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IN VITRO REACTIVITY TO CONCANAVALIN A OF BOVINE LYMPHOCYTES AFTER CRYOPRESERVATION

H. KUIL

Department of Tropical Veterinary Medicine and Protozoology, State University of Utrecht (The Netherlands)

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ABSTRACT

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Cryopreservation of bovine peripheral lymphocytes and its effect on the in vitro response to concanavalin A tested in a microculture system is described. Using DMSO as cryoprotectant in the medium, the cells were cooled to -30°C at 1.3°C/minute and further to -80°C at 6°C/minute and then rapidly to -196°C by dropping in liquid nitrogen. The cells were recovered by rapid thawing in water at 30-35°C and washed twice before use in the stimulation test. Ten percent DMSO had a much better protective effect than 5%; addition of 25% fetal bovine serum to the freezing had no favourable effect. In most of the 16 animals used in the experiments the frozen lymphocytes gave the same or a higher response to Con A than those kept in the DMSO containing medium at 4°C for two hours.
The responses of the frozen cells were comparable to those of fresh lymphocytes (kept at 4°C for two hours in medium without DMSO).

INTRODUCTION

In vitro reactivity of lymphocytes has become an important parameter to evaluate various functional capacities of these cells in man and animals. Adequate storage of these cells during which these capacities are retained is of great importance. It opens the possibilities to study the in vitro responses of sequential samples under identical cultural conditions and it provides means to reproduce observations.

There are several reports of succesful cryopreservation of human lymphocytes (Gross et al., 1975, Grant, 1976, Weiner, 1976, Birkeland, 1976) and lymphocytes of laboratory animals (Jennings et al., 1978) dog (Gluckman et al., 1977) and cattle (Kleinschuster et al., 1979, Tate and Cram, 1982).

Results of cryopreservation of bovine peripheral lymphocytes and its effect on the in vitro response to concanavalin A are

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reported here.

MATERIALS AND METHODS

Isolation of lymphocytes

Lymphocytes were isolated from blood a-septically collected from the jugular vein of Dutch-Friesian heifers. Heparin (Thromboliquine, Organon, Holland) 50 I.U./ml was used as anticoagulant. The freshly collected blood was kept on ice for 10 minutes and thereafter centrifuged for 10 minutes at 200 g. in a refrigerated centrifuge. Plasma and buffy coat were collected and diluted with equal parts of washing medium. The washing medium consisted of $NaHCO_3$ buffered RPMI 1640 (Flow Laboratories, Irvine, UK) containing penicillin (100 I.U./ml), streptomycin (0.1 mg/ml) and heparin (5 I.U./ml). The mixture was layered on half the volume Ficoll (Pharmacia, Uppsala, Sweden)-Isopaque (Nijegaard, Oslo, Norway) density 1.080 g/ml., and centrifuged for 45 minutes at 400 g. The cells were collected from the interface and washed twice in the washing medium by centrifugation for 10 and 5 minutes at 160 g. During the whole isolation procedure the cells were kept at 0° - 4°C.

The lymphocytes were counted electronically (Sysmex Cc 108, TOA, Japan) or in a haemocytometer.

Freezing procedure

The cells were frozen in the washing medium without heparin, either with 25% fetal bovine serum (FBS) or without and with dimethyl sulfoxide (DMSO) 5% or 10% as freezing protectant. Lymphocyte suspensions in this medium were obtained by adding slowly (dropwise) cold medium containing twice the desired final concentration of DMSO to an equal volume of a suspension of 2.10^7 lymphocytes per ml. The suspensions were frozen in aliquots of 1 or 2 ml in polyethylene screw capped vials (Cryotube, NUNC, Denmark). The tubes were frozen in a Cryoson B4 programmable biological freezer with liquid nitrogen cooling (Cryoson, Benelux, Holland). They were cooled at a rate of 1.3°C per minute until -30°C, providing extra cooling to compensate for crystalization heat during phase transition. From -30°C until -80°C cooling took place at a rate of 6°C per minute. For storage the tubes were transferred to a liquid nitrogen storage vessel.

Thawing procedure

For thawing the tubes were immersed in water at 30°-35°C. The contents was diluted 1:10 with cold washing medium containing 5% FBS. The cells were washed twice in this medium, resuspended in culture medium and counted. For all the experiments the lymphocytes were thawed on the day they were frozen. The effect of storage of the frozen cells for any length of time was not examined.

