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CRYOPRESERVATION OF HUMAN LYMPHOID CELLS FROM VARIOUS TISSUES *

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Cryopreserved cells of both animals and humans have been shown to retain their functional characteristics, including lymphoproliferative responses, cytotoxicity, mixed lymphocyte culture reactivity [2,3] and the ability to restore haematopoietic and immunological function in irradiated animals [5]. In view of the fact that few observations on the immunological characteristics of cryopreserved human pathological lymphoid cells have been reported, we evaluated the influence of cryogenic preservation on the T and B cell surface markers and on the proliferative response to mitogens of lymphoid cells from various, normal and pathological sources.

MATERIALS AND METHODS

Lymphoid cells were collected from the lymph nodes, spleen, bone marrow and peripheral blood of patients with various lymphoproliferative disorders. In addition, lymphoid cells from fetal thymus, normal lymph nodes and normal peripheral blood were used in this study (tab. 1).

Lymphoid cell separation

Cells from solid tissues were obtained by mincing and shaking the tissues in Hanks' balanced salt solution (HBSS). Blood and bone marrow heparinized samples were incubated at 37 °C for 1 h, and the cells were removed from the cell-enriched plasma. In all cases the mononuclear cells were separated by using a Ficoll-hypaque

Key-words: Cryopreservation; E rosettes; Immunofluorescence; Lymphoid cells; Lymphoproliferative disorders; Phytohaemagglutinin (PHA); Pokeweed mitogen (PWM).

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source	diagnosis	fresh lymphoid cells		21 days cryopreserved			35 days cryopreserved		
		Er (%)	Ifl (%)	recovery (%)	Er (%)	Ifl (%)	recovery (%)	Er (%)	Ifl (%)
lymph node	WDDL	13	70	65	22	60	75	19	59
lymph node	WDDL	3	65	53	6	70	67	7	nd
lymph node	PDDL	6	70	86	10	80	90	4	64
lymph node	PDDL	70	5	32*	75	5	33*	69	5
lymph node	reactive non-specific follicular hyperplasia	58	16	55	53	22	45	61	13
lymph node	reactive non-specific follicular hyperplasia	58	19	63	53	17	50	50	22
lymph node	reactive non-specific follicular hyperplasia	63	12	38*	55	7	nd	nd	nd
spleen	WDDL	66	10	69	69	8	55	56	10
bone marrow	CLL	3	60	nd	nd	nd	50	2	63
bone marrow	ALL	3	3	nd	nd	nd	61	3	3
bone marrow	WDDL	78	5	25*	64	3	nd	nd	nd
bone marrow	ALL	80	1	68	82	2	nd	nd	nd
peripheral blood	ALL	74	6	71	75	3	nd	nd	nd
peripheral blood	CLL	9	65	63	3	68	nd	nd	nd
peripheral blood	CLL	11	80	90	10	76	nd	nd	nd
peripheral blood	CLL	7	70	78	3	69	nd	nd	nd
peripheral blood	NC	76	10	68	78	9	nd	nd	nd
peripheral blood	NC	82	12	67	84	9	nd	nd	nd
peripheral blood	NC	75	18	30*	75	15	27*	70	11
peripheral blood	NC	69	13	25*	70	11	21*	65	11
fetal thymus	—	90	0	60	90	0	nd	nd	nd
fetal thymus	—	97	0	50	89	0	nd	nd	nd
fetal thymus	—	60	0	70	63	0	nd	nd	nd

Er = E rosettes; Ifl = immunofluorescence; WDDL = well differentiated diffuse lymphoma; PDDL = poorly differentiated diffuse lymphoma; CLL = chronic lymphoid leukaemia; ALL = acute lymphoblastic leukaemia; NC = normal control; nd = not done; * = without fetal calf serum (see text).

Tab. 1. - Comparison of the surface markers between fresh lymphoid cells and lymphoid cells cryopreserved for 21-35 days.

gradient, according to the method of BOYUM [1]. After counting, about 1 ml of the lymphoid cell suspension was used for immunological tests, while the remaining cells were then frozen.

