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B. DeSales Lawless[a]; Joann Williams[b]
[a] Department of Cellular Physiology and Immunology the Rocke, feller University New York, New York [b] Nasa-Ames Research Center Moffett Field, California

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# Carbonyl Iron Particles in Magnetic Cell Sorting

B. DeSALES LAWLESS*
DEPARTMENT OF CELLULAR PHYSIOLOGY AND IMMUNOLOGY
THE ROCKEFELLER UNIVERSITY
NEW YORK, NEW YORK 10021

JoANN WILLIAMS
NASA-AMES RESEARCH CENTER
MOFFETT FIELD, CALIFORNIA 94035

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## ABSTRACT

Iron–dextran microspheres are easily and inexpensively made by coating uniformly sized carbonyl iron particles with dextran. The iron–dextran spheres can be conjugated with a variety of ligands (antibodies, lectins, avidin, etc.) and used with a permanent rare earth magnet to remove cells, organelles, or macromolecules from mixtures and suspensions. The spheres can be stored indefinitely for use when they are needed. Results obtained with iron–dextran particles in magnetic cell sorting are comparable to those with commercial products. They are particularly helpful when large numbers of cells are to be sorted or when a large panel of antibodies is needed.

*Key Words.* Carbonyl iron; Dextran; Magnetics; Cell separation

## INTRODUCTION

Magnetic affinity cell sorting (MACS) is an efficient technique for removing or recovering specific cell types from a heterogeneous cell suspension (1). The procedure works equally well with macromolecules. When a suspension of mixed cells, some of which have been magnetically labeled, is placed in a magnetic field, the magnetically complexed cells are

* To whom correspondence should be addressed at Box 280, The Rockefeller University, 1230 York Avenue, New York, New York 10021-6399. Telephone: (212) 327-8114. FAX: (212) 327-8875.



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immobilized and the noncoupled cells can be decanted or aspirated. When the magnetic field is removed, the nonlabeled cells are freed.

Investigators have reported successful cell fractionations using several types of magnetic microspheres: polystyrene (2), polyacrolein (3), albumin (4). Many types of magnetic spheres are commercially available. Iron–dextran particles have been prepared from precipitates of ferrous and ferric chlorides which were then coated with a dextran polymer (5). These particles were used with an electromagnet. The preparation of carbonyl iron–dextran spheres described here is both rapid and inexpensive and is used with a rare-earth permanent magnet.

## MATERIALS AND METHODS

### The Permanent Magnet

A rare-earth, samarium-cobalt permanent magnet (Edmunds Scientific Co., Barrington, New Jersey) 1 × 1 × 5 cm is used. Its residual induction is 8500 G and its coercive force is 8000 oersted. The rare-earth MagniSort (4″ × 6″) is used with microtiter plates (DuPont Biotechnology Systems, Wilmington, Delaware 19898).

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### Test Tubes

Falcon 2050 polystyrene test tubes (Becton Dickinson Co., Oxnard, California) 12 × 75 mm are used in separations. In some situations, we found 96-well U-bottomed polystyrene microtest plates suitable (Corning Glass Works, Corning, New York 14831).

### Cell Preparations

Peripheral blood mononuclear cells (PBMC) are prepared from human venous blood of normal healthy volunteers drawn in heparinized syringes. The cells are obtained from the blood by density gradient centrifugation (Lymphocyte Separation Medium, Litton Bionetics, Kensington, Maryland 20795). The buffy coat cells are washed and resuspended in phosphate buffered saline–1% BSA–0.02% azide (PBS-BSA-AZ) pH 7.4. The concentration is adjusted to 1 × $10^6$ cells/mL. Cells are counted on a hemacytometer, and viabilities are determined by trypan blue exclusion (6).

### Cell Labeling

One milliliter of the cell suspension is centrifuged at 1800 rpm (Sorvall RT 6200) for 3 minutes at 4°C. The supernatant is decanted and 100 μL of a 1:5 dilution of antibody culture supernatant is added, and the tubes

or plates are incubated for 45 minutes on ice. In the study reported here, Leu-3a (anti T-helper/inducer) or Leu-2 (anti T-cytotoxic/suppressor) are used as primary antibodies to label cells.

## Preparation of Iron–Dextran Microparticles

One hundred milligrams of carbonyl iron (Sigma Corp., St. Louis, Missouri) is vortexed for 5 minutes to produce particles with an average size of 1 μm; 100 mg of 40K Dextran (Pharmacia Fine Chemical Co., Piscataway, New Jersey) is added and the mixture is vortexed for 1 minute and then suspended in 10 mL of 7.5% ammonium hydroxide. The suspension is brought to 65°C for 15 minutes, cooled, and washed with PBS-BSA-AZ.

