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FRACTIONATION OF BONE-MARROW CELLS BY COUNTER-CURRENT DISTRIBUTION IN AQUEOUS POLYMER TWO-PHASE SYSTEMS

RELATION BETWEEN SETTLING TIME AND THE EFFICIENCY OF SEPARATION

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SUMMARY

The fractionation of heterogeneous populations of rat and human bone-marrow cells has been studied by counter-current distribution in a charged 5% dextran—4% poly(ethylene glycol) two-phase system. The subfractionation into two broad populations has been achieved at a low top/bottom phase volume ratio by increasing (up to 20 min) the settling time allowed for the phases to be separated after each mixing step. No effect of this parameter on a homogeneous population of erythrocytes has been observed. However, hetereogeneous cell separations can be improved by exploiting different phase settling times.

INTRODUCTION

Bone marrow of adult mammals such as rabbits, mice and rats provides the most suitable cells for a biochemical study of erythropoiesis due to its accessibility to sampling and its capacity for synthesis of both a unique protein (haemoglobin) and a specific metabolite [2,3-biphosphoglycerate (glycerate-2,3-P_2)]. Since cellular heterogeneity and interactions between the different cell types in the bone marrow make detailed investigation difficult, several red blood cell separation techniques that supply the necessary material for tackling this work have been developed [1, 2].

Thin-layer counter-current distribution (CCD) in dextran—poly(ethylene glycol) (D—PEG) two-phase systems is one such technique [3—7]. It has been

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used to separate and/or subfractionate blood cell populations according to subtle alterations in their surface properties, which occur as a function of abnormal and normal in vivo processes or in vitro treatments [5—11]. The distribution, according to age, of homogeneous populations of circulating erythrocytes from adult humans [5, 6], rats [5, 6, 10] and chickens [10], as well as the subfractionation of heterogeneous populations of bone-marrow cells from rats [8, 9] and erythrocytes from young chickens [11], have been reported.

We describe here the influence of settling time (i.e. the time allowed for the separation of the phases after each mixing step) on the fractionation by CCD of bone-marrow cell populations from rats and humans, using a charged 5% D—4% PEG two-phase system.

MATERIALS AND METHODS

Male Wistar rats (*Rattus norvegicus*) weighing 150—200 g were used. Animals were anaesthetized with ether and killed by exsanguination. Pools of bone-marrow cells (from at least five rats) were prepared from the femur and tibia as previously described [12], using a saline solution of 0.137 mol/l sodium chloride, 3.5 mmol/l potassium chloride, 0.5 mmol/l magnesium chloride, 1.4 mmol/l potassium dihydrogen phosphate, 8.1 mmol/l sodium biphosphate and 1 U/ml heparin, pH 7.4 (SSP). Blood was collected in heparinized tubes and, after centrifugation (400 *g*, 10 min) at 4°C, cells were washed with SSP. For radioactivity experiments, rats were intraperitoneally injected with 50 μCi/kg of [^{59}Fe]ferric citrate (Nuclear Iberica, Spain). They were killed at 3, 7, 17, 24 and 48 h after injection and pools of bone-marrow cells were prepared as before.

Human bone-marrow cells (acute lymphoblastic leukaemia in remission) were obtained from patients under conventional multiple-drug therapy (Department of Haematology, "Puerta de Hierro" Hospital, Madrid). The samples, which were normal by morphological criteria, were collected in SSP solution. After washing twice, the pellets were resuspended in a minimum volume of SSP.

The following stock solutions (in water), to be used for the preparation of phase systems, were: 20% (w/w) Dextran T-500 (Pharmacia, Uppsala, Sweden), standardized by polarimetry; 40% (w/w) PEG 6000 (Carbowax 6000, Serva, Heidelberg, F.R.G.); 1 mol/l sodium chloride and 0.2 mol/l sodium phosphate buffer, pH 6.8 (composed of equimolar quantities of mono- and dibasic phosphates). 5% D—4% PEG charged two-phase systems containing 0.03 mol/l sodium chloride and 0.09 mol/l sodium phosphate buffer (pH 6.8) were prepared by weight from the above stock solutions in a sufficient quantity (800 g) for several experiments. Care was also taken to use the same polymer batches for the experiments being compared [4—6]. Phase systems were allowed to equilibrate for 24 h at 4°C in a separatory funnel and top and bottom phases were then separated.

Fractionation of cells by the CCD procedure has been described previously [3, 5]. Various versions of the CCD apparatus with 60-cavity rotors, made at the Universities of Madrid (Spain), Umeå (Sweden) and Sheffield (U.K.), were employed.

