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Study of human cord blood lymphocytes by immobilized metal ion affinity partitioning[1]

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Abstract

The potential of immobilized metal ion affinity partitioning (IMAP) using dextran–PEG+PEG–IDA–M(II) systems to separate mononuclear cells from cord blood has been evaluated. The distribution of B cells, T cells, monocytes and hematopoietic stem cells between PEG and dextran phases was determined by flow cytometry with fluorochrome-labelled specific antibodies. Comparing these values with the post-Ficoll repartition resulted in the determination of enrichment factors, for each subpopulation, in the different phases. We were able to distinguish the partition pattern of B cells, T cells, monocytes and stem cells in different IMAP systems. Their partition was affected by the nature and the concentration of the metal used, but no specificity in distribution for the subpopulations was found.

Keywords: Aqueous two-phase systems; Partitioning; Lymphocytes; Cord blood

1. Introduction

Aqueous two-phase partitioning of proteins and cell organelles was introduced and developed by Albertsson [1]. This technique is based upon the ability of aqueous soluble polymers, such as dextran and poly(ethylene glycol) (PEG), to form biphasic systems. In such systems, the partitioning of the cellular components will reflect their overall surface properties, such as hydrophobicity, surface net charge, etc. Introduction of various affinity ligands into the PEG phase gives rise to partitioning due to the interaction between the ligand and the molecule or the supramolecular structure. Although ligands such as fatty acids [2], peptides [3] and triazine dyes [4] have been used for protein separation, in the case of whole cells only antibodies [5] and fatty acids [6] have been used usually as affinity ligands and only in a rather limited manner.

The immobilized metal ion affinity (IMA) concept described by Porath et al. [7] was extended to phase partitioning by grafting a chelator, iminodiacetate (IDA) onto PEG. This ligand is able to chelate transition metal ions, e.g. Cu(II), Ni(II), Zn(II) and Co(II). Proteins in solution, or in membrane structures, will establish coordination bonds with the immobilized metal ion via accessible and deprotonated histidine residues [8,9]. This technique has

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[1]This article is dedicated to P.Å. Albertsson and H. Walter on their 65th birthdays.

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been successfully used for cell separation with the advantage of a shear-free system [10].

In this manner, healthy and pathological cells have been studied by IMAP and it was possible to differentiate malarial red blood cells, cancerous fibroblasts and lymphoma cells from their healthy counterparts using PEG–IDA–Ni(II) as an affinity ligand [11], with excellent viability and with little modification of the cell surface.

In this work, we studied the usefulness of IMAP systems to segregate low abundance cells, e.g. human cord blood mononuclear cells, or more precisely hematopoietic stem cells, from other cell types in a complex cell mixture. These cells, representing less than 1% (0.3 to 0.7%) of the total mononuclear cells of human cord blood, are characterized by the expression of the CD_{34} surface antigen. It is known that CD_{34}^{+} cells are a heterogenous population, representing the first step of hematopoiesis. Nevertheless, all of them carry the specific CD_{34} antigen and are capable of producing erythropoietin in a culture system. They are able to proliferate and to differentiate into blood cells and the most important feature of these cells is that they have the capacity for self renewal in their totipotent state. The purification of these cells is of great importance for the study of the earliest stages of hematopoiesis and also for the therapy of some blood diseases, like leukaemia. Major sources of hematopoietic stem cells are the bone marrow, peripheral blood and cord blood. We chose cord blood due to its availability and because it contains a potentially large population of CD_{34}^{+} stem cells. Techniques actually used for CD_{34}^{+} cell purification are based on the binding of monoclonal antibodies raised against CD_{34}. They are very specific, giving high yield, but they are very expensive and the amount of blood that can be treated is limited. In addition, high binding strength of the antibodies could modify the cell surface properties and may activate stem cell differentiation into a specific blood cell-line. The pseudobiospecific ligands used in IMAP systems have lower binding strength and do not seem to activate cell differentiation [11]. Moreover, they are easily removed from the cell surface. We describe here the application of this technique for enrichment of unmodified CD_{34}^{+} cells.

2. Experimental

2.1. Reagents and chemicals

Dextran T500 and Ficoll were purchased from Pharmacia (Uppsala, Sweden), PEG 6000 was obtained from Serva Feinbiochemica (Heidelberg, Germany), monomethoxy poly(ethylene glycol) 5000 was from Sigma (St. Louis, MO, USA). Fluorescence-labelled antibodies for flow cytometry were supplied by Becton Dickinson (Le Pont De Claix, France). RPMI medium was purchased from ICN (Costa Mesa, CA, USA). All reagents used were of analytical grade.