Freezing procedure

A controlled rate freezer was used in all the experiments. Liquid nitrogen was used for freezing, and dimethyl-sulphoxide (DMSO) was added as a cryoprotective agent.

The cell suspension was handled at the temperature of melting ice and was distributed in 1 ml ampoules in 0.5 aliquots of the following medium: 80% HBSS, 20% fetal calf serum (FCS).

As the concentration of the cells did not appear to be very critical, various numbers of cells were cryopreserved per freezing tube. This cell suspension was slowly diluted with an equal volume of 80% HBSS and 20% DMSO, up to a final concentration of 80% HBSS, 10% FCS and 10% DMSO/ml.

The cells were cooled at a rate of 1 °C/min till –40 °C, and the transition phase time was shortened to 2-3 min. Thereafter, a more rapid rate of 5-7 °C/min to –100 °C was achieved (fig. 1). The freezing tubes were then transferred to the liquid nitrogen vessel.

Thawing

After 21 and 35 days of storage the ampoules were removed from the deep freezer immediately before use and plunged into a water bath at 37 °C with constant agitation until the last ice crystals had disappeared (1-2 min). DMSO was diluted by stepwise addition of HBSS, containing 10% FCS, to lower the DMSO concentration, at a rate of 1%/min [5].

The cells were washed twice. After counting, the cell concentration was adjusted to about 10 x 10^6 cells/ml, and the viability was evaluated by the trypan blue exclusion test.

E-rosette test

Purified lymphoid cells, 0.25 ml at about 4 x 10^6 cells/ml, were mixed with 0.25 ml of a suspension of sheep red blood cells (SRBC) at about 4 x 10^8 cells/ml. The mixed cell suspension was incubated at 37 °C for 15 min, centrifuged at

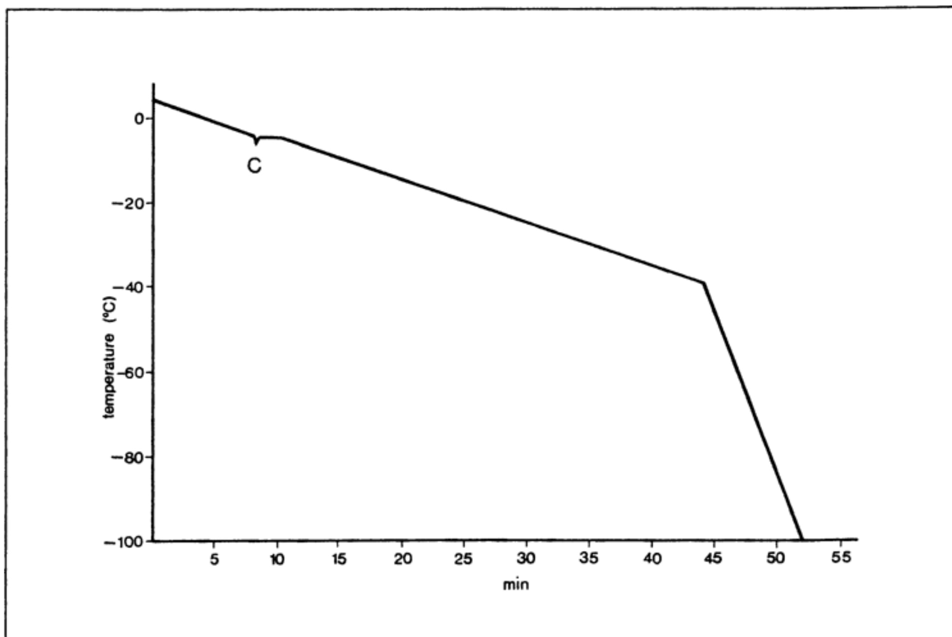

Fig. 1 - Freezing programme curve. The freezing procedure starts at about 4 °C, the temperature is reduced by 1 °C/min to –40 °C, passing the critical point (C), where the phase transition time is shortened to 2-3 min (see text).