The first three or five cavities of the rotor (depending on the concentration of the cell suspension) each received a mixture of 0.6 ml of bottom phase plus 0.1 ml of the cell suspension, while the remaining 57 or 55 cavities each received 0.7 ml (i.e. 90% lower chamber volume) of bottom phase. Top phase (0.9, 0.7 or 0.4 ml in different experiments) was then added to the cavities in order to produce specific phase volume ratios, L (L = 1.3, 1 or 0.6, respectively). A partition cycle is formed by a 20-s shaking time followed by one of the three different phase settling times being tested (5, 10 or 20 min). After each cycle, a transfer is carried out. With each transfer, the cells in the top phase are carried forward where they are re-extracted with fresh bottom phase; those cells at the interface are re-extracted with fresh top phase. The transfers (55 or 57, depending on the number of cavities receiving cell suspension) were repeated at 4° C. At the end of the CCD run, 1 ml of saline solution (SSP) was added to each cavity of the rotor to transform the system into one phase. The contents of each cavity were collected and cell fractions were directly analysed. Distribution of cells in the different cavities along the extraction train (i.e. CCD profile) is given in terms of protein (Lowry method [3], haemoglobin [13], absorbance at 410 nm or 540 nm (Uvikon 810 spectrophotometer, Kontron), radioactivity of ^{59}Fe (Nuclear Chicago well-scintillation counter) or total iron (Perkin Elmer 2380 atomic absorption spectrophotometer). All chemicals used were of analytical grade.

RESULTS AND DISCUSSION

Fractionation of rat bone-marrow cells and erythrocytes

As initially reported, the CCD method subfractionates bone-marrow erythroid cells according to their relative stages of development. A top/bottom phase volume ratio in each cavity, L = 1.3, and a settling time for the separation of the two phases after each mixing step of the CCD process, t = 5—6 min, were used [8, 9]. Younger cells have a lower partition coefficient, K, than the older ones and, therefore, were less displaced to the right (i.e. slow-moving cells) in the CCD profile. However, these populations were not clearly separated. Since the subfractionation of a heterogeneous population of erythrocytes from young chickens was only achieved at a lower L value and higher phase settling time [11], a search for similar favourable CCD operating conditions was then initiated in our laboratory using bone-marrow cells from normal rats. This obvious and simple approach has not yet been exploited to improve CCD bone-marrow cell fractionation.

According to theory [3], a displacement of CCD profiles and a separation between them should be possible when a heterogeneous population (i.e. cells having a different partition coefficient K) is fractionated by CCD at a low top/bottom phase volume ratio L. The influence of three different L values (1.3, 1 and 0.6) on the fractionation efficiency of rat bone-marrow cells was first studied (results not shown). In spite of the displacement of CCD profiles, increased efficiency of separation was not observed. A single peak was repeatedly obtained under all three L values. The most convenient profile was found to be that for L = 0.6, located around cavity 40.

The influence of the phase settling time on fractionation efficiency was then

studied. It is well established that D—PEG two-phase systems need a relatively long setting time before reaching equilibrium conditions, due to the high viscosity of the phases and their small density differences. Settling time decreases by increasing polymer concentration, reducing the temperature and decreasing the depth of the phases [3, 5, 14]. The cavities of the CCD rotor are designed to produce short-phase settling times by reducing the depth of the phases [3]. However, equilibrium distribution is not absolute at the time that the top phase is transferred in CCD procedure and cell partitioning is thus carried out before equilibrium is reached [7]. This is also supported by the well-known time-dependence of cell partition observed in single-tube experiments [7, 15—17].

CCD fractionations were carried out here using three phase settling times (5, 10 or 20 min) and the lowest top/bottom phase volume ratio (L = 0.6). Results for rat bone-marrow cells (heterogeneous population) are shown in Fig. 1. As clearly observed, single peak populations are obtained when either 5 or 10 min are used. Two broad but well-defined subpopulations were achieved when the longest settling time (20 min) was employed, i.e. as the difference in K between cells is enhanced. This profile has been obtained repeatedly using a wide variation of total cells ($1—7 \cdot 10^8$) and different CCD rotors. For comparative reasons, the results for rat erythrocytes appear in Fig. 2. As expected, no fractionation was achieved for such a homogeneous population. In all