2.2. Preparation of mononuclear cells (MNC) from human cord blood

Cord blood samples were collected just after birth in citrate phosphate dextrose anticoagulant. Mononuclear cells were prepared by density gradient centrifugation on Ficoll as described by Boyum [12].

The cells were washed twice with RPMI medium and twice with isotonic phosphate buffered saline (PBS, 0.1 *M* phosphate, 1.5 *M* NaCl, pH 6.8) before partition experiments were carried out.

2.3. Synthesis of M-PEG–IDA–M(II)

M-PEG 5000 was chlorinated, using thionyl chloride under anhydrous conditions, to yield M-PEG–Cl. This compound was then derivatized with iminodiacetic acid yielding M-PEG–IDA as described previously [13]. The ligand was charged as described in [9] by adding 4 g of M-PEG–IDA to 20 ml of a 50 m*M* solution of $CuSO_4$, $NiSO_4$ or $ZnSO_4$, in 50 m*M* sodium acetate buffer, pH 4.0. The resulting M-PEG–IDA–M(II) was then extracted from the aqueous solution with chloroform and the solvent was removed in a vacuum evaporator.

2.4. Preparation of biphasic systems

Two-phase systems were prepared from stock solutions of dextran T500 (20%, w/w), PEG 6000 (40%, w/w) and PBS. To include M-PEG–IDA–M(II) in the system, we prepared stock solutions by

substituting PEG with M-PEG–IDA–M(II) in a range of 0 (naked systems) to 7% (w/w) (IMAP systems) of the total PEG.

Each system had a global composition of 5% dextran and 4.5% PEG and was made isotonic using PBS. The weight of the systems was 7.8 g which was completed with cell samples (from a cell suspension of 10^7 cells/ml) to 8 g. The systems were homogenized and allowed to settle before adding the cell suspension.

2.5. Partitioning of mononuclear cells in IMAP systems

A 200-μl aliquot of mononuclear cell suspension (10^7 cells/ml) was added in each biphasic system. Homogenization was performed by inverting the test tube 20 times, then the systems were allowed to settle in a vertical position at 20°C for 30 min before 4 ml of the upper phase, 0.8 ml of the interphase and 2.9 ml of the lower phase were collected, representing more than 95% of the volume of the three phases.

2.6. Flow cytometric analysis of mononuclear cell subpopulations

The cell population distribution was monitored by cytofluorimetry using four surface antigens; CD_{34}, CD_{19}, CD_{14} and CD_2 that are associated with stem cells, B lymphocytes, monocytes and T lymphocytes, respectively. The cells of the three samples obtained from each biphasic system were washed twice with PBS and then resuspended in 200 μl of PBS.

Three 50-μl aliquots of each sample were taken and incubated with fluorescent-labelled antibody for 30 min at 4°C. $HPCA_2$-RD1, My_4-FITC and B_4-RD1 + T_{11}-FITC antibodies specific for CD_{34}, CD_{14}, CD_{19} and CD_2 antigens, were used. After incubation, the cells were washed twice and resuspended in 200 μl of PBS. These suspensions were analyzed in an EPIC Profile cytofluorimeter (Coulter). By using an isotypic control for each antibody it was possible to discriminate between specific and non-specific fluorescence.

The cell population distribution was evaluated both in the post-Ficoll fractions prior to IMAP, and in the three phases (lower, inter and upper) obtained after the IMAP step. One blood sample was tested for each ligand concentration.

3. Results and discussion

The mean composition of the post-Ficoll mononuclear cells was 32.5% of CD_2^+ (T lymphocyte); 8.7% of CD_{14}^+ (monocytes); 5.2% of CD_{19}^+ (B lymphocytes) and 0.5% of CD_{34}^+ (hematopoietic stem cells). In order to appreciate any selectivity of IMAP for the different cell populations, we have calculated the enrichment of each cell type after partition, by dividing the percentage obtained after recovery of all the three (upper, inter and lower) phases from IMAP systems, by the percentage obtained after Ficoll gradient centrifugation.

Different metal ions [e.g., Cu(II), Ni(II) and Zn(II)], the most frequently used in the IMAP system were charged to PEG–IDA. They were used as the ligand in the systems and have different patterns of cell distribution in all three phases. We did not use Co(II) because Zn(II) and Co(II) have a very similar recognition pattern to histidine residues [14].

