパ球の凍結保存に関する問題点，免疫学及び臨床への応用の可能性を検討した．

細胞の凍結は，細胞膜を境として，細胞内外で異なった凍結像を描くので，細胞膜の凍結障害の影響を防ぎ最小にすることは，至適凍結保存条件の決定に重要な指標となる．

リンパ球はその膜上に多数の抗原や，各種のレセプターをもっている．したがって，凍結保存がリンパ球膜に及ぼす影響は，Eロゼットレセプター，補体レセプター，Fc レセプター，細胞表面免疫グロブリンなどの表面マーカーについて検討されてきた．全般的に，これらの表面マーカーは凍結保存による影響を受けないとの報告が多い．[8-11] しかし，Jewett

surface markers are not affected by cryopreservation. However, after cryopreservation, Jewett et al[12] reported an increase of E-rosette and a decrease of EAC-rosettes, while Callery et al[13] reported a decrease of E-, EA_γ-, and EA_μ-rosettes and an increase of EAC-rosette. It is considered that differences among these results occurred because rosette-forming tests are easily affected by differences of SRBC lot, presence of AET treatment and neuraminidase treatment, temperature of reaction, reaction period, and mechanical handling after rosette formation as well as different conditions of cryopreservation used.

ら[12]は凍結保存後，Eロゼットの増加，EACロゼットの減少，Callery ら[13]は E, EA_γ, EA_μ ロゼットの減少，EACロゼットの増加を報告している．これらの結果の相違は，ロゼット形成試験が，SRBCのロット差，AET処理やneuraminidase処理の有無，反応温度，反応時間，ロゼット形成後の機械的操作の影響を受けやすく，凍結保存条件の違いも加わって生じたと考えられる．

One of the newest and most reliable methods of identifying lymphocyte surface markers is the use of monoclonal antibodies, such as those against Leu-1, Leu-2a, Leu-3a, Leu-7, HLA-DR, Mo2, and B1 antigens. For the proportions of lymphocyte subpopulations detected by these antigens, no difference was observed before and after cryopreservation as in the case of the E- and EA-rosette-forming tests. However, because changes in lymphocyte subpopulations classified by surface markers do not necessarily correlate perfectly with changes in their function, the effects of cryopreservation on the biological function of lymphocytes was studied further.

一方，リンパ球表面マーカーを識別する最新の最も信頼できる方法として，Leu-1, Leu-2a, Leu-3a, Leu-7, HLA-DR, Mo2, B1 抗原に対するモノクローナル抗体を用いる方法がある．これらで検出されるリンパ球subpopulationの割合を検討したところ，Eロゼット形成試験や，EAロゼット形成試験と同様に，凍結保存前後においてその割合に変化を認めなかった．しかし，表面マーカーで分類したリンパ球subpopulationの変動が，必ずしもその機能の変動と完全に相関するわけではないので，更に，リンパ球の生物学的機能に関して，凍結保存の影響を検討した．

In the previous paper, we reported the effect of cryopreservation on lymphocyte blastogenesis stimulated by phytohemagglutinin, concanavalin A, and PWM and mixed lymphocyte culture. In the present paper, analyses were made on the capacity for antibody production and cytotoxicity of cryopreserved lymphocytes. B lymphocytes, responding to various antigens and in the presence of helper T cells and macrophages, proliferate, differentiate, and mature, to finally become antibody-producing cells. Donaldson et al[14] studied antibody production of cryopreserved lymphocytes using the Jerne plaque assay and found no significant changes in capacity of lymphocytes to produce antibody. Here we made such measurements using radioimmunoassay and obtained results similar to theirs. Under the cryopreservation conditions reported here, cryopreservation was possible with functions of B cells, T cells, and macrophages for antibody production to be kept intact.

