

Evaluation of Eluents from Separations of CD34+ Cells from Human Cord Blood Using a Commercial, Immunomagnetic Cell Separation System

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Human CD34+ cells from cord blood were separated in a two-step process using a commercial, immunomagnetic cell retention system. The performance of the system was evaluated by analyzing a number of eluents from the separations with a number of analytical techniques. In addition to cell counts and flow cytometry analysis, a new experimental technique that is undergoing development, cell tracking velocimetry (CTV), was used. CTV measures the degree to which a cell is immunomagnetically labeled, known as the magnetophoretic mobility, of a population of cells on a cell-by-cell basis and presents the results in the form of a histogram similar to flow cytometry data. The average recovery and purity of CD34+ cells from 10 separations was 52% and 60%, respectively. CTV analysis indicated that the mean magnetophoretic mobility of the positively enriched CD34 cells was $9.64 \times 10^{-5} \text{ mm}^3/\text{T-A-s}$, while the mean mobility from negative eluents was $-2.02 \times 10^{-6} \text{ mm}^3/\text{T-A-s}$, very similar to the mobility of unlabeled cells. Within the positive eluents, the range of magnetophoretic mobility was approximately 50-fold, representing a plausible 50-fold range in surface CD34 antigen expression. CTV analysis also indicated that in some separations, positive cells were not retained by the immunomagnetic cell retention system. Finally, preliminary studies indicate that monocytes might be a primary cause in the lower purities and recoveries seen in this study. It is suggested that the monocytes phagocytose the magnetic nanobeads and become sufficiently magnetized to be retained within the Miltenyi column, reducing the purity of the positive eluent.

Introduction

Batch separation of specific cells using immunomagnetic labels has become a significant preparative technology in clinical and research settings. A recent computer search of the biological literature (Medline) indicated over 750 articles referencing batch, immunomagnetic separations in at least part of the experimental protocols. Examples of the various types of separations include separation of CD34+ peripheral blood stem cells (PBSC) (Despres et al., 2000), the separation of rare, circulating cancer cells from human cancer patients (Siewert et al., 2001; Racila et al., 1998), the separation of fetal cells from peripheral maternal blood (Geifman-Holtzman et al., 2000), the separation of glial cells from central nervous tissue (Wright et al., 1997), and the separation of microorganisms in food (Uyttendaele et al., 2000).

Two basic parameters can be used to evaluate the performance of a batch, immunomagnetic cell separation system: (1) the fractional (or percent) recovery of the target cells in the final product with respect to total number of target cells in the original feed and (2) the fractional (or percent) purity of the target cells in the final

product. Obviously, these two parameters are dependent on accurate determination of the presence of the desired cell in both the feed and final product. However, to fully understand and potentially optimize the performance of the separation system, measurements of other performance parameters are needed.

For a batch, immunomagnetic cell separation process, one can further evaluate the performance of the system by making the following divisions: (1) the ability of the magnetic retention device to retain the labeled cells (i.e., the cells are sufficiently magnetic to be retained), (2) the specificity of the magnetic labels for the target cells, and (3) the ability of the retention device to retain only the magnetically labeled cell (i.e., to not retain unlabeled cells).

Once labeled, the cell suspension is passed through a magnetic retention device, which typically consists of a flow chamber surrounded by a powerful magnetic gradient. The magnetic gradient is assumed strong enough to retain the immunomagnetically labeled cells yet allow the unlabeled cells (or weakly labeled) to pass through. The actual devices used in which the magnetic energy field gradient is focused range from flasks to flow channels resting on top of or surrounded by a magnet to small columns, packed with small steel spheres (Zborowski et al., 1996; Racila et al., 1998; Despres et al., 2000). After the magnetically labeled cells have been

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retained by the magnetic field, the flow channel is removed from the magnetic field. The retained cells are then either analyzed on the deposited surface or are allowed to flow out of the device and are subsequently studied.

The actual separation process within the retention device can be thought of as a two step process: (1) the attraction and subsequent movement of the immunomagnetically labeled cells to the retention surface, and (2) the immobilization (or retention) of the immunomagnetically labeled cells on the retention surface. A sufficient force must be present in order to retain the immobilized cells as the fluid viscous forces act to remove the cells from the surface. For obvious reasons, the actual design of the flow chamber, the type of and distribution of packing material (if present), and the related fluid mechanics within the flow channel are very important.

The magnetic labeling process can be either a one- or two-step antibody binding process, where one of the antibodies is covalently bound to a magnetic particle. A magnetic particle can range in size from several microns (Dynal beads) to a colloid of 30–200 nm (Miltenyi Biotec, Auburn, CA; Immunicon Corporation, Huntington Valley, PA). In some cases an avidin–biotin binding step can replace one of the antibody binding steps.

Theory of Imparting a Magnetic Force on a Cell. Quantitatively, the magnetic force operating on a single magnetic bead is defined as

$$F_b = \Delta\chi V_m \frac{\nabla B^2}{2\mu_0} \quad (1)$$

where F_b is the magnetic force acting on a single magnetic carrier, $\nabla B^2/2\mu_0$ is the magnetic energy field gradient, μ_0 is the magnetic permeability of free space, V_m is the volume of the paramagnetic material in a magnetic carrier, and $\Delta\chi$ is the difference in magnetic susceptibility between the carrier and the suspending medium (Zborowski, 1997). When immunomagnetic colloidal labels are used (Miltenyi, Immunicon), large numbers ($>10^4$) of antibody bead complexes can bind to a cell (McCloskey et al., 2000). In these cases, the magnetic force operating on a paramagnetically labeled cell in a magnetic energy field gradient is given by

$$F_m = F_b \beta ABC \quad (2)$$

where the lumped term βABC represents the magnetic particle binding capacity of a cell (McCloskey et al., 2000).