3.1. IMAP of cord blood mononuclear cells with different PEG–IDA–M(II)

Fig. 1 shows a plot of the enrichment factor of CD_{34}^+, CD_{14}^+, CD_{19}^+ and CD_2^+ cells as a function of PEG–IDA–Cu(II) concentration. In the upper phase of the systems (Fig. 1a), we did not see any discrimination of the different subpopulations of mononuclear cells at any ligand concentration tested. This implies that there is no selectivity for PEG–IDA–Cu(II) towards a single subpopulation, in our systems.

However, CD_{34}^+ cells were significantly enriched at the interphase of systems containing PEG–IDA–Cu(II) (Fig. 1b). Enrichment reached a maximum value of 7.8 at 6% PEG–IDA–Cu(II).

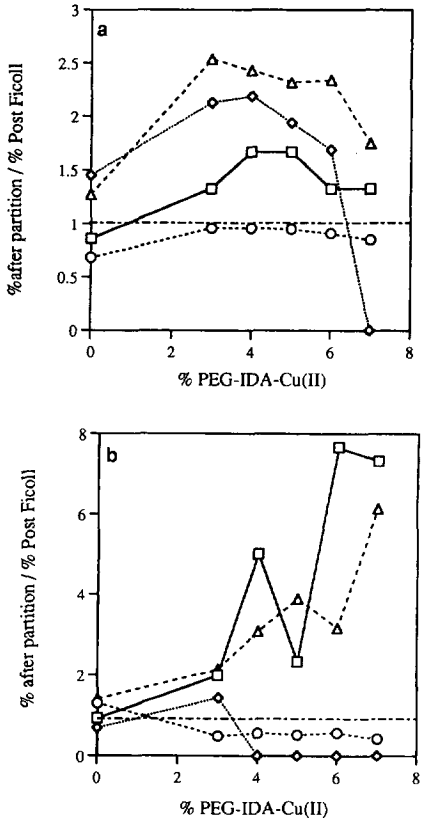

Fig. 1. Partition of human cord blood mononuclear cells in IMAP systems with PEG–IDA–Cu(II). Global composition of the system is 5% dextran–4.5% PEG in 0.01 *M* phosphate buffer, 0.15 *M* NaCl, pH 6.8. The cell distribution in (a) the upper phase and (b) the interphase, compared with the composition of mononuclear cells obtained after Ficoll fractionation, is shown. □=CD^+_{34} cells; ◇=CD^+_{14} cells; ○=CD^+_2 cells and △=CD^+_{19} cells.

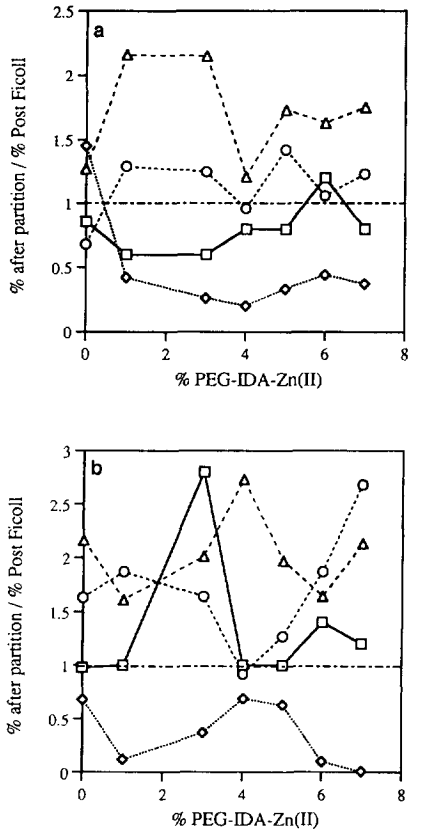

Fig. 2. Partition of human cord blood mononuclear cells in IMAP systems with PEG–IDA–Zn(II) and with the composition of the system as shown in Fig. 1. Cell distribution in (a) the upper phase and (b) the lower phase compared with the composition of mononuclear cells obtained after Ficoll fractionation, is shown. □=CD^+_{34} cells; ◇=CD^+_{14} cells; ○=CD^+_2 cells and △=CD^+_{19} cells.

