HIGH GRADIENT MAGNETIC SEPARATION OF ERYTHROCYTES

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ABSTRACT The high gradient magnetic separation technique has been applied to separate paramagnetic erythrocytes from a cell suspension that also contained diamagnetic cells. Paramagnetism was induced in the red blood cells by oxidizing the iron atoms in the cell hemoglobin to the ferric state (methemoglobin). Diamagnetic cells were either untreated erythrocytes, containing oxyferrohemoglobin, or leukocytes in a suspension of mouse spleen cells. Cell suspensions were passed through a column containing 40 μm diameter stainless steel wire in a high magnetic field (33 kG). The paramagnetic cells were retained on the surface of the wire while the diamagnetic cells passed through. Elution of the paramagnetic cells was accomplished by removing the column from the magnet, in effect turning off the field.

INTRODUCTION

The first step in the analysis of complex biological systems is the separation and isolation of different components of interest. In the case of suspensions of intact cells, separations are readily achieved by centrifugation and sedimentation techniques when there are differences in size or specific gravity that can be exploited. In addition, separations on the basis of cell surface properties are frequently possible by affinity chromatography methods. Recently, it has also been shown that magnetic forces can be employed in cell separations. Molday et al. (1) have achieved separations of cells that selectively bind ferromagnetic microspheres using a small permanent magnet. Melville et al. (2) have used high gradient magnetic separation (HGMS)[1] to remove erythrocytes from whole blood. The cells were treated with isotonic sodium dithionite to insure that the endogenous hemoglobin was in the deoxygenated reduced (Fe^{2+}) state, which is paramagnetic.

We have applied the HGMS technique to erythrocytes that were made paramagnetic by a relatively mild procedure: incubation at 0–4°C for 10–40 min in phosphate-buffered saline (PBS) to which 20 mM sodium nitrite has been added. This oxidizes between 50 and 90% of the hemoglobin iron in the cell from the ferrous to the paramag-

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[1] *Abbreviations used in this paper:* Hb, hemoglobin; HGMS, high gradient magnetic separation; PBS, phosphate-buffered saline; RBC, red blood cells.

netic ferric state (methemoglobin). Untreated cells, having >95% ferrohemoglobin, are diamagnetic in air-saturated buffer because ferrohemoglobin liganded to molecular oxygen has no magnetic moment.

The physical retention of paramagnetic cells is effected by flowing a cell suspension through a region of high magnetic field in a column packed with fine steel wire. Magnetization is induced in both the steel wire and the paramagnetic cells. If the magnetic forces of attraction between a cell and the wire matrix are greater than the viscous forces in the flowing medium, the cell will be retained. Diamagnetic cells, on the other hand, flow essentially unhindered through the loosely packed column. The paramagnetic cells can be eluted by turning off the magnetic field or by removing the column from the magnet.

In this report we will describe two examples of retention of erythrocytes containing paramagnetic methemoglobin from cell suspensions that also contained diamagnetic cells. In the first case, the diamagnetic cells were untreated erythrocytes. In the second case, the diamagnetic cells were leukocytes in a suspension of mouse splenocytes. The spleen cell suspension was treated with sodium nitrite to oxidize hemoglobin in the erythrocyte fraction; the viability of the leukocytes was unaffected by the nitrite treatment and passage through the magnetic system.

MATERIALS AND METHODS

Magnet

The superconducting magnet, the liquid helium Dewar flask, and the column containing the stainless steel wire matrix are shown in Fig. 1. The Dewar flask held the liquid helium (temperature 4.2°K) which maintained the magnet in the superconducting state. A vertical room tem-

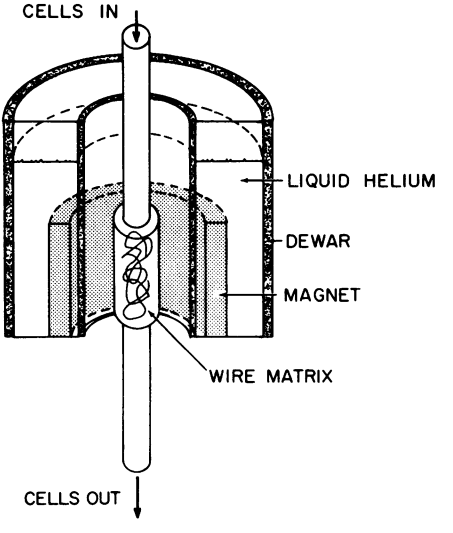

FIGURE 1 Geometry of the HGMS column and magnet. The superconducting magnet solenoid is mounted in a Dewar flask that has vertical room temperature flow-through access for the column.

perature bore has been provided in which a column can be suspended through which samples can flow. The Dewar flask (Superconducting Technology, Mountain View, Calif.) had a height of 12 cm, an inside diameter of 5 mm, and an outside diameter of 9 cm. The magnet was run in a persistent mode with a current of 46 A which generated a central field of 33 kG.