Lymphocyte stimulation

The lymphocyte stimulation was carried out following the method described by van Dam et al. (1978). In brief: lymphocytes were cultured in round bottom microtiter plates, 300,000 cells per well in 0.2 ml culture medium containing varying concentrations of concanavalin A (Con A; Sigma-St Louis, U.S.A.). The culture medium consisted of the washing medium containing heparin 1 I.U./ml and 5% F.B.S. All tests were carried out in triplicate. The plates were incubated at 37°C in a CO_2 incubator, 5% in a humidified air atmosphere. After 72 hours 0,25 µ Ci of methyl-(^{3}H)-thymidine (Amersham International, Amersham, England) (specific activity 0.5 Ci/m. mole) was added to each well. After another 24 hours of incubation, the cultures were harvested with a semi-automatic multiple cell culture harvester (Skatron, Norway). The incorporation of (^{3}H) thymidine was determined with a liquid scintillation spectrometer (L.K.B. - Wallace L.S.C., L.K.B., Bromma, Sweden) and expressed as disintegrations per minute (d.p.m.). To obtain mean values those of the triplicate wells were averaged. Net d.p.m. values were obtained by subtracting the mean d.p.m. of control cells (no Con A in medium) from that of stimulated cells. Stimulation indexes (s.i.) were calculated dividing the mean d.p.m. of stimulated lymphocytes by the mean d.p.m. of control cells.

Broad outline of the experiments

Reactivity to Con A of lymphocytes after one of the following three treatments were compared: freezing in medium with DMSO 5% or 10% (frozen cells), two hours at 4°C in the same medium with DMSO (unfrozen cells) and two hours at 4°C in this medium but without DMSO (fresh cells).

RESULTS

Recovery of the lymphocytes

After washing of the lymphocytes to remove the DMSO, by two centrifugations in cold washing medium, losses varying from 30%-70% were observed. These losses occurred both in frozen lymphocyte batches and those kept at 4°C, without difference in the rate of these losses. Lymphocytes of 10 heifers kept in freezing medium containing 10% DMSO and 25% FBS for two hours at 4°C showed a recovery of 59 ± 13% after two washings, compared with 69 ± 21% of cells which had been frozen in the same medium.

Effect of DMSO concentration on cryopreservation

With lymphocytes of four heifers the effect of two DMSO concentrations, 5% and 10% was examined. The results are given in Table I. Ten percent DMSO had a much better protective effect than 5 percent. Further experiments were therefore carried out with 10% DMSO.

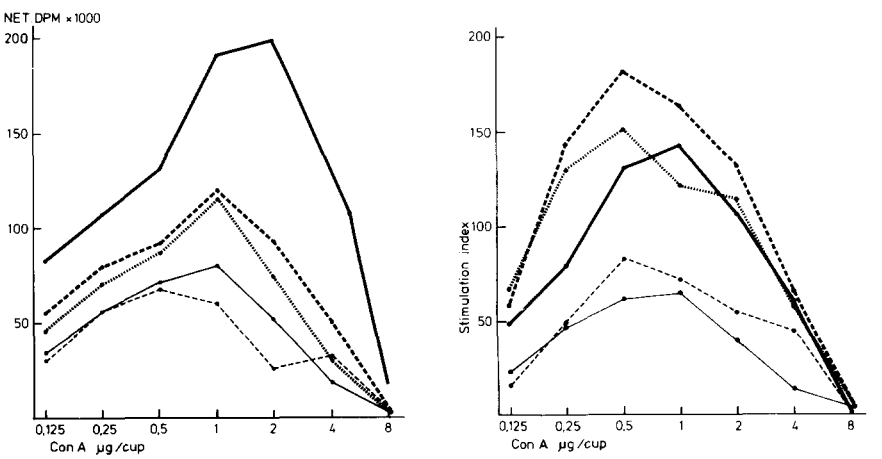

Fig. 1. Effect of cryopreservation on the response of lymphocytes (of one animal) to various concentrations of Con A.
•---------• cells in DMSO 10% kept at 4°C, •----------• cells in DMSO 10% and frozen, •——— cells in DMSO 10% + FBS 25% kept at 4°C, •·····• cells in DMSO 10% + FBS 25% and frozen, ——— cells in FBS 25% and kept at 4°C (fresh cells).

The effect of concanavalin A concentration

In 3 experiments with lymphocytes of two animals each, the effect of freezing on the reactivity of lymphocytes was tested at various Con A concentrations. In all experiments the optimum stimulation, both of frozen and non-frozen cells were obtained at the same Con A concentration level of 0.5 - 2 µg/well (Fig. 1).

The freshly collected, untreated cells responded in most cases best to the Con A when the net d.p.m. values are considered. Based on these values the DMSO-treated and frozen cells gave the same results or slightly better than the cells which were exposed to DMSO, not frozen but kept at 4°C (Fig. 1a). When the results are considered according to the stimulation index values the frozen lymphocytes gave in most cases the best responses (fig.1b). Addition of 25% FBS to the freezing medium did not improve the responses to Con A both with and without freezing (Fig.1, Table I).

