Enzyme-linked immunosorbent assay for measuring serum IgE

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Summary. Serum IgE was measured by a sandwich method using polystyrene beads coated with anti-IgE γ-globulin and peroxidase labelled anti-IgE γ-globulin. The method was simple, and as sensitive as the radioimmunoassay.

Enzyme-linked immunosorbent assays (ELISA) are being applied widely in the medical field. We have developed an ELISA method for measuring the serum IgE level as described below.

Serum obtained from a patient with IgE myeloma (PS) was kindly supplied by Dr K. Ishizaka. IgE was purified from the serum using DEAE cellulose column chromatography followed by gel filtration. After elution of IgG with 0.005 M phosphate buffer, pH 8.0, from a DEAE cellulose column, E myeloma protein was eluted with 0.025 M phosphate buffer, pH 8.0. The solution was applied to a Sephadex G-200 column and eluted with borate buffered saline, pH 8.0. E myeloma protein was digested with papain to obtain an Fc fragment. The Fc fragment gave a single precipitin band with anti-IgE supplied by Ishizaka. A rabbit was immunized every 2 weeks with 1 mg of the IgE-Fc fragment in complete Freund's adjuvant and was bled 7 days after being immunized 5 times. This rabbit antiserum was absorbed on a Sepharose 4B column coupled with normal IgG, and showed a single precipitin line with the myeloma protein in immunoelectrophoresis. Rabbit γ-globulin fraction was obtained from this antiserum by the duplicate sodium sulfate precipitation method, first at 18% and then at 12%. Polystyrene beads used in an ELISA for insulin[2] in our laboratory were sensitized with 10 µg/ml of the previously obtained anti-IgE class-specific γ-globulin in 0.076 M, pH 6.4 phosphate buffered saline for 1 h at 37 °C by the method of Voller[3]. Horseradish peroxidase was coupled with the same anti-IgE γ-globulin as a second antibody by the method of Nakane[4]. The coupling ratio of globulin/enzyme in the conjugate used was about 1:2 in moles.

A volume of 0.025 ml of the serum sample being tested, and a standard IgE solution, were added to 0.5 ml of phosphate buffered saline containing 30% rabbit serum. An anti-IgE coated bead was added to each assay tube, and the tube was shaken thoroughly and incubated for 3 h at room temperature, followed by washing 3 times with 5 ml of saline containing Tween 20. Then 0.5 ml of peroxidase-labelled anti-IgE γ-globulin was added to each tube and incubated at 4.0 °C for 16 h. The experimental procedures thereafter were the same as in the ELISA for insulin[2].

The minimum and maximum concentrations of IgE measurable by ELISA were 6.25 and 1600 units/ml, respectively. The intraassay coefficient of variation was 6.2% at about 100 units/ml (n = 5) and 3.5% at about 400 units of IgE/ml (n = 5). The curve obtained from various serum dilutions ran parallel with a standard curve.

38 sera from normal subjects and atopic patients were tested with ELISA and with a commercially available radioimmunoassay (RIA) kit for IgE (Pharmacia). The correlation coefficient between the values measured by ELISA(Y) and RIA(X) was the regression equation $Y = 0.992 \times - 4.71$ ($\gamma = 0.990$). In summary, this ELISA is simple, and as sensitive an assay system as the radioimmunoassay.

ELISA methods for IgE have been reported by Hoffmann[5], Guesdon et al.[6], and Weltman et al.[7]. The first is a competitive immunoassay and requires a large amount of IgE for testing. A sandwich method similar to ours using a cellulose disc or single radial diffusion is employed in the latter two. They use glutaraldehyde for coupling. As compared with theirs, our procedure is simpler, and its sensitivity is higher.

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2 M. Yoshioka, H. Taniguchi, A. Kawaguchi, T. Kobayashi, K. Murakami, M. Seki, A. Tsutou, M. Tamagawa, H. Minoda and S. Baba, Clin. Chem. *25*, 35 (1979).

3 A. Voller, D.E. Bidwell and A. Bartlett in: Protides of the Biological Fluids vol. 24, p. 751. Ed. H. Peeters. Pergamon Press, Oxford 1976.

4 P.K. Nakane and A. Kawaoi, J. Histochem. Cytochem. *22*, 1084 (1974).

5 D.R. Hoffman, J. Allergy clin. Immun. 51, *303* (1973).

6 J.L. Guesdon, R. Thierry and S. Avrameas, Clin. exp. Immun. *25*, 180 (1976).

7 J.K. Weltman, A.R. Frackelton, R.P. Szaro and B. Rotman, J. Allergy clin. Immun. *58*, 426 (1976).

Cryoprotection of human bone marrow committed stem cells (CFU-c) by dextran, glycerol and dimethyl sulfoxide[1]

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Summary. Dextran, glycerol and dimethyl sulfoxide (DMSO), alone or in combination, were used for cryoprotection of human bone marrow cells. The viability of cryopreserved cells was assessed by culture of myelopoiesis-committed stem cells (CFU-c) in vitro. A significantly better protection against freezing injury was obtained by 9% dextran in combination with 3 or 5% DMSO, and also with 5 or 10% DMSO alone, than with either 15% glycerol or 9% dextran with 1% DMSO.