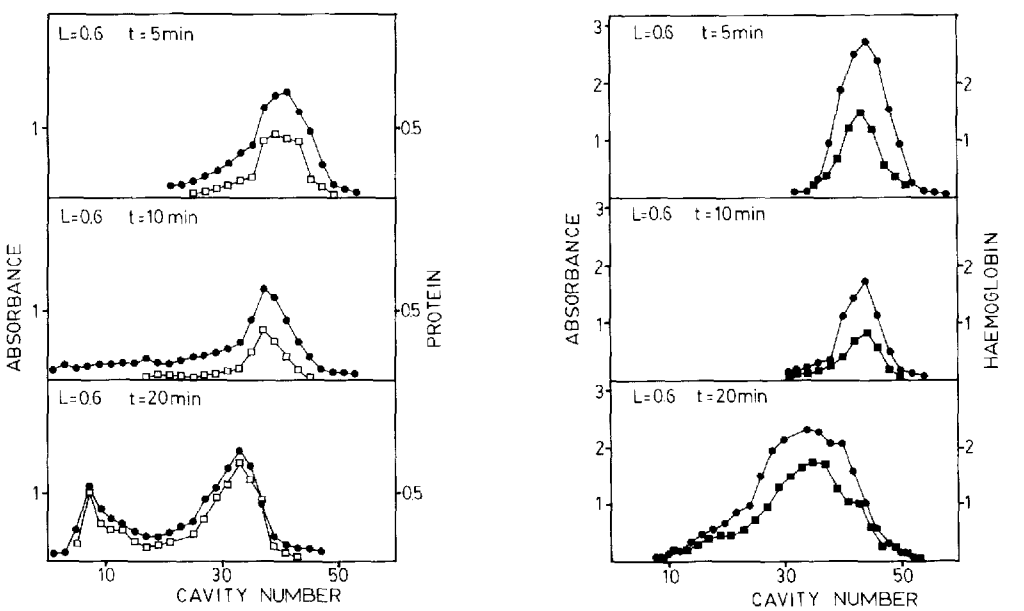

Fig. 1. Influence of the phase settling time on the CCD profiles of normal rat bone-marrow cells. Distribution of cells is given in terms of absorbance (410 nm) (•) and protein (mg/ml) (□). Phase system contained 5% (w/w) D, 4% (w/w) PEG, 0.03 mol/l sodium chloride and 0.09 mol/l sodium phosphate buffer, pH 6.8; 57 transfers were completed at 4– 5°C. See text for details.

Fig. 2. Influence of the phase settling time on the CCD profiles of normal rat erythrocytes. Distribution of cells is given in terms of absorbance (540 nm) (•) and haemoglobin (mg/ml) (▪). Conditions for CCD were as in Fig. 1.

experiments (Figs. 1 and 2) it is observed that cells are less displaced from the origin with time as the system approaches the equilibrium distribution.

The influence of phase settling time on CCD fractionation of heterogeneous populations results from the association of cells with microscopic globules of the lower phase that persist in the PEG-rich top phase for some time after the horizontal interface has formed. This mechanism has been described as an important determinant of the partitioning behaviour of cells [7, 16, 17]. It implies that the separation of cells by CCD depends on non-equilibrium conditions. Our present data support such a possibility and make interesting use of a 20-min settling time to resolve bone-marrow cell subpopulations, as preliminarily reported [18].

The suggestion can also be made that differences in cell size of haematopoietic cells could be an additional factor in the increase of fractionation efficiency observed when the phase settling time is increased (Fig. 1). In agreement with this suggestion, a homogeneous cell size population (such as circulating erythrocytes) is not fractionated at all when identical phase settling times are applied (Fig.2).

The fact that for $L = 0.6$ and $t = 20$ min the mature circulating erythrocytes (Fig. 2) are located in coincidence with the fast-moving cells of bone marrow (Fig. 1), seems to support the idea that the more mature erythroid cells are mainly located in this subpopulation. This is also supported by the increase of the slow-moving cell subpopulation (around cavity number 8) obtained when erythroid-rich bone-marrow populations (obtained from anaemic animals or prepared from normal rats after Percoll gradients) are fractionated by the CCD procedure [19].

^{59}Fe-labelled bone-marrow cell fractionation

Subfractionation according to age of circulating erythrocytes has been demonstrated by combining the CCD procedure with the ^{59}Fe-labelling technique [6, 10]. Thus, an attempt was made to extend such an approach in order to locate the presence of erythroid cells in the two broad subpopulations (Fig. 1).

Bone-marrow cell suspensions were prepared from rats killed at different times (3, 7, 17, 24 and 48 h) after injection of [^{59}Fe]ferric citrate. Subfractionation in two broad peaks is repeatedly observed. Haemoglobin and iron atomic absorption determinations (at zero time), or haemoglobin measurement and radioactivity iron counts (at 3, 7, 17, 24 and 48 h), show that different types of erythrocytic cells (Fig. 3) are present in both subpolulations. As suggested before, more mature erythroid cells seem to be predominant in the fast-moving subpopulation. Since erythroid cell precursors are transformed into erythrocytes after 24—48 h, radioactivity (^{59}Fe) incorporated at the time of isotope injection disappears progressively in both subpopulations after such a period of time (see Fig. 3).