In free suspension within a magnetic field, the magnetic force will result in an induced velocity of the labeled cell. This magnetic force will be opposed by a drag force. For a Reynolds number less than 0.1, the drag force can be approximated using Stokes' law:

$$F_d = 3\pi\eta D_c v_c \quad (3)$$

where η is the fluid viscosity, D_c is the cell diameter, and v_c is the cell velocity. Since the magnetic and drag forces are typically orders of magnitude higher than the acceleration that a cell would experience in a magnetic energy field gradient (Reddy et al., 1996), an equality of the magnetic and drag force can be solved for the induced velocity. This velocity can be normalized by dividing by the magnitude of the magnetic energy field gradient to

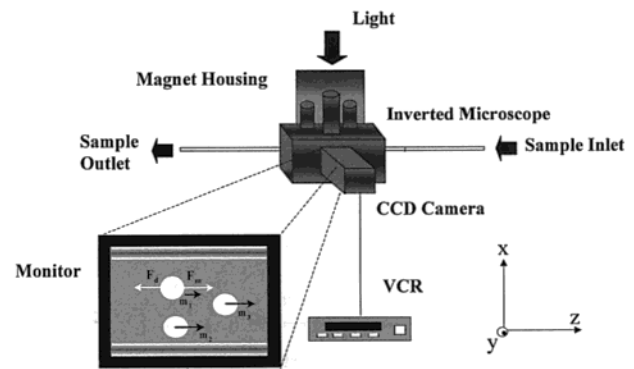


Figure 1. Overview of the cell tracking velocimetry apparatus.

obtain a term referred to as the *magnetophoretic mobility*, m . Mathematically, m can be expressed as

$$m = \frac{v_c}{S_m} = \frac{\Delta\chi V_m}{3\pi D_c \eta} \beta ABC \quad (4)$$

where S_m , the magnitude of the magnetic gradient, is defined by

$$S_m = \frac{|\nabla B^2|}{2\mu_0} \quad (5)$$

and v_c is the velocity of the labeled cell, D_c is the diameter of the cell, and η is the viscosity of the fluid.

Cell Tracking Velocimetry. To experimentally measure the magnetophoretic mobility, an instrument has been developed that measures the magnetophoretic mobility on a cell-by-cell basis (McCloskey et al., 2001; Nakamura et al., 2001; McCloskey et al., 2000; Moore et al., 2000; Chalmers et al., 1999). This technique, referred to as cell tracking velocimetry (CTV), uses a microscopic imaging system, computer imaging technology, and particle tracking programs to quantify the movement of immunomagnetically labeled cells or microbeads (beads on the order of microns) in a specifically designed, constant magnetic gradient (Figure 1). Calibration and control studies indicate that mobilities calculated with this system are reproducible and accurate (Nakamura et al., 2001; Moore et al., 2000). Magnetophoretic mobilities are reported in absolute units, which allows comparisons from day to day and, given the appropriate assumptions or measurements, from cell type to cell type.

The original objective of this study was to determine the magnetophoretic mobility of immunomagnetic labeled CD34+ cells. However, the performance of the actual separation process by the Miltenyi system was also considered. The quantification of immunomagnetic labeling consisted of using the CTV instrument to measure the mean and distribution of magnetophoretic mobility in the positive and negative eluents of the batch separations. In addition, using cell counts and flow cytometry, estimates of the recovery and purity of CD34+ cells in the final product were made. Finally, a discussion of the challenges of separating rare cells from cord blood and heterogeneous cell suspensions in general is presented.

Materials and Methods

Cells. Umbilical cord blood was used for these studies and was obtained through informed consent as approved by Ohio State University's Institutional Review Board. The mononuclear cell (MNC) layers were isolated by density gradient centrifugation over Ficoll-Hypaque (1.077

Table 1. Summary of Performance

run	feed					second, positive eluent		
	vol (μL)	concn (cells/ μL)	total no. cells	CD34 purity	total no. CD34+	recovery of CD34+ cells	CD34 purity	fraction of total cells recovered ^a
1	300	2.5×10^5	7.5×10^7	4.0×10^{-3}	3.0×10^5	1.14	0.76	5.53×10^{-1}
2	300	1.8×10^5	5.3×10^7	1.0×10^{-3}	5.3×10^4	0.55	0.29	9.28×10^{-1}
3	300	1.5×10^5	4.4×10^7	1.0×10^{-3}	4.4×10^4	0.00	0.00	6.94×10^{-1}
4	600	3.3×10^5	2.0×10^8	5.0×10^{-3}	10.0×10^5	0.39	0.86	6.50×10^{-1}
5 ^c	300	3.1×10^5	9.3×10^7	1.3×10^{-2}	1.2×10^6	0.19	0.57	2.87×10^{-1}
6 ^c	600	3.3×10^5	2.0×10^8	9.0×10^{-3}	1.8×10^6	0.67	0.76	9.34×10^{-1}
7 ^c	600	3.3×10^5	9.4×10^8	1.5×10^{-2}	3.0×10^6	0.21	0.65	9.33×10^{-1}
8	600	3.3×10^5	1.4×10^9	3.0×10^{-3}	6.0×10^5	1.01	0.81	9.34×10^{-1}
9	600	3.3×10^5	6.3×10^8	2.0×10^{-3}	4.0×10^5	0.78	0.78	5.61×10^{-1}
10 ^b	600							
11 ^c	600	3.3×10^5	2.0×10^8	1.5×10^{-2}	3.0×10^6	0.08	0.53	1.07
ave		2.9×10^5	3.8×10^8	6.8×10^{-3}	1.1×10^6	5.02×10^{-1}	6.00×10^{-1}	7.5×10^{-1}

^a The sum of the total number of cells in the first and second negative eluent and the second positive eluent divided by the total number of cells fed. ^b In this experiment, the FACS results were not available. ^c Indicates that the column was "pulsed".

g/cm³, Accurate Chemicals, Westbury, NY) at 400g for 30 min at 18 °C. The MNC layers were collected, washed with Ca²⁺, Mg²⁺-free Dulbecco's phosphate-buffered saline (PBS) containing 1% HSA. The MNC layers were then resuspended in degassed PBS containing 0.5% HSA and 2 mM EDTA (buffer). Cell were washed by adding 5–10 \times of the original volume in milliliters of buffer solution and centrifuged at 400g for 7 min.

Cell Counts. The concentration of cells in a specific suspension was determined by using a Unopette test (Becton Dickinson, San Jose, CA) and a hemocytometer.

Magnetic Labeling of CD34+ Progenitor Cells. The MNC layers were resuspended in buffer to obtain a concentration of 10⁸ total cells/300 μL . To this cell suspension was added Fc receptor blocker (human IgG, Miltenyi Biotech, Auburn, CA) in the amount of 100 μL /10⁸ total cells. Simultaneously, a hapten conjugated CD34 antibody in the amount of 100 μL /10⁸ total cells was added (Reagent A2, Miltenyi Biotech). This suspension was incubated for 15 min at 6–12 °C. After incubation, the cell suspension was washed with buffer and resuspended in buffer to obtain a cell suspension at a concentration of 10⁸ total cells/400 μL . To this suspension was added MACS nanobeads recognizing the hapten-conjugated CD34 antibody (Reagent B, Miltenyi Biotech) in the amount of 100 μL /10⁸ total cells. This suspension was once again incubated at 6–12 °C for 15 min. Finally, in preparation of loading into the magnetic column, the cell suspension was washed and resuspended in buffer to obtain a concentration of approximately 10⁸ total cells/500 μL . The initial concentrations for each separation run are presented in Table 1.