Modern antimitotic chemo- and radiotherapy of patients with malignant tumours frequently causes a life-threatening bone marrow aplasia. The haemopoietic depression can be successfully reversed by an infusion of cryopreserved autologous bone marrow cells[2,3]. According to experimental evidence, obtained on animals exposed to supralethal doses of irradiation, the repopulation of the aplastic marrow and functional recovery are positively correlated with

the quantity and viability of the infused peripheral blood or bone marrow nucleated cells[4–8].

The viabiltiy of cryopreserved haematopoietic progenitor cells and their proliferative potential can be assessed by several in vivo and in vitro assays[9–15]. We have compared the effects of several cryoprotectors on human myelopoiesis-committed stem cells (CFU-c) by the application of a clonal CFU-c assay in agar plate cultures.

Ashwood-Smith[16] has shown that the addition of dextran to DMSO and glycerol improves the cryoprotection of Chinese hamster ovary cells. In a preliminary communication we compared[17] the recoveries of human bone marrow cells and CFU-c after freezing in the presence of glycerol or DMSO alone or in combination with 2 extracellular cryoprotectors (dextran and hydroxy ethyl starch). Since DMSO, apart from its unpleasant odour, causes local pain at the site of i.v. application and exerts various toxic effects on living cells[16,18–20], a reduction of its concentration in cryopreserved cell suspensions by the addition of dextran seems to be desirable. Therefore, we extended our previous experiments by testing combinations of 9% dextran with 1, 3 and 5% DMSO, and compared their cryoprotective effects with those of 5 and 10% DMSO, as well as with 15% glycerol.

Materials and methods. Aspiration of bone marrow and separation of cells have been carried out as described previously[21,22]. Samples from 11 haemotologically normal individuals (usually patients undergoing neurosurgery) were collected after obtaining informed consent. The light density (<1.077 g/ml) fraction of human bone marrow cells was used for the culture experiments.

Cryoprotectors and freezing procedure. After addition of an equal volume of double-concentrated cryoprotector in Hank's solution to the separated bone marrow cells, the suspension was mixed thoroughly, and aliquots dispensed into glass ampoules which were sealed. The following cryoprotectors were used:

Dimethyl sulfoxide puriss., $d_{20}=1.10$ (Serva, Heidelberg); Glycerol 85%, Ph.H. VI/Eur., $d_{20}=1.225$ (Steinfels, Zurich); Dextran, M.W. 70.000 (Fisons Ltd., Loughborough).

The concentration of cryoprotector used in the experiments was as follows: DMSO alone (5 and 10%), glycerol alone (15%) and combinations of dextran (9%) with 1, 3 and 5% DMSO. The freezing of cells was carried out following a well established procedure[22], and the ampoules were stored in liquid nitrogen.

Standardized CFU-c assay. 3 concentrations of mononuclear bone marrow cells (2.5, 5.0 and 7.5×10^4 cells/ml), immobilized in 0.25% agar, were used in all experiments. Human placenta conditioned medium[23] and feeder layers containing 2×10^6 cryopreserved and irradiated mononuclear cells from peripheral blood of healthy human donors[22] were used seperately as a source of stimulation. Aggregates containing more than 50 cells were counted as colonies on the 12th day of incubation. The cultures were incubated at 37 °C in a fully humidified atmosphere containing 3% CO_2.

Determination of the post-freezing recovery of CFU-c. Mononuclear cells separated from human bone marrow, washed and resuspended in McCoy 5A modified medium containing 20% fetal calf serum, were divided into several aliquots. Cryoprotectors were added to the experimental samples which were frozen, and stored in liquid nitrogen for at least several h. In the meantime the controls, containing no cryoprotector but an equivalent volume of medium and fetal calf serum instead, were diluted 5-fold[24] and plated. The frozen samples were thawed, and treated in the same way as the controls.