前回の報告で phytohemagglutinin, concanavalin A, PWMで惹起されるリンパ球の幼若化反応と，mixed lymphocyte cultureに及ぼす凍結保存の影響を述べた．今回は，凍結保存リンパ球の抗体産生能と細胞障害活性について調べた．Bリンパ球は，種々の抗原に反応して，helper T細胞，macrophageの存在下で，増殖，分化，成熟して最終的に抗体産生細胞となり，抗体を産生する．凍結保存リンパ球による抗体産生については，Donaldson ら[14]が，Jerne plaque assayを用いて検討したところ，リンパ球の抗体産生能の変動は有意でなかった．著者らは，radioimmunoassayを用いて測定したが，彼らと同様の結果を得た．著者らの凍結保存条件では抗体産生に関与するB細胞，T細胞，macrophageは機能を保持し，凍結保存が可能であった．

Next, review was made on cytotoxicity which had been considered to be sensitive and susceptible to low temperature. ADCC means cytotoxicity by nonsensitized leukocytes which are activated by combining with target cells through specific antibodies and their cell-surface Fc receptors. With regard to ADCC of cryopreserved lymphocytes, some reported high values[11] after cryopreservation and some others low values.[13] Miller et al,[15] using ^{3}H-proline cytotoxicity assay, reported that ADCC activity decreased when the E:T ratio was low, but that no difference was observed when E:T ratio was high. We observed no effect of cryopreservation by the plaque assay using SRBCs as target cells, but did observe a decrease in ADCC activity after cryopreservation by the same ^{3}H-proline cytotoxicity assay as used by Miller et al, using monolayer adherent T-24 cells as target cells. One reason for these inconsistencies is that ADCC response itself differs remarkably with the kind of target cells, various antibodies, and kind and number of effector cells used. The reason why Miller et al[15] observed no difference in ADCC between before and after cryopreservation when using high E:T ratios, is probably that there are not such changes in results as reflected in ADCC in the presence of more than a certain number of killer cells. Therefore, when cryopreserved lymphocytes are used for measuring ADCC, it is necessary to make measurements under the condition that the number of cells is sufficient for ADCC.

NK cells, which possess nonspecific cytotoxicity capabilities, are considered to function as a primary defense against heteroantigen (carcinoma) in an organism and to have an important role in immunologic surveillance. Thus, effects of cryopreservation on NK cells were studied from the aspects of the proportion of NK cells and NK cell activity. The results obtained showed that there was no difference in the proportion of NK cells before and after cryopreservation, but that NK cell activity decreased after cryopreservation. This indicates that the expression of membrane surface markers, at least the Leu-7 antigen, does not correlate directly with function. This may be because the Leu-7 surface antigen on NK cells which was used to determine the proportion of NK cells, did not act as a functional receptor and remained intact, whereas it is possible that functional receptors on NK cells were

次に，従来から低温に感受性が高く影響を受けやすいと考えられている細胞障害活性について検討を加えた．ADCC とは，非感作白血球が特定の抗体とその細胞表面の Fc レセプターを介して標的細胞に結合し，細胞障害を行う現象である．凍結保存リンパ球による ADCC については，凍結保存後高値，[11] あるいは低値[13] を示したという報告のほか，Miller ら[15] の ^{3}H-proline 法によると E:T 比が低いときは，ADCC 活性は低下するが，E:T 比が高いときは差を認めなかったと報告している．著者らは SRBC を標的細胞としたプラーク法では，凍結保存による影響は認めず，Miller らと同じ方法である単層付着 T-24 細胞を標的細胞とした ^{3}H-proline 法では，凍結保存後 ADCC 活性の低下がみられた．このように各報告が一定でない原因には，ADCC 反応自体が，標的細胞の種類，抗体の種類，エフェクター細胞の種類と数によって著しい多様性を示すことが挙げられる．しかし，Miller ら[15] のいう高い E:T 比を用いた ADCC で凍結保存前後に差がないということは，一定以上のキラー細胞が存在すれば，ADCC 活性に反映される結果には変化がなくなるためではないかと考えられる．したがって，ADCC に，凍結保存リンパ球を使用する際には，ADCC に必要な細胞数を満たした条件で，測定を行わなければならない．

