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A new micromethod for determination of interleukin-1 production from frozen human blood mononuclear cells

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A microtechnique is described for inducing IL-1 activity in vitro from frozen human blood mononuclear cells (BMNC) using the lymphocyte-activating factor (LAF) method. When compared to the conventional culture system, this micromethod offers several advantages: (1) the complete assay requires a total of 1.0×10^5 mononuclear cells which is easily obtained from 1 ml of whole blood; (2) the method of isolation and freezing of cells preserves their viability and ability to produce IL-1 for several weeks; (3) when compared to the conventional test using fresh BMNC, frozen cells produce the same amount of IL-1. The microtechnique described here is highly reproducible and provides a useful tool for evaluating the production of IL-1 from BMNC of human subjects in clinical studies including those with immunodeficiencies, malnutrition and malignant diseases.

Key words: Interleukin-1; Micromethod; Lipopolysaccharide; Lymphocyte-activating factor; Mononuclear cell, frozen

Introduction

Interleukin-1 (IL-1) is a multifunctional cytokine which stimulates T and B lymphocytes and mediates many components of the acute-phase response (Dinarello, 1984). Mononuclear phagocytes are a primary source of IL-1 and these cells can be stimulated by a variety of agents including microorganisms, microbial products, inflammatory agents, plant lectins and antigens. Endotoxin (or lipopolysaccharide, LPS) from Gram-negative bacteria appears to be the most potent soluble inducer of this cytokine.

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IL-1 can be assayed by several different assay systems which measure IL-1 functional activity (Dinarello, 1984). When assaying its ability to promote the proliferation of mouse thymocytes, IL-1 has been known as lymphocyte-activating factor (LAF) (Gery et al., 1972). This test is the simplest method for estimating IL-1 activity from isolated blood mononuclear cells (BMNC) and is routinely used in many laboratories for determining IL-1 activity. The LAF assay is also specific for IL-1 in that another cytokine, tumor necrosis factor (or cachectin) which shares many biological properties with IL-1 has no effect in the LAF assay (Dinarello and Krueger, 1986). We have developed a new microtechnique for the production of IL-1 in vitro requiring a small (1×10^5) concentration of frozen BMNC cells.

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Materials and methods

Isolation and freezing of BMNC

Venous blood was obtained with preservative-free heparin as an anticoagulant (10 U/ml; Elkins-Sinn, Cherry Hill, NJ) from randomly selected healthy donors. Blood mononuclear cells were isolated using the Ficoll-Hypaque gradient (Böyum, 1968). The cell interphase was aspirated, washed three times in 0.9% NaCl and resuspended in minimal essential medium (MEM; M.A. Bioproducts, Walkersville, MD) containing antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cell suspensions were counted and adjusted to various final concentrations. After centrifugation the cells were resuspended in heat-inactivated fetal calf serum (FCS; Hyclone Laboratory, Logan, UT) containing 5% dimethyl sulfoxide (DMSO; A.R. Mallinckrodt, Paris, KY) and stored in 1 ml aliquots at $-70\,^{\circ}$C for 1 month.

Production of IL-1 in vitro

The BMNC were thawed in a water bath at 37 °C, washed twice in 30 ml of MEM and resuspended in complete MEM (containing antibiotics, 2 mM L-glutamine and 0.01 M Hepes buffer) supplemented with 1% heat-inactivated (56 °C × 30 min) human AB serum. As above, cell counts and the number of recovered viable cells after freezing were determined. 100 μl of this mononuclear cell suspension was dispensed to each well of a 96-well microtiter place (Nunc, Roskilde, Denmark) and mixed with 100 μl of MEM containing different LPS concentrations. Suspensions of unstimulated cells (control culture) received the same volume of MEM without LPS. After incubation at 37 °C in 5% CO_2 for 24 h, the total IL-1 was obtained by three freeze-thaw cycles, and the supernatant medium was measured in the LAF assay.