Magnetic Separation. After the two-step immunomagnetic labeling procedure, the cells were immediately introduced into a prefilled (with PBS buffer) MS+ column (Miltenyi Biotech). The actual process, as recommended by the manufacturer, consisted of introducing the labeled cell suspension into the top of the MS+ column, followed by 1.5 mL of buffer. The added cell suspension was allowed to flow through the column and was collected at the bottom exit. This cell suspension was referred to as the first, negative eluent. Next, the MS+ column was removed from its magnetic housing and 1.0 mL of buffer solution was forced through the column by using a plunger supplied by the manufacturer to elute the retained cells. This collected cell suspension was referred to as the first, positive eluent. This first, positive eluent was used as a feed to a second prefilled column mounted in the magnetic housing, and the process was repeated a second time, producing a second, negative eluent and

a second, positive eluent. Figure 2 presents a diagram of the process.

In runs 5–7 and 11 the first feed did not flow continuously through the column. Consequently, as suggested by the manufacturer, the plunger was attached and pressure was applied to force the cell suspension through the column. The experimental runs in which this pressure was needed are marked in both the table and figures.

Flow Cytometry. To determine the performance of the separation system, initial aliquots of cord blood MNC were taken prior to immunomagnetic labeling for flow cytometry background IgG and autofluorescence studies and to determine the initial percentage of positive CD34 cells in the sample. In addition, aliquots were removed from all eluents for flow cytometry analysis. These aliquots ranged in size from 1×10^4 to 3×10^5 cells and were resuspended in 80–100 μL of buffer. For studies in which the percentage of CD34+ cells present in a specific eluent was desired, a sample was removed in which 20 μL /10⁶ cells of anti-human CD34/FITC conjugated antibody (Becton-Dickinson) was added. In all cases the final cell concentration was 10⁶ cells/100 μL and the cell suspensions containing the fluorescent antibodies were incubated for 30 min at 6–12 °C. After incubation, the cell suspension was washed in 1–2 mL of buffer, centrifuged, and resuspended in the appropriate amount of buffer. These various cell suspensions were analyzed on a Beckman-Coulter EPICS Elite II ESP flow cytometer. Both autofluorescence and isotype controls were performed to produce the appropriate gates to quantify the positive cell populations.

Cell Tracking Velocimetry. CTV was used to determine the mean and distribution of the magnetophoretic mobility (eq 4) of unlabeled cord blood and representative cell samples from the second, positive eluent and the first, and in some cases, the second, negative eluent. From the magnetophoretic mobility of the unlabeled cord blood, threshold values or gates were chosen such that cells with a mobility above this gate were considered positively labeled with respect to the magnetic labels. This procedure allowed the percentage of positively labeled cells to be determined. The operation of the CTV instrument has been discussed previously and will only be briefly explained here (Nakamura et al., 2001; Chalmers et al., 1999a,b,c).

The suspension of cells to be analyzed are pumped into a square borosilicate glass channel (1.0 mm i.d., 1.4 mm o.d.). This channel passes through a specially designed magnet that contains a microscope objective (Figure 1).

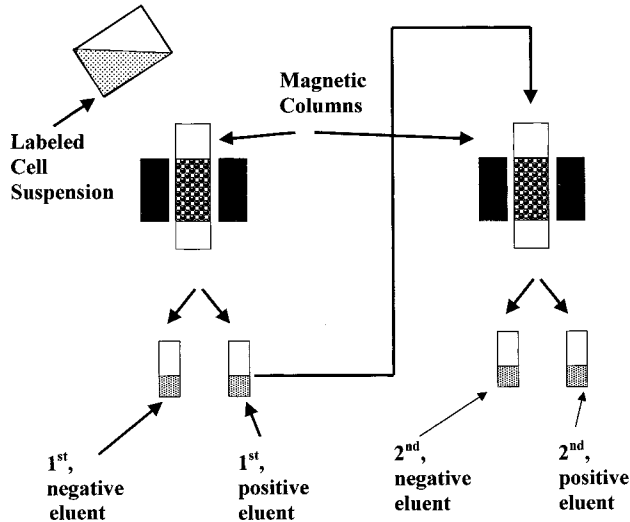


Figure 2. Schematic diagram of the cell separation process.

The magnetic gradient, S_m , is nearly constant within this microscopic viewing area with a magnitude of 198 T-A/mm² (Chalmers et al., 1999a); consequently, the magnetically induced velocity remains nearly constant with a direction perpendicular to gravity in the microscopic viewing region. This constant, magnetically induced velocity greatly simplifies later computer analysis. The movement of cells within this viewing region are then recorded and processed using a video imaging and computer hardware and software instrumentation. The final output is a histogram that presents the distribution of the magnetophoretic mobility of a cell suspension. This histogram format facilitates the gating of the data in a manner similar to FACS.

Results

A total of 11 separations of human cord blood, using the protocol described previously, were conducted, and the overall performance is presented in Table 1. As is observed, a significant range in overall recovery and purity of CD34+ cells in the final, second positive fraction was obtained.

Total cell recovery was calculated by dividing the sum of the total number of cells in each of the recovered eluents (first and second negative, and second positive) by the total number of cells added to the system. The total number of cells in each eluent was obtained by knowing the volume of the collected eluent in calibrated tubes (also by keeping track of the total amount of fluid added to the column) and multiplying by the cell concentration as determined by a hemocytometer. A calibration test of the reproducibility of the hemocytometer data resulted in a coefficient of variation (CV) of 22% (data not shown).

The final recovery of CD34+ cells was calculated using the following relationship:

$$R_{CD34+} = \frac{F_{CD34+,2+e} \cdot N_{tot,2+e}}{F_{CD34+,f} \cdot N_{tot,f}} \quad (6)$$

where F_{CD34+} is the fraction of positive cells as measured by flow cytometry, N_{tot} is the total number of cells in a given cell sample as measured by hemocytometer and cell suspension volume, 2+e refers to the second, positive eluent, and f refers to the feed.