Column

A glass column with a cross-sectional area of 0.70 cm^2 held a 4-cm-high bed of randomly packed 40-μm diameter stainless steel wire. The 3-ml bed volume contained 1 g of wire that had a length of approximately 130 m and a surface area of approximately 150 cm^2, and filled about 5% of the bed by volume. The column was silicone-treated to minimize physical adherence of cells.

The wire in the column matrix was "hard-drawn" stainless steel in the 300 series of alloys. It was magnetic, which is typical of these metals until they have been annealed. In contrast, when the column was filled with common nonmagnetic stainless steel wire, no cell retention was observed. For HGMS application to slurries of mineral particles, ordinary stainless steel wool is most often used because this is usually magnetic. For cells, however, Melville et al. (2) have reported, and we have also observed, that the rough surfaces of the steel fibers cause excessive cell lysis.

A syringe pump was used to drive samples through the column at a uniform rate with minimal pulsation. The cell suspension was pumped at rates between 40 and 120 ml/h, which corresponded to velocities from 1–3 cm/min for fluid and cells passing through the wire matrix.

Separations were achieved by allowing the sample cell suspension to flow onto the column at room temperature in the magnet until the emerging buffer became essentially clear of cells. The column was then removed gently and suspended outside the magnet for the elution of the cells that had been retained by the wire matrix in the field. This procedure was adopted because it is inconvenient to change the field in the superconducting magnet.

Cells

Washed, packed cells from freshly drawn human whole blood were used in the separation of erythrocytes containing methemoglobin (metHb) from those containing oxyHb. For the separation of erythrocytes from leukocytes, a fresh suspension of spleen cells from a BALB/c mouse was prepared, washed at least twice, incubated 10–40 min at 0–4°C in PBS containing 20 mM sodium nitrite, washed again, and run onto the column.

To quantitate numbers of leukocytes, red cells were removed by lysis in 0.75% ammonium chloride 17 mM Tris buffer for 2 min at 4°C. Unlysed cells were counted in a hemacytometer, and viability was determined by the trypan blue exclusion test.

Hb assays were used as an indicator for relative numbers of red cells in various cell suspensions. Each fraction was first centrifuged, and the (colorless) supernate discarded. Lysis of the pellet cells and conversion of the Hb to metHb liganded to cyanide was done in one step with a commercial reagent (no. 116, Hycel, Inc., Houston, Texas) and calibrated with a commercial standard (no. 117, Hycel, Inc.). Absorbances were read on a Bausch and Lomb-Shimadzu UV210 spectrophotometer (Bausch & Lomb, Scientific Optical Products Div., Rochester, N.Y.).

RESULTS

Erythrocyte Separations

Data showing the separation of erythrocytes containing metHb and oxyHb are presented in Table I, and the time-course of the elution of material from the column is

TABLE I

HEMOGLOBIN ASSAYS OF RBC PASSED AND HELD BY MAGNETIC FILTER

Sample	Hb passed	Hb held	Total Hb	Hb passed/total Hb
	mg	*mg*	*mg*	*%*
50 μl oxy RBC	14.4	1.1	15.5	92
50 μl met RBC	0.3	11.6	11.9	2
50 μl oxy RBC + 50 μl met RBC	15.4	13.1	28.5	54

Samples were passed through the magnetic matrix at 2.1 cm/min (1.5 ml/min) in a field of 33,000 G. Cells collected while the column was in the magnet were assayed for Hb content, and the results summed to yield the Hb passed. Cells collected after the column was removed from the magnet are shown under Hb held. This value includes the cells eluted in a final rapid flush that typically provided about 5% of the fraction held.

