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Review

Surface affinity chromatography of human peripheral blood cells

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Abstract

Column packings with chemically bonded polyethylene glycol (PEG) and polypropylene glycol (PPG) were prepared and the separation of human peripheral blood cells by an aqueous polymeric two phase system could be performed in a partition chromatography. In this chromatographic system, the interaction of blood cells with bonded PEG, PPG stationary phases is apparently selective, and granulocytes and lymphocytes are more strongly retained on the column than erythrocytes and platelets. The hydrophobic interactions between the hydrophobic region on the cell surface membrane and the bonded PEG, PPG phases is probably the main factor affecting the retention of lymphocytes and granulocytes. The selective separation of human peripheral platelets, granulocytes and lymphocytes was investigated using another type of column packing bonded with methoxyethoxymethyl (MEM) groups. The isolation of platelets and lymphocytes from human leukocyte-rich plasma was performed with a MEM–Sephadex column and elution with 0.27 *M* sucrose solution. Platelets could be selectively collected from the same column by elution with 0.31 *M* methyl-α-D-mannoside at the high recovery of 100%.

Keywords: Reviews; Blood cells; Partitioning; Polymeric two-phase systems

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1. Introduction

Improvements in the fractionation methods used for the investigation of cells have long been required. In column chromatographic methods, which seem to have a potential ability for cell fractionations, many practical difficulties are experienced, such as the adsorption of a large number of cells on the column. The subsequent separation of blood cells according to their adherence characteristics is exemplified by the technique of adherence chromatography, which uses a column of siliconized glass beads about 200 μm in diameter [1]. However, it was impossible to separate human lymphocytes from erythrocytes by the use of the glass bead column. The recovery from the column is about 60–70% for small lymphocytes and 35–45% for polymorphonuclear leukocytes [1]. Several reports have described the chromatographic behavior of certain types of mammalian cells on columns packed with agarose beads [2], polymethylmethacrylate beads [3] and cross-linked dextran beads [3,4]. However, only a few papers have described the adherence of cells to packing materials [3,5].

Aqueous-polymer two-phase systems for the partitioning of biological macromolecules including several kinds of cells were first established by Albertsson [6]. Partitions of macromolecules, such as viruses [7,8], bacteria [9,10], cell organelles [11] and blood cells [12–17] have been carried out by the use of various aqueous polymer phase systems. Among the many types of the aqueous–aqueous polymer phase systems available, the dextran–polyethylene glycol (PEG) system has been the most commonly used for the partition of the cells. In the 1960s, Albertsson introduced the thin-layer counter-current distribution apparatus that can conveniently carry out multi-stage partitioning with the polymer phase systems in a unit gravitational field. However, in this thin-layer counter-current distribution apparatus, the partition process is carried out by three operational steps of mixing, settling and transferring the mobile phase, hence it requires relatively long separation times. If one of the phases could bind to the support material, it is clear that the partition in the aqueous-polymer two-phase system could be carried out in a column as a liquid partition chromatography.

Several column packings with chemically bonded polyethylene glycols (PEGs) and polypropylene glycols (PPGs) as the stationary phase were prepared. Using these packings, fractionation of human peripheral blood cells was attempted by means of column chromatography, based on the partition between chemically bonded PEG, PPG stationary phase and dextran constituting the aqueous mobile phase [18]. The influence of the mobile phase composition on the chromatographic behavior of human erythrocytes, platelets, granulocytes and lymphocytes has been studied [19]. The retention volumes of the blood cells at a physiological pH of 7.4 depended on the average molecular mass of the bonded PEG in the range of 400–20 000, increasing with the increasing molecular mass of PEG [20]. Further, the same tendency for the retention behavior of blood cells on the PEG-bonded phase was observed for the isoelectric points which were determined by using the cross-partition method. The relationships between surface hydrophobicity of human peripheral blood cells and their retention behavior on PEG- and PPG-bonded columns were also investigated [21–24]. Consequently, it was clear that hydrophobic interactions play an important role in the retention and separation of granulocytes and lymphocytes on PEG- or PPG-bonded column packings.

Methoxyethoxymethyl (MEM) groups which are similar to the two oxyethylene groups of PEG, were also introduced into the column packing materials, such as Cellulofine (cellulose beads) and Sephadex (cross-linked dextran beads), used for size-exclusion chromatography. Cellulofine consists of a D-glucose polymer in which the D-glucose units are bound in

β(1→4) linkages. Sephadex consists of a D-glucose polymer in which the linkage between the D-glucose units is α(1→6). The selective separation of human peripheral blood cells was examined by using MEM-bonded Cellulofine and Sephadex columns. Isotonic mono- and disaccharides and methyl-α-D-pyranosides solutions were used as the mobile phases. The adsorption and the elution behavior of human peripheral blood cells, especially platelets, granulocytes and lymphocytes, on the MEM-bonded columns were also studied [25].

The details of the blood cells retention and separations along with the hydropobicity of the surface of the cells and the column packing gels will be described in this review.

2. Preparation of column packings

2.1. PEG–Sepharose 6B column packings

PEG-bonded Sepharose 6B packings were prepared as follows [18]. One gram of epoxy-activated Sepharose 6B was washed on a sintered-glass filter funnel (G3) with ca. 100 ml of water and then mixed with PEG solution. The solution was prepared by dissolving 10 g of either PEG 6000 or 20 000 (20M) in 20 ml of a solution adjusted to pH 9.0, 10.0 or 11.0 with sodium hydrogen carbonate–sodium carbonate buffer, and pH 12.0 and 13.0 with sodium hydroxide solution. The reaction mixture was shaken for 16 h at 32.5, 40 and 45°C in a thermostat. The coupled product was washed on a sintered-glass filter funnel successively with water, 0.1 *M* sodium tetraborate–boric acid–0.5 *M* sodium chloride buffer (pH 8.0) and 0.1 *M* sodium acetate–acetic acid–0.5 *M* sodium chloride buffer (pH 4.0) until PEG was no longer detected in the filtrate. The product, suspended in 25 ml of 1 *M* 2-aminoethanol, was shaken for 12 h at room temperature, thoroughly washed with water and dried under vacuum at 105°C for 48 h. PEG 20M was coupled to agarose beads through the mediation of bisoxiran [1,4-bis(2,3-epoxypropoxy)butane] as shown in Fig. 1. A terminal hydroxy group in the PEG 20M was directly coupled to an active oxirane group of epoxy-activated Sepharose 6B and a stable hydrophilic uncharged ether linkage was formed between PEG and Sepharose 6B.

As the coupling conditions depend greatly on the properties of the ligand and the functional group to be coupled, suitable conditions for the oxirane coupling of PEG 20M with epoxy-activated Sepharose 6B were investigated at 32.5, 40.0 and 45.0°C for 16 h in aqueous solution adjusted to pH 9.0, 10.0, 11.0, 12.0 and 13.0, respectively. The amount of PEG 20M coupled was determined from the amount of uncharged PEG recovered in the reaction mixture and washings of the products. As shown in Fig. 2,

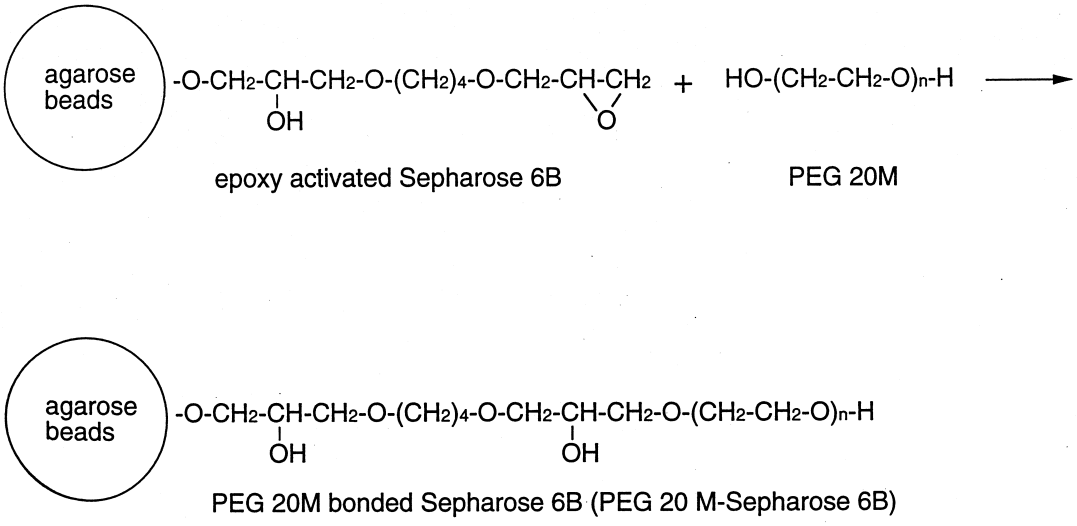

Fig. 1. Preparation of PEG 20M-bonded Sepharose 6B column packings.