LAF assay

Total IL-1 was measured as follows. Mononuclear cell supernate was diluted 1 : 5 in complete RPMI 1640 supplemented with 5% heat-inactivated FCS and 100 μl were dispensed in triplicate into wells of a 96-well microtiter plate. D10.G4.1 cells, a cloned murine T-cell line (Kaye et al., 1984) was split 1 : 4 and used between day 7 and 14 of culture. The concentration of cells was adjusted to 2×10^5/ml in RPMI 1640/5% FCS containing PHA (Burroughs Wellcome Co., Research Triangle Park, NC) at a concentration of 2 μg/ml. 100 μl of the T cell/mitogen suspension were added to each well and microcultures were incubated for 72 h at 37 °C in a humidified atmosphere with 5% CO_2. 24 h prior to harvesting, each culture was pulsed with 1 μCi of tritiated thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA). Lysed cellular material was collected on glass fiber filter paper (934-AH, Whatman) with a Mash cell harvester (Cambridge Technology, Cambridge, MA) and placed in organic solvent (Ready-solv, Beckman Instruments, Fullerton, CA). Incorporated radioactivity was determined with a liquid scintillation spectrometer. Results are expressed as the mean ($\pm$SD) of triplicate counts per minute (cpm).

Results

A systematic study was undertaken to define the optimal culture conditions for IL-1 production from frozen cells.

Recovery of viable mononuclear cells

Three different concentrations of BMNC from five normal subjects were studied (Table I). Recovery of viable cells after thawing ranged between 44 and 55% of the original number. DMSO and storage at $-70\,^{\circ}$C efficiently preserved the cells during 1 month, since viability of the thawed cells was over 96% as determined by trypan blue dye-exclusion test. Most of the cells retained their typical morphology.

Influence of cellular concentration on IL-1 production

To determine the minimal cell density needed for adequate IL-1 response to LPS stimulation when frozen cells were thawed, different concentrations of frozen and fresh cells from one subject were tested (Fig. 1). The cell suspensions were varied from 0.1 to 10.0×10^4 cells/microtiter well and all microcultures were stimulated with 1.0 ng/ml of LPS. 5.0×10^4 cells/well was used

TABLE I

RECOVERY OF BMNC AFTER FREEZING

BMNC were mixed with FCS-DMSO and stored in 1 ml aliquots at −70°C for 1 month. The cells were thawed at 37°C, washed twice, counted and their viability determined using exclusion of trypan blue.

Before freezing [a]	After freezing [a]						Efficiency (%) of cell recovery [b]
	A	B	C	D	E	Mean ± SEM	
5.0	2.9	2.8	2.6	2.5	3.0	2.76 ± 0.09	55
1.0	0.5	0.5	0.4	0.4	0.5	0.46 ± 0.02	46
0.5	0.3	0.2	0.2	0.2	0.2	0.22 ± 0.02	44

[a] Expressed as BMNC $\times 10^6$/ml.
[b] Mean values of viable cells from normal donors (A–E).

in control cultures. IL-1 production was highest when 5.0×10^4 cells/well were used for either frozen and fresh cells.

Dose-response curve to LPS

Fig. 2 shows the production of IL-1 from frozen and fresh BMNC (1×10^4 cells/well) from one donor to four concentrations of LPS (0.01–10.0 ng/ml). IL-1 production was readily detected at all doses and no difference was observed between fresh and frozen cells. As shown in Fig. 2, a concentration as low as 0.01 ng/ml of LPS was sufficient to induce IL-1 production from frozen BMNC with a response approximately five times higher than the respective control culture.