In an attempt to understand the cause(s) of the large range in the recovery and final purity of CD34+ cells,

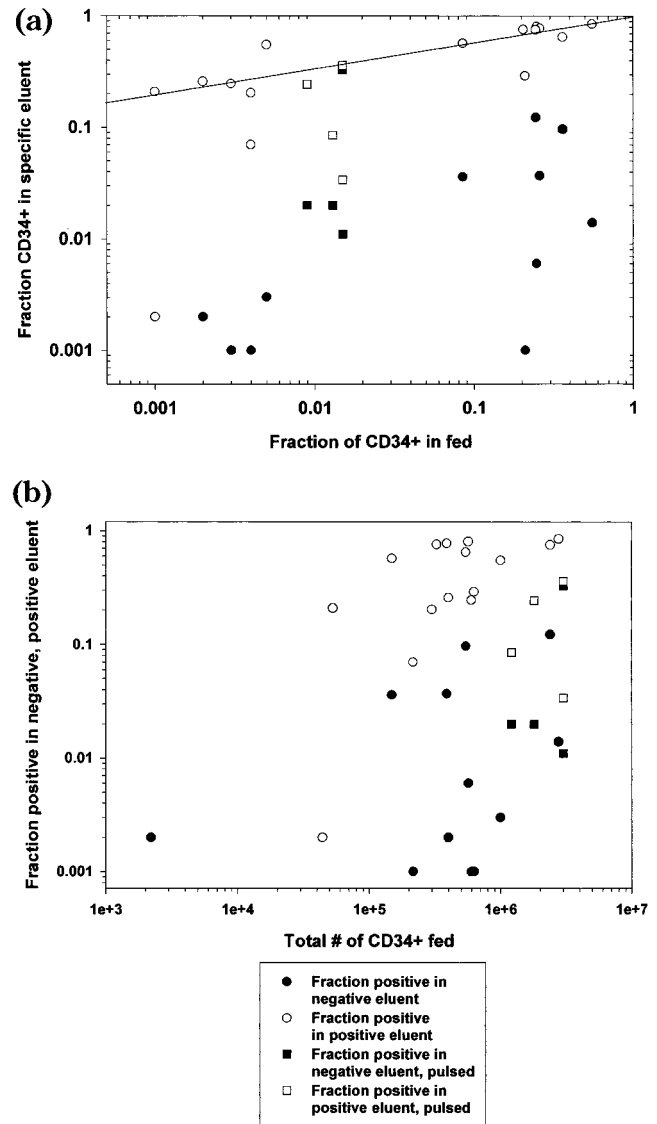


Figure 3. Fraction of CD34+ in the positive and negative eluent as a function of the fraction positive in (a) the feed and (b) the total number of CD34+ cells loaded.

both values were plotted as a function of initial, total cell loading to the column and as a function of initial number of CD34+ cells loaded (plots not shown). The only trend that could be observed is that both the purity and recovery appear to reach a peak when the initial, total number of CD34+ cells is in the approximate range of 1×10^5 to 1×10^6 cells. Taking the average of the recovery and purity in the range of 9×10^4 to 1×10^6 CD34+ cells, one obtains significantly higher values of purity and recovery of 0.82 and 0.68, respectively.

Of the 11 total runs, four had sufficiently slow flow through the column such that the plunger had to be applied to the top of the MS column to create a slight pressure to resume flow. These runs, referred to as being pulsed are marked with a "c" in Table 1 and presented as open and solid squares in all of the figures.

To further study the separation performance, the process was divided into two independent stages. This was possible since flow cytometry analysis was also conducted on the first, negative eluent sample and the first, positive eluent sample. These data are presented in a plot of the fraction of CD34+ cells in the negative and positive eluents (y -axis) as a function of the fraction of CD34+ in the feed (x -axis) (Figure 3a). Ideally, one

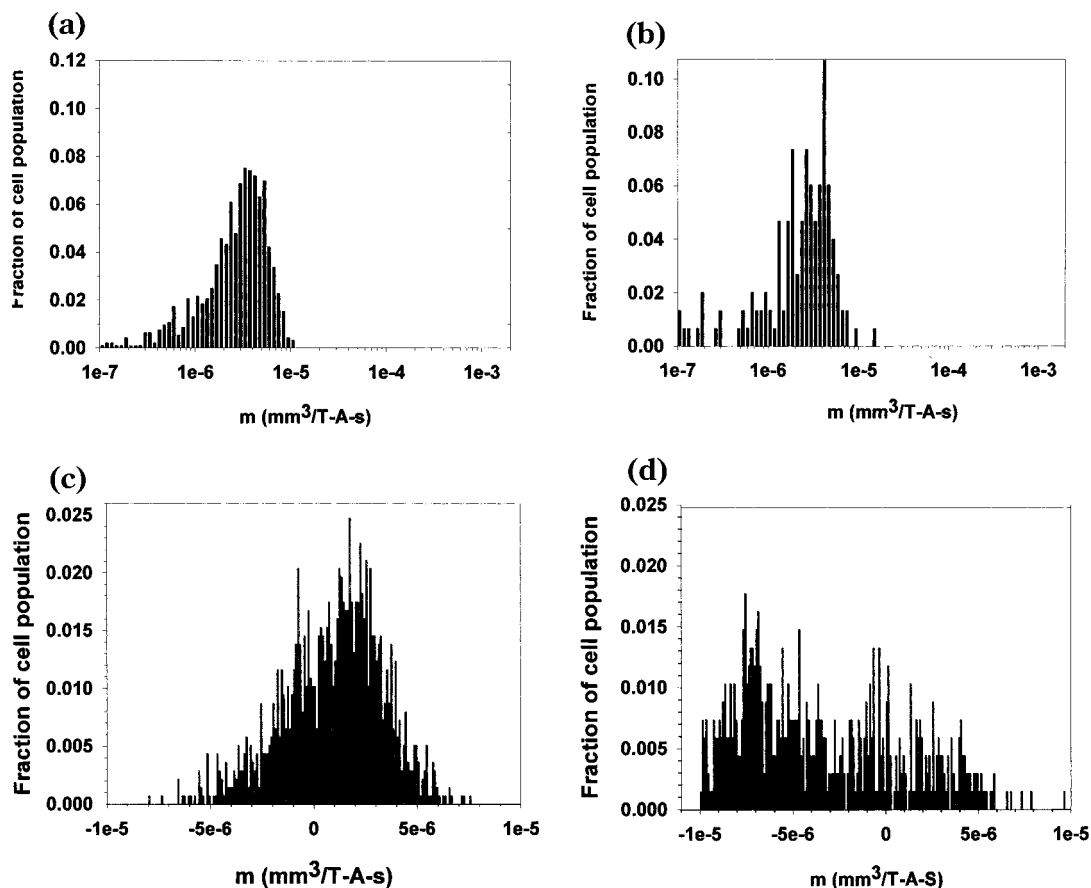


Figure 4. Histograms of the magnetophoretic mobility of (a and c) monodisperse 2.7 μ m calibration beads and (b and d) fresh, Ficoll-Hypaque separated, unlabeled cord blood. Note that a and b are in a log mode and c and d are in a linear mode.

would expect that the fraction of CD34+ cells in the positive eluent (open symbols) would not vary and remain near a value of 1.0 and that the fraction of CD34+ cells in the negative eluent (closed symbols) would remain low (below a fraction of 0.001) and also not vary with the fraction of CD34+ cells in the feed. However, in practice this was not observed. Although there is a noticeable increase (as can be observed by the slope of the solid line) in the fraction of CD34+ cells in the positive eluent with increasing fraction of CD34+ in the feed, no trends in the fraction of CD34+ in the negative eluent can be observed.