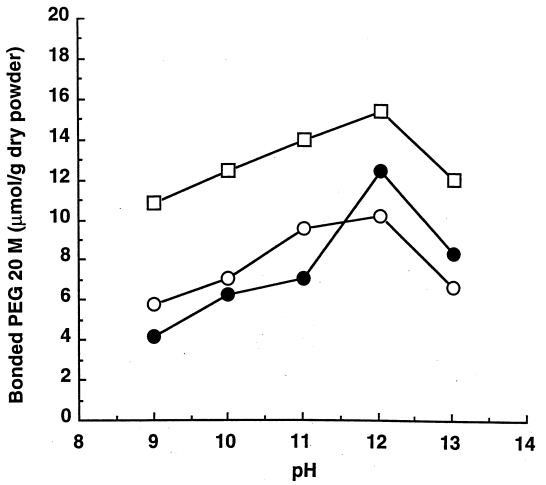

Fig. 2. Effect of pH of the coupling solution on the amount of bonded PEG 20M to epoxy-activated Sepharose 6B at 32.5°C (●), 40°C (□) and 45°C (○). Carbonate–hydrogen carbonate buffers were used in the pH range from 9 to 11 and sodium hydroxide solution were used for pH 12 and 13.

the optimum coupling condition for PEG 20M was a 16-h period at 40°C in a solution of pH 12.0. The amount of PEG coupled under the conditions as the bonded stationary phase was 15.7 μmol per gram of dry powder (314 mg per gram of the dry powder). In addition, the amount of PEG 20M bound was also expressed by means of the carbon and nitrogen content of the products, the latter obtained from the hydroxyethylamino substituent which was introduced by treatment with 1 *M* 2-aminoethanol after the coupling, for the purpose of blocking residual free epoxy groups. The analysis of the dried product prepared under optimal conditions was C: 47.1, H: 6.6 and N: 0.3%. The carbon content was the highest and the nitrogen content the lowest compared with the contents in the products prepared according to the bisoxirane coupling conditions shown in Fig. 2. On the other hand, epoxy-activated Sepharose 6B hydrolysed under optimal coupling conditions gave values of C: 45.1, H: 6.7 and N: 0.5% as a control.

The PEG 400-, 4000-, 6000- and 20M-Sepharose 6B column packings were also prepared under the same conditions as above.

2.2. PPG–Chromagel A4 column packings

Oxirane coupling of diglycidyl ether of PPG 200, 400 and 950 to agarose beads (Chromagel A6) was performed by a method similar in principle to that of Hjertén et al. [26]. Fig. 3 shows the oxirane coupling with agarose and hydrolysis of the residual epoxy groups. A 100-ml volume of wet agarose beads was transferred to a glass filter funnel and washed with 100-ml portions of each solvent, water–dioxane (4:1), water–dioxane (3:2), water–dioxane (2:3),

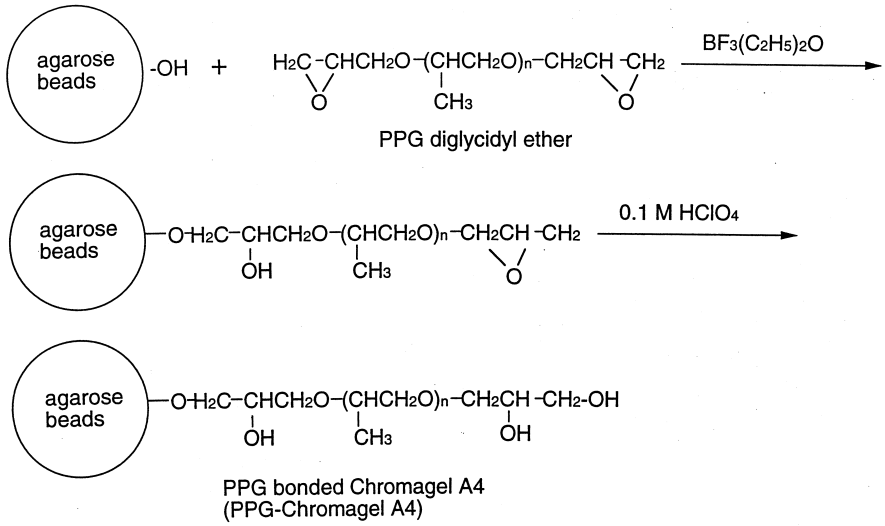

Fig. 3. Preparation of PPG-bonded Chromagel A4 column packings.

water–dioxane (1:4) and seven times with dioxane. After exchanging the water in the gel beads with dioxane, the beads were transferred to a reaction vessel equipped with a stirrer, and 100 ml of dioxane and 2 ml of 48% boron trifluoride etherate in diethyl ether were added. The mixture was stirred for 5 min and the following amounts of PPG diglycidyl ether in 10 ml of dioxane were added dropwise: PPG 200 diglycidyl ether, 1.1 g (3.5×10^{-3} mol); PPG 400 diglycidyl ether, 7.1 g (1.4×10^{-2} mol); and PPG 950 diglycidyl ether, 20.8 g (2.0×10^{-2} mol). The reaction mixture was stirred for a further 40 min at room temperature. After the coupling reaction, the products were separated by filtration, and were shaken with 0.1 *M* perchloric acid for 1 h at room temperature to hydrolyse the residual epoxy groups. The prepared PPG-bonded Chromagel A4 was transferred to a glass filter funnel and washed with dioxane, followed by dioxane–water according to the same procedure as described above but in the reverse order, and finally washed with distilled water.

The determination of the amount of bonded PPG was performed as follows [24]: PPG-Chromagel beads were carefully washed with acetone on a glass filter-funnel and about 5 g of the beads were dried in vacuo (P_2O_5) overnight at 70°C. A 40-ml volume of 88% (w/v) formic acid was added to about 200 mg of dried gels and the mixture was heated for about 4 h on a boiling water-bath for complete hydrolysis and then evaporated to dryness under reduced pressure at 70°C. The hydrolysed product was dissolved in ^2H_2O and lyophilized. This deuterium-exchange procedure was repeated three times. Finally, 100 mg of lyophilized powder was weighed and dissolved in 0.5 ml of [$^2H_6$] dimethyl sulfoxide for ^1H NMR measurement. The oxypropylene residue content of PPG in the gel (mg/g dry powder) was obtained from the integrated peak intensity of the methyl proton (Fig. 4A) using a calibration graph (Fig. 4B).

2.3. MEM–Cellulofine GH 25, MEM–Sephadex G25 column packings

(β-Methoxyethoxymethyl) triethylammonium chloride (MEM TEACl) is known as a reagent which can protect the hydroxy groups of several compounds during chemical reactions. The hydroxy groups of the glucose in the Cellulofine and Sephadex were modified with MEM TEACl as follows [26]: 1 g of dry Cellulofine GH-25 or Sephadex G25 was mixed with 10 ml of acetonitrile containing 0.3–5.0 g (0.003–0.02 *M*) of MEM TEACl and refluxed for 6 h. After the reaction, the coupled products were washed on a sintered glass filter funnel successively with water and sieved to obtain a uniform size of beads (usually 105–210 μm). The MEM–Cellulofine GH-25 and the MEM–Sephadex G25 were hydrolyzed with 20.2% hydrochloric acid–d_2 (^{2}HCl) and the amount of MEM groups was determined by NMR spectroscopy. The methoxy proton signal from

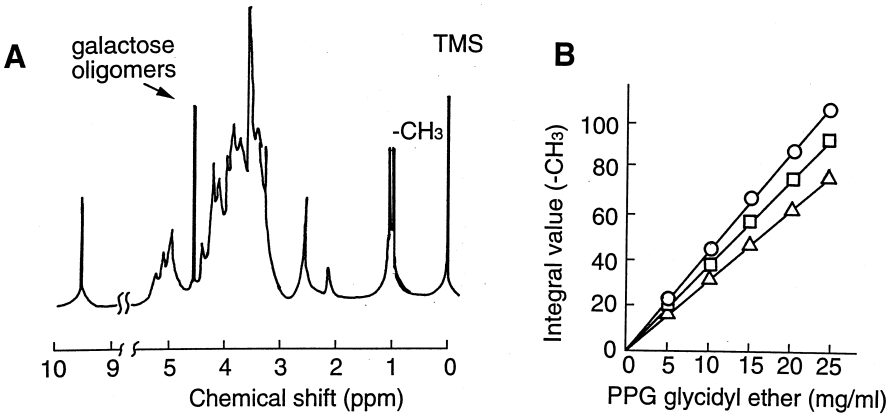

Fig. 4. ^1H NMR spectrum of hydrolysed PPG 400–Chromagel A4 in [$^2H_6$] dimethyl sulphoxide at 200 MHz (A) and calibration graphs of integral value of methyl proton of PPG 200 (○), 400 (□) and 950 (△) glycidyl ethers (B). Measurement conditions: spectral width, 2000 Hz; pulse width, 5 μs; pulse recycle time, 20 s; accumulation, 4 times.

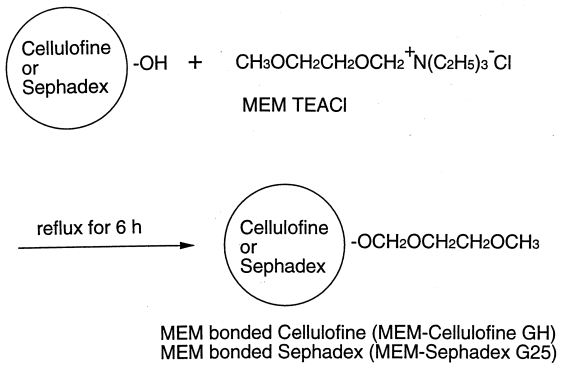

Fig. 5. Preparation of MEM-bonded Cellulofine and Sephadex column packings.

the MEM group is observed at ca. 4.0 ppm (data not shown). The peak area of the methoxy proton signal was determined from a calibration graph obtained with a series of concentrations of MEM TEACl as a standard.

MEM groups were directly coupled to the hydroxyl groups in the Cellulofine and Sephadex as shown in Fig. 5. The coupling conditions depend greatly on the amount of MEM TEACl added to the reaction mixture. The contents of MEM groups in the MEM–Cellulofine, MEM–Sephadex range from 0.37 to 0.48, and from 1.25 to 1.95 mmol MEM groups in one gram of the dry powder, respectively. The packing with the highest MEM content is favorable for the retention of blood cells, especially platelets. Thus the packing having the highest content of MEM groups was used for the chromatographic selective separation of blood cells.

3. Collection of blood cells from human peripheral blood

Human blood was drawn from normal male adult donors by venous puncture and 0.05 ml of a 1000 U/ml heparin was added to 10 ml of blood except for the collection of platelets. Siliconized glassware was used in the all procedures.

3.1. Erythrocytes

After whole blood was centrifuged at 500 *g* for 10 min, the supernatant and the buffy coat layer were removed. The cells were washed three times with 0.9% (w/v) saline.

3.2. Platelets

A centrifugation isolation technique based on that of Leeksma and Cohen [27] was employed. A 10-ml volume of whole blood was mixed with 1 ml of 1% (w/v) disodium ethylenediamine tetraacetic acid in 0.9% (w/v) saline, and was centrifuged at 65 *g* for 20 min after filtration through an absorbent gauze. The supernatant (platelet-rich plasma, PRP) was pipetted into another tube and centrifuged at 250 *g* for 20 min and the sedimented platelets were collected and washed twice with 0.9% (w/v) saline.

3.3. Granulocytes

The sodium metrizoate–dextran sedimentation technique of Bøyum was used [28]. A mixture of 10 ml of blood and 2 ml of 0.9% physiological saline was carefully layered on 10 ml of a mixed solution of 32.8% (w/w) sodium metrizoate (*N*-methyl-3,5-diacetamido-2,4,6-triiodobenzoate)–6% (w/w) dextran T500 (1:2). The system was allowed to stand for 1–2 h until the sedimentation of erythrocytes was complete. The supernatant was centrifuged at 130 *g* for 10 min and precipitated cells were then washed three times with 5-ml volumes of saline.

3.4. Lymphocytes

A sodium metrizoate–Ficoll sedimentation technique based on that of Thorsby and Bratlie was used [29]. A 5-ml volume of blood was defibrinated by rotating the test tube vertically for 10 min with five glass spheres of 5-mm diameter, and 5 ml of the defibrinated blood were mixed with an equal volume of saline in a centrifuge tube and carefully layered on 7.5 ml of Lymphoprep [a mixture of 9.6% (w/v) sodium metrizoate and 5.6% (w/v) Ficoll; Nyegaard, Oslo, Norway]. The tube was centrifuged at exactly 400 *g* for 30–40 min. The lymphocytes layer was separated and the cells were suspended with about 2 ml of saline and centrifuged at 500 *g* for 10 min. The precipitated cells were washed three times with five volumes of saline.

3.5. Leukocyte-rich plasma

The blood was centrifuged at 65 *g* for 15 min. After the erythrocytes were all sedimented, the supernatant (leukocyte-rich plasma, LPP) was centrifuged at 250 *g* for 20 min. Most of the sediment consisted of platelets, granulocytes and lymphocytes.

4. Determination of the isoelectric point of human peripheral blood cells

It has been reported that the cross-partition in aqueous two-phase systems [30] can be applied to the determination of the isoelectric point not only of soluble proteins but also of subcellular organelles [11,31]. It has been shown that the cross-partition method can also be applied to determine the isoelectric point of human erythrocytes, platelets, granulocytes and lymphocytes. Further, on the basis of the retention behavior of the blood cells at their isoelectric point pH ($pH_{c.p.}$), it may be possible to characterize the hydrophobic interactions between the cell surfaces and the bonded PEG stationary phases.

The partition phase systems used were as follows [32].

System A_1: 7.5% (w/w) of dextran T500, 7.5% (w/w) of PEG 4000, 90 mmol/kg of sodium phosphate buffer and 30 mmol/kg of sodium chloride.

System A_2: The same composition as in system A_1 but without the sodium chloride.

System B_1: 7.5% (w/w) of dextran T500, 7.5% (w/w) of PEG 4000, 10 mmol/kg of lithium phosphate buffer and 120 mmol/kg of lithium chloride.

System B_2 : The same composition as in system B_1 but with 60 mmol/kg of lithium sulfate instead of the lithium chloride.

System C_1: 6% (w/w) of dextran T500, 6% (w/w) of PEG 4000, 320 mmol/kg of sucrose, 5 mmol/kg of sodium citrate–phosphate buffer and 100 mmol/kg of sodium chloride.

System C_2: The same composition as in system C_1 but with 50 mmol/kg of sodium sulfate instead of the sodium chloride.

The pH of system A ranged from 5.0 to 7.5, and of systems B and C from 4.0 to 7.0. Each system was thoroughly mixed and portions were pipetted into each tube. A number of 1.3×10^6–1.7×10^6 erythrocytes were added to systems A_1 and A_2. A number of 1.2×10^5–3.3×10^5 platelets or 2.7×10^5–6.5×10^5 granulocytes were also added to systems B_1 and B_2. A number of 1.2×10^5–2.3×10^5 lymphocytes were added to the systems C_1 and C_2.

The phase systems were thoroughly mixed by inverting the test-tubes, which were allowed to stand for 20 min. After phase separation, 0.1 ml of the upper phase was carefully pipetted from the systems in each tube and diluted with 10 ml of Isoton (aqueous electrolyte diluent for blood cells counting; Coulter Diagnostics, Hialeath, FL, USA). The number of cells distributed in the upper phase was counted with a Coulter counter. The distribution of blood cells in the two-phase systems was expressed as the percentage of the number in the upper phase with respect to the total added to the systems.

In Fig. 6, the percentage of the number of cells in the upper phase of the two-phase systems composed of 7.5% (w/w) dextran T500–7.5% (w/w) PEG 4000 containing several salts are plotted against the pH value of the systems. The two sets of the curves in each instance correspond to the two different salt solutions of the two-phase systems.

Human platelets and granulocytes were only slightly partitioned in the PEG-rich upper phase of the dextran T500-PEG 4000 phase systems containing sodium phosphate (A_1 and A_2) because of their low surface charge compared with that of lymphocytes and erythrocytes (data not shown). To obtain reasonable partition into two-phases, we used two sets of lithium chloride and lithium sulfate systems (B_1 and B_2, respectively) for platelets and granulocytes (Fig. 6b and c). For the same reason, systems containing chloride and sulfate buffered with sodium citrate–phosphate were chosen as appropriate two-phase systems (C_1 and C_2) for lymphocytes (Fig. 6d).

Sulfate, phosphate and citrate are known to increase the partition coefficient of negatively charged proteins in PEG phases, as a consequence of the interaction between dextran and these anions. It has also been shown that the value of the cross-point determined from the intersection of two curves varies only slightly with different salts in the partition systems.

The partition curves of erythrocytes in the PEG-rich upper phase in systems A_1 and A_2 cross with

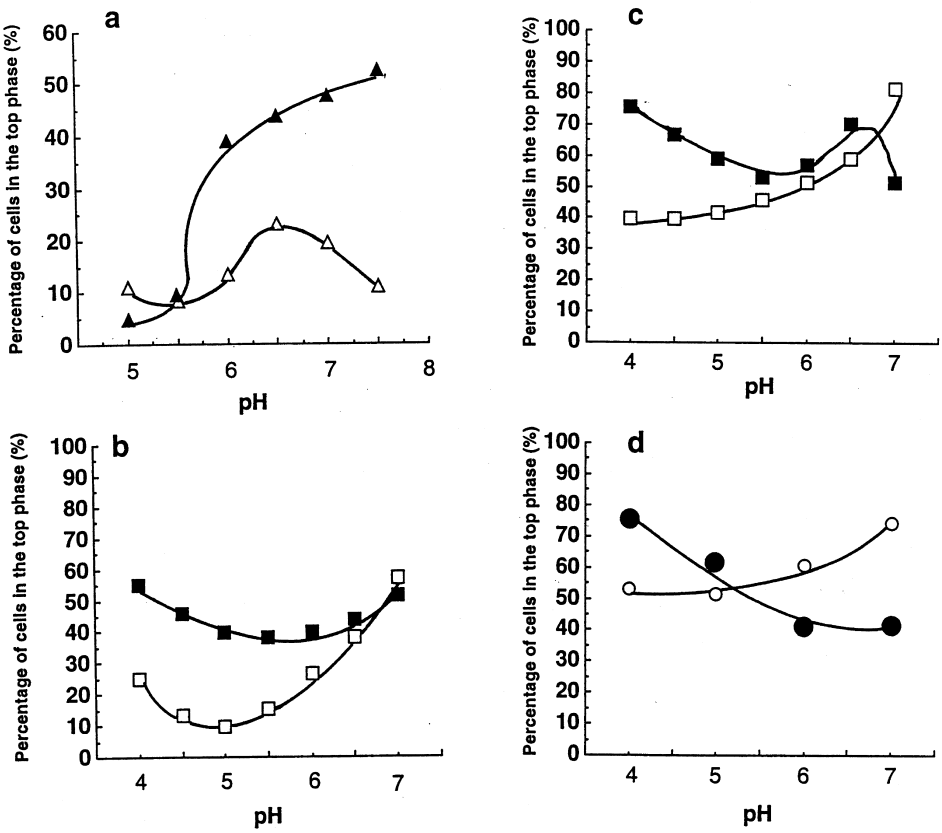

Fig. 6. Cross-partition curves of human erythrocytes (a), platelets (b), granulocytes (c) and lymphocytes (d). Relative ratio as a percentage of the cells in the upper phase is plotted as a function of the pH of the two-phase systems. Systems A_1 and A_2 for erythrocytes containing NaCl (△) or nothing (▲). Systems B_1 and B_2 for platelets and granulocytes containing LiCl (□) or Li_2SO_4 (■). Systems C_1 and C_2 for lymphocytes containing NaCl (○) or Na_2SO_4 (●).

each other at pH 5.5 (Fig. 6a). The symbol $pH_{c.p.}$ is used to express the pH value at this cross-point. The $pH_{c.p.}$ values for erythrocytes is 5.5, for platelets and granulocytes are 6.8 and for lymphocytes, is 5.2 respectively.

5. Chromatography of human peripheral blood cells on PEG-Sepharose column

Each PEG 400, 4000, 6000 and 20M-Sepharose 6B column packing was suspended in each of the mobile phases, and packed into the column (25×0.9-cm I.D.) with the slurried packing. The column was thoroughly washed with the mobile phase to equilibrate the chemically bonded PEG phase, using a peristaltic pump. To obtain the retention volume of the blood cells, the total amount of platelets, granulocytes, lymphocytes and erythrocytes prepared as above was suspended in 0.6 ml of the mobile phase. A 0.5-ml volume of each cell suspension containing 6.6×10^5–2.4×10^6 platelets, 1.4×10^5–2×10^5 granulocytes, 9.5×10^4–1.5×10^5 lymphocytes or 2.5×10^4–3.4×10^4 erythrocytes was independently loaded on the column, and the column was eluted with each of the mobile phase. In the case of the separation studies, the above cells are mixed artificially and loaded on the column. The chromatography was

performed at room temperature. A flow-rate of 3–12 ml/h was maintained by the use of the peristaltic pump. The absorbance of the eluate at the desired wavelength was monitored continuously with a effluent monitor. The fractions were collected in glass vials every 10 or 15 min, the volume of each fraction being about 0.75–2.0 ml. Each fraction was diluted with 5–10 ml saline and the number of blood cells was counted with a Coulter counter. The recovery of the eluted cells was calculated from the combined number in each fraction compared with the cells loaded on the column.

The viability of the cells eluted from the column was checked by trypan blue dye exclusion. A 1-ml volume of isotonic dye solution (4 mg/ml) was added to the 1-ml fractions obtained by chromatography. The mixture was allowed to stand for 5 min until the dead cells were stained. The viable cells were counted under the microscope. In the case of the platelets, the capability of aggregation was checked by microscopy. A 25-μl volume of thrombin solution containing 1.25 U of enzyme was added to a 0.2-ml volume of the chromatographed platelets fraction. The aggregation of platelets was observed under microscopy.

5.1. Effect of the mobile phase composition on the retention volume of blood cells

5.1.1. Concentration and molecular mass of dextran in the mobile phase

Table 1 shows the retention volumes and separation factors for erythrocytes, platelets, granulocytes and lymphocytes eluted independently from the PEG 20M-Sepharose column. All reported retention volumes of the blood cells are the average of triple or further examinations. It can readily be seen that the elution order of the blood cells from the column is erythrocytes, platelets, granulocytes and lymphocytes, except for the use of the mobile phase V in which dextran was absent. Among the various factors investigated, the concentration and the molecular mass of dextran in the mobile phase had a significant effect on the retention volumes of the blood cells. As shown in Table 1, the retention volumes of erythrocytes, platelets and granulocytes increased with decreasing the concentration of dextran T40 from 8.0 (mobile phase I) to 4.5% (II). However, except for the granulocytes eluted with the mobile phase III, when the concentration of dextran T40 and T500 was decreased from 4.5 (mobile phase II or VI) to 2.0%

Table 1
Effect of mobile phase composition on the retention volumes and the separation factors of human erythrocytes (*e*), platelets (*p*), granulocytes (*g*) and lymphocytes (*l*)

Mobile phase	Concentration (%, w/w)			Retention volume (ml)				Separation factor		
	Dextran T40	Dextran T500	DEAE–dextran	*e*	*p*	*g*	*l*	*p*/*e*	*g*/*p*	*l*/*g*
I	8.0	–	–	3.8	7.6	8.3	17.5	2.00	1.09	2.10
II	4.5	–	–	5.4	8.9	9.0	16.5	1.64	1.01	1.83
III	2.0	–	–	5.8	7.4	13.2	16.2	1.28	1.78	1.23
IV	1.0	–	–	5.6	5.7	6.1	10.6	1.02	1.07	1.74
V	–	–	–	5.1	5.7	6.2	4.9	1.12	1.15	1.04 (e/l)
VI	–	4.5	–	ads	11.6	12.1	18.0	–	1.04	1.49
VII	–	2.0	–	ads	8.3	10.6	12.5	–	1.28	1.18
VIII	4.5	–	0.5	8.7[a]	8.6	14.0	15.4	0.99	1.63	1.10
IX	2.0	–	0.5	5.9[a]	11.0	15.4	18.8	1.86	1.40	1.22
X	–	2.0	0.5	10.3[a]	10.3	12.6	14.8	1.00	1.22	1.17

Column: PEG 20M-bonded Sepharose 6B (25×0.9-cm I.D.).
Mobile phases I–X containing 0.09 *M* potassium phosphate at pH 7.4.
ads: adsorbed on the column.
[a] Adsorbed a portion of the erythrocytes at the top of the column.

(III or VII), the retention volumes of platelets and lymphocytes were decreased. The retention volumes of erythrocytes, platelets and granulocytes became very similar with the mobile phase IV containing 1.0% (w/w) dextran T40, whereas lymphocytes were still appreciably retained with the eluent. Obviously, in the absence of dextran in the mobile phase (mobile phase V), all cells were only weakly retained on the stationary phase.

Among the seven kinds of the mobile phase which contained no DEAE–dextran (mobile phase I–VII), mobile phase III, containing 2.0% (w/w) of dextran T40 produced the best separation factor of 1.78 between platelets and granulocytes, whereas that of erythrocytes and platelets was 1.28. An increase in the molecular mass of dextran from T40 (mobile phase II or III) to T500 (VI or VII) appreciably increased the retention of the platelets and decreased that of lymphocytes. The best separation factors between granulocytes and lymphocytes of 2.10 and that between erythrocytes and platelets of 2.00 were obtained by use of the mobile phase I.

In order to demonstrate the capability of the PEG 20M–Sepharose column, an artificial mixture of human granulocytes and lymphocytes was chromatographed, using mobile phase I. As shown in Fig. 7, the two kinds of cells were well separated and eluted within 10 h. The recoveries of granulocytes and lymphocytes from the column were 67 and 82% of the number of the cells applied on the column.

Fig. 8 shows an elution pattern for a synthetic mixture of human erythrocytes and lymphocytes

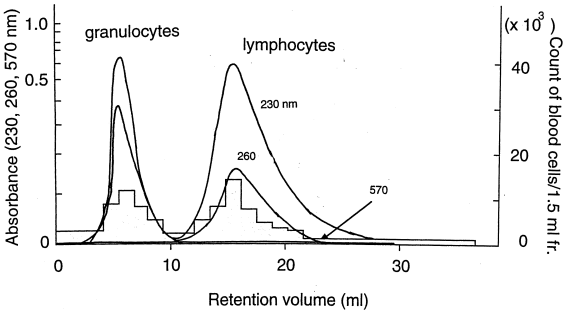

Fig. 7. Elution profile of human granulocytes and lymphocytes. Column: PEG 20M–Sepharose 6B (25×0.9-cm I.D.); eluent: 8% (w/w) dextran T40–0.09 *M* sodium phosphate buffer at pH 7.4; flow-rate: 3.0 ml/h; sample: a mixture of 2.4×10^4 granulocytes and 3.9×10^4 lymphocytes in 0.5 ml of the eluent.

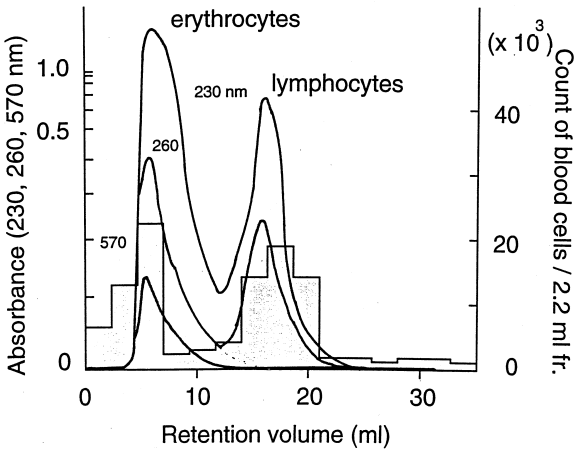

Fig. 8. Elution profile of human erythrocytes and lymphocytes. Column: PEG 20M–Sepharose 6B (25×0.9-cm I.D.); eluent: 8% (w/w) dextran T40–0.09 *M* sodium phosphate buffer at pH 7.4; flow-rate: 4.4 ml/h; sample: a mixture of 2.3×10^4 erythrocytes and 5.8×10^4 lymphocytes in 0.5 ml of the eluent.

obtained by using the same column and the mobile phase. The two kinds of cells were fairly well separated and eluted within about 4.6 h. The recovery of the erythrocytes from the column was 50%.

The selectivity of the chemically bonded PEG 20M stationary phase for chromatographic separation is further illustrated in Fig. 9, which shows an elution profile for a synthetic mixture of human erythrocytes, granulocytes and lymphocytes using mobile phase I. Three kinds of blood cells were well separated within 13.5 h.

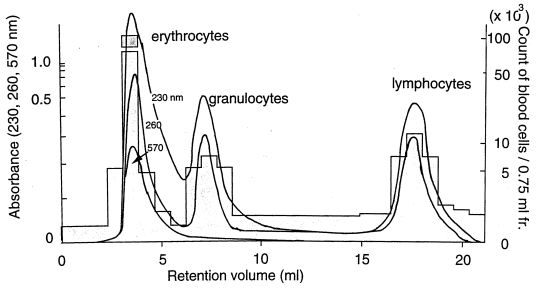

Fig. 9. Elution profile of human erythrocytes, granulocytes and lymphocytes. Column: PEG 20M–Sepharose 6B (25×0.9-cm I.D.); eluent: 8% (w/w) dextran T40–0.09 *M* sodium phosphate buffer at pH 7.4; flow-rate: 1.5 ml/h; sample: a mixture of 1.9×10^6 erythrocytes, 2.6×10^4 granulocytes and 2.7×10^4 lymphocytes in 0.5 ml of the eluent.

5.1.2. Effect of DEAE–dextran in the mobile phase on the retention volume of the blood cells

In order to examine the influence of polycationic polymer in the eluents, three kinds of mobile phases containing 0.5% (w/w) DEAE–dextran in addition to either dextran T40 or T500 were also used. It has been pointed out in APTP systems that dextran–PEG systems can be modified by incorporation of DEAE–dextran to enhance the separations based on the surface charge of mammalian erythrocytes of a number of species [13,33]. Table 1 shows that the addition of 0.5% (w/w) of DEAE–dextran to a mobile phase containing 4.5% (w/w) dextran T40 (mobile phase VIII) decreased the retention volumes of platelets and lymphocytes and decreased the separation factors between platelets and erythrocytes, and between granulocytes and lymphocytes as compared with those obtained with the mobile phase II. On the other hand, the cationic polymer increased the retention of all cells except for erythrocytes in the mobile phases containing 2.0% (w/w) of either dextran T40 (mobile phase IX) or T500 (mobile phase X) in comparison with those values obtained with mobile phase III or VII. In every case, the separation factor between platelets and granulocytes was appreciably decreased because of the increased retention volume of the platelets. The usefulness of the mobile phase systems containing DEAE–dextran was demonstrated by the fact that one can discriminate between erythrocytes and platelets due to the retarded elution of the latter cells when using the mobile phase IX.

5.1.3. Effect of sodium chloride in the mobile phase on the retention volume of the blood cells

The effect of neutral salts, such as sodium chloride, in the mobile phases on the retention of the human peripheral blood cells was investigated. Each suspension of platelets, granulocytes and lymphocytes was eluted independently from the PEG 20M–Sepharose column (25×0.9-cm I.D.) with the mobile phase composed of 2% (w/w) dextran T40, sodium chloride and sodium phosphate buffer at pH 7.4. The retention volume of platelets, granulocytes and lymphocytes were plotted against the concentration of sodium chloride in the mobile phase (Fig. 10). An increase in the sodium chloride concentration was accompanied by a concomitant decrease in the phosphate buffer concentration to maintain the isotonicity of the mobile phase. As the concentration of sodium chloride increased in the mobile phase, the retention volume of the platelets decreased. On the other hand, the retention volumes of the granulocytes and the lymphocytes decreased using the mobile phase containing 0.07 *M* NaCl, and a portion of these cells were adsorbed on the column. In particular, the affinity of granulocytes and lymphocytes for the stationary phase was significantly increased as the NaCl was increased to the highest concentration of 0.13 *M*.

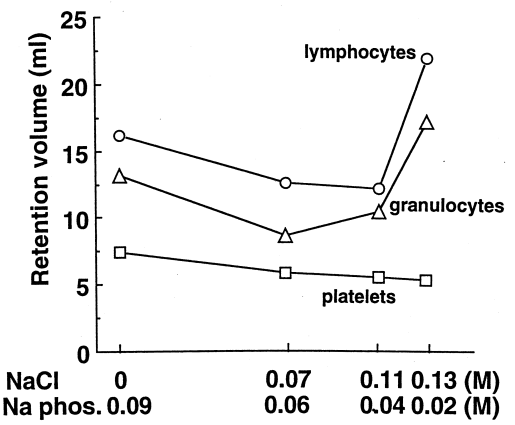

Fig. 10. Influence of sodium chloride concentration on the retention volume of human platelets (□), granulocytes (△) and lymphocytes (○). Column: PEG 20M–Sepharose 6B (25×0.9-cm I.D.); eluent: 2% (w/w) dextran T40–0.09 *M* sodium phosphate buffer at pH 7.4.

The effect of the addition of NaCl to the mobile phase shows that the negative charge on the cell surface is one determinant of the retention of blood cells on the column. The contribution of hydrophobic interactions between the surface of the granulocytes and lymphocytes and PEG-bonded phase should be taken into consideration.

5.2. Effect of the molecular mass of PEG stationary phase on the retention of human blood cells

In the previous section, addition of the neutral salts to the mobile phase while maintaining the isotonicity resulted in an increase in the retention volume of granulocytes and lymphocytes. In the

hydrophobic affinity chromatography of serum albumin and enzymes, it is well known that the addition of neutral salts to the mobile phase increases the hydrophobic interaction between the stationary phase and the proteins. It is presumed that the hydrophobic interactions partly contribute to the retention of blood cells, especially granulocytes and lymphocytes on the PEG 20M–Sepharose 6B column.

To confirm the contribution of hydrophobic interactions to the retention of blood cells, PEGs with different molecular mass (PEG 400, 4000, 6000 and 20M) are coupled to the epoxy-activated Sepharose 6B. The affinity of the blood cells to the bonded PEG stationary phase was investigated by using the four kinds of column packings, which differ from one another in hydrophobicity.

Table 2 shows the effects of the molecular mass of bonded PEG stationary phase on the retention volume and the separation factors of human blood cells using the mobile phase at pH 7.4. It can be seen that the order of elution from the column is erythrocytes, platelets, granulocytes and lymphocytes except for a few cases. The retention volumes of the four kinds of blood cells increase along with increasing the molecular mass of bonded PEG. Good separation factors between erythrocytes and platelets and between granulocytes and lymphocytes were obtained with the use of the PEG 20M–Sepharose 6B column. The bonded PEG phase of higher molecular mass seems to give a better separation than the lower one.

In practice, however, because of the negative charge of the surface of the blood cells is based predominantly on sialic acid, there is a risk that electrostatic interactions may occur between the cells and the bonded PEG stationary phase in which inorganic electrolytes are distributed unequally relative to the mobile phase. On the basis of the retention behavior of the blood cells at their isoelectric point pH values, it may be possible to characterize the hydrophobic interactions between the cells and the bonded PEG stationary phase.

Table 2
Retention volumes and separation factors of human erythrocytes (*e*), platelets (*p*), granulocytes (*g*) and lymphocytes (*l*) using different molecular mass of PEG as the bonded phase

Bonded phase	Retention volume (ml)				Separation factor		
	e	*p*	*g*	*l*	*e*/*p*	*p*/*g*	*g*/*l*
PEG 400	4.7	5.6	9.4	10.3	1.19	1.68	1.10
PEG 4000	5.3	5.5	11.1	11.4	1.04	2.02	1.03
PEG 6000	5.3	6.4	11.3	12.7	1.20	1.77	1.12
PEG 20M	5.8	7.4	13.2	16.2	1.28	1.78	1.23

Column: PEG-bonded Sepharose 6B (25×0.9-cm I.D.).
Mobile phase: 2% (w/w) dextran T40–0.09 *M* potassium phosphate at pH 7.4.

The $pH_{c.p.}$ of blood cells determined by the cross-partition method is at pH 5.5 for erythrocytes, pH 6.8 for platelets and granulocytes and pH 5.2 for lymphocytes (Fig. 6). The retention volumes of human blood cells, such as erythrocytes, platelets, granulocytes and lymphocytes under the absence of the electrostatic interaction were obtained at their $pH_{c.p.}$ and compared with the retention volumes obtained at pH 7.4. The retention volumes of the four kinds of blood cells at pH 7.4 and $pH_{c.p.}$ are plotted against the molecular mass of PEGs bonded on the Sepharose columns (Fig. 11). It can be seen that the retention volumes of erythrocytes, granulocytes and lymphocytes obtained at pH 7.4 are greater than those at $pH_{c.p.}$. There are a few exceptions for platelets with the PEG 4000–Sepharose and for lymphocytes with the PEG 20M–Sepharose column. When using PEG of higher molecular mass, such as PEG 6000 and 20M, as the bonded stationary phase, the retention volume of platelets obtained at pH 7.4 is higher than that obtained at $pH_{c.p.}$ 6.8. It seems that the retention volume of granulocytes shows a similar tendency when the molecular mass of bonded PEG is increased from 6000 to 20M. The retention volume of granulocytes increases with increasing molecular mass of bonded PEG from 400 to 6000 at $pH_{c.p.}$ 6.8, and that of lymphocytes also increases more markedly at $pH_{c.p.}$ 5.2. The above results suggest that the affinity between blood cells and the chemically bonded PEG phase at $pH_{c.p.}$ can be interpreted in terms of the hydrophobic interactions between the cells and the stationary phases.

6. Chromatography of human peripheral blood cells on PPG–Chromagel columns

In the previous section, the retention volumes of granulocytes and lymphocytes showed a marked dependence on the molecular mass of the PEG

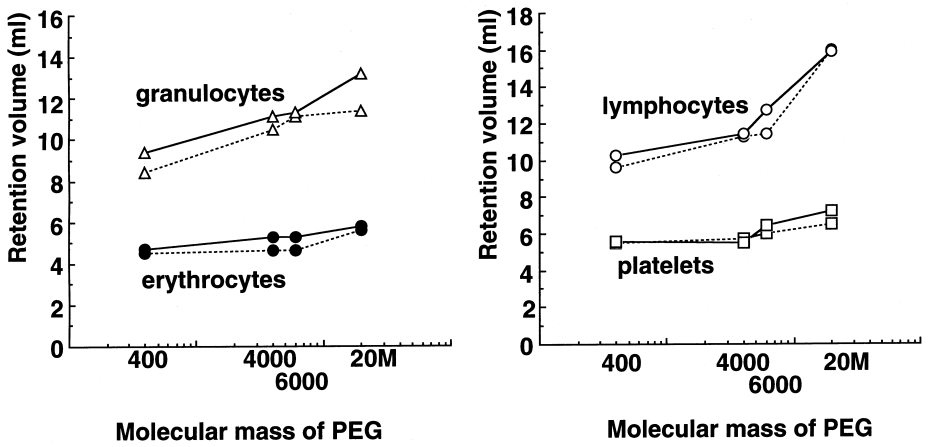

Fig. 11. Effect of pH of the mobile phase on the retention volumes of human erythrocytes (●), platelets (□), granulocytes (△) and lymphocytes (○). Solid lines indicate the changes of the retention volumes of blood cells at pH 7.4. Dotted lines indicate those obtained at $pH_{c.p.}$.

stationary phase at the isoelectric points of blood cells, increasing with increasing the molecular mass of PEG.

In this section, to confirm the hydrophobic interaction between the surface of the granulocytes, lymphocytes and bonded stationary phase, polypropylene glycol (PPG), which is more hydrophobic than PEG, bonded Chromagel packings were prepared and the relationship between the retention volumes of blood cell and the oxyethylene residue contents were discussed.

Table 3 shows the retention volumes of human blood cells determined by eluting from the PPG 200-, 400- and 950–Chromagel A4 with 0.09 *M* sodium phosphate buffer ($pH_{c.p.}$) containing 2% (w/w) dextran T40. Granulocytes and lymphocytes show a strong affinity for the column packings that have the highest oxypropylene residue contents (Nos. 3, 6 and 9) in each group (Nos. 1–3, 4–6 and 7–9). Lymphocytes especially show a notable increase in the retention on the columns that have about 16 oxypropylene units (PPG 950) (Nos. 7–9). Further, the retention volumes of the four kinds of blood cells were determined on the columns packed with PPG–Chromagels having nearly the same oxypropylene residue contents with a different number of oxypropylene units (Nos. 2, 5 and 7). As the number of oxypropylene units increases from about 7 (No. 2) to 9 (No. 5), the retention volumes of granulocytes increase, but those of lymphocytes remain unaltered. However, the retention volumes of erythrocytes and platelets exhibit only small but non-systematic changes with regard to the oxypropylene residue content and the number of oxypropylene units.

In conclusion, it is clear that hydrophobic interactions play an important role in retaining the four kinds of blood cells at $pH_{c.p.}$ and PPG–Chromagel A4 columns retain both granulocytes and lymphocytes sufficiently even if they contain a small amount of oxypropylene residues. On the other hand, ery-

Table 3
Retention volumes of human peripheral erythrocytes (*e*), platelets (*p*), granulocytes (*g*) and lymphocytes (*l*)

No.	Bonded phase	Bonded PPG (mg/g dry gel)	Retention volume (ml)			
			e	*p*	*g*	*l*
1	PPE 200	15.5	4.9	5.7	7.3	15.6
2		37.0	5.0	5.1	8.2	16.2
3		86.6	5.0	4.6	11.8	20.2
4	PPG 400	6.8	4.6	4.5	9.5	14.4
5		36.9	4.7	5.0	10.1	16.3
6		73.8	4.6	4.5	12.7	16.5
7	PPG 950	36.4	4.8	5.8	10.4	16.3
8		72.0	3.8	5.4	10.6	18.9
9		100.6	4.8	7.3	15.4	26.2

Column: PPG-bonded Chromagel A4 (25×0.9-cm I.D.).
Mobile phase: 2% (w/w) dextran T40–0.09 *M* potassium phosphate at isoelectric point of each cell.

throcytes and platelets are retained slightly on these columns but are not separated from each other. It is thought that the hydrophobicity of the PPG–Chromagel beads is too low to separate these two kinds of cells.

7. Chromatography of human peripheral blood cells on MEM-bonded columns

The chromatographic separations of human granulocytes and lymphocytes were achieved by using polyethylene glycol (PEG) and polypropylene glycol (PPG)-bonded agarose columns with a phosphate buffered mobile phase containing dextran. However, the separation of erythrocytes and platelets could not be achieved on these column packings because the retention volumes of erythrocytes and platelets are too small.

In this section, methoxyethoxymethyl (MEM) groups, which are similar to the two oxyethylene groups of PEG, were introduced into two types of column packings, such as Cellulofine and Sephadex, used for size-exclusion chromatography. Cellulofine (cellulose beads) consists of a D-glucose polymer in which the D-glucose units are bound in β(1→4) linkages. Sephadex (cross-linked dextran beads) consists of a D-glucose polymer in which the linkage between the D-glucose units is α(1→6). The selective separation of human peripheral blood cells was investigated by using MEM-bonded Cellulofine GH-25-c (MEM–Cellulofine) and MEM-bonded Sephadex G25 (MEM–Sephadex) columns. Isotonic mono- and disaccharides and methyl-α-D-pyranosides solutions were used as the mobile phases. The elution behavior of human peripheral blood cells, especially platelets, granulocytes and lymphocytes, on the MEM-bonded column packings and the recoveries of these cells from the columns were investigated.

7.1. MEM–Cellulofine GH-25 column

Since erythrocytes are easily separated from human blood by centrifugation, in the present studies, the separations of human peripheral platelets, granulocytes and lymphocytes were examined. Each suspension of platelets, granulocytes and lymphocytes was chromatographed on a MEM–Cellulofine GH-25 column (25×0.9-cm I.D.). Eight kinds of isotonic solutions including 0.9% (0.154 *M*) sodium chloride solution were used for the mobile phase. Table 4 shows the retention volumes and the recoveries of the platelets, granulocytes and lymphocytes eluted from the MEM–Cellulofine GH-25. The amount of the MEM groups in the packings used in the chromatography is 0.48 mmol/g dry powder. In the Table, 'ads' indicates that no cells could be recovered from the column.

A little difference in the retention volumes of the

Table 4
Retention volumes of human peripheral platelets (*p*), granulocytes (*g*) and lymphocytes (*l*) on MEM-bonded Cellulofine column

Mobile phase[a]	Concentration (*M*)	Retention volume (ml)		
		p	*g*	*l*
NaCl	0.154	5.8 (32.0)[b]	5.5 (6.9)	6.1 (64.7)
Sucrose	0.270	4.2 (100.0)	ads[c]	ads
D-Glucose	0.310	4.4 (88.9)	ads	ads
D-Galactose	0.310	13.4 (64.3)	6.7 (34.6)	12.8 (22.9)
D-Mannose	0.310	13.9 (5.6)	5.5 (44.3)	15.2 (25.4)
Methyl α-D-glucoside	0.300	4.7 (80.6)	ads	ads
Methyl α-D-galactoside	0.300	14.7 (40.0)	5.3 (4.5)	ads
Methyl α-D-mannoside	0.300	6.0 (8.4)	ads	7.1 (7.7)

Column; MEM–Cellulofine GH-25-c (25×0.9-cm I.D.).
The recovery of the eluted cells was calculated from the combined number in each fraction compared with the cells loaded on the column.
[a] Mobile phase is isotonic solution at pH 7.4.
[b] Recoveries of cells from column indicate in parentheses.
[c] ads: no cells are recoverable.

platelets, granulocytes and lymphocytes was obtained by using the physiological saline (0.154 *M* NaCl) as a mobile phase, because only weak interactions between the column packing beads and these three kinds of cell surfaces could occur. On the other hand, when we used the mobile phase which containing 0.27 *M* sucrose, 0.31 *M* D-glucose and 0.30 *M* methyl-α-D-glucoside, the granulocytes and lymphocytes are adsorbed on the column. It can be seen that the selective separation of the platelets is easily performed by using these three mobile phases. The recoveries of the platelets eluted with sucrose, D-glucose and methyl-α-D-glucoside were 100, 88.9 and 80.6%, respectively. The eluted platelets can be aggregated by adding thrombin solution to the chromatographic fraction, then the viability of the platelets was confirmed.

Among the mobile phases containing the mono- and disaccharides and methyl-α-D-pyranosides, it was possible to recover over 20% of the lymphocytes, and 30% of the granulocytes from the column by eluting with both D-galactose and D-mannose isotonic solutions; 0.31 *M* D-mannose gives the highest recovery of 44.3% of the granulocytes. The viability of the eluted granulocytes and lymphocytes was checked by their capability to exclude trypan blue dye. Over 90% of the cells are alive after surface affinity chromatography. In order to demonstrate the applicability of the MEM–Cellulofine column to the selective separation of granulocytes and lymphocytes, a mixture of these blood cells was chromatographed with D-mannose solution. Fig. 12 shows the elution pattern of the granulocytes and the lymphocytes using mannose solution as the mobile phase. Granulocytes and lymphocytes obtained from 10 ml of blood were mixed together and eluted with the mannose solution from the MEM–Cellulofine column (25×0.9-cm I.D.) at a flow-rate of 20 ml/h. The number of cells is indicated in the histogram. The maximum absorbance of the elution curves at 254 and 580 nm coincide with the maximum peaks in the histogram of the cells in the fractions. Retention volumes of 6–7 ml and 12–14 ml, corresponding to the center cuts of the first and the second peaks in the chromatogram, contain over 95% of granulocytes and lymphocytes, as confirmed by giemsa staining. These two types of cells separated well within 1.2 h.

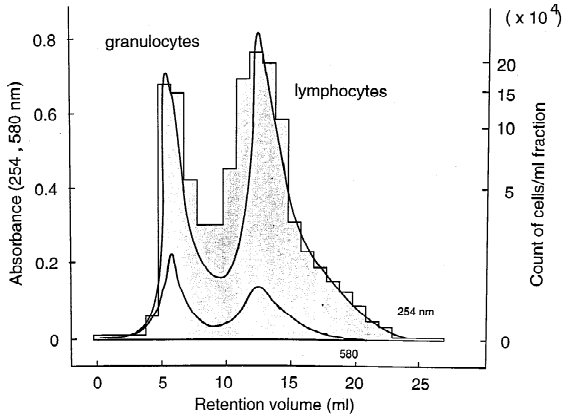

Fig. 12. Elution profile of human peripheral granulocytes and lymphocytes. Column: MEM–Cellulofine GH-25 (25×0.9-cm I.D.); mobile phase: 0.31 *M* D-mannose solution at pH 7.4; flow-rate: 20 ml/h; sample: 1.5-ml cell suspension of granulocytes and lymphocytes.

7.2. MEM–Cellulofine GH-25 column

Table 5 shows the retention volumes and the recoveries of the platelets, granulocytes and lymphocytes from the MEM–Sephadex G25 column (25× 0.9-cm I.D.). The packing with the highest content of MEM groups (1.95 mmol/g dry powder) prepared in this study was used for the chromatography. In the table, 'ads' also means that most of the blood cells adsorbed onto the column. The retention volumes of the platelets, granulocytes and lymphocytes are nearly equal using physiological saline (0.154 *M* NaCl) as a mobile phase. As with the MEM–Cellulofine column, it seems that the interactions between the MEM stationary phase or the surface of the Sephadex beads and the surface of the cells are only very small in the saline solution. The results indicate that the mono- and disaccharides and methyl-α-D-pyranoside are necessary in the mobile phase for the adsorption and separation of human blood cells. Using the methyl-α-D-glucoside and methyl-α-D-mannoside isotonic solutions, platelets were separated selectively, because the interaction between the surfaces of the granulocytes and the lymphocytes and the MEM–Sephadex G25 column packing beads are too strong to elute the cells from the column. The recovery of the platelets using the methyl-α-D-mannoside is 100%, which is higher than

Table 5
Retention volumes of human peripheral platelets (*p*), granulocytes (*g*) and lymphocytes (*l*) on MEM-bonded Sephadex column

Mobile phase[a]	Concentration (*M*)	Retention volume (ml)		
		p	*g*	*l*
NaCl	0.154	5.5 (100.0)[b]	5.6 (30.8)	5.4 (17.9)
Sucrose	0.270	13.4 (72.9)	ads[c]	6.3 (41.4)
D-Glucose	0.310	4.2 (6.3)	ads	13.2 (7.3)
D-Galactose	0.310	ads	6.7 (34.6)	21.8 (8.5)
D-Mannose	0.310	13.9 (14.0)	5.5 (44.3)	5.1 (40.4)
Methyl α-D-glucoside	0.300	14.6 (18.5)	ads	ads
Methyl α-D-galactoside	0.300	13.6 (31.7)	5.3 (4.5)	14.4 (6.6)
Methyl α-D-mannoside	0.300	6.1 (100.0)	ads	ads

Column: MEM–Sephadex G 25 (25×0.9-cm I.D.).
The recovery of the eluted cells was calculated from the combined number in each fraction compared with the cells loaded on the column.
[a] Mobile phase is isotonic solution at pH 7.4.
[b] Recoveries of cells from column indicate in parentheses.
[c] ads: no cells are recoverable.

using a methyl-α-D-glucoside solution. It is found that the methyl-α-D-mannoside isotonic solution is excellent for the purification of platelets on the MEM–Sephadex column. The eluted platelets were also tested by checking their capability to aggregate upon the addition of thrombin solution. More than 90% of the cells viable in this experiment. The highest recovery of 41.4% of the lymphocytes is obtained by elution with the 0.27 *M* sucrose solution. To demonstrate the capability of the MEM–Sephadex column packings to separate and purify the platelets and lymphocytes from human blood, human leukocyte-rich plasma was eluted from the column by an isotonic sucrose solution. Fig. 13 shows the elution pattern of the human leukocyte-rich plasma on the MEM–Sephadex column (25×0.9-cm I.D.) using a sucrose solution at pH 7.4. The sample solution (leukocyte-rich plasma) contained mainly platelets, granulocytes and lymphocytes. The eluted cells from the MEM–Sephadex column were identified on the basis of their retention volumes compared with the retention volumes of platelets and lymphocytes eluted independently. Separation was performed within 1.5 h and two types of cells were well separated from the leukocyte-rich plasma. Retention volumes of 6–8 ml and 13–16 ml, corresponding to the center cuts of the first and second peaks, contain over 95% of platelets and lymphocytes. The viability of the blood cells eluted from the column was evaluated by confirmation of trypan blue dye exclusion under the microscope; over 90% of the platelets and lymphocytes are confirmed to be viable cells. It seems that the chromatographic separation procedures described in this section are very useful for the fractionation of granulocytes and lymphocytes, because contamination with erythrocytes is low compared with the conventional Ficoll gradient method. Furthermore, the chromatographic system does not require special apparatus, e.g. a cell separator, and does not need a special technique, e.g. the attachment of beads to the cell surface for the separation by a magnetic field.

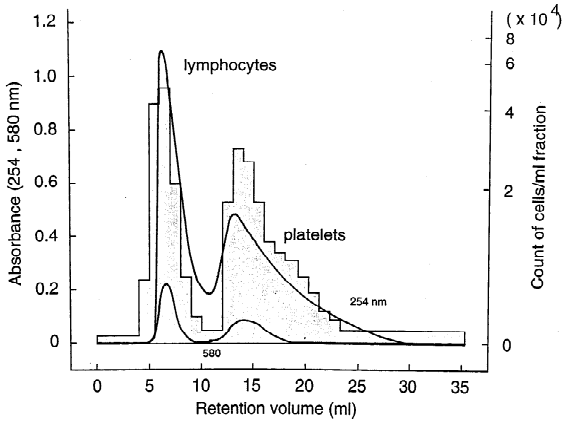

Fig. 13. Elution profile of human leukocyte-rich plasma. Column: MEM–Sephadex G25 (25×0.9-cm I.D.); mobile phase: 0.27 *M* sucrose solution at pH 7.4; flow-rate: 20 ml/h; sample: 1.5 ml of human leukocyte-rich plasma.

8. Conclusions

The chromatographic behavior of human blood cells was investigated by using a PEG 20M–Sepharose 6B column and several mobile phases. It was shown that an increase in sodium chloride concentration in the mobile phase resulted in an increase in the retention volumes of granulocytes and lymphocytes. In the hydrophobic affinity chromatography of plasma proteins, viruses and even whole cells (baker's yeast) [26], these materials are eluted from the column by decreasing the ionic strength of the mobile phase. On the basis of these facts, it was assumed that the major affinity between blood cells and the bonded PEG 20M phase is hydrophobic interactions.

The retention volumes of the blood cells, except for erythrocytes, increase in order of increasing molecular mass of the bonded PEG (PEG 400, 4000, 6000 and 20M) and PPG (PPG 200, 400 and 950) phases. This implies that the enhancement of the hydrophobicity of these column packings based on an increase in the molecular mass of bonded PEG and PPG causes longer retentions for platelets, granulocytes and lymphocytes, especially the last two cells. The retention volumes of the four kinds of blood cells at pH 7.4 were, in general, slightly greater than those at respective $pH_{c.p.}$ values. This indicates that the electrostatic interaction makes a relatively small contribution to the retention of the cells, compared with hydrophobic interactions.

The use of the MEM-bonded column packings (MEM–Cellulifine and MEM–Sephadex) and isotonic solutions of the mono- and disaccharides and methyl-α-D-pyranosides are very useful for the selective separation of human blood platelets, granulocytes and lymphocytes. It was found that the selective separation and purification of human peripheral blood cells was performed on the basis of the different affinities between the MEM stationary phase, the surface of the packings and the blood cell surfaces.

9. List of abbreviations

APTP	Aqueous polymer two phase system
DEAE	Diethylaminoethyl
LRP	Leukocyte-rich plasma
MEM	Methoxyethoxymethyl
MEM TEACl	(β-Methoxyethoxymethyl) triethylammonium chloride
NMR	Nuclear magnetic resonance
PEG	Polyethylene glycol
PPG	Polypropylene glycol
PRP	Platelets-rich plasma

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