

Chromatography of Erythroblasts on Immobilized Transferrin¹ (41530)DAVID A. JUCKETT² AND DONALD E. HULTQUIST³*Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, Michigan 48109*

Abstract. Affinity chromatography with immobilized transferrin has been used to effect a partial purification of erythroblasts and reticulocytes from bone marrow. Human transferrin was covalently attached to Sepharose CL-2B, Bio-Gel A-1.5, A-0.5, P-20, P-6, P-4, and P-2, and Sephadex G-75. Nucleated erythroid cells bound to the greatest degree to the polyacrylamide P-6 bead, whereas reticulocytes had the greatest affinity for the Sepharose CL-2B bead. The polyacrylamide P-6 bead has a greater density of surface groups than the Sepharose bead, and this correlates to the greater density of transferrin receptors on erythroblasts. The beads were used in a column which contained a central stirring bar with multiple crosspieces. The stirrer provided a method to uniformly agitate the beads to facilitate elution of the cells bound to the matrix. Approximately $1-2 \times 10^8$ bone marrow cells could be applied to a 25-ml matrix bed. This procedure resulted in a considerable resolution of cell types and gave a high yield of reticulocytes and erythroblasts from rat and rabbit bone marrow.

Our interest in studying the role of proteases during erythroid maturation has led us to attempt to isolate immature erythroid cells from bone marrow and to separate these erythroid cells on the basis of the degree of maturation. Whereas small numbers of pure erythroblasts can be readily obtained from tissue culture, a sufficient number of cells for enzymatic studies (10^8-10^9 cells) cannot be easily obtained by this method. High erythroblast to leukocyte ratios can be obtained from very anemic laboratory animals, but our goal was to isolate large numbers of erythroblasts from normal bone marrow of any animal source. Isokinetic centrifugation would then be used on the pure erythroblasts to separate them according to size, which is correlated to the degree of maturity (1, 2).

We have made progress toward the purification of erythroblasts by taking advantage of the presence of transferrin receptor sites on the surfaces of immature erythroid cells. Reticulocytes have been reported to be partially purified from whole blood using affinity chromatography with transferrin bound to Sepharose 4B (3). We have extended this technique to the purification of erythroblasts and

reticulocytes from normal rabbit and rat bone marrow. We have systematically tested several bead porosities to determine the optimal matrix and we have incorporated a glass stirrer in the column to provide mechanical agitation which facilitates elution of bound cells.

Methods. Materials. Sephadex G-75, 50-100 mesh, Sepharose CL-2B, and human transferrin were obtained from Sigma Chemical Company. Bio-Gel P-2, P-4, P-6, and P-20 polyacrylamide beads, all 50-100 mesh, and Bio-Gel A-0.5m and A-1.5m Agarose beads, 100-200 mesh, were obtained from Bio-Rad Laboratories. RPMI 1640 powdered tissue culture media was obtained from GIBCO Laboratories. All other chemicals were reagent grade.

Isolation of bone marrow cells. Bone marrow cells were obtained from Sprague-Dawley rats and New Zealand rabbits. The marrow was flushed from the rear leg bones using a 16-gauge needle and a solution containing 120 mM NaCl, 5 mM KCl, 20 mM glucose, and 7 mM phosphate buffer, pH 7.4. The cell suspension was forced several times through an 18-gauge needle to break up the clumps and the cells were washed several times in the salt-glucose buffer. In preparation for chromatography, the cells were transferred to an 8.3 mM Tris-Cl buffer, pH 7.4, containing 10.4 g of RPMI 1640 powdered media and 1 g of NaCl per liter.

Identification of cells. Morphological identification was obtained from slides stained with

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benzidine–Giemsa (4) or with Wright's stain. For the benzidine stain, slides were flooded for 1 min with 1% benzidine in methanol. A 1-min exposure of the slides to a solution of 52% ethanol + 7.5% H₂O₂ stained hemoglobin-containing cells golden yellow. After counterstaining with Giemsa for 30–45 sec, erythrocytes and reticulocytes appeared yellow to brown, nucleated erythroid cells showed green nuclei and yellow cytoplasm, and leukocytes appeared blue with pink granules.