200 x *g* for 5 min and incubated at 4 °C overnight. The supernatant was discarded, and the pellet was gently resuspended. A drop was placed on a haemocytometer for examination. Lymphoid cell binding more than three SRBC was considered as positive [6].

Surface immunoglobulin staining

Fifty μl of the lymphoid cell suspension, about 10 x 10^6 cells/ml, were mixed with 50 μl of fluorescein-labelled anti-human polyvalent immunoglobulin. After incubation for 45 min at 4 °C, the cells were washed three times with cold HBSS plus 10% FCS, and a drop of the cell pellet was placed on a slide and mounted under a cover slip.

The immunofluorescence was examined with a Zeiss microscope, equipped with incident fluorescent light, and at least 200 cells were counted. Cells were considered positive when showing either a bright fluorescent ring or bright spots on the cell membrane [4].

Cell cultures

Lymphoid cells, at a concentration of 2 x 10^6 cells/ml, were cultured in RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin and 20% heat-inactivated FCS. Triplicate samples of 0.1 ml of this cell suspension were incubated in microwells at 37 °C, 100% humidity and 5% CO_2 for 72 h, in the presence or absence of 5 μg, in 0.1 aliquots, of phytohaemagglutinin (PHA) or pokeweed mitogen (PWM). For the evaluation of mitogen reactivity, 1 μCi/well of tritiated thymidine (TMM 48 B, specific activity 28 Ci/mM) was added for the last 16 h of culture. The cultures were harvested by a Skatron multiple cell culture harvester, the cells being lysed with distilled water. The radioactivity was measured by a Beckmann Beta liquid scintillation counter. Activation values were expressed as the mean cpm of triplicate culture.

RESULTS

Lymphoid cells were collected from various sites in 23 cases. During the process of freezing and thawing, FCS was added to the medium in 18 cases, while in 5 cases it was not (tab. 1). The cell recovery after 21 and 35 days of storage was 67% and 62%, respectively, when 10% FCS was used, while a recovery of 30% and of 27% was found when FCS was omitted. The absence of FCS, as a protein source in the medium, seems to represent a limiting factor for the cell recovery percentage after freezing and thawing. However, the ratio of T and B cells in the total number of cells recovered after cryopreservation appeared to be very similar for all the cases studied, independently of the presence of FCS during freezing and thawing. The percentage of T and B cell surface markers recovered does not appear to be influenced by the cryopreservation technique and the long period of storage. In addition, we did not observe any difference in the samples tested after three or five weeks (tab. 1). The viability of the cryopreserved lymphoid cells after these storage periods was greater than 75% in all the cases studied.

Table 2 compares the results of the mitogenic response between fresh lymphoid cells and lymphoid cells frozen for 21 days from various pathological sources.

source	diagnosis	fresh lymphoid cells		21 days cryopreserved	
		PHA (cpm ± SE)	PWM (cpm ± SE)	PHA (cpm ± SE)	PWM (cpm ± SE)
lymph node	PDDL	23,344 ± 5,330	16,804 ± 2,340	32,320 ± 8,604	20,204 ± 3,300
lymph node	WDDL	12,304 ± 3,307	9,500 ± 1,902	16,300 ± 4,202	6,803 ± 3,120
lymph node	HD	108,653 ± 10,409	35,300 ± 8,102	171,784 ± 7,230	44,040 ± 9,200
bone marrow	ALL	31,400 ± 2,301	8,600 ± 1,420	26,000 ± 3,660	9,400 ± 2,430
bone marrow	CLL	21,200 ± 3,320	11,356 ± 2,304	16,600 ± 3,200	8,943 ± 1,920
peripheral blood	CLL	18,000 ± 4,370	6,800 ± 2,302	12,332 ± 2,607	5,220 ± 3,320
peripheral blood	NC	123,334 ± 7,204	53,230 ± 3,321	155,640 ± 5,200	45,327 ± 4,302
peripheral blood	NC	89,963 ± 8,902	38,343 ± 5,204	102,230 ± 9,200	31,221 ± 5,300
peripheral blood	NC	148,634 ± 9,600	31,230 ± 3,490	156,523 ± 4,230	41,090 ± 5,360

HD = Hodgkin's disease; for other abbreviations, see tab. 1.

