Separation of cells labeled with immunospecific iron dextran microspheres using high gradient magnetic chromatography

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Immunospecific magnetic microspheres, consisting of ferromagnetic iron dextran conjugated to Protein A, were used to specifically label red blood cells (RBC) for cell separation studies using high gradient magnetic chromatography (HGMC). When 10^7–10^8 RBC labeled with Protein A–iron dextran microspheres were applied to a column containing 30 mg stainless steel wire placed in a 7.5 kilogauss magnetic field, 96 ± 2% of the cells were retained in the column. These cells could be eluted by removing the magnetic field and mechanically agitating the column. The retention of labeled cells by HGMC was shown to be dependent on the applied magnetic field and the amount of wire packed into the column. HGMC in conjunction with cell labeling with immunospecific iron dextran microspheres have useful applications for the separation of specific cell types.

Cell separation *Magnetic microsphere* *Red blood cell* *Iron dextran* *Immunolabeling* *Magnetic chromatography*

1. INTRODUCTION

The separation of specific cells from mixed populations is essential for a complete understanding of the role of these cells in complex normal and pathological processes. Over the years a variety of cell separation techniques have been developed based on differences in physical properties of cells such as size, charge, density and adhesion as well as differences in cell surface antigens [1]. The latter techniques in which cell-specific antibodies are used with immunolabeling methods have proven most useful in separating cells into subclasses. This is most evident in the area of cellular immunology where lymphocytes displaying cell specific antigens have been detected and separated by automated fluorescence cell sorting methods [2] and immunoaffinity chromatography [3].

Several laboratories have explored the possibility of using magnetic fields to separate cells. In one area of research high gradient magnetic separation has been used to separate cells based on their paramagnetic properties. Authors in [4,5] and [6] have shown that red blood cells containing paramagnetic hemoglobin can be quantitatively retained on a high gradient magnetic column containing stainless steel wool. This technique has been recently applied to the separation of rosette-forming cells [7]. In a different approach, magnetic microspheres conjugated to antibodies have been used to label and separate specific cells using simple permanent magnets or electromagnets [8,9]. Immunospecific ferromagnetic iron dextran microspheres consisting of a magnetic Fe_3O_4 core coated with dextran and conjugated to Protein A [10] have proven to be particularly useful reagents for labeling and separating cells.

Here, we describe the application of the high-gradient magnetic separation technique with im-

Abbreviations: HGMC, high gradient magnetic chromatography; Ig, immunoglobulin; PBS, phosphate-buffered saline; RBC, red blood cells; WGA, wheat germ agglutinin

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munospecific ferromagnetic iron dextran particles for the separation of cells. We refer to this technique as HGMC. In this initial study parameters affecting magnetic retention of labeled cells are investigated using red blood cells as a model system.

2. MATERIALS AND METHODS

2.1. *Preparation of diaminoethane-derivatized dextran*

A dextran solution containing 20 g Dextran-T40 (Pharmacia) in 100 ml of 0.05 M acetate solution (pH 6.5) was oxidized with 2.14 g sodium periodate for 1.5 h at 23°C. The solution was then dialyzed overnight against 2 l distilled H_2O at 4°C. The oxidized dextran-T40 was diluted with water to 180 ml and mixed with 20 ml of 6 M diaminoethane which had been previously titrated to pH 8.7 with glacial acetic acid. The coupling reaction was stirred for 1 h at 23°C and quenched by reduction with 0.75 g $NaBH_4$. The solution was then extensively dialyzed and finally lyophilized. Presence of amino groups on the dextran was confirmed qualitatively by the 2,4,6-trinitrobenzene sulfonate test [11].

2.2. *Synthesis and purification of ferromagnetic iron dextran microspheres*

Diaminoethane-derivatized ferromagnetic iron dextran microspheres were synthesized by modification of the procedure in [10]. Briefly stock aqueous dextran solution was prepared by mixing 1 vol. of 50% (w/w) diaminoethane derivatized dextran with 4 vols of 50% (w/w) underivatized Dextran T-40. A freshly prepared 5-ml aqueous iron chloride solution containing 0.75 g $FeCl_3 \cdot 6H_2O$ and 0.32 g $FeCl_2 \cdot 4H_2O$ was mixed with 5 ml of the stock dextran solution. The mixture was stirred vigorously at 23°C and 10 ml of 7.5% (v/v) NH_4OH was rapidly added. The black suspension was stirred continuously for 1 h at room temperature and subsequently centrifuged at 17300 × *g* for 10 min to remove aggregated material. The ferromagnetic iron dextran microspheres were separated from unbound dextran as in [10].

2.3. *Conjugation of proteins to the iron dextran microspheres*

Diaminoethane-derivatized iron dextran microspheres were conjugated to proteins including *Staphylococcus aureus* Protein A, goat antirabbit Ig antibody and wheat germ agglutinin by the two-step glutaraldehyde reaction [12]. Routinely, 2 ml diaminoethane derivatized iron dextran microspheres (7–10 mg/ml) were reacted with 0.2 ml of 25% aqueous glutaraldehyde for 1.5 h at 23°C. The solution was then dialyzed at 4°C against three 1-l changes of 0.01 M sodium phosphate buffer (pH 7) over 15 h to remove excess glutaraldehyde. Conjugation of the protein to the glutaraldehyde-activated microspheres was carried out by adding 2 mg purified protein to 2 ml of the microspheres. After stirring continuously for 12–16 h at 23°C, the reaction was stopped by the addition of 0.05 M glycine.

The protein–ferromagnetic iron dextran conjugates were separated from unbound protein by gel filtration chromatography on 1.5 × 25 cm Sephacryl S-300 column in PBS. The conjugates collected as the peak fraction in the void volume had an iron dextran concentration of 3–4 mg/ml as measured by dry weight.

2.4. *Red blood cells*

For most magnetic separation studies 6 ml fresh human blood were washed twice with 15 ml PBS by repeated centrifugation. The final pellet was resuspended in 6 ml PBS and saturated with carbon monoxide. The RBC were lightly fixed by the addition of 30 ml of 0.25% glutaraldehyde in PBS. After 30 min at 25°C, the cells were washed 3 times with PBS by repeated centrifugation, resuspended in PBS containing 0.05 M glycine for 1 h and finally washed again in PBS.

An aliquot of fixed red blood cells were iodinated by oxidation with chloromine T. Briefly, 2 ml fixed human RBC containing 4×10^9 cells were mixed with 0.75 mCi $Na^{125}I$ (Amersham). Chloramine T (4 mg/ml) was added in three 50-μl aliquots at 5-min intervals. After 15 min the reaction was quenched by the addition of 0.1 ml sodium metabisulfite (8 mg/ml). The ^{125}I-labeled RBC were extensively washed by repeated centrifugation to remove unreacted ^{125}I. The ^{125}I-labeled RBC were mixed with noniodinated RBC to give a spec. act. of $2–3 \times 10^4$ dpm/10^7 RBC.

2.5. *Labeling of cells with immunoferromagnetic iron dextran*

The indirect labeling procedure was routinely

used to label antigens on cells with ferromagnetic iron dextran conjugates. In RBC studies, routinely 1.4×10^8 ^{125}I-labeled RBC in 150 μl were incubated with 100 μl rabbit antihuman RBC antiserum (Cappel Lab) for 30 min at 25°C. The cells were then washed 3 times with 2 ml PBS by centrifugation at 1500 rpm for 5 min in a Beckman TJ-6 centrifuge. Aliquots of 2×10^7 cells in 200 μl were then treated with 100 μl of either Protein A or goat antirabbit Ig iron dextran microspheres at 3–4 mg/ml for 30–60 min at 23°C. Finally, the cells were washed 3 times in PBS as described above and the final pellet was resuspended in 0.5 ml PBS.

2.6. *High gradient magnetic column*

A custom-made electromagnet regulated by a variable transformer was capable of generating a magnetic field of up to 1 tesla (10 kilogauss) midway between 2 cm diameter pole faces set at a gap of 10 mm. A glass column with an inside diameter of 0.6 cm and a length of 10 cm, was loosely packed with 25 μm diameter stainless steel wire (type 302, Goodfellow Metals, Cambridge) and placed in the electromagnet such that all of the wire was between the pole faces. In most studies, 30 mg wire was used; in some studies, however, the amount of wire was varied. The flow rate from the passage of cells through the column was controlled by a peristatic pump (Gilson Minipuls II) and fractions were collected with a Gilson Fractionator. A diagram of the high gradient magnetic chromatography apparatus is illustrated in fig.1.

2.7. *High gradient magnetic chromatography*

A known quantity of cells, usually 10^7–10^8 cells in 0.5 ml of PBS or Hank's balanced salt supplemented with 0.5% (w/v) bovine serum albumin was applied to the top of the column. With the magnet on, the cells were passed through the column with buffer at a flow rate of 2 ml/min. A volume of 8–12 ml was collected containing the cells which were not retained by the magnet. After the magnet was turned off and the column was removed from the magnet, cells were eluted from the column with 8 ml buffer. Finally, residual cells bound to the wire were mechanically dislodged by placing the column against a vortex mixer for 30 s. These cells were then eluted with an additional 12 ml buffer. These two fractions contained the

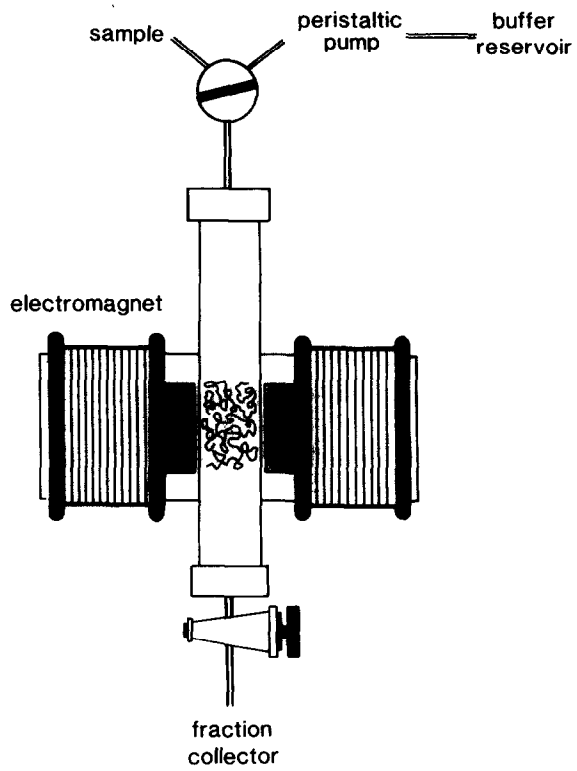

Fig.1. Diagram of the HGMC system.

cells which were initially retained by the magnet. In many studies direct elution after the magnet was turned off and elution after vortexing were combined into a single operation. Cells recovered in the fractions were centrifuged at 2000 cpm for 5 min, resuspended in a known volume of buffer and measured either for radioactivity in a Beckman 8000 gamma counter or for cell number in a hemocytometer.

3. RESULTS

3.1. *Red blood cells as a model system*

Human red blood cells were selected as a model system to investigate parameters affecting high gradient magnetic chromatography of immunospecific iron dextran-labeled cells. However, since authors in [4] and [5] have shown that RBC containing paramagnetic deoxyhemoglobin or ferrihemoglobin are retained in a high gradient magnetic field, it was necessary to first determine the extent of retention of RBC under varying conditions of hemoglobin oxidation and ligand coor-

dination by the high gradient magnetic column used in this study.

When RBC containing hemoglobin predominantly in its diamagnetic state, i.e., freshly-drawn RBC saturated with oxygen or carbon monoxide, were passed through a high gradient magnetic column packed with 30 mg of wire in an applied magnetic field of 0.75 tesla, 97 ± 2% of the cells passed through the column. This was comparable to the a value of 98 ± 2% obtained when RBC were passed through this column in the absence of an applied magnetic field. Glutaraldehyde-fixation of carbon monoxide-saturated cells and/or radioiodination with ^{125}I had no significant effect on the passage of cells through the high gradient magnetic column. Carbon monoxide stabilizes the heme iron of hemoglobin in its diamagnetic state whereas glutaraldehyde fixation prevents RBC lysis during long term storage and minimizes RBC agglutination by the primary antiserum, i.e., rabbit antihuman RBC serum. Labeling of RBC with ^{125}I permitted rapid and quantitative analysis of cells for HGMC.

When RBC were treated with sodium nitrate under conditions which cause oxidation of hemoglobin with the heme iron in a paramagnetic state, the fraction of cells passing through the high gradient field was reduced to 77%.

3.2. *HGMC of immunoferromagnetic iron dextran-labeled cells*

Magnetic chromatography of fixed, CO-saturated, [^{125}I]RBC labeled with immunoferromagnetic iron dextran conjugates is illustrated in fig.1. When 10^7 [^{125}I]RBC sensitized with rabbit antihuman RBC antibodies and subsequently labeled with Protein A–ferromagnetic iron dextran conjugates were applied to a column packed with 30 mg stainless steel wire and placed in an applied magnetic field of 0.75 tesla, over 96% of the cells were retained on the column. A similar result was obtained when goat antirabbit Ig–ferromagnetic iron dextran conjugates were used instead of the Protein A conjugates. Approx. 40–60% of retained cells could be eluted after removing the column from the magnetic field. Most of the remaining cells could be eluted after mechanically agitating the column on a vortex mixer. Total recovery of cells applied to the column was 98%. Similar results were obtained if the Protein A–iron dextran conjugate was diluted by a factor of ten, if the number of labeled cells applied to the column was increased to 6×10^8, or if unfixed RBC were used.

The specificity for retention of [^{125}I]RBC labeled with Protein A–ferromagnetic iron dextran conjugates was confirmed in control experiments. When [^{125}I]RBC were directly treated with the Protein A–ferromagnetic iron dextran conjugates or first treated with nonspecific antiserum prior to treatment with the conjugate, 97% of the [^{125}I]RBC passed directly through the column (fig.2). Results are summarized in table 1.

Labeling of RBC with Protein A–ferromagnetic iron dextran conjugates was also confirmed by transmission electron microscopy. In the experimental system, specifically-labeled RBC which were retained by the magnetic field were densely coated with the electron dense iron dextran particles as in [10]. In the control experiments RBC which were only treated with the Protein–iron dextran conjugate and which were not retained by the magnetic field were devoid of particles.

3.3. *Parameters affecting magnetic chromatography*

The effect of applied magnetic field on the passage of immunoferromagnetic iron dextran-labeled cells through the high gradient magnetic

Table 1

Magnetic retention of immunoferromagnetic iron dextran-labeled cells

Treatment of RBC	% Cells recovered		% Total cell recovered
	Passed fraction	Retained fraction	
α RBC antiserum + Protein A–iron dextran	4 ± 2	96 ± 2	95 ± 2
α RBC antiserum + goat antirabbit Ig–iron dextran	2 ± 1	98 ± 2	97 ± 2
Protein A–iron dextran	96 ± 2	4 ± 2	96 ± 3
Goat antirabbit Ig–iron dextran	95 ± 2	5 ± 2	98 ± 2
Buffer	97 ± 2	3 ± 2	98 ± 2

column containing 30 mg wire is shown in fig.3. The percentage of RBC passing through the column reached a lower value of 4% at an applied field greater than 0.6 tesla. Cells which passed through the column under these conditions showed a lower degree of immunoferromagnetic iron dextran labeling compared to the retained cells as visualized by transmission electron microscopy (not shown). Furthermore, when cells which had been retained on the column at fields greater than 0.6 tesla were eluted and reapplied to the column under similar magnetic field conditions, the percentage of cells which passed through the column was reduced to less than 1%. A linear relationship was obtained when the percentage of labeled cells which passed through the magnetic field was plotted on a log scale as a function of the applied field (inset, fig.3).

Fig.4 shows the effect of the fraction of the column volume occupied by the wire on the percentage of cells which passed through the column at constant applied field and constant flow rate. When the percentage of cells which passed through the field was plotted on a log scale as a function of the fractional volume occupied by the wire, a linear relationship was observed.

3.4. *Studies on other systems*

Fixed or unfixed human RBC labeled with wheat germ agglutinin–ferromagnetic iron dextran microspheres were fully retained on the high gradient magnetic column. However, when RBC were treated with WGA–iron dextran reagents in the presence of the inhibitor *N,N'*-diacetylchitobiose, 95% of the cells passed through the column. Bovine rod outer segments labeled with WGA–iron dextran microspheres were also quantitatively retained in the magnetic column in the absence of inhibitor.

In other studies when rat thymocytes were sequentially treated with mouse monoclonal antibody OX-7 against the Thy surface antigen, rabbit anti-mouse Ig and Protein A–iron dextran

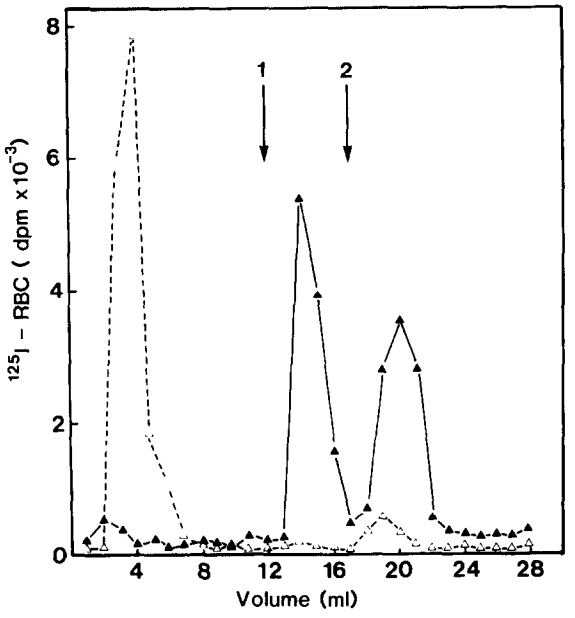

Fig.2. HGMC of RBC treated with immunospecific ferromagnetic iron dextran microspheres. [^{125}I]RBC sequentially treated rabbit antihuman RBC antiserum, and Protein A–ferromagnetic iron dextran microspheres (▲—▲) were applied to the HGMC column in the presence of a 0.75 tesla field and eluted with 12 ml buffer. The column was removed from the field and further eluted with 5 ml buffer (arrow 1). Finally the column was placed against a vortex mixer for 1 min and eluted with an additional 11 ml buffer (arrow 2). RBC treated only with Protein A–ferromagnetic iron dextran microspheres (△—△) were chromatographed in the same way.

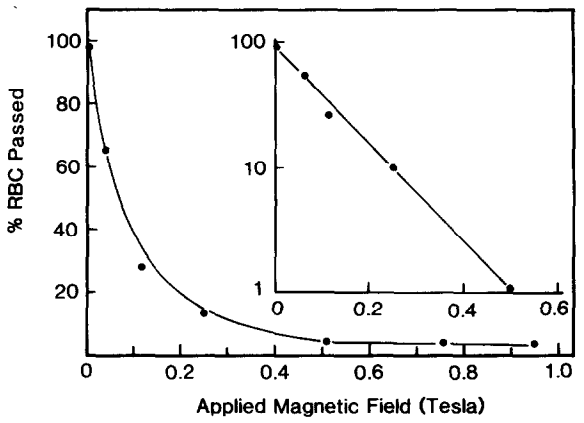

Fig.3. The effect of applied magnetic field on the percentage of labeled RBC which passed through the HGMC column. 10^7 [^{125}I]RBC labeled with immunospecific iron dextran were applied to a column containing 30 mg stainless steel wire and were subjected to various applied magnetic fields. The number of cells which passed through was determined by counting.

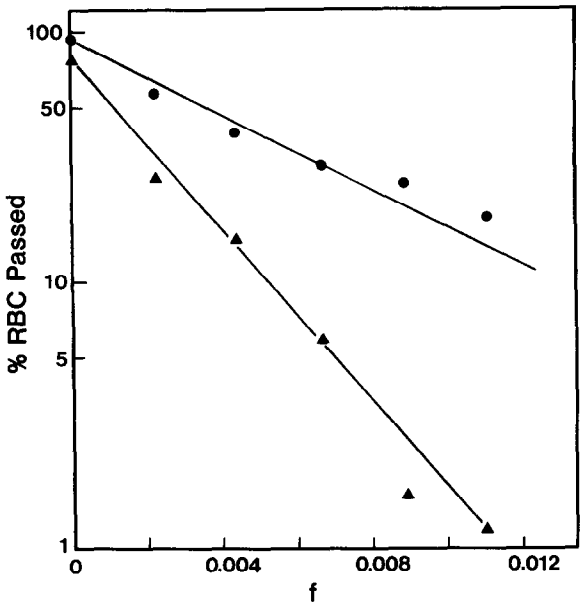

Fig.4. The effect of the fraction of volume occupied by the stainless steel wool (f) on the percentage of RBC which passed through the HGMC column in the presence of magnetic fields of 0.25 tesla (▲—▲) and 0.65 tesla (●—●).

microspheres, 90% of the cells were retained on the column.

4. DISCUSSION

Here ferromagnetic iron dextran particles prepared from diaminoethane-derivatized dextran were conjugated to antibodies or lectins by the two-step glutaraldehyde reaction. These reagents were found to be highly active in labeling cell surface antigens and receptors and retained their ferromagnetic properties. An important advantage of these reagents over the ferromagnetic iron dextran conjugates prepared by the periodic acid oxidation–borohydride reduction method [10], however, is their stability in buffers. Conjugates prepared by the glutaraldehyde reaction were stable in PBS whereas conjugates prepared by periodate oxidation were found to aggregate in this buffer over a period of several days.

The high gradient magnetic chromatographic technique was highly effective in retaining red blood cells and thymocytes heavily labeled with immunospecific ferromagnetic particles. Under optimal conditions over 95% of the cells were retained in the magnetic field. These cells could be eluted by removing the magnetic field and mechanically dislodging the cells from the wire. Control studies confirmed the specificity of labeling and retention of labeled cells in a magnetic field.

As predicted from the theory of paramagnetic particle capture in a high gradient magnetic field [5,13] a linear relation between the percentage of cells passed and either the magnetic field strength or the fraction of the volume occupied by the wire was observed. Other parameters including the flow rate can also be varied to optimize retention of cells [14].

These studies demonstrate the feasibility of using high gradient magnetic chromatography to efficiently separate cells labeled with immunospecific ferromagnetic iron dextran particles from unlabeled cells. Further studies, however, are required to determine the relationship between the number of magnetic microspheres bound per cell and to retention of labeled cells in a magnetic field. Studies exploring ways to remove the immunoferromagnetic particles from cells are also required.

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