NK 細胞は，抗原に未感作の状態で，細胞障害活性を有している細胞で，生体の異種抗原（癌腫）に対する最初の防禦機能として働くものと考えられており免疫監視機構において重要な役割を担っている．そこで凍結保存が NK 細胞に及ぼす影響を，NK 細胞の割合と NK 細胞活性について検討した．著者らの結果では，NK 細胞の割合は凍結保存前後に変動はなかったが，NK 細胞活性は凍結保存後は低下していた．これは膜表面マーカーの表現，少くとも Leu-7 抗原と，機能は単純に相関しないことを示すものである．この原因として，NK 細胞の割合の検出に用いた NK 細胞表面抗原の Leu-7 は機能的レセプターとして機能しないでそのまま保持されるが，一方 NK 細胞上の機能レセプターには凍結保存による不活性

inactivated and damaged by cryopreservation. According to our results, the proportion of cells capable of binding to target cells decreased immediately after thawing, and this provides evidence for the possibility that functional receptors have been damaged by cryopreservation.

化や障害等が生じている可能性が考えられる．著者らの実験，すなわち標的細胞とのbinding assayの結果によれば，凍結保存融解直後その割合が低下しており，凍結保存により機能レセプターに障害が生じている可能性を裏付けるものであろう．

Effect of cryopreservation on NK cell activity has thus far been made using various assay systems. Though no effect of cryopreservation has been observed in experiments using M14,[13] F-265,[10] T-24, and RT4[16] as target cells, it has been reported that a decrease in NK cell activity is caused by cryopreservation when K-562 cells, a widely-used NK sensitive cell line, is used.[9,13] We also found that NK cell activity against K-562 decreased immediately after cryopreservation, though a correlation in activity was observed before and after cryopreservation.

現在までに，NK細胞活性に及ぼす凍結保存の影響は，種々のassay系で検討されている．標的細胞としてanti M 14 adherent cell,[13] F-265,[10] T-24, RT4[16]を用いた実験では，凍結保存による影響はみられなかったが，現在広く用いられているNK細胞高感受性株であるK-562を用いると，凍結保存によりNK細胞活性が低下すると報告されている．[9,13] 著者の成績でも，K-562を用いた凍結保存直後のNK細胞活性は凍結前の活性と相関関係を認めたが，その活性は低下していた．

Various conditions of cryopreservation such as freezing rate and serum component of freezing medium, and E:T ratio as a condition for NK cell activity measurement were tested, but no improvement was observed in NK cell activity after cryopreservation. However, a recovery of NK cell activity was observed after preincubation of cryopreserved lymphocytes at 37°C for 18 hours. These are considered to be valuable data because few similar results have been reported. Mechanisms of recovery of NK cell activity by preincubation are presumed to be: 1) recovery of NK cell function damaged by cryopreservation, 2) induction of NK cells during preincubation, and 3) a relative increase in effector cells incurred in the processing of preincubation.

そこで，凍結保存条件（凍結速度，凍結保存液の血清成分）及びNK細胞活性の測定条件（E:T比）を変えて検討したが，凍結保存後のNK細胞活性の改善はみられなかった．しかし，凍結保存リンパ球を37°C，18時間preincubation後に，NK細胞活性の回復を認めた．これらのことは現在まで他に報告が少なく，貴重な資料と考える．Preincubationによる NK細胞活性回復の機序として考えられるものとしては次のとおりである．1) 凍結保存により障害されたNK細胞機能の回復，2) preincubation中のNK細胞の誘導，3) preincubationの細胞操作中に生じるエフェクター細胞の相対的な増加である．

With regard to 2), Zielske et al[17] reported that FCS has mitogenic function against human lymphocytes and that blastogenic lymphocytes show cytotoxicity. Also, Ortaldo et al[18] observed NK cell activity after culture for five or more days after removing cells possessing Ig Fc receptors. However, cytotoxic response did not increase after culture for 18 hours in either case. In these studies, we screened FCS to use lots not mitogenic against human lymphocytes in all the experiments, and cultured fresh lymphocytes in medium containing these FCS for 18 hours for confirmation, and observed no difference in NK cell activity before and after culture. Further, no difference was