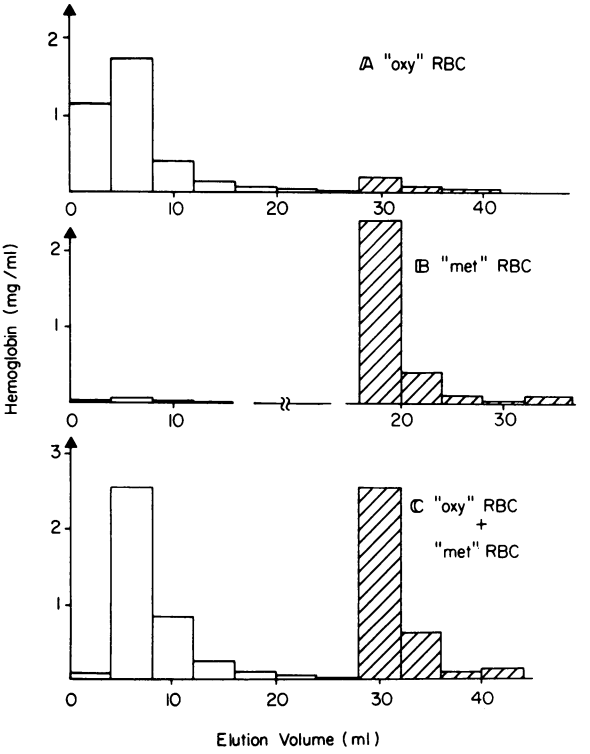

FIGURE 2 Elution profiles for red blood cells (RBC). Unshaded bars represent cells emerging from the column in the presence of the magnetic field (33 kG); the shaded bars show elution of cells at the same flow rate (2 cm/min) after the column was removed from the magnet. The last bar represents a rapid flush of the column to dislodge trapped cells.

shown in Fig. 2. From Table I it is clear that more than 90% of the cells in a diamagnetic cell sample are passed by the column, and more than 90% of paramagnetic cells are retained by the column. This is true both for the pure samples (A and B) and for the mixture of the two types of cells (sample C). The total recovered material accounts for better than 90% of the cells in the initial sample.

The graphs in Fig. 2 show the profiles of elution of erythrocytes from the column. In each case buffer flowed through the column until the eluant appeared clear, at which time the flow was interrupted briefly and the column gently removed from the magnet. When the flow was resumed at the original speed, cells that had been retained in the magnetic field promptly came off the column. Each fraction was spun down, and the pellet assayed for total Hb content. The supernate was colorless in every case; there was no evidence of any cell lysis resulting from passage through or retention by the wire matrix.

It was clear from the color of the cells passed and held by the magnetic filter that the oxidation-reduction state of the cell Hb was the basis of the separation. The cells eluted while the column was in the magnet were bright red due to oxyferrohemoglobin, whereas those that came off only after the column was removed from the magnet were brown due to metHb. A spectrum of Hb contained by cells in the peak of the diamagnetic fraction (the second tube in Fig. 2 C) is shown in Fig. 3. From the absence of significant absorption at 630 nm one can infer that $<5\%$ of the Hb in this fraction is in the ferric state.

In addition to the data shown, we have measured the percent retention of red blood cells (RBC) containing metHb at other flow rates, and at two other values of the applied field. The degree of retention drops with any increase in fluid flow velocity or with any decrease in magnetic field. At flow rates from 1.4 to 3.3 cm/min the percentage of cells retained varied from 86 to 96%, in a 30-kG field. When the field was lowered to 15 kG, the same flow rates yielded smaller retention values, from 76 to 92%.

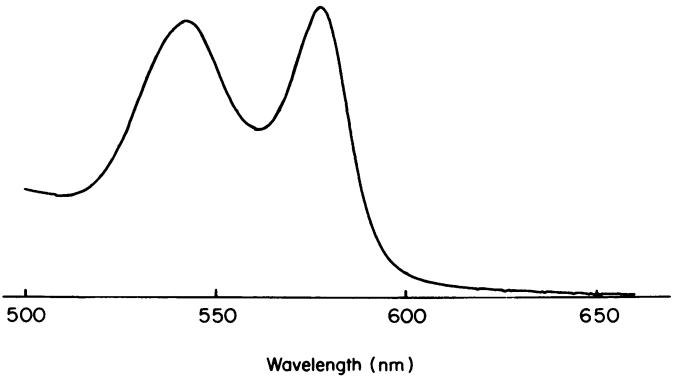

FIGURE 3 Spectrum of the oxyHb in a lysate of cells passed by the magnetic filtration system (the second tube of Fig. 2 C). Although metHb comprised 40–50% of the original sample, none is detectable in this fraction.