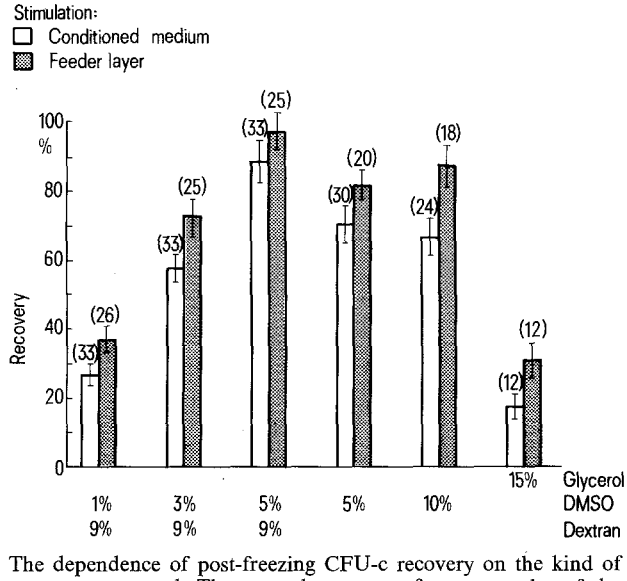
In the first few experiments additional non-frozen controls for each cryoprotector were run in parallel to the frozen probes. Since no significant difference was found in the CFU-c recovery after this short incubation at room temperature, these controls were omitted.

The number of colonies obtained by the plating of cells after freezing was compared to the colony count of the respective nonfrozen control taken as a reference (100%). All the platings were done in duplicate or triplicate.

The recoveries of CFU-c after freezing with the various cryoprotectors or combinations of cryoprotectors were tested with up to 11 samples of bone marrow cells. Glycerol had been tested in a previous study, and poor recoveries of CFU-c (10–20%) had been found[17]. Therefore, in the present study, the cryoprotective effects of glycerol were tested on 4 bone marrow samples only.

Results and discussion. The results of CFU-c recovery obtained with 1 cryoprotector and a single kind of stimulation were calculated as mean values from 24–99 results (duplicates or triplicates from 4–11 samples of bone marrow cells in 3 different cell concentrations), and are separately presented in the figure. Feeder layers provided a better stimulation than conditioned medium in all experiments. This is in agreeement with the results of our previous study[22].

The post-freezing recovery of CFU-c, measured by its clonal proliferation in vitro, depends on many factors. The presence of mature granulocytes, platelets, fibrinogen and calcium ions contribute to cell aggregation ('clumping') before, and particularly after freezing. Therefore, bone marrow cells were subjected to different cell separation procedures[25–28], plasma was replaced by homologous AB serum, and ACD solution was added to the dilution fluid to remove calcium ions[27]. The selection of an appropriate cryoprotector is, however, by far the most important factor for successful preservation of bone marrow cells by freezing. The 2 most often used cryoprotectors, glycerol and DMSO, have been shown to differ considerably in their cryoprotective effectiveness[16,17,29–31].



The dependence of post-freezing CFU-c recovery on the kind of cryoprotector used. The controls were nonfrozen samples of the corresponding bone marrows ($n=4-11$) containing no cryoprotector. The assay was carried out on 3 concentrations of target cells (2.5, 5.0 and 7.5×10^4 cells/ml) and the results were pooled (number of experiments in brackets). The columns represent mean values of all determinations done with 1 stimulus (bars indicating ±1 SD. All readings done in duplicate or triplicate).

All experiments in this study were carried out on 3 concentrations of bone marrow cells in order to remain in the optimal range of colony counts[32]. The chosen cell concentrations were on the linear part of the curve which expresses the relationship between the amount of plated cells and the resulting counts of colonies.

The plating of the same cell concentration before and after freezing often results in higher colony counts after freezing. The increase of numbers of CFU-c's after cryopreservation could be explained by a higher resistance of lymphocytes and stem cells against cryogenic injury. This can consequently lead to an enrichment in stem cells of the bone marrow samples after freezing. Therefore, in this study the cells were diluted in an identical way before and after freezing, and the same volume of cell suspension was used for the CFU-c assay.

It can be concluded from the data in the figure that the addition of dextran significantly ($p < 0.01$) improved the recovery provided by 5% DMSO alone. With this combination the results were at least as good as with 10% DMSO. This means that the concentration of DMSO can safely be reduced to 5%, i.e. to a lower level of toxicity. However, a further reduction of the concentration of DMSO is followed by a linear decrease of the recovery, in spite of the addition of dextran. Therefore, the combination of 5% DMSO with 9% dextran seems to be the optimum cryoprotector.