Covalent attachment of transferrin to beads. Derivatization of Sepharose, Bio-Gel agarose, and Sephadex beads followed the procedure of March *et al.* (5). Washed beads (25 ml) were suspended in 25 ml of 2 M Na₂CO₃ at 0° and stirred rapidly as 3 ml of CNBr/CH₃CN (50 ml of CH₃CN + 100 g CNBr) was added. After 2 min the beads were washed on a funnel with 500 ml of 0.2 M bicarbonate buffer, pH 9.5. The moist gel was transferred to 50 ml of 0.2 M bicarbonate, pH 9.5, containing 25 mg of the iron complex of human transferrin. The beads incubated overnight at 4° while rotating. The beads were removed by filtration and the amount of unbound transferrin in the filtrate was determined photometrically at 470 nm. Usually 100% of the transferrin was bound after 24 hr. The beads were washed immediately with 0.2 M bicarbonate buffer, pH 9.5. In order to ensure that no activated sites remained, beads were incubated in some experiments with 5 mM ethanolamine in 0.2 M bicarbonate buffer, pH 9.5, for 4 hr at 4°. Ethanolamine treatment did not change the effectiveness of the gel. Beads were then washed with 8.3 mM Tris–Cl buffer, pH 7.4, containing 0.14 M NaCl. The beads were stored at 4° in the presence of 0.01% sodium azide.

The polyacrylamide beads were derivatized by the method of Inman (6). A quantity of dry beads (enough to make 25 ml when swollen) was added to 30 ml of 6 M hydrazine at room temperature. The suspension was stirred slowly for 30 min and then washed on a funnel with 2 liters of 0.2 M NaCl. The gel was suspended in 1 N HCl and cooled to 0°. After addition of 2.5 ml of a cold solution of 1 M NaNO₂, the gel was stirred for 3 min and then washed on a funnel with approximately 400 ml of 0.2 M NaCl at 0°. The gel was immediately transferred to 25 ml of a 0.05 M so-

dium borate buffer, pH 8.8, containing 0.05 M KCl and 1 mg/ml transferrin and allowed to rotate at 4° overnight. The remaining active sites were blocked by incubation with a solution of 3 M NH₄Cl + 1 M NH₄OH for 4 hr at 4°. Usually less than 10% of the transferrin bound to the gel, due to the inability of the transferrin to penetrate the bead.

Chromatographic separation of cells. Twenty-five milliliters of derivatized beads was placed in a glass column (2.5 cm in diameter) and was supported by a nylon mesh screen covered with a 2-mm layer of glass beads (approx. 700 μm diameter). A glass stirring rod ran down the center of the column (see Fig. 1). Crosspieces of the stirrer were spaced 0.5 cm apart and alternated at right angles to each other. The column was equilibrated with the Tris–Cl, NaCl, RPMI 1640 medium. Cell suspensions of 1–2 × 10⁸ cells in 1-ml volume were added to the top of the column using a pipet. The cells were allowed to flow into the matrix and then the flow was stopped for 1–2 min in order to allow the cells to settle onto the beads and bind. Nonadherent cells were then washed from the column using a flow rate of 1 ml/min which was maintained by a peristaltic pump. Adherent cells were removed from the column by gently stirring the matrix at approximately 30 rpm and eluting the column at a flow rate of 3–4 ml/min. All procedures were performed at room temperature. Fractions were collected and counted, and the contributions of each cell type were determined.

