

Lymphocyte Chalone from Calf Thymus: Problems of Large Scale Extraction and Assay

Reinhard Maschler and H. Rainer Maurer

Pharmazeutisches Institut der Freien Universität Berlin, Königin-Luise-Straße 2–4,
D-1000 Berlin 33

Z. Naturforsch. **36 c**, 568–571 (1981); received March 4/April 24, 1981

Lymphocyte Chalone, Calf Thymus Freezing, Lymphocyte Responsiveness, Cryopreservation,
Colony Assay

Large scale extraction and assay of the lymphocyte chalone (LC), the natural lymphocyte proliferation inhibitor, require optimized, reproducible and standardizable methods. To this end we compared the effects on LC yield of storing thymus at different temperatures after collection from calves: Both quick freezing on solid CO₂ and slow freezing at –50 °C led to a 30% loss of LC yield relative to that from unfrozen tissue kept at 0 °C. Moreover we found that the apparent decrease or total loss of LC activity upon storage of a purified LC fraction may result from an occasional lack of LC-responsiveness of human blood lymphocytes depending on the donor's physiological state, in spite of normal PHA reactivity. This suggested the use of LC-responsive, cryopreserved lymphocytes, the advantages of which are documented and discussed in detail.

Introduction

We have shown [1] that calf thymus contains a factor which specifically inhibits T-lymphocyte colony growth in a reversible, non-toxic manner, thus meeting the definition [2] of a lymphocyte chalone (LC). Several reports [1, 3, 4] suggest that LC may be a low molecular weight peptide contained in lymphoid tissues in very low concentrations. In order to isolate LC on a large scale with optimal yields, which will be published, it is essential to determine and evaluate optimal extraction conditions. We have repeatedly found that LC yields may vary and that partially purified fractions with LC of high specific activity do not remain stable upon storage. This could result from the conditions of thymus processing and/or from the reproducibility of the assay system, among other possibilities. We therefore investigated the influence on LC yield of storing thymus frozen and unfrozen after collecting from calves. We also studied the reproducibility of the T-lymphocyte colony assay [5] and found the state of human donor's lymphocytes to be a major variable. The consequences of our findings will be discussed.

Abbreviations: cv, coefficient of variation; GC, granulocyte chalone; ID₅₀, inhibitory dose reducing the colony number to 50% of the control; LC, lymphocyte chalone; PEG, polyethylene-glycol; PHA, phytohemagglutinin; sem, standard error of the mean.

Reprint requests to Prof. Dr. H. R. Maurer.

0341-0382/81/0700-0568 \$ 01.00/0

Materials and Methods

Chemicals and reagents

See [1, 5, 6] for details.

Preparation of extracts

Acetone powder preparation, aqueous extraction, ultrafiltration and gel chromatography were carried out as described previously [1].

Assay systems

The T-lymphocyte colony assay and the granulocyte colony assay have been published elsewhere [1, 6, 7].

Storage of calf thymus

Fresh calf thymus was manually dissected from connective and fatty tissue and stored on ice for 6 to 8 h. Three aliquots were stored separately on water-ice, on CO₂-ice and at –50 °C for about 18 h. Frozen tissue pieces of walnut-size were thawed in saline containing 0.02% NaN₃, and homogenized at 0–4 °C using an electric blender. The homogenate was treated with acetone and processed as described previously [1]. ID₅₀-units are defined as doses of Biogel P6 pool fraction K_{av} ~ 0.7 inhibiting lymphocyte colony growth by 50% [1].