3.2. PEG–IDA–Zn(II)

Fig. 2 represents the plot of the enrichment factor of CD^+_{34}, CD^+_{14}, CD^+_{19} and CD^+_2 cells as a function of PEG–IDA–Zn(II) concentration. In the upper phase of these systems (Fig. 2a) an enrichment of CD^+_{19} cells was observed at concentrations of ligand between 1 and 3%. It should also be noted that all cell types, except CD^+_2, are impoverished at these concentrations of ligand.

At 4% PEG–IDA–Zn(II), a sudden decrease of CD^+_{19} cell enrichment is observed in the upper phase. This was correlated with the rise of enrichment observed in the lower phase of the same system (Fig. 2b), thus for 4% ligand, there is almost a repulsion (negative affinity), preferentially pushing the CD^+_{19} cells from the upper phase to the lower phase.

3.3. PEG–IDA–Ni(II)

Fig. 3 shows a plot of the enrichment factor of CD^+_{34}, CD^+_{14}, CD^+_{19} and CD^+_2 cells as a function of PEG–IDA–Ni(II) concentration. In the upper phase of systems with 3% of ligand (Fig. 3a), we observed an enrichment of CD^+_{19} cells in this phase, other cell subpopulations were less abundant than in the post-Ficoll population, which indicates a repulsion for these cells at 3% of PEG–IDA–Ni(II). For 4, 5, 6

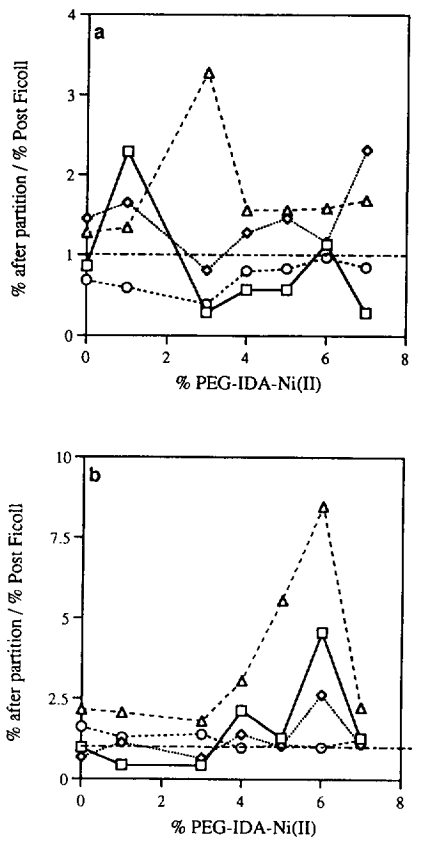

Fig. 3. Partition of human cord blood mononuclear cells in IMAP systems with a PEG–IDA–Ni(II) composition of the systems as shown in Fig. 1. Cell distribution in (a) the upper phase and (b) the lower phase compared with the composition of mononuclear cells obtained after Ficoll fractionation, is shown. □=CD^+_{34} cells; ◇=CD^+_{14} cells; ○= CD^+_2 cells and △=CD^+_{19} cells.

and 7% of PEG–IDA–Ni(II), one can see that the enrichment of CD^+_{19} cells is as low as 1.5 in the upper phase and that this was accompanied by a dramatic increase in the percentage of CD^+_{19} cells present in the lower phase (up to an eight-fold increase compared to the post-Ficoll value) (Fig. 3b).

The partition behaviour of mononuclear cell subpopulations is very different from that of normal adult lymphocytes and lymphoma cells observed in previous studies [11]. Segregation of normal and pathological lymphocytes (lymphomas) was maximal when the concentrations of PEG–IDA–Ni(II) were between 10 and 20%, showing an increase of selectivity with increasing ligand concentrations, typical of an affinity phenomenon. This work shows that discrimination of different mononuclear cell subpopulation is more effective either in the upper phases of systems with 3% PEG–IDA–Ni(II) or in the lower phases of systems with 5 or 6% PEG–IDA–Ni(II). This discrimination is based on the fact that only one subpopulation is enriched while the others have the same or lower abundance than in the post-Ficoll population. All the IMAP systems studied seem to be very effective in the isolation of CD^+_{19} cells. In previous studies of lymphocytes carried out with the same systems [11], cells obtained from Ficoll fractionation were determined without distinguishing between T and B lymphocytes.

For CD^+_{34} cells, the best enrichment values were observed in interphases of systems with 4, 6 or 7% PEG–IDA–Cu(II), but in these phases we also obtained an enrichment in CD^+_{19} cells. In Table 1, the percentage of CD^+_{34} cells present in each phase of selected systems (6% copper, 3% zinc and 1 or 6% nickel), obtained after single-step partition experiments compared with post-Ficoll cell populations, to determine if IMAP systems were able to separate this low abundance subpopulation, is summarized.