Results and Discussion. The profiles of four cell types eluted from agarose beads are shown in Fig. 2. A large number of erythrocytes and leukocytes passed through the column without binding but most of the erythroblasts and reticulocytes adhered to the column and were subsequently eluted with stirring. The erythroblasts were identified as primarily middle and late normoblasts. Attempts to elute the bound cells by addition of soluble transferrin were unsuccessful. Physical agitation of the column matrix was always necessary for elution, as was previously reported for separation of bone marrow cells using immobilized wheat germ agglutinin columns (7) and peripheral blood cells using concanavalin A immobilized on beads and fibers (8). Nonspecifically bound leukocytes (mostly myeloid cells) were

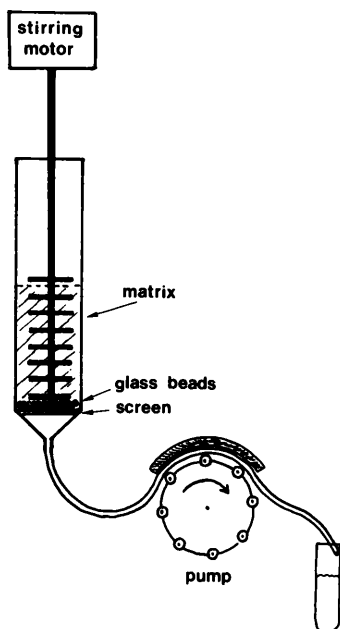


FIG. 1. Diagram of column showing the matrix of devivatized beads, matrix support, stirring ladder, and pump. Column diameter was 2.5 cm and matrix volume was 25 ml. The stirring rate was 30 rpm during elution of bound cells.

also eluted upon stirring and therefore contaminated the eluted fraction. Bone marrow from phenylhydrazine-treated rats contained a lower leukocyte to erythroblast ratio and, when these were used, a smaller percentage of contaminating leukocytes were present in the eluted fraction.

Results of experiments with three types of agarose beads, four types of polyacrylamide beads, and one type of Sephadex bead are shown in Table I. The same degree of purification and yield were found with rat and rabbit bone marrow. For each cell type the results are given as percentage recovery, overall purification, and the change in erythroblast to leukocyte ratio. The recovery refers to the number of that cell type in the fraction eluted by stirring, compared to the number that was applied to the column. The purification factor is the ratio of the fraction of a cell type in the eluant to its fraction of the cells added to the column.

The Bio-Gel P-6 polyacrylamide bead gave the highest recovery and purification of erythroblasts. Yields of approximately 7.5×10^6

erythroblasts were obtained from 1×10^8 bone marrow cells. The purification, whether measured as percentage of total cells or as an increase in the ratio of erythroblasts to leukocytes, decreased as bead porosities became either greater or less than that of the P-6 bead. Reticulocytes were best purified on the most porous bead, Sepharose CL-2B. In those experiments using polyacrylamide beads in which the reticulocytes were counted (data not shown), the highest porosity gave the best purification. A second passage of reticulocytes through a column resulted in only a 1.5-fold purification and a 50% yield. A second passage of nucleated erythroid cells gave no further purification. The behavior during the second purification may be due to damage of cell receptors and cell membranes.

In contrast to the results with the transferrin-immobilized beads, underivatized Bio-Gel P-20 and Sepharose CL-2B beads bound and then released only small numbers of cells. Likewise, albumin-derivatized Sepharose CL-2B yielded few cells. These procedures resulted in no purification of erythroid cells, suggesting that transferrin is essential in effecting the purification of the cells. The ef-

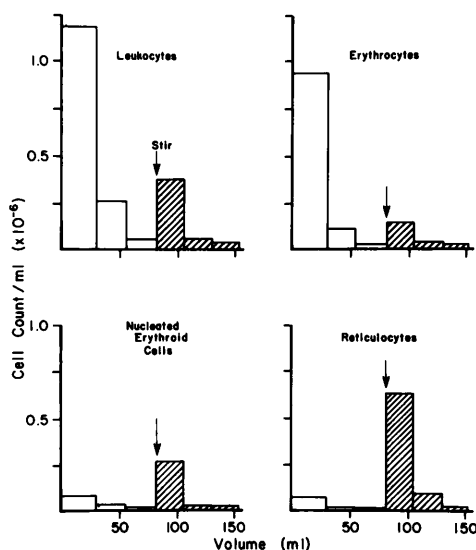


FIG. 2. Elution profiles of cells from a typical experiment on the Agarose A-1.5 m matrix. A suspension of 1×10^8 cells in 1 to 2 ml was applied to the column. Six fractions of approximately 25 ml were collected and analyzed. Stirring was begun after collection of the third fraction. The separation was performed at 20 to 25°.