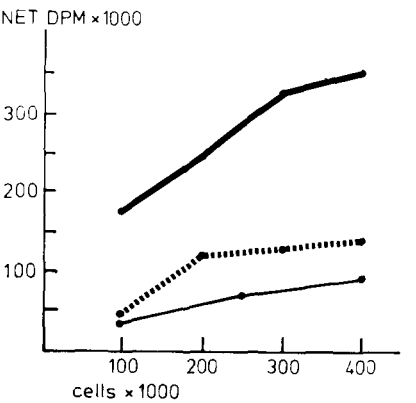

Fig. 2. Effect of cryopreservation on the response to Con A. (1.5 µg/cup) at various cell concentrations. Result of one animal.

——— cells in DMSO 10% + FBS 25% kept at 4°C,
••••••••••• cells in DMSO 10% + FBS 25% and frozen,
——— cells in FBS 25% kept at 4°C (fresh cells).

Effect of lymphocyte concentration in the culture

The effect of the cell concentration in the culture was studied with lymphocytes of two animals. In both cases the responses of the fresh cells were the best, whereas for DMSO treated cells there was not much difference between the cells

TABLE I

The effect of freezing and the addition of dimethylsulfoxide (DMSO) and fetal bovine (FBS) serum to the freezing medium on the response of lymphocytes to Con A 1.5 µg/well.

Heifer	DMSO %	FBS %	Net DPM* ± SD 4°C	Net DPM* ± SD frozen	DMSO %	FBS %	Net DPM* ± SD 4°C	Net DPM* ± SD frozen
1			33.2 ± 3.5	0.1			29.2 ± 2.0	0.1
2	0	0	62.5 ± 9.6	0.1	0	25	31.4 ± 1.5	0.1
3			12.3 ± 1.9	0.1			15.0 ± 2.1	0.1
4			12.9 ± 2.0	0.1			13.2 ± 2.6	0.1
1			44.8 ± 2.8	13.9 ± 0.3			23.0 ± 1.4	20.3 ± 2.1
2	5	0	82.3 ± 8.2	24.5 ± 1.0	5	25	23.3 ± 1.5	14.0 ± 0.9
3			20.6 ± 1.8	3.5 ± 1.0			27.1 ± 3.7	8.7 ± 0.7
4			21.2 ± 3.3	3.5 ± 0.9			30.5 ± 4.8	7.0 ± 3.4
1			27.6 ± 3.7	28.3 ± 1.3			21.6 ± 1.8	26.5 ± 2.0
2	10	0	49.7 ± 3.7	49.5 ± 3.9	10	25	32.4 ± 5.4	55.0 ± 3.8
3			15.0 ± 0.4	13.6 ± 2.4			27.7 ± 5.3	31.0 ± 2.1
4			14.0 ± 0.6	12.7 ± 1.3			29.4 ± 7.0	31.8 ± 1.8

* Net disintegrations per minute x 1000. Means of triplicate wells.

TABLE II

Response to Con A (1 g/well) of lymphocytes (300,000/well) of 10 heifers before and after freezing.

Nr.	DMSO 0%, FBS 0% 4°C (fresh cells) Net DPM* S.D.	S.I.**	DMSO 10%, FBS 25% 4°C Net DPM* ± S.D.	S.I.**	DMSO 10%, FBS 25% frozen Net DPM ± S.D.	S.I.**
1	23.8 ± 3.0	59	32.7 ± 1.0	110	93.8 ± 4.3	95
2	75.0 ± 2.7	57	71.3 ± 9.8	55	154.3 ± 7.5	58
3	69.1 ± 17.6	77	98.9 ± 6.5	48	134.9 ± 5.3	54
4	78.1 ± 7.4	26	81.4 ± 2.7	20	125.5 ± 12.0	32
5	56.0 ± 3.8	25	59.6 ± 19.4	50	123.3 ± 12.0	37
6	74.0 ± 1.6	28	77.4 ± 7.5	32	83.7 ± 8.0	12
7	108.1 ± 1.2	117	114.4 ± 8.0	96	149.4 ± 15.4	146
8	117.7 ± 8.0	92	127.9 ± 6.2	130	170.8 ± 12.4	100
9	157.7 ± 11.9	178	162.0 ± 1.1	152	134.7 ± 15.8	233
10	118.6 ± 14.5	91	121.2 ± 22.2	52	157.5 ± 6.1	92
MEAN	87.8 ± 38.1	75 ± 48	94.7 ± 38.0	75 ± 44	132.8 ± 27.6	86 ± 65

* Net disintegrations per minute x 1000. Means of triplicate wells.
** Stimulation index.

kept at 4°C and the frozen cells (Fig. 2).

