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BRIEF COMMUNICATION

A Convenient and Economical Freezing Procedure for Mononuclear Cells

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Cryopreservation techniques have become essential in longitudinal evaluation of lymphocyte function. This study describes a convenient method for freezing of peripheral blood mononuclear cells, using a small cryocontainer. Analysis of cell function, assayed by lymphoproliferation to recall antigens and by cytotoxic capacity of activated lymphocytes, was performed in parallel on fresh and frozen–thawed cells. Although cryopreservation did affect lymphocyte function, our results indicate that this freezing method performed equally well compared to a computer controlled device. We conclude that the cryocontainer has proved to be a suitable and practical tool in clinical studies and is an economical alternative to conventional methods. © 1995 Academic Press, Inc.

The use of cryopreserved cells has become inevitable in field studies or longitudinal surveys. Successive cell samples from different donors, taken at different timepoints or at distant locations, can be evaluated simultaneously and experimental errors, due to day to day variability in the biological assays, can be minimized. The freezing procedure itself is critical to preserve optimal functionality of the cells. Computer controlled freezing devices generate reproducible temperature curves with compensation for crystallization heat (3). However, they are expensive, stationary, and inconvenient for field conditions. We used a cryocontainer with isopropyl alcohol placed in a −80°C freezer, which approximates a temperature decrease of −1°C/min (5). We performed all functional assays in parallel on fresh and frozen–thawed lymphocytes.

MATERIALS AND METHODS

Cell Separation and Cryopreservation

Peripheral blood mononuclear cells (PBMC) were purified by density gradient centrifugation. PBMC were prepared for cryopreservation by aliquoting 5–10 × 10^6 in 1 ml ice-cold serum and by gentle addition of 1 ml ice-cold Me_2SO (20% in RPMI). Cells were kept on ice until the freezing process began.

Freezing of PBMC was performed three different ways. The first method used the Cryo 1°C freezing container (Nalgene Catalog No. 5100-0001). The cell aliquots were placed in the container, transferred to a −80°C freezer, and placed in liquid nitrogen after overnight storage. The exact temperature gradient inside the Nalgene cryobox could not be registered.

This method was compared with an automatic controlled freezer (Planer Kryo 10-1.7) with two different programmed temperature curves. The temperature decline was monitored inside the cooling chamber and inside a control vial containing cells.

The first curve (designated "fixed curve" hereafter) generated a fixed temperature decrease in the cooling chamber from 0 to −80°C at −1°C/min. The second curve (named "adapted curve" hereafter) was generated to ensure a temperature gradient of −1°C/min inside the sample. This was

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obtained in a four-step procedure: (I) 0 to −8°C at −1°C/min, (II) −8 to −40°C at −30°C/min, (III) −40 to −18°C at 7°C/min, and (IV) to −80°C at −1°C/min. After reaching −80°C with both curves, the samples were quickly transferred into liquid nitrogen.

Thawing was performed by immersion in a 37°C water bath. Cells were washed twice and resuspended in the appropriate culture medium. Viability was determined by exclusion of eosin.

Lymphocyte Proliferation Assay

PBMC from four normal healthy donors were cultured for 6 days in 10% pooled normal human serum (pNHS) in the presence of medium, varicella zoster antigen (VZ, 1/200; Behring Hoechst), tetanus toxoid (TT, 1/200; Wyeth Laboratories), *Candida albicans* antigen (CA, 1/200; Pasteur), or pokeweed mitogen (PWM, 1/200; Gibco).

PBMC from persons chronically infected with hepatitis B virus (HBV) were stimulated for 6 days in 10% pNHS with medium, VZ, or HBV-specific antigens (rHBsAg, 3 μg/ml, provided by SKB Rixensart; rHBcAg and rHBeAg, 2 μg/ml, provided by Organon Teknika).

Proliferation of PBMC was determined by measuring incorporation of [^{3}H]thymidine (0.4 μCi/well, sp act: 5.0 Ci/mmol; Amersham, UK) during the final 8 h of the culture. Results were expressed as mean cpm ± standard deviation or as stimulation index (mean cpm of stimulated cultures/mean cpm of unstimulated cultures). A stimulation index of ≥2 was considered as positive.

Cytolytic Assay

In order to investigate whether the freezing procedure could influence (a) the cytotoxic capacity of already stimulated lymphocytes and (b) the induction of cytotoxicity in unstimulated frozen–thawed cells, we compared the cytolytic capacity of effector PBMC, which had been stimulated before or after the freezing procedure with IL-2 (10 U/ml) and IL-6 (100 U/ml) (8). Cytolytic function was determined in an anti-CD3 redirected chromium release assay using P815 as target cells (10).

RESULTS AND DISCUSSION

PBMC, either fresh or frozen with the cryocontainer or with the controlled freezer, were compared for lymphoproliferation after a 6-day culture with control antigens (TT, VZ, and CA) and PWM. In all frozen–thawed PBMC, lower proliferative responses were observed compared to fresh PBMC, but the cryobox method performed equally well compared to the conventional freezing procedure with fixed or adapted curve (Fig. 1).

HBV-specific stimulation was performed on PBMC from three chronically infected patients of whom two were asymptomatic HBsAg carriers and one had signs of active viral replication and disturbed liver functions. Fresh PBMC were compared with PBMC frozen in the cryobox (Table 1). In all patients, proliferative responses to HBeAg and HBcAg (the latter not shown) were maintained after freezing and HBsAg-induced proliferation remained absent. Similar to the control antigen stimulation, the HBV antigen-induced cell growth was lowered when expressed as stimulation index.