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2 P.R. Graze and R.P. Gale, Transpl. Proc. *10,* 177 (1978).
3 A.B. Deisseroth and R.A. Abrams, Cancer Treatment Rep. *63,* 461 (1979).
4 W. Calvo, T.M. Fliedner, E. Herbst, E. Hügl and C. Bruch, Blood *47,* 593 (1976).
5 W. Nothdurft, C. Bruch, T.M. Fliedner and E. Rüber, Scand. J. Haemat. *19,* 470 (1977).
6 B.H. Peterson, Scand. J. Haemat. *18,* 235 (1977).
7 N.C. Gorin, G. Herzig, M.I. Bull and R.G. Graw, Blood *51,* 257 (1978).
8 F.R. Appelbaum, G.P. Herzig, R.G. Graw, M.I. Bull, C. Bowles, N.C. Gorin and A.B. Deisseroth, Transplantation *26,* 245 (1978).
9 J.E. Till and E.A. McCulloch, Radiation Res. *14,* 213 (1961).
10 T.R. Bradley and D. Metcalf, Aust. J. exp. Biol. med. Sci. *44,* 287 (1966).
11 B.L. Pike and W.A. Robinson, J. Cell Physiol. *76,* 77 (1970).
12 R.G. Worton, E.A. McCulloch and J.E. Till, J. Cell Physiol. *74,* 171 (1969).
13 J.R. Stephenson, A.A. Axelrad, D.C. McLeod and M.M. Shreeve, Proc. natl Acad. Sci. *68,* 1542 (1971).
14 H.B. Benestad, Scand. J. Haemat. *7,* 279 (1970).
15 D.W. Golde and M.J. Cline, Blood *41,* 45 (1973).
16 M.J. Ashwood-Smith, Ann. N.Y. Acad. Sci. *243,* 246 (1975).
17 M. Bettler, R. Odavic, K.A. Deubelbeiss and U. Bucher, Schweiz. med. Wschr. *107,* 1459 (1977).
18 N.A. David, A. Rev. Pharmac. *12,* 353 (1972).
19 N. Schoenfeld, O. Epstein, L. Nemesh and A. Atsmon, Israel J. med. Sci. *13,* 466 (1977).
20 E.G. Bamberger, M.C. Aggio, C.B. Lozzio and B.B. Lozzio, Leukemia Res. *2,* 305 (1978).
21 We are indebted to physicians from the Department of Neurosurgery at our hospital for their kind co-operation and to the numerous patients who readily accepted to donate bone marrow.
22 R. Odavic, J. Blom and E.A. Beck, Acta haemat. *61,* 243 (1979).
23 A.W. Burgess, E. Wilson and D. Metcalf, Blood *49,* 573 (1977).
24 U.W. Schaefer and K.A. Dicke, in: La cryoconservation des cellules normales et néoplasiques, p. 63. Ed. R.S. Weiner, R.K. Oldham and L. Schwarzenberg. Colloque Inserm, Villejuif 1973.
25 J.R. Wells, W.G. Ho, P. Graze, A. Sullivan, R.P. Gale and M.J. Cline, Exp. Hemat. *7,* Suppl. 5, 12 (1979).
26 G. Spitzer, D.S. Verma, A. Zander, K.B. McCredie and K.A. Dicke, Blood *52,* Suppl. 1, Abs. 474, 232 (1978).
27 R.S. Weiner, J.S. Tobias and R.A. Yankee, Biomedicine *24,* 226 (1976).
28 J.R. Wells, A. Sullivan and M.J. Cline, Cryobiology *16,* 201 (1979).
29 M.J. Ashwood-Smith, Ann. N.Y. Acad. Sci. *141,* 45 (1967).
30 A.H. Ragab, E. Gilkerson and S.C. Choi, Cancer Res. *34,* 942 (1974).
31 M. Harada, C. Ishino and K. Hattori, Transplantation *25,* 204 (1978).
32 R. Odavic, J. Blom, C.M.H. Harrisson and R. Mini, manuscript submitted for publication.

Early B lymphocytes in man[1]

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Summary. We have confirmed in man the presence of a subpopulation of B lymphocytes which cannot reexpress their immunological receptors after challenge with antibodies. These early B lymphocytes were studied in peripheral blood, in cord blood and also, with anti-idiotypic sera, in the peripheral blood of a myeloma patient.

The reported existence of a subpopulation of mouse B lymphocytes unable to re-express surface immunoglobulins (Ig) after antigenic or antibody challenge in vivo and/or in vitro[2-4] tends to support the clonal abortion theory of B lymphocyte tolerance[5,6]. This theory postulates that, during maturation, B lymphocytes proceed through an early stage in which they are susceptible to inactivation mediated by the ligand antigen (or antibody) -surface receptor. Such inactivation could play an important role in self-tolerance in adults and neonates.

Recently, Ault and Unanue[7] have demonstrated that human IgM and IgD bearing B blood lymphocytes display a similar pattern of defective receptor resynthesis in vitro after antibody challenge, in contrast to human spleen and tonsil B cells. Human blood lymphocytes have therefore been considered to be relatively immature cells.

In this communication we present data concerning the in vitro biosynthetic capacity of human adult and cord blood lymphocytes after induction of capping-endocytosis of surface receptors by anti-Ig sera and 24 h of culture[8]. In both cases, the biosynthetic capacity decreased on passing from IgG/IgA to IgM/IgD bearing cells. Moreover, adult lymphocytes displayed a much greater resynthetic ability. This lends further support to the view that such a phenomenon is due to a subpopulation of early B lymphocytes. Furthermore, the existence of such a population was looked for