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Cryopreservation of PHA-stimulated human lymphocytes

Cryopreservation of Isopaque-Ficoll (Pharmacia) isolated and PHA-M (Difco) stimulated lymphocytes [1] (from 300–600 ml fresh human blood) using PEG 20000 as a cryoprotector was carried out in a BV4-liquid-N₂-freezer (Cryoson) with a 30 min lymphocyte program. For details see ref. 3. Cryopreserved cells were thawed at 37 °C with gentle shaking until about 1/5 volume of ice was left in the 1 ml ampoule. The cell suspension was diluted about 1:10 with Dulbecco's Eagle modified medium and washed (10 min, 400 × g, 20 °C). The pellet was dissolved in 13 ml of the same medium and washed under the same conditions a second time. The pellet was resuspended in medium giving a final concentration of 1.2×10^6 cells/ml for the assay.

The lymphocytes were immediately compared with an aliquot of fresh lymphocytes from the same preparation for their ability to react to the Biogel-P6 fraction $K_{av} \sim 0.7$. Only those lymphocytes were used for further experiments which responded sufficiently well ($\pm 15\%$) to a fully active P6-standard fraction. No loss of responding activity was seen over a period of at least 6 months [8]. The colony yield of cryopreserved lymphocytes ranged between 35 and 50 per 30 μ l agar gel.

Results

Storage of calf thymus prior to extraction

Three methods of storing fresh calf thymus prior to extraction were compared with respect to their LC yields: Storage frozen on solid CO₂, at –50 °C or unfrozen at 0 °C on ice. Table I shows the calculated yields expressed in ID₅₀-units; these were obtained from dose-response curves of the colony assays carried out in glass capillaries [1]. The assays were performed with liquid-N₂-cryopreserved lymphocytes. A loss of more than 30% of ID₅₀-inhibition was seen with both freezing methods compared to the 0 °C-method (Table I, row 2), while there was little effect on granulocyte colony growth (Table I, row 3). Considering this inhibition to be unspecific [1], one may subtract it from the LC-inhibition; this however, does not significantly change the yield row 6).

The volume yields of the preparations were 66 and 51% for the CO₂ and –50 °C method, respec-

Table I. The effect on LC yield of storing calf thymus at various temperatures before preparation of an acetone powder. LC yield is expressed as ID₅₀ units of Biogel P6 fraction at $k_{av} \sim 0.7$ [1]. The extract volume (line 7) is referred to 10 kg of fresh thymus, equivalent to 1.73 kg of acetone powder.

Yield	Storage conditions		
	CO ₂	– 50 °C	0 °C
LC-ID ₅₀ units	2835	2796	4209
in %	67.4	66.4	100
GC-ID ₅₀ units	318	180	623
in %	51.0	28.9	100
Difference (LC-GC units) in %	2517	2616	3586
	70.2	73.0	100
Extract volume in %	66.4	50.7	100
Total unit yield (LC-GC) (%)	46.6	37.0	100
Total LC unit yield (%)	44.8	33.7	100
Time factor	2	2	1

tively (Table I, row 7). The total yield after CO₂ storage was 47% and for –50 °C storage 37% as compared to the 0 °C storage (Table I, row 8). The time expended was double for both freezing experiments as compared with the 0 °C experiment. This was due to the slow ultrafiltration rates.

Responding and non-responding lymphocytes for the lymphocyte colony assay

Previously [1] we have shown that an aqueous extract from calf thymus acetone powder yields, after partial purification upon Biogel P6 chromatography, a fraction at $K_{av} \sim 0.7$ which contains LC activity (Fig. 1). This fraction, when tested after various periods (weeks) and storage at 4 °C, was found to lose its inhibitory activity to different degrees. However, we also found an increase of activity upon storage. First we attributed this change to physicochemical influences of the storage conditions (oxidation, adsorption etc.) to which the fraction was exposed. However, we recently discovered that the reactivity of the lymphocyte assay to LC may greatly vary according to blood-batch, suggesting that this latter variation is more likely to be the cause for the activity change, rather than any chemical alteration of the LC fraction. Thus, colony growth of the lymphocyte of a 29 years-old man was only partially inhibited by fraction 0.7 (Fig. 1), although these fresh lymphocytes showed a normal