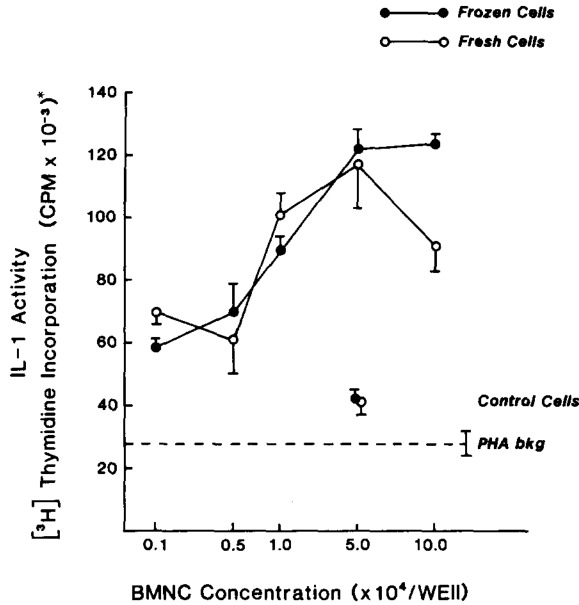

Fig. 1. Comparison of IL-1 production from frozen vs. fresh BMNC. Density of 5.0×10^4 cells/well was used in control culture and all microcultures were stimulated with 1.0 ng of LPS/ml. * Mean ±SD of triplicate of microcultures. The PHA background (PHA bkg) represents [^{3}H]thymidine incorporation into mouse D10.G4.1 cells supplied only with PHA. BMNC incubated alone (control cells) produced low background levels of IL-1.

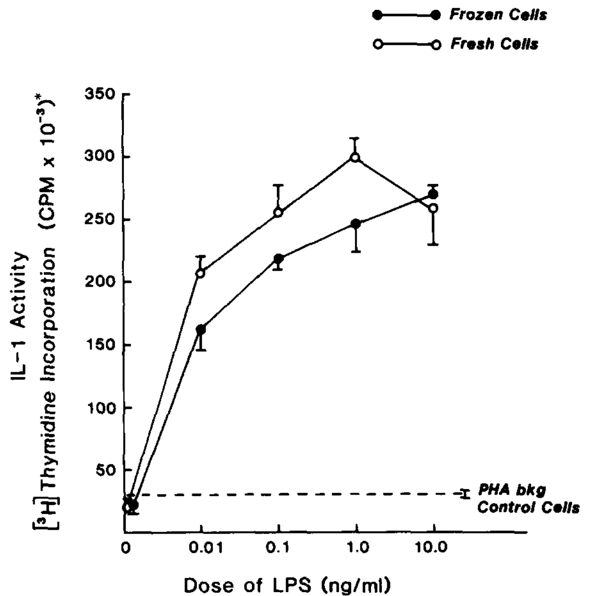

Fig. 2. Dose-response of LPS on IL-1 production from frozen BMNC: comparison with fresh cells. * Mean ±SD of triplicate of microcultures. The PHA background (PHA bkg) represents [^{3}H]thymidine incorporation into mouse D10.G4.1 cells supplied only with PHA. BMNC incubated alone (control cells) produced low background levels of IL-1.

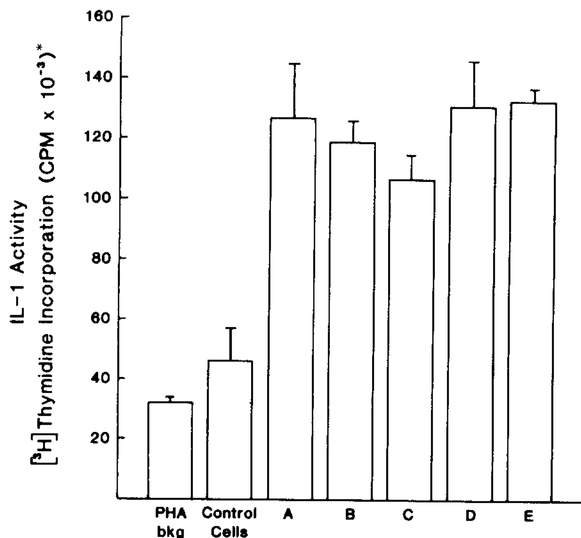

Fig. 3. Reproducibility of the microtechnique. 1.0×10^4 frozen-thawed BMNC/well from five healthy donors (A–E) were stimulated with 1.0 ng of LPS/ml. Unstimulated cells from subject C were used as control (unstimulated) culture. * Mean ± SD of triplicates. The PHA background (PHA bkg) represents [^{3}H]thymidine incorporation into mouse D10.G4.1 cells supplied only with PHA. BMNC incubated alone (control cells) produced low background levels of IL-1.