Compared to fresh cells, a marked and specific loss of monocytes in frozen–thawed cells was revealed by phenotypic analysis (data not shown) irrespective of the freezing procedure used. This observation confirms previous reports (4, 9) and can account for differences in proliferative capacity observed after stimulation of fresh and frozen–thawed cells, since it is known that proliferative responses to mitogen and antigen are monocyte-dependent (1, 6).

All freezing methods reduced the cytolytic capacity: the OKT3-specific lysis at a 10:1 effector–target ratio dropped from 60 to less than 20% in fresh and frozen–thawed

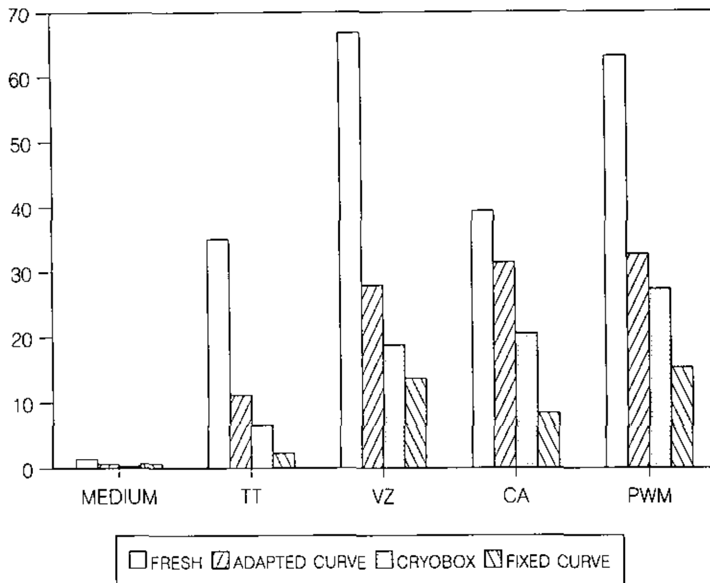

FIG. 1. Proliferation of fresh PBMC and PBMC frozen three different ways (adapted curve, cryobox, and fixed curve). Stimulation was performed with medium (negative control), TT, VZ, CA, or PWM for 6 days in 10% pNHS. The results from one representative experiment are expressed as cpm × 10E-3.

effector cells, respectively. The loss of cytolytic T cell function was similar in cells frozen–thawed before or after activation (Fig. 2). This clear-cut loss of cytotoxic capacity has also been reported for lymphokine activated killer cells and natural killer cells (2, 7).

The cryobox method was successfully applied to investigate lymphocyte functions with results comparable to conventional freezing devices. The cryobox method provides a low-cost alternative for use in longitudinal clinical studies and in field conditions.

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TABLE 1
Proliferative Responses of Fresh and Frozen PBMC to Stimulation with HBV Antigens

	Medium	HBsAg (SI)[a]	HBeAg (SI)
I[b]	1071 ± 410[c]	2192 ± 484 (2.0)	9779 ± 2402 (9.1)
I*	2896 ± 723	1362 ± 351 (0.5)	14127 ± 4002 (4.9)
II	3903 ± 342	3932 ± 15 (1.0)	24563 ± 1691 (6.3)
II*	8311 ± 1921	13141 ± 4997 (1.6)	43759 ± 11416 (5.3)
III	1374 ± 332	1703 ± 270 (1.2)	26592 ± 3598 (19)
III*	8384 ± 3280	11200 ± 7696 (1.3)	45119 ± 7591 (5.4)

[a] SI, stimulation index (medium = 1).

[b] Fresh PBMC and frozen PBMC (*) from three different donors were compared. Two asymptomatic carriers (I, II) and one chronic active (III) hepatitis were examined.

[c] Proliferative responses of fresh PBMC and PBMC frozen with the cryobox after a 6-day stimulation with medium (negative control), HBsAg (3 μg/ml), and HBeAg (2 μg/ml).

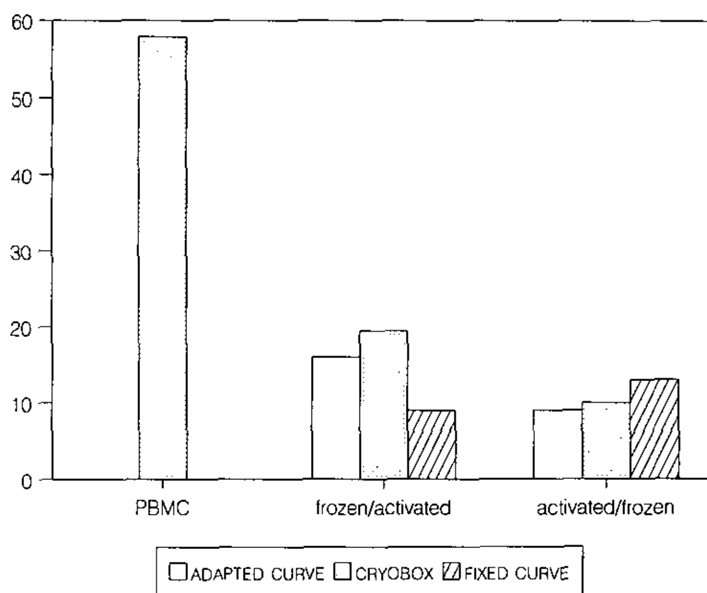

FIG. 2. Cytotoxic capacity of fresh and frozen–thawed PBMC defined as percentage of OKT3-specific lysis of P815. Fresh PBMC are compared with PBMC frozen according to the three procedures (cryocontainer and two automated freezer methods). Results are expressed as mean percentage of Cr51 release obtained from triple wells at an E/T ratio of 10:1.

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