An alternative to Figure 3a is to plot the fraction positive in the positive and negative eluent as a function of the total number of CD34+ loaded onto the column, Figure 3b. There appears to be a general increase in the fraction of CD34+ cells in the negative eluent as the number of total CD34+ cells fed to the column increases. While care should be taken in interpreting log-log plots, this trend has analogies to the breakthrough concept in chromatography, namely, that there is a limited number of binding sites for the cell to be retained (bound) and that once those sites are occupied, further positive cells cannot bind and are thus not retained.

The previous analysis was based on total cell counts using a hemocytometer, volumes of cell suspension measured with a pipet, and fractions of a cell population positive for CD34 cells through flow cytometry analysis. These 11 experiments, which were conducted over a period of approximately one and three-quarters years, were primarily performed to provide CD34+ cells for magnetophoretic mobility analyses by the CTV. During this period significant progress was made in the develop-

ment of the CTV instrument (Chalmers et al., 1999a; Nakamura et al., 2001).

Figures 4a and 4c are histograms of the CTV determined magnetophoretic mobility of 2.7 μ m, nonparamagnetic polymeric microspheres, and 4b and 4d are histograms of fresh, separated, unlabeled cord blood. Figures 4a and 4b are log presentations of the data, whereas 4c and 4d are linear presentations of the data. Unlike a typical flow cytometer in which the photons emitted from the fluorescent label are amplified by a photo multiplier tube and a log amp, resulting in an output in the form of relative fluorescence intensity, the raw data from the CTV system is the velocity of the tracked cell or particle, which is measured in a linear mode. Consequently, the data from CTV can be plotted on either a linear or log scale, while the data from a flow cytometer can only be presented in the manner in which it was acquired (either logarithmically or linearly). The 2.7 μ m, nonmagnetic polymeric microspheres histograms are from a larger study of a set of polymeric microspheres doped with various amounts of paramagnetic material used to test the accuracy of the CTV system (Moore et al., 2000).

A significant point with respect to the CTV histograms of the unlabeled cord blood cells (4b and 4d) is the highest mobility that the cells achieve, approximately 1×10^{-5} (mm³/T-A-s). This maximum mobility can be used as a threshold or gate, such that any cell with a mobility higher than this value is considered positively labeled with respect to immunomagnetic labels.

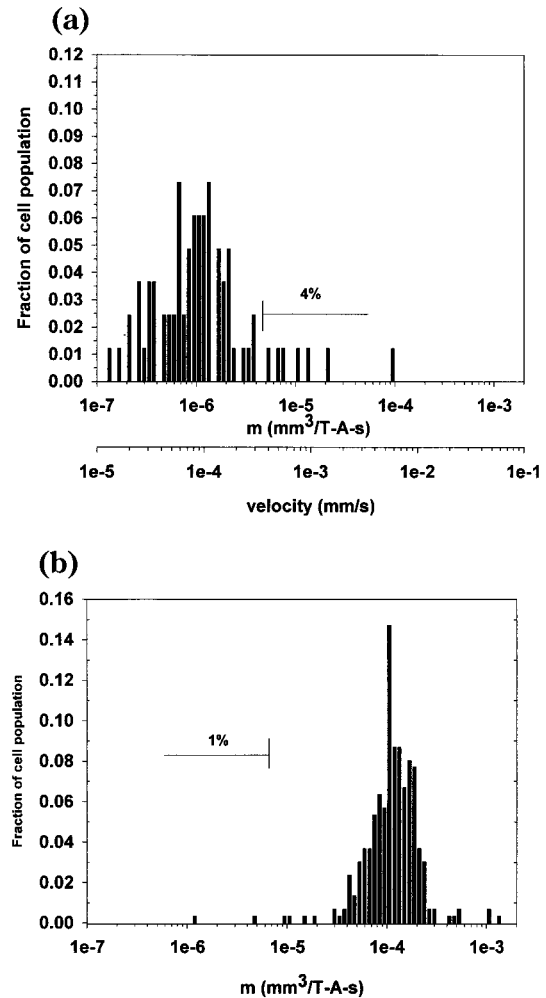
Table 2 presents the number of particles or cells tracked, N , and the mean mobility (mm³/T-A-s) of the two populations presented in Figure 4a-d, as well as the data for two other nonlabeled cord blood samples. Both the

Table 2. Mean Mobilities of Polymeric Microspheres and Nonlabeled Cord Blood Cells

sample	N	mobility (mean, mm ³ /T-A-s)
unlabeled cord blood	203	-8.10×10^{-7}
unlabeled cord blood	450	-2.60×10^{-6}
unlabeled cord blood	677	-4.24×10^{-6}
mean	443	-2.55×10^{-6}
polystyrene calibration beads	1373	9.18×10^{-7}

