

# Effects of Antibody Concentration on the Separation of Human Natural Killer Cells in a Commercial Immunomagnetic Separation System

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**Background:** The magnetic separation of a cell population based on cell surface markers is a critical step in many biological and clinical laboratories. In this study, the effect of antibody concentration on the separation of human natural killer cells in a commercial, immunomagnetic cell separation system was investigated.

**Methods:** Specifically, the degree of saturation of antibody binding sites using a two-step antibody sandwich was quantified. The quantification of the first step, a primary anti-CD56-PE antibody, was achieved through fluorescence intensity measurements using a flow cytometer. The quantification of the second step, an anti-