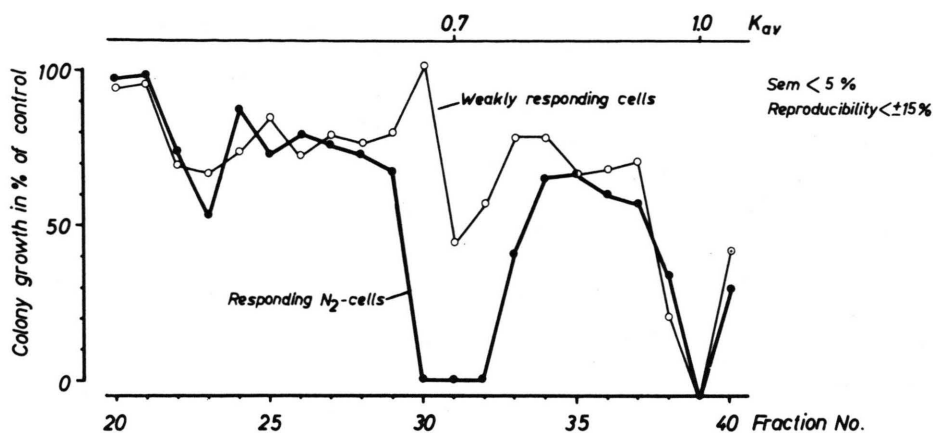


Fig. 1. Testing of Biogel P6 chromatography fractions of a calf thymus extract using weakly responding, fresh human lymphocytes (open circles) and fully responding, cryopreserved human lymphocytes (closed circles) in the colony assay. Controls: Weakly responding cells 52 ± 1 , fully responding cells 43 ± 2 colonies/capillary. Statistics: Sem < 5%, reproducibility by cv < $\pm 15\%$.

PHA stimulation rate (52 ± 1 colonies/capillary = 100%). Three weeks after this assay, the fresh lymphocytes from the same donor responded by 100% inhibition to a corresponding fraction which had been stored meanwhile at 4°C . Fig. 2, presenting dose-response curves of fraction 0.7, emphasizes this phenomenon on the basis of 3 independent experiments for each curve (sem < 4%, cv < $\pm 15\%$). We concluded that lymphocytes can be obtained from healthy donors which either will, will not or will only

partially respond to LC in spite of full PHA reactivity.

It should be noted that any toxic factors such as NaN_3 or salts do inhibit colony growth of LC responding and non-responding lymphocytes equally well (Fig. 1, fraction 39 $\cong k_{av} = 1.0$). The great variation in LC response of fresh, normal lymphocytes suggested some method of standardization of the LC colony assay. Therefore, lymphocytes from healthy blood bank donors were PHA stimulated, frozen by liquid nitrogen in the presence of PEG 20000 and thawed [8]. Samples of the same lymphocytes were tested before and after cryopreservation for LC response in the T-lymphocyte colony assay. Only those lymphocytes were (and are now) taken for further LC testing which fully responded to a pre-tested batch of fraction 0.7. Figs. 1 and 2 demonstrate the effects of fully responding N_2 -cryo-preserved lymphocytes which are equal to those of fully responding, fresh lymphocytes.

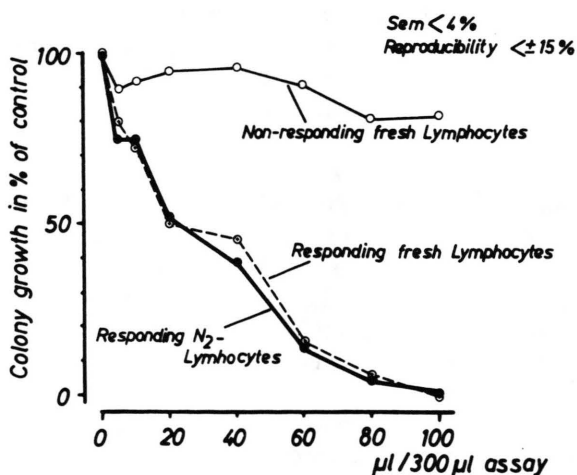


Fig. 2. Dose-response curves of Biogel P6 fraction $k_{av} = 0.7$ using non-responding, fresh human lymphocytes (control: 46 colonies), responding, fresh human lymphocytes (control: 39 colonies) and responding, cryopreserved human lymphocytes (control 44 colonies/capillary). Statistics: Sem < 4%, reproducibility by cv < $\pm 15\%$.