numerical value of mobility and visual observations of the location of peaks in Figures 4c and 4d indicate an average negative mobility in the nonlabeled cord blood relative to the nonmagnetic polymeric microspheres. This average negative mobility probably represents the diamagnetic properties of the blood cells. Diamagnetism is the opposite of paramagnetism, and theoretically, biological cells should be slightly diamagnetic as a result of the diamagnetic properties of carbon, nitrogen, and oxygen (Chikov et al., 1991; Kuznetsov et al., 1997; Winoto-Morbach et al., 1994, 1995). This slight diamagnetic nature of biological cells has also been observed in other cell types in the CTV apparatus (data not shown). Figures 5–8 present the results of CTV analysis of the first, negative eluent (5a, 6a, 7a, 8a) and the second, positive eluent (5b, 6b, 7b, 8b) from runs 7, 8, 10, and 11 in Table 1. Using a mobility of 1×10^{-5} (mm³/T-A-s) as the threshold value to distinguish between negatively and positively labeled cells, the fraction of positive cells in a negative eluent and fraction of negative cells in a positive eluent are presented. To assist in the analysis and facilitate a comparison between CTV and flow cytometry, Table 3 presents the number of cells tracked, the mean mobility, and the percent positive by CTV and flow cytometry for the first negative, second negative, and second positive eluents. As a point of reference, a second *x*-axis was added to Figure 5a with units of mm/s. It should be remembered that this velocity is a function of the magnetic gradient (eq 4); hence this velocity is achieved with the specific magnetic gradient present in the CTV instrument. However, this velocity axis clearly indicates that the assumption of Stokes' law is valid. Assuming that the suspending buffer has a density and viscosity of water and using a cell diameter of 5 μ m, the cell would have to move at a velocity of 20 mm/s to achieve a Reynold number of 0.1, the limit of Stokes' law. As can be observed in all of the CTV Figures, 20 mm/s is over 2 orders of magnitude higher than the highest velocity observed.

Table 3 indicates that not all of the eluents were analyzed by CTV because of the enhancement of the CTV instrument during the period of time that the studies were conducted. Nevertheless, a number of observations can be made. First, the mean mobility of the first, negative eluent was effectively the same as the unlabeled control samples (Table 1). Second, a significant range (approximately 50-fold) exists in the mobility of the

**Figure 5.** Histograms of the magnetophoretic mobility of (a) the first, negative eluent in run 7 and (b) the second, positive eluent.

positively labeled cells (Figures 5b, 6b, 7b, 8b). Third, Figures 5a, 6a, 7a, and 8a indicate some leakage of positive cells into the negative eluents while Figures 5b, 6b, 7b, and 8b indicate the presence of negative cells in the second, positive eluent, based on application of the threshold value of 1×10^{-5} mm³/T-A-s.

The range in magnetophoretic mobility of labeled cells is consistent with the range in fluorescence intensity shown by the flow cytometer histograms. Specifically, Figure 9a is a histogram of the flow cytometry analysis of the second, positive eluent from one of the cord blood separations. The cells were labeled with anti-human CD34+ FITC. Figure 9b is the magnetophoretic mobility of the second, positive eluent presented in Figure 7b (Note, the histograms in 9a and 9b did not come from the same experiment). A broad distribution is repre-

Table 3. CTV and FACS Analysis of the Various Eluents from the MiniMACS MS Column Separations

run	negative eluent 1				negative eluent 2				positive eluent 2			
	<i>N</i>	mean mobility	% pos by CTV	% pos by FACS	<i>N</i>	mobilities	% pos by CTV	% pos by FACS	<i>N</i>	mean mobility	% pos by CTV	% pos by FACS
7	876	-2.18×10^{-6}	4.00	33.00	na	na	na	na	412	1.31×10^{-4}	99.00	65
8	831	-2.45×10^{-6}	6.00	1.00	na	na	na	na	462	7.70×10^{-5}	98.00	81
9	616	-2.12×10^{-6}	0.69	2.00	na	na	na	na	230	7.70×10^{-5}	77.00	80
10	691	-6.04×10^{-7}	0.81	na	1956	2.01×10^{-6}	2.00	na	823	5.68×10^{-5}	92.50	na
11	972	-2.76×10^{-6}	12	1.1	381	-3.75×10^{-6}	10	1.4	381	4.85×10^{-5}	66	53
av		-2.02×10^{-6}							482	8.55×10^{-5}	91.6	75.3

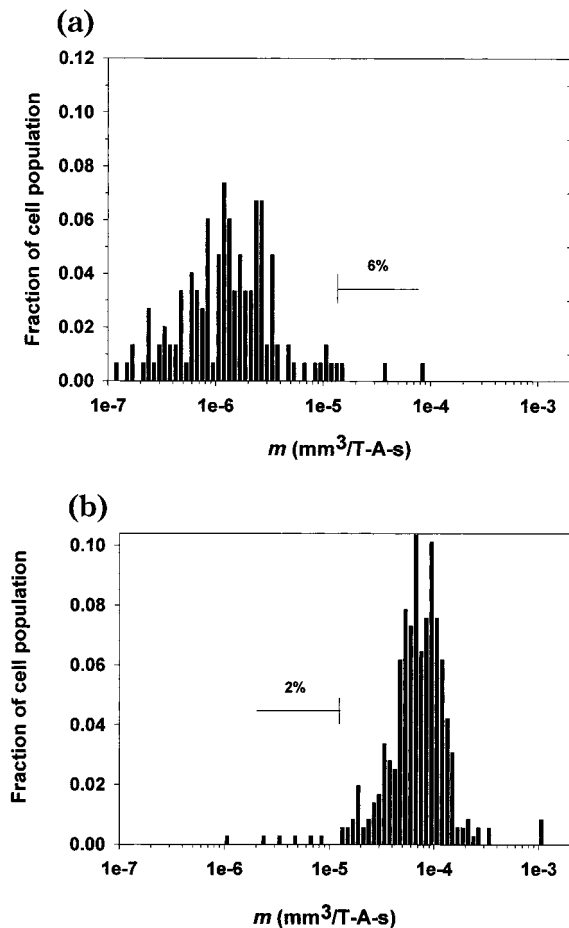


Figure 6. Histograms of the magnetophoretic mobility of (a) the first, negative eluent in run 8 and (b) the second, positive eluent.

sented in both the CTV and the flow cytometry histogram.

Discussion

The original objective of this study was to determine the magnetophoretic mobility of immunomagnetically labeled CD34+ cells from human cord blood. Since these cells are typically rare (0.1–3%), a commercial, batch immunomagnetic cell retention system was used initially to enrich the cells prior to CTV analysis. As a result, the collection of data obtained in the process of enrichment of CD34+ cells allowed preliminary studies on the actual separation process itself.