Tab. 2 - Comparison of the mitogenic response between fresh lymphoid cells and lymphoid cells cryopreserved for 21 days. Results are expressed in cpm ± standard error (SE).

Although in some cases there was a wide dispersion in the results between the blastic response of the fresh and frozen cells, the cryopreserved lymphoid cells maintained the capability to respond to PHA and to PWM as well as the fresh cell suspension.

DISCUSSION

It has been demonstrated by other workers [2,7] that peripheral blood lymphocytes can be stored in liquid nitrogen without losing their immunological characteristics. We studied the effect of cryopreservation on lymphoid cells collected from different sources and containing either pure thymocyte cells, such as those from fetal thymus, or both T and B lymphoid cells, such as those from lymph nodes, spleen, bone marrow and the peripheral blood in malignant lymphoma cases.

From our results we can conclude that:

a. the application of the freezing and thawing procedure to abnormal lymphoid cells does not alter the proportion of T and B cells originally found in the fresh suspension;

b. fetal calf serum, as reported by THORPE et al. [8], appears to be an important factor for cell protection during the freezing and thawing phases;

c. the time of storage in liquid nitrogen does not interfere either with the percentage of cell recovery or with the maintenance of the immunological properties of the lymphoid cells.

The ability to store pathological lymphoid cells without loss of their immunological properties should stimulate new approaches to immunotherapy in a variety of diseases. Furthermore, autologous cryopreserved cells could be used to evaluate, *in vitro*, the immune status of patients on long-term chemotherapy.

SUMMARY

Normal and pathological lymphoid cells, collected from different sources, were cryopreserved using a programmed freezing procedure. With this cryopreservation technique, the percentage of T and B cell surface markers and the proliferative response to mitogens were not influenced by 21 and 35 days of storage in liquid nitrogen. The recovery percentage of the lymphoid cells was satisfactory when fetal calf serum was added, as a protein source, to the medium during freezing and thawing phases, while a very low percentage of cells was recovered if the fetal calf serum was omitted.

REFERENCES

1. Boyum A.: Separation of leucocytes from blood and bone marrow - Scand. J. clin. Lab. Invest. *21* (Suppl. 97), 77, 1968.
2. Du Bois M. J. G. J., Schellekens P. Th. A., De Wit J. J. F. M., Eijsvoogel V. P.: *In vitro* reactivity of human lymphocytes after cryopreservation using a programmed cooling device - Scand. J. Immunol. *5* (Suppl. 5), 17, 1976.
3. Factor M. A., Strong D. M., Miller J. L., Sell K. W.: Cell-mediated lymphocytotoxicity following *in vitro* sensitization of frozen stored human mononuclear cells in heterologous serum - Cryobiology *12*, 521, 1975.
4. Pernis B., Forni L., Amante L.: Immunoglobulin spots on the surface of rabbit lymphocytes - J. exp. Med. *132*, 1001, 1970.
5. Schaefer U. W., Dicke K. A., Van Bekkun D. W.: Recovery of hematopoiesis in lethally irradiated monkeys by frozen allogenic bone marrow grafts - Europ. J. clin. biol. Res. *17*, 483, 1972.
6. Special Technical Report: Identification, enumeration and isolation of B and T lymphocytes from human peripheral blood - Scand. J. Immunol. *3*, 521, 1974.
7. Thierry C., Turc J. M., Valles H., Serrou B.: Routine technique of lymphocyte cryopreservation evaluated by *in vitro* tests of immune response - Europ. J. Cancer *13*, 367, 1977.
8. Thorpe P. E., Knight S. C., Farrant J.: Optimal conditions for the preservation of mouse lymph node cells in liquid nitrogen using cooling rate techniques - Cryobiology *13*, 126, 1976.

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