In an experiment to determine the capacity of the column to retain paramagnetic RBC, a sample of 250 μl packed cells was run. Approximately 200 μl were held by the column matrix. No overloading of the matrix was detectable in samples of 25 and 50 μl; in these samples identical fractions of the cells were retained. For a sample of 100 μl packed cells, however, the fraction retained decreased, indicating that a sample of this size started to overload the capacity of the matrix.

Erythrocyte-Leukocyte Separations

A second application of the magnetic separation technique was an extraction of cells containing Hb from a suspension of mouse spleen cells that had been treated with sodium nitrite. At a flow rate of 1 cm/min, the passed fraction typically contained 6–20% of the amount of Hb in the original cell suspension and 60–90% of the original number of small nucleated cells that resisted lysis in Tris-ammonium chloride. As measured by trypan blue exclusion, the 85–95% viability of the white cells was unimpaired by nitrite treatment and passage through the column. The total number of white cells recovered in the fractions eluted inside and outside the magnet was 85–90% of the original. Recovered RBC amounted to 60–80% of the original.

DISCUSSION

In this study HGMS in a large magnetic field has been applied to RBC made paramagnetic by oxidation of the cell Hb to the ferric (metHb) state. HGMS (3–6) is a generally useful technique for separation of paramagnetic particles suspended in a fluid medium, whereas an ordinary magnet will suffice for ferromagnetic particles. (Ferromagnetic objects, such as the microspheres in ref. 1, have a permanent magnetic moment. Paramagnetic particles, on the other hand, have no permanent magnetization, although a magnetic moment can be induced in the presence of an external magnetic field.) By comparison with slurries of mineral particles (4), cells are weakly paramagnetic, which leads to smaller magnetic forces, and they are large in diameter, which leads to larger viscous forces. As a result, the retention of cells requires greater magnetic fields and slower fluid flow rates than are typical in mineral applications.

We have used a magnetic field of 33 kG and flow velocities of 1–3 cm/min for cells through the column. These values are consistent with the experimental parameters employed by Melville et al. (2) who used a 17.5-kG field and a flow velocity of approximately 0.6 cm/min. Our higher field allows the use of somewhat more rapid flow rates and a slightly coarser grade of steel wire in the column matrix (40 μm diameter as opposed to the 25 μm wire used by Melville et al.). In principle, even higher fields would allow cell separations with an even greater throughput. In practice, however, it would be easier to increase the diameter of the column to accommodate greater volumes at the same fluid speed through the wire matrix.

The observed retention of paramagnetic cells in our experiments could be varied in a way that agreed qualitatively with the deviations one would expect from the theory of HGMS when the field, flow rate, and diameter of steel wire in the matrix were al-

tered. We conclude that the small fraction of paramagnetic cells that pass through the magnetic filter (sample B in Table I, and similar experiments) is due to our choice of flow rates rather than to imperfections in the column matrix or overloading the matrix with cells.

One can see from Table I that the flow rates we have used are rapid enough to avoid undue physical trapping of diamagnetic cells, while still allowing a high degree of retention of paramagnetic cells. In general, it was found, however, that separations of mouse splenocytes were of comparable quality only when the cells were washed well before being run on the column. It is possible that the 5–20% of the initial Hb-containing cells that typically came through the column may reflect a clumping effect. If a fraction of the splenic erythrocytes had been associated with other cells or solid material as they flowed through the column, this would have increased the viscous flow forces relative to the magnetic forces, and these cells would have been eluted.

Although we have shown data for the magnetic retention of RBC having approximately 90% or more of the internal Hb converted to the metHb form, we can now vary the experimental parameters to decrease the percentage of Hb in a cell that must be in the met form for a cell to be retained. This threshold will be lowered by the use of a stronger magnetic field, slower flow velocities, and an improved matrix in the column, which will contain finer wires to generate larger magnetic forces in the vicinity of the wire surface.

On the basis of the observation (7) that older circulating erythrocytes contain greater amounts of metHb than young cells (up to 8% observed [7]), one can consider HGMS as a technique for removing older erythrocytes from circulating blood. This may have clinical application to chronic anemia where multiple transfusions lead to excess iron deposition in tissue and hemochromatosis as transfused cells age and are broken down by the body. If cells could, instead, be removed from circulation artificially shortly before they are removed naturally, this build up could be largely circumvented without significantly compromising the therapeutic effect of the transfusions.[2]

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