Human bone-marrow cell fractionation

Due to the interest that the separation of bone-marrow cells has for the clinical laboratory, the above CCD experimental conditions were also extended to human bone-marrow cells. Therefore, the distribution of such cells by using

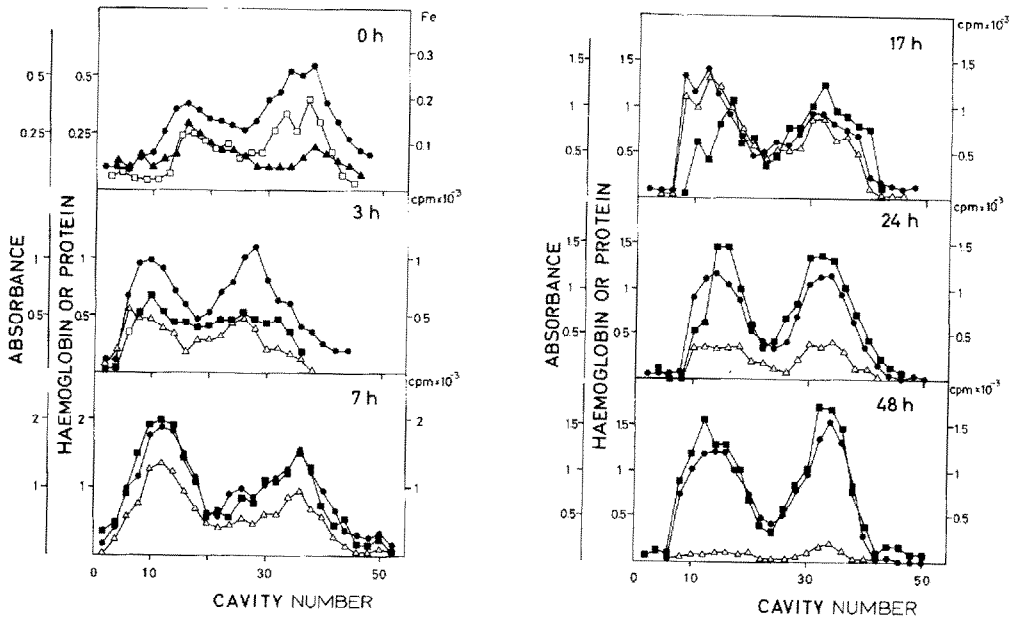

Fig. 3. CCD profiles of bone-marrow cells from rats injected with [^{59}Fe]ferric citrate and killed at different times (3, 7, 17, 24 and 48 h) after isotope injection. Distribution of cells is given in terms of absorbance (410 nm) (•), protein (mg/ml) (□), haemoglobin (mg/ml) (■), iron (ppm) (▲) and ^{59}Fe (cpm) (△). Phase settling time 20 min. All other conditions for CCD as in Fig. 1.

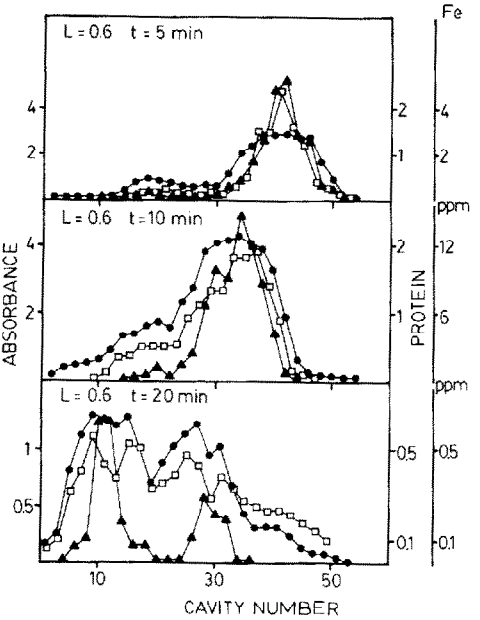

Fig. 4. CCD profiles of human bone-marrow cells at different phase settling times. Distribution of cells is given in terms of absorbance (410 nm) (•), protein (mg/ml) (□) and iron (ppm) (▲). Phase settling time 20 min. All other CCD conditions as in Fig. 1.

a low phase volume ratio ($L = 0.6$) and three different phase settling times (5, 10 or 20 min) was studied. Results are shown in Fig. 4. The single peak around the cavity 40, observed at 5 min, was displaced towards a slow-moving position when time allowed for the phase separation was higher (10 min). Only at the nearest to equilibrium experimental conditions (20 min) did human bone-marrow cells become distributed along the CCD profile, either in terms of absorbance or protein (mg/ml). To confirm the presence of erythrocytic cells, iron content was also measured. These values seem again to indicate that erythrocytic cells are present in both subpopulations. This is a similar finding to that observed with rat bone-marrow cells (Figs. 1 and 3). The fractionation of human bone-marrow cells by using this procedure could then be of clinical importance.

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