TABLE I. EFFECT OF GEL TYPE ON DEGREE OF PURIFICATION AND RECOVERY OF VARIOUS CELL TYPES^a

Derivatized gel	No. of experiments	Erythrocytes		Reticulocytes		Erythroblasts		Leukocytes		Increase in ratio of erythroblast/leukocyte (n-fold)
		Recovery (%)	Purif. (n-fold)	Recovery (%)	Purif. (n-fold)	Recovery (%)	Purif. (n-fold)	Recovery (%)	Purif. (n-fold)	
Sephadex CL-2B	5	13	0.51	49	2.14	35	1.47	16	0.70	2.13
Agarose A-1.5 m	5	11	0.54	43	2.02	36	1.77	15	0.75	2.25
Agarose A-0.5 m	6	10	0.58	29	1.61	34	1.91	13	0.72	2.53
Sephadex G-75	2	2	0.14	9	0.78	19	1.73	12	1.20	1.70
Erythrocytes plus reticulocytes										
		Recovery (%)		Purif. (n-fold)						
Bio-Gel P-20	6	26	0.81	53	1.77	30	0.86	2.5		
Bio-Gel P-6	6	23	0.81	72	2.47	19	0.75	3.4		
Bio-Gel P-4	4	27	0.87	50	2.00	17	0.73	3.1		
Bio-Gel P-2	2	5	0.59	14	1.40	9	1.02	1.5		

^a Cells were separated by a single passage on a column in the absence of EDTA as described in the text. Purification is expressed as "fold," where 1.0 represents no purification.

fectiveness of human transferrin in the procedure is not surprising in light of the reported affinity of rabbit erythroid receptors for human transferrin (9). The similarity of our results with rabbit and rat bone marrow suggests that rat and rabbit receptors have similar affinities for the human protein.

The tendency of reticulocytes and erythroblasts to bind preferentially to beads of different porosity may be due to the difference in the density of transferrin receptors on the two cell types. The receptor densities are about $6 \times 10^{10}/\text{cm}^2$ and $19 \times 10^{10}/\text{cm}^2$ for reticulocytes and erythroblasts, respectively (10). The difference in bead porosity represents a difference in the degree of the packing of the sidechains of the bead polymer. At lower porosity, the sidechains are more closely packed which allows for a higher possible density of bound transferrin. The P-6 bead would be expected to have a higher density of possible transferrin binding sites whereas the CL-2B bead would have a lower density. This correlates to the density of receptors on the two cell types and their preferential binding to the two beads. The results further suggest that the binding of a large number of receptors per cell is required in order for the cell to adhere.

Several variations in the composition of the cell suspension buffer were made in order to optimize purification. Only the addition of 1 mM EDTA was found to have a positive effect. EDTA reduced the nonspecific binding of leukocytes but did not affect the affinity of the erythroblasts for the matrix. Incorporation of bovine serum albumin or complete calf serum to the buffer did not substantially change the results of the chromatography. Calf serum levels of 30%, however, caused the erythroblasts to form clumps. Allowing these clumps to settle might prove to be a simple way to improve the erythroblast to leukocyte ratio before applying the sample to the column. Vortexing, repeated pipetting, or addition of 30 mM lactose dissociated the clumps. (Lactose is believed to compete for the natural "erythroid developmental agglutinin" that facilitates the aggregation of erythroblasts in the bone marrow (11).)

In summary, an improved method for isolating immature erythroid cells from bone marrow has been developed using affinity chromatography on immobilized transferrin.

We have systematically examined the binding of erythroblasts to various transferrin-bound matrices. There exists an optimum matrix type which is correlated to the side group density of the bead and the cellular receptor density. Release of adherent cells was accomplished with a stirring system which gently and uniformly agitated the whole column matrix *in situ*. These findings will be of benefit for the further application of this technique for cell separation.

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