Results with lymphocyte of 10 heifers

The results obtained with lymphocytes of 10 heifers frozen in RPMI medium containing 10% DMSO and 25% FBS are given in Table II. The best responses were obtained with the frozen cells. The difference between the net DPM values of the frozen and the non-frozen cells are significant ($P < 0.01$) those of the s.i. values are not.

DISCUSSION

Recovery of the cells after freezing was not different from that after being in the DMSO containing freezing medium at 4°C for 2 hours. Klein Schuster et al., (1979) and Weiner (1976) found somewhat higher recovery percentages of bovine and human lymphocytes respectively. Weiner, however, found the losses only in the cells which were frozen and not in those incubated in a DMSO containing medium for 30 minutes. The time spent in the unfrozen DMSO containing medium - in our experiments 2 hours - could also be of influence. Apart from that, centrifugation is believed to be a very crucial procedure.

Ten percent DMSO gave a better protection during freezing than 5%. This is in agreement with Weiner (1976) who found the optimal yield of viable human lymphocytes at a DMSO concentration of 7.5 - 12.5%. Our results also indicate that addition of serum, at a concentration of 25% does not result in better cryoprotection of a medium containing 10% DMSO. This is also in agreement with Weiner (1976) who observed no cryoprotective effect of serum added to human lymphocytes when DMSO concentration of 7.5% or higher were used.

The frozen lymphocytes of some animals responded more to Con A than those kept in the same medium at 4°C for two hours. A possible explanation for this observation could be a longer exposure to the toxic effect of DMSO of the cells kept at 4°C. This effect was observed by Malinin (1973) for kidney cells and by Schreck et al. (1967) for lymphocytes.

The responses of the frozen lymphocytes were comparable to those of the fresh cells. The results of the individual animals varied, also depending which value was compared, net. d.p.m. or

s.i. (Table II, Fig. 1).

Whether the losses of the cells and the lower responses to Con A observed in some animals do occur at random or selectively remains to be examined by including other mitogens in the stimulation test and carrying out a mixed lymphocyte culture (M.L.C.). Kleinschuster et al. (1979) who included 3 mitogens (Con A, P.H.A. and P.W.M.) in their test and also determined the percentages of B- and T-lymphocytes and monocytes before and after freezing did not find indications that freezing causes selective losses. Tate and Cram (1982) observed no change in the percentages of lymphocytes binding anti-IgG and of those forming spontaneous rosettes with sheep red blood cells after freezing whole blood.

Our results indicate that cryopreservation of bovine lymphocytes offers good possibilities to store these cells without loosing essential immunological capacities.

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REFERENCES

Birkeland, S.A., 1976. The influence of different freezing procedures and different cryoprotective agents on the immunological capacity of frozen-stored lymphocytes. Cryobiology 13: 442-447.

Gluckman, J.C., Dubois, M. and Leon, S., 1977. The use of cryopreserved lymphocytes for measuring cell-mediated immunity to allo-antigens in dogs by a microcytoxicity assay. Cryobiology 14: 529-533.

Grant, G.K., 1976. A simple method for the cryopreservation of lymphocytes. Retention of specific immune effector functions by frozen stored cells. Clin.exp.Immunol. 23: 166-174.

Gross, R.L., Levin, A.G., Peers, F.G., Steel, C.M. and Tsu,T., 1975. Spontaneous rosette and rosette-inhibition test on fresh and cryopreserved lymphocytes. Cryobiology 12: 455-462.

Jennings, J.J., Yanari, S.S. and Reid, R. 1978.Immune reactivity of frozen-thawed murine spleen cells. Cryobiology 15: 272-278.

Kleinschuster, S.J., Van Kampen, K.R., Spendlove, R., Athuru, D., Zupancic, M.L. and Muscaplat, C.C., 1979. Cryopreservation of bovine mononuclear leucocytes. Am.J.Vet.Res. 40: 1649-1651.

Malinin, G.L., 1973. Cytotoxic effect of dimethylsulfoxide on the ultrastructure of cultured rhesus kidney cells. Cryobiology 10: 22-32.
Schreck, R., Lloyd, M., Elrod, M. and Batra, K.V., 1967. Cytocidal effect of dimethyl sulfoxide on normal and leukemic lymphocytes. Ann. N.Y. Acad. Sci., 141: 202-213.
Tate, E.H. and Cram, L.S., 1982. A method for simultaneous isolation and cryopreservation of bovine lymphocytes, Cryobiology, 19: 136-146.
Van Dam, R.H., van Kooten, P.J.S. and van der Donk, J.A.., 1978. In vitro stimulation of goat peripheral blood lymphocytes: optimization and kinetics of the responses to mitogens and to allogenic lymphocytes. J. Immunol. Method., 21: 217-228.
Weiner, R.S., 1976. Cryopreservation of lymphocytes for use in vitro assays of cellular immunity. J.Immunol. Meth. 10: 49-60.