The results presented in Table 1 and Figure 3 indicate the complexity of evaluating the separation performance of rare cells from human cord blood. Although the average recovery and purity of the final product is relatively high, it is somewhat lower than other literature reports, (Schwinger et al., 1999). At this point it should be noted that published literature reports (de Wynter et al., 1999; Lang et al., 1999) and studies in our laboratory (data not shown) routinely demonstrate higher recoveries and purities using MACS systems on peripheral blood than what is reported in this study using cord blood. A likely cause is the inaccuracy of determining the number of positive cells in the original feed (and potentially in the final product). It has been suggested that this inaccuracy is increased by the presence of nucleated red blood cells in cord blood (Hubl et al., 1998). However, the leakage of positive cells and retention of negative cells in the column is also a possibility. Also, as discussed

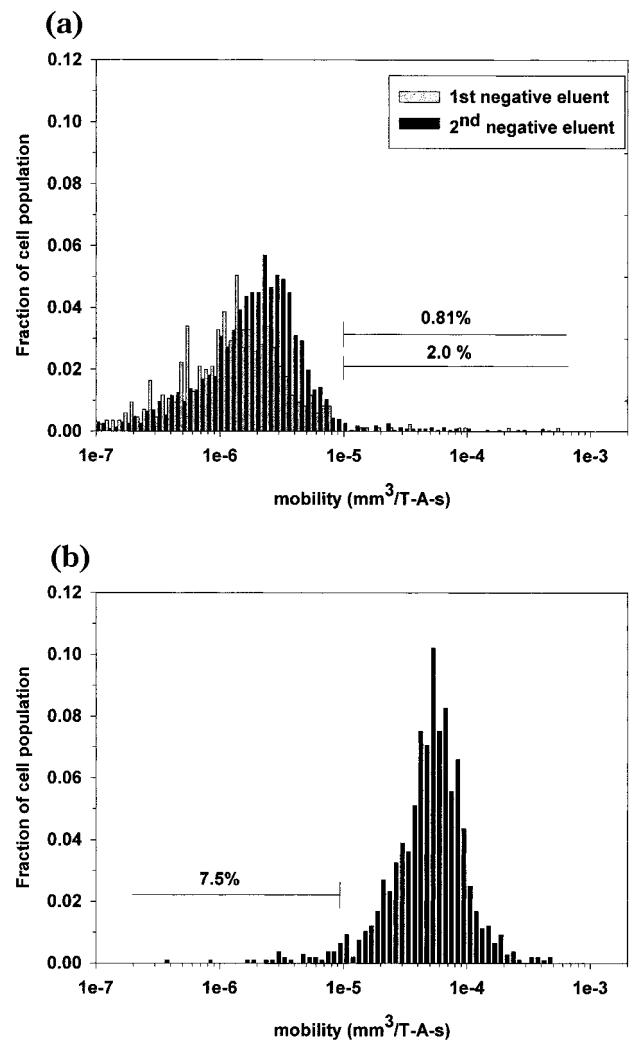


Figure 7. Histograms of the magnetophoretic mobility of (a) the first and second negative eluent in run 10 and (b) the second, positive eluent.

previously, if the initial number of CD34+ cells loaded into the MS+ column was in the range of 1×10^5 to 1×10^6 , significantly higher values of recovery and purity were obtained.

Ongoing research in our laboratory has focused on the role of monocytes in cord blood during the antibody/MACS nanoparticle labeling process. Monocytes play vital roles in multiple host-defense mechanisms such as phagocytosis of microorganisms as well as phagocytosis of effete cells, cellular debris, and other particulate matter. A preliminary study was performed in our laboratory to determine if monocytes would phagocytose MACS nanoparticles without having a primary antibody attached to the cellular surface. A sample of cord blood was divided into two separate aliquots: one that contained monocytes and the other where the monocytes had been depleted. Both of these samples were then incubated with only MACS nanoparticle reagent present and then run through the Miltenyi MS+ columns. CTV analysis indicated that the feeds containing the monocytes had significant mobilities over the 1×10^{-5} threshold. When this sample was passed through a MiniMACS MS+ column, recoveries on average were about 50%, suggesting that cells were retained in the column. However, the feeds where the monocytes had been removed resulted in a mobility similar to unlabeled cord blood, indicating no uptake of the MACS nanoparticles. When the mono-

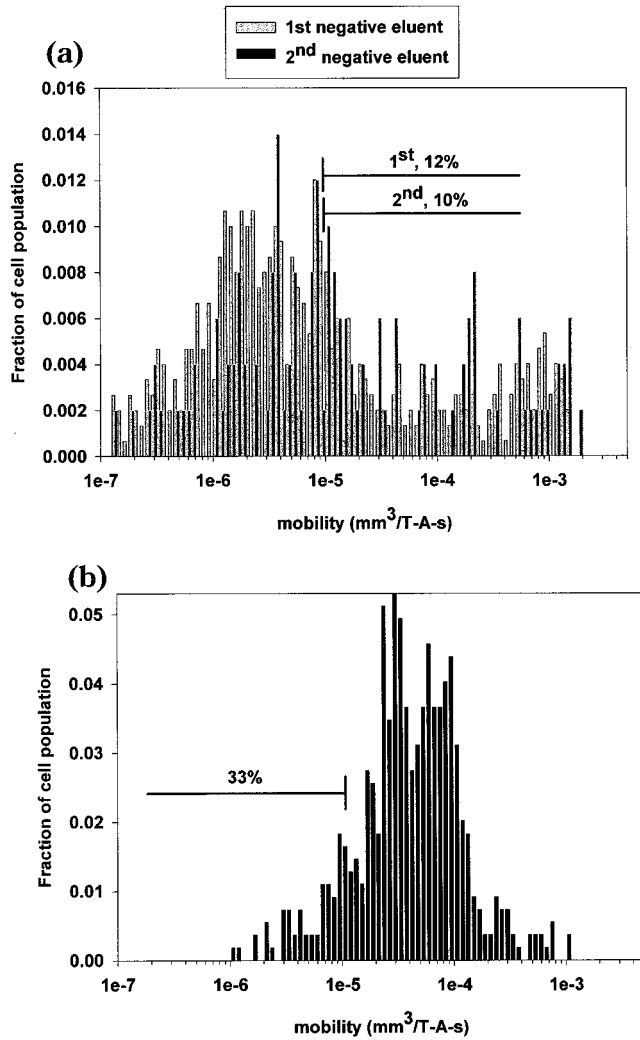


Figure 8. Histograms of the magnetophoretic mobility of (a) the first and second negative eluent in run 11 and (b) the second, positive eluent.

cyte depleted sample was passed through a MiniMACS MS^+ column, recoveries averaged around 85%.

While this preliminary study indicates that monocytes phagocytize MACS nanoparticles, further studies are needed to verify this observation. Nevertheless, these initial results indicate that the depletion of monocytes is a crucial step to better overall recovery and purity when working with cord blood stem cells and the MiniMACS system. It should also be noted that none of the cord blood samples used in the studies presented in Figures 3–8 and Tables 1–3 had the monocytes depleted.