In each phase of the system containing 6% PEG–IDA–Cu(II) (Table 1), one can see that CD^+_{34} cells are preferentially partitioned into the interphase. This

Table 1
Best percentage values of CD^+_{34} from human cord blood mononuclear cells after a single step partition experiment

Composition of system	Post-Ficoll	Lower phase	Interphase	Upper phase
6% PEG–IDA–Cu(II)	0.3	N.D	2.3	0.4
3% PEG–IDA–Zn(II)	0.5	1.4	0.3	0.3
1% PEG–IDA–Ni(II)	0.7	0.3	0.6	1.6
6% PEG–IDA–NI(II)	0.7	3.2	0.7	0.8

Values are expressed as the percentage of the total mononuclear cells in the sample and are compared to the percentage in the starting population of mononuclear cells.
N.D. = percentage not determined because of the low number of cells found in the phase.
Note: The post-Ficoll preparations were different in each case due to differences in the initial composition of CD^+_{34} cells.

means that the affinity of the ligand for the cell surface is not sufficient to partition them into the upper phase. In systems with 1% PEG–IDA–Ni(II) (Table 1), CD_{34}^{+} cells are preferentially distributed in the upper phase. We observed a shift of this cell population to the lower phase in systems that had a higher concentration (6%) of the same ligand (Table 1). CD_{34}^{+} cells are also preferentially enriched in the lower phase of systems with 3% PEG–IDA–Zn(II) (Table 1).

The results obtained after single step partition experiments have shown that cell supopulations display different behaviour. Despite the influence of chelated metal ions on the partition behaviour, we did not find any specificity of these ligands towards a specific cell surface determinant and hence towards a targeted cell subpopulation.

Immobilized metal ion affinity partitioning (IMAP) is a concept similar to immobilized metal ion affinity chromatography (IMAC). Partition is influenced not only by the relationship between the cell surface and the physical properties of the phases, but more selectively, by the affinity of the immobilized metal ions to the histidine residues of membrane-associated proteins.

According to previous results obtained in our laboratory [11], we put forth the hypothesis that glycophorins played an important role in the affinity partitioning of human red blood cells. Nevertheless, on mononuclear cells of cord blood, surface proteins are more diversified and it is more difficult to identify a target molecule for the metal chelate.

Hematopoietic stem cells are an heterogeneous population of cells, characterized by the CD_{34} surface antigen. CD_{34} is expressed abundantly in hematopoietic stem cells and also in pathological cells, such as leukaemia cells, but is absent from mature normal blood cells [15].

Structural studies [16] have shown that CD_{34} is a glycoprotein of an apparent molecular mass of 110 kDa, with nine potential N-glycosylation sites, additional O-glycosylation sites and with a long extracellular segment. Partial sequence and amino acid composition have shown that over 35% of the amino acids in the extracellular domain are either serine or threonine, thus O-linked carbohydrates seem to be abundant, although the precise number and the nature of these sites is not known. Based on size fractionation studies, they have been shown to consist predominantly of sialic acids and they could confer an overall negative charge onto the cell.

The partial sequence of the N-terminal segment of CD_{34} does not contain any histidine or tryptophan residues and therefore no conventional site for a metal chelate. In addition, the carbohydrate moiety may have an occluding effect, i.e., preventing the accessibility of the ligand to the protein surface. Furthermore, the negative charge, due to sialic acids, can cause a repulsion between the ligand and the potential target sites on the protein surface. Such repulsive effects, due to negative charge, have been reported previously in IMAC systems [17]. Enrichment of CD_{34}^{+} cells in the upper phase of some IMAP systems, without any correlation to the ligand concentration, can indicate the influence of electrostatic effects due to sialic acids and hydrogen bonds, that are due to serine and threonine residues in the cell partition.

In conclusion, we have observed some differences in the behaviour of different subpopulations of human cord blood mononuclear cells, as a function of the nature and concentration of the ligand used. Until now, the enrichment of some cell subpopulations, in the metal-containing phase, could not be attributed to many specific interactions of the ligand with the cell surface proteins. Nevertheless, purely from an application point of view, based on these preliminary results, we propose a multi-step extraction protocol (counter-current distribution) using different PEG–IDA–M(II) ligands, at certain concentrations, to obtain enriched fractions of specific mononuclear cell subpopulations.

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