2) については，Zielskeら[17]は，FCSはヒトリンパ球に対しmitogenicな作用をもち，芽球化リンパ球が細胞障害活性を示すと報告している．またOrtaldoら[18]は，Ig Fcレセプター保有細胞除去後5日間以上培養してNK細胞活性を認めている．しかし，いずれの場合も18時間培養では細胞障害活性は認めていない．著者らは以前よりFCSをスクリーニングして，ヒトリンパ球に対してmitogenicでないロットを全実験に使用しているが，これらのことを再確認するために，新鮮リンパ球をFCSを含む培養液で18時間培養したが，培養前後にNK細胞活性の差を認めなかった．更に，preincubation前後で，凍結

observed in the proportion of subpopulations of cryopreserved lymphocytes before and after preincubation (Table 8), so that an increase in cytotoxicity caused by increase in the numbers of NK cells is negated.

With regard to 3), though there was no difference in viability of cryopreserved lymphocytes before and after preincubation (Table 8), because of the decrease in cell number by about 10% due to cell killing during preincubation and because of technical loss caused by preincubation, the final concentration of NK cells in cell suspension used in the experiments might have increased and false recovery of NK cell activity might have resulted.

However, it is considered that the strongest reason for the recovery of NK cell activity is as noted in 1), recovery of NK cell function. NK cell activity is considered to manifest itself through three steps, i.e., binding of NK cells and target cells, NK cell activation, and finally damage to target cells caused by cytotoxic molecules released from NK cells. Thus, recovery of NK cells through these steps is considered to be involved in the recovery of NK cell activity. Among these, we studied the recovery of intercellular binding capacity, and observed that the ratio of cells binding with target cells increased after preincubation. Although all the target-binding cells may not be NK cells,[19] it is considered that recovery of binding capacity with target cells is necessary for the recovery of NK cell activity after preincubation. Our experimental results clearly indicate this relationship. Recovery of intercellular binding strongly suggests an eventual recovery of the other two steps mentioned above, and it is probably appropriate to consider that recovery of NK cell activity by preincubation represents functional recovery of NK cells themselves.

Therefore, it seems possible to recover many immunologic parameters and functions after cryopreservation, but as the NK cell activity study indicates, it may be necessary to assess the effect of cryopreservation on particular lymphocyte functions of interest before extensive studies are undertaken.

保存リンパ球の subpopulation の割合に変動はなく（表８），NK 細胞の増加による細胞障害活性の増加は否定される．

次に 3) については，preincubation 前後で凍結保存リンパ球の生存率は不変であったのに対し（表８），細胞数は約10％減少し，preincubation 中に死滅したり，preincubation による機械的損失などが生じたため，実験に用いた細胞浮遊液中に占める NK 細胞の最終濃度が増加して，NK 細胞活性の見掛け上の回復をもたらしたのかもしれない．

しかしながら，最も考えられる NK 細胞活性回復の要因としては，1) に挙げた NK 細胞の機能回復によるものが考えられる．NK 細胞活性発現に関しては，NK 細胞と標的細胞の結合，NK 細胞活性発現，NK 細胞から放出される細胞障害酵素による標的細胞障害の３段階が挙げられている．したがって，NK 細胞活性の機能回復にはこれらの段階での回復が考えられる．著者らは，このうちの細胞間結合能の回復について調査した．すなわち，preincubation によって，標的細胞に結合する細胞の割合が増加することを認めた．Target-binding cell のすべてが NK 細胞ではないが，[19] preincubation 後の NK 細胞活性の回復には，標的細胞への結合能の回復が必要条件であると考えられる．著者らの実験結果はこれらの関係を明確に裏付けるものである．細胞間結合能が回復する事実は，ひいては他の残り２段階でも機序が回復することを強く示唆するものであり，preincubation によってもたらされた NK 細胞活性の回復は NK 細胞自身の機能回復によるものと考えるのが妥当であろう．

以上のように，多くの免疫学的パラメーターや機能は，凍結保存後の回復が可能であるが，NK 細胞活性の研究が示したように，広範な調査を手掛けるときは，前もって目的の検査ごとに凍結保存の特定リンパ球機能に与える影響について検討することが必要であろう．

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