Intersubject variability of the micromethod

To establish the variability of the microtechnique among different subjects, five donors were evaluated for reactivity to LPS using 1.0×10^4 frozen BMNC/well with 1.0 ng/ml of LPS. As shown in Fig. 3, unstimulated control cells (from donor C) did not produce IL-1. However, IL-1 production was similar from stimulated cells of all subjects (A–E), and was approximately four times higher than the control microculture. In all the studies unstimulated frozen cells did not produce IL-1.

Discussion

The polypeptide IL-1 plays a critical role in immunoregulation, and studies of in vitro production of IL-1 have received a great deal of attention in recent years. It is generally agreed that the LAF assay, a measurement of the proliferative response of mouse thymocytes or D10.G4.1 cells to suboptimal concentrations of mitogens, is the most specific and sensitive assay for IL-1 (Dinarello, 1986). Measurement of IL-1 production in vitro offers an opportunity to evaluate responses to infection, injury and several immunological reactions (Dinarello, 1984). The peripheral blood monocytes are probably the most important and potent source of IL-1, although B and natural killer cells from the peripheral blood also produce IL-1 (Matsushima et al., 1985; Scala et al., 1984). By means of study described here, we have demonstrated the utility of frozen BMNC in a simple, sensitive and reproducible microtechnique for evaluating IL-1 production. The main difference between this microtechnique and the conventional method is the number of post-freezing cells needed for an acceptable response.

IL-1 production by BMNC stimulated with LPS or other substance has only been reported using freshly obtained cells. The value of a simple and rapid micromethod requiring small number of cells is obvious, as cells can be obtained, frozen, and stored at −70°C with maintenance of normal function until they can be processed at the laboratory at a later time. In addition, the small number of cells needed permits studies in human subjects in whom it is not possible to obtain large blood volumes.

This microtechnique can be used to evaluated IL-1 production from a total of 1.0×10^5 frozen BMNC, which are easily obtained from 1 ml of whole heparinized blood. Thus, even young children may be studied, as well as infected and/or malnourished subjects and patients with immunodeficiencies and/or malignancies.

One application of this method may lie in the preparation of cultures after collecting blood from several subjects at the same time. The isolation and freezing of cells would allow experiments to be set up at a later time, perhaps when additional information was available. Another application might be in field studies, since samples can be easily transported on dry ice and their viability preserved in order to determine IL-1 production at a central laboratory.

LPS stimulation of human BMNC results in increased mRNA and large amounts of intracellular IL-1 (Auron et al., 1984; Lepe-Zuniga and Gery, 1985). Therefore, we measured total IL-1 (intracellar plus extracellular). The concentration

of 5.0×10^4 frozen cells/well and 1.0 ng/ml of LPS were considered optimal; however, even a cellular concentration ten times lower (0.5×10^4 cells/well) stimulated with the same LPS dose showed significant IL-1 levels when compared with control cells. The unstimulated cells did not show spontaneous stimulation after mixing with FCS-DMSO and storage at $-70\,^{\circ}C$ for 1 month and therefore these procedures did not falsely induce IL-1.

In late 1984, two forms of IL-1 had been cloned and the complete amino acid sequences, derived from the cDNAs, were described (Auron et al., 1984; Lomedico et al., 1984). These forms are called beta and alpha in human blood monocytes and in mouse macrophage cells line P388D, respectively. In the present studies concentrations of recombinant human IL-1 beta varying from 3.75 to 375 pg/ml (Dinarello et al., 1986a) were used as positive controls in the LAF assay. LPS-stimulated frozen cells produced comparable amounts, that is approximately 30 pg of IL-1 beta/10^4 cells.

Our results agree with those reported by other investigators (Duff and Atkins, 1982; Dinarello and Krueger, 1986), since cells after freezing responded to LPS concentration as low as 50 pg/ml. We suggest the routine use of three different concentrations of stimulant, rather than a single optimal dose, to detect possible abnormalities of IL-1 production.

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