Discussion

The chemical identification of LC requires large scale extraction procedures which must be optimized. Hence we studied the effects on LC yield of storing calf thymus at different temperatures after recovery in the abattoir. Our data showed that fresh calf thymus kept in water ice gives the best yield. However, since 100 kg quantities of thymus could not be collected within a few h, freezing was in-

avoidable. Both quick freezing of thymus pieces on CO₂-ice and slow freezing at -50 °C produced a loss of about 30% in LC yield relative to unfrozen material (Table I). No significant difference between the two freezing procedures was noted; thus CO₂ itself does not affect LC activity chemically. The 30% loss may be acceptable for practical and economic reasons. We found the extraction of frozen thymus pieces with acetone preferable; thawed pieces contained fibrous material which was difficult to manipulate. When acetone powder from frozen thymus extracted with aqueous solutions, a finely-dispersed homogenate was obtained which required twice as much time for ultra-filtration as compared with an extract from unfrozen thymus.

The LC yield was expressed as the ID₅₀ units recovered with a Biogel P6 fraction at $K_{av} \sim 0.7$ containing the required LC specificity [1]. However unspecific inhibitor activity was always found as revealed by the granulocyte colony assay; this was substrated from the LC yield to determine the LC specific activity. Ultrafiltrate YM10F-UM2R with molecules between 10000 and 1000 d contained in many cases high quantities of non-specific inhibitors so that Biogel chromatography proved necessary to demonstrate LC specificity.

Thus, freezing of calf thymus for storage seemed possible at the expense of about 30% loss of LC yield; total loss of LC activity could not be due to the freezing process, however.

A partial or total lack of response of a healthy donor's lymphocytes to a known LC preparation does occur in only about 5% of all cases tested. Thus, normally we tend to have confidence in the validity of the assay system, particularly when the PHA reactivity is normal. However, lymphocytes may not respond on LC as demonstrated in Figs. 1 and 2, for reasons which we cannot yet explain satisfactorily. It

may be that subclinical infections or stress phenomena play a role which do not affect the PHA reactivity, thus questioning this reaction as to its clinical significance. The LC insensitivity at normal PHA-stimulation rates and its reaction to toxic compounds such as NaN₃ (Fig. 1, $k_{av} = 1.0$) suggested that there exists a specific interaction between LC (or subsequent reaction products) and some factor(s) of the donor's lymphocytes or plasma.

In consequence, the use of a standardized lymphocyte population kept in large quantities for repeated testing seemed essential. Lymphocytes from blood bank donors were cryopreserved in liquid nitrogen after PHA stimulation, thus avoiding any variability and saving time for assaying. Instead of using DMSO, a common cryoprotector, we preferred PEG (of 20–40 000 d at 7.5–10% w/v) because it provided a good stimulation index [8] and did not affect the LC response. However, it is clear that each batch of lymphocytes to be cryopreserved for large scale assaying must be tested before freezing and after thawing with the particular test substance. Such cryopreserved cells are very useful when shown to be responsive to a known substance. They are, however, of little use for the purpose of screening unknown factors (or new drugs), since the cells may not react to the latter. As the cryopreserved lymphocytes were found to be active after storage up to at least 6 months, the advantages of the cryopreservation technique are self evident.

Acknowledgements

We thank Mrs. Waltraud Metzger, Miss Jutta Rath, and Mr. Christoph Dietrich for excellent technical assistance. We are indebted to the Bundesminister für Forschung und Technologie for generous support.

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