The heterogeneity of cord blood cells and the difficulty in estimating the fraction of rare cells in a blood sample by flow cytometry is a well discussed observation (Brecher et al., 1996; Johnsen et al., 1996; Gratama et al., 1997). This difficulty has led to the establishment of a number of standardized protocols (i.e., ISHAGE, Sutherland et al., 1996) and commercial, standard kits (ProCOUNT assay, Becton Dickinson Immunocytometry Systems, San Jose, CA) for enumeration of CD34^+ cells. In general, these protocols use multiple color flow cytometry analysis to more accurately determine both the number of CD34^+ cells and the number of negative cells. However, even when using these standardized protocols, the same samples of peripheral blood and apheresis product sent to different labs using the protocols mentioned resulted in CVs of 18–30%, and gating errors increased the CVs

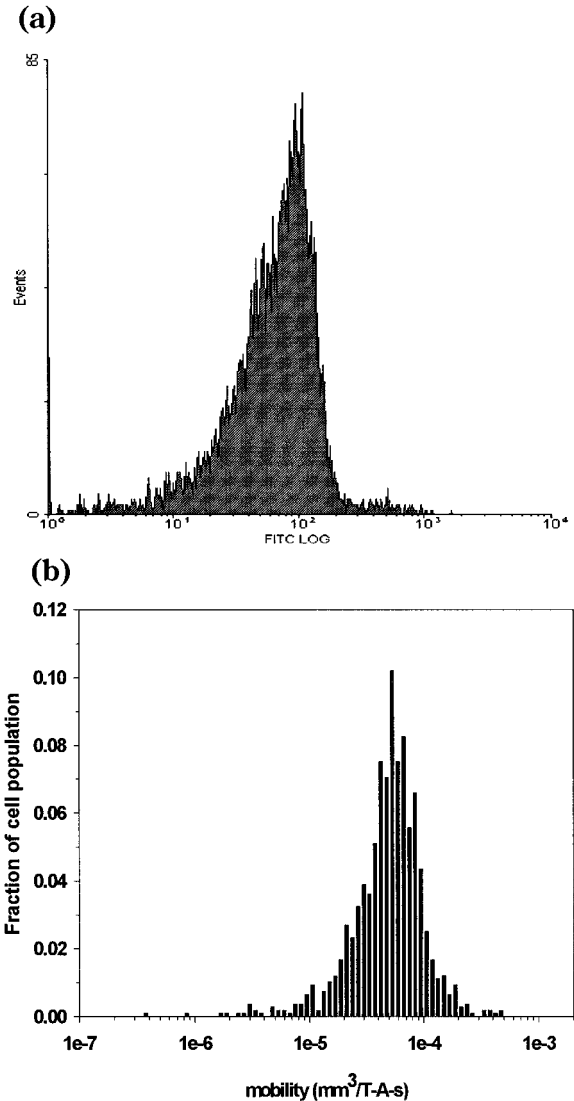


Figure 9. Flow cytometry histogram (a) of a representative sample of the second, positive eluent. The cells were labeled with anti-human CD34^+ FITC. For comparison, the histogram in 7b is presented in 9b.

to 82% (Gratama et al., 1997). In addition, it has been reported that at least one of these protocols might not work well for cord blood because of the relatively high content of nucleated red cells (Hubl et al., 1998).

Because these relatively advanced protocols can still produce significant variability and at least one apparently does not work well with cord blood, the relatively simple, single color method of CD34^+ cell detection with flow cytometry was chosen. In addition, the protocols described above required significantly more events (typically 50,000–75,000) than the 10,000 that we used. Also, our primary focus was the quantification of the magnetophoretic mobility of immunomagnetically labeled CD34^+ cells. The CTV system was and continues to be under development, so it was decided that as many CD34^+ cells as possible should be available for CTV analysis rather than flow cytometry analysis.

Given the various errors associated with the flow cytometry measurements, especially with estimates in the number of positive cells in the feed (in all but one case less than 1%), it is not surprising that significant variability is observed in the calculated recovery of CD34^+ cells. However, this reasoning cannot be applied as easily to account for the low purity in the final product

since flow cytometry is usually much more accurate when the percent positive is high. Interestingly, the percent of positive cells in the second, final eluent are consistently higher with the CTV results when compared to the flow cytometry results. This discrepancy could be the result of a difference in the selectivity of the antibody-magnetic colloid conjugate versus the antibody-FITC conjugate for the CD antigen epitope or differences in how the two instruments differentiate between positive and negative cells.

The CTV results present a clear difference between the mean magnetophoretic mobility in the negative eluents and the second, positive eluent. However, the CTV results (Figures 5–8) also indicate the presence of positive cells in the negative eluents and negative cells in positive eluents using our threshold value. Even the relatively low amount of positive cells in the negative eluent can have a significant, detrimental effect when attempting to recover rare cells. The presence of positive cells in the negative eluents and negative cells in positive eluents also indicates that the actual magnetic cell separation procedure could be further optimized.

A final observation with respect to the CTV results is the large range in magnetophoretic mobility of the positively labeled cells (Figures 5b, 6b, 7b, and 8b). Equation 4 and the work of McCloskey et al. (2000) indicate that the magnetophoretic mobility, m , within certain limits, is proportional to the antibody binding capacity, ABC, of the cell. If one assumes that the ABC is proportional to the number of antigen sites on the cell, then the cells in the second, positive eluent have a range of CD34 antigen expression levels of 50-fold.

A magnetic cell separation apparatus has been developed, referred to as the dipole magnetic flow sorter (DMFS), which can exploit such a range of expression levels (magnetophoretic mobility) by fractionating the immunomagnetically labeled cell populations into different exit flow channels based on different magnetophoretic mobilities (Moore et al., 1998). In conclusion, the work presented in this report represents a work in progress involving the evaluations of several aspects of immunomagnetic cell separation. Ongoing and future work in our laboratories is focusing on each of the aspects presented in this report: the ability of the magnetic retention device to retain only the paramagnetically labeled cells, the selectivity of the paramagnetic labels for the target cell, and the quantification of the factors involved in making the cells sufficiently paramagnetic. In addition, potential cell function associated with the range in magnetophoretic mobility of CD34+ cells from human cord blood is being studied in the DMFS.

Acknowledgment

This work was supported by the National Cancer Institute (R33 CA81662-01 to J.J.C., RO1 CA62349 to M.Z., and CA16058-25 to OSU), the National Science Foundation (BES-9731059 to J.J.C.), and Novamedics Inc. (Cleveland, OH). We would like to thank Mr. Andy Oberszyn at the Ohio State University Analytical Cytometry Laboratory for performing FACS analysis and making technical suggestions.

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Accepted for publication July 16, 2001.

BP010079R