## Preparation of Iron–Dextran Complexes

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The dextran vicinal diol moity coating the carbonyl iron is first oxidized to aldehyde by periodate oxidation. A Schiff base is then formed with ethylenediamine to produce particles with active amino groups on the surface. The carbonyl-iron–dextran particles are activated by suspending them in 10 mL of 0.1 N sodium acetate, 0.15 N NaCl, pH 6.5, made 5 mM in sodium periodate, and rotating them at room temperature for 60 minutes. Using the permanent magnet, the spheres are washed in PBS and suspended in 20 mM borate, pH 8.5, 8 N ethylenediamine, and the mixture is rocked for 1 hour at room temperature. The reaction is stopped with 0.05 M glycine and then treated with cyanoborohydride to form a stable Schiff base.

The antibody to be conjugated to the activated spheres is first oxidized by periodate following the procedure for the dextran activation. The carbohydrate on the immunoglobulin Fc region is converted to aldehyde.

Iron–dextran–amino spheres (2.0 mg) are rocked at room temperature for 1 hour with 1.0 mg activated antibody. Glycine (0.05 M) is added to stop the reaction, and then the bond is stabilized by washing with 0.1 mM cyanoborohydride. The antibody conjugated spheres are then suspended in PBS, pH 7.4. If they are to be stored, 0.02% sodium azide is added.

## Fractionation Procedures

Before each cell depletion the test tubes or microtiter plates are washed with PBS-BSA-AZ to minimize nonspecific cell binding. In direct separations, the cells are incubated with a primary antibody attached to iron–dextran. In indirect separation, the cells are first incubated with a primary antibody or culture supernatant containing an antibody for 30 minutes on

ice, washed, and then incubated with a secondary goat-antimouse antibody conjugated iron–dextran particles.

A particle-to-cell ratio of 40:1 was found optimum for negative depletions. To an aliquot of $10^6$ cells of which half are targeted cells, $2 \times 10^7$ iron–dextran goat-antimouse particles are added (200 μL). For larger cell volumes, either multiple wells of the microtiter plate or the 5.0-mL test tubes are used. The particle conjugates are prepared so that concentration is approximately 1 mg particles/mL. There are approximately $10^8$ particles/mg. The cells and antibody conjugates are gently rocked for 15 minutes, 4°C. The volume is then increased 5× with PBS-BSA-AZ. The test tubes are rested on the permanent bar magnet and the microtiter plates on the Magnisort plate magnet for 3 minutes. Nonadherent cells are removed by decanting or by aspiration while holding the tube or plate to the magnets. Adherent cells are recovered when the tubes or plates are removed from the magnetic field. Both cell populations are washed, counted, and analyzed by flow cytometry.

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## Flow Cytometry

Approximately 50,000 cells are pelleted and 1 μL of the Becton Dickinson simultest fluorescent antibody mixture is added. The simultest mixture contains FITC-Leu-3 and PE-Leu-2. Leu-3 is a mouse antihuman CD4 helper/inducer cell antibody; Leu-2 is a mouse antihuman CD8 T-cytotoxic/suppressor cell antibody. The cells are incubated with the simultest mixture in the cold for 20 minutes, washed, and analyzed on the Becton Dickinson FACScan Flow Cytometry Analyzer using either the Consort-30 or Lysys software programs. The cells are fixed with 3.7% formaldehyde for 10 minutes on ice if they are not to be analyzed on a future day.

## RESULTS

The results reported illustrate the average of numerous depletion studies in which carbonyl iron–dextran particles conjugated with goat-antimouse antibodies are used to deplete CD4+ and CD8+ cells from human peripheral blood mononuclear cells.

Figure 1 is a graphic presentation using Becton Dickinson FACScan Lysys computer program. Histograms show relative number of cells on the *y* axis and fluorescence intensity on the *x* axis. Merged histograms (left) show depletion of fluoroisothiocyanate-labeled CD4+ cells. Merged histograms (right) show removal of phycoerythrin-labeled CD8+ cells.

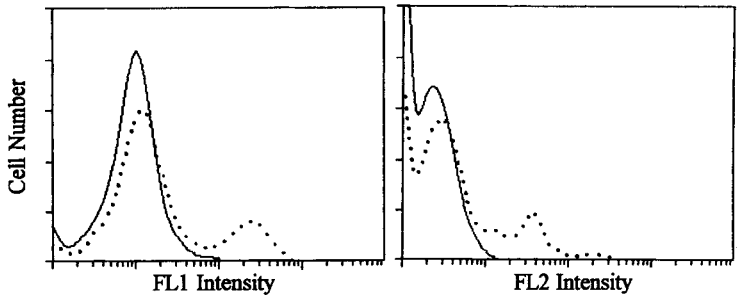


FIG. 1 Histograms of flow cytometry analysis. Left: CD4+ cells labeled with fluoroisothiocyanate (FL1). Right: CD8+ cells labeled with phycoerythrin (FL2). Depleted (——), undepleted (···).

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Figure 2 shows the dot plot distribution of the cells using the Becton Dickinson Consort 30 FACScan program. The upper left quadrant in each figure contains the CD8+ cells which are stained with PE-Leu-2; the lower right quadrant contains CD4+ cells stained with FITC-Leu-3a. The dot plots indicate near complete removal of the CD4+ cells in (b) and of CD8+ cells in (c).

In Fig. 3 the first bar graphs indicate the percentage removal of CD4+ cells using commercially available polystyrene magnetic spheres. These particles were used as a comparison study with our iron–dextran preparations. The middle graphs indicate the percentage removal of CD4+ cells, and the graph at the right the removal of CD8+ cells using the goat-antimouse conjugated iron–dextran microspheres. The average number of cells used in the experiments are shown on the *y* axis.

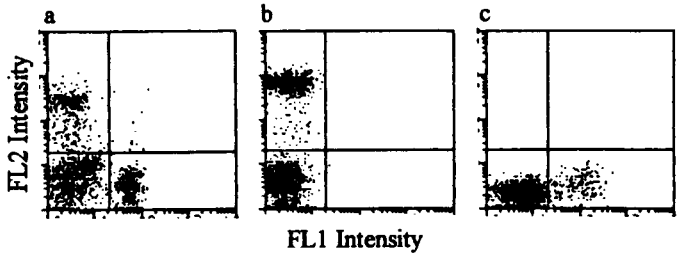


FIG. 2 Dot plots of flow cytometry analysis: CD4+ cells labeled with fluoroisothiocyanate (FL1); CD8+ cells labeled with phycoerythrin (FL2); UL quadrant = CD8+ cells; LR quadrant = CD4+ cells. (a) Undepleted, (b) CD4+ depleted, (c) CD8+ depleted.

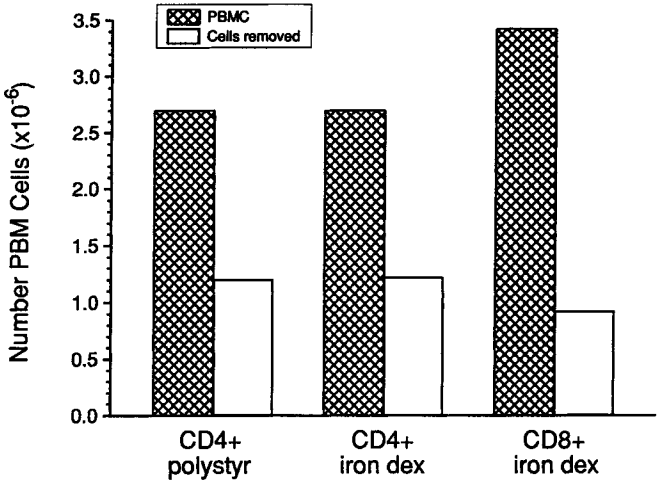


FIG. 3 Depletion of peripheral blood mononuclear cells (PBMC).

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## DISCUSSION

The efficiency of immunomagnetic cell separation procedures depends on affinity of the antibody for the cells. The antibodies should be carefully titered to optimize the system (7, 8). When complete removal of the desired population is not achieved, several modifications of the system can be made. The protocol can be repeated a second or third time. We have obtained excellent results using a biotinylated antibody followed by avidin conjugated to spheres. Monoclonal antibodies can be biotinylated using NHS-biotin (*N*-hydroxy-succinimidobiotin) (9). Rather than use NHS-biotin, we use an analog, NHS-S-S-biotin. The disulfide link is readily broken by reduction and used in positive cell recovery.

Magnetic cell sorting has some advantages over cell sorting by flow cytometry. The fluorescence activated cell sorter (FACS) is expensive. Its capacity is about $10^7$ cells/h (10). Magnetic affinity cell sorting (MACS) can be completed in less than an hour. Labeling of the oligosaccharide moieties of the antibodies with dextran–iron spheres insures that the label will be far from the site of antigen attachment (11). This conjugation is by periodate oxidation, which has been shown to have minimal effects on antibody avidity and immunoreactivity (12).

A panel of antibodies can be conjugated to carbonyl iron–dextran spheres and stored indefinitely. If unconjugated primary antibodies are

used, then only a single secondary antibody needs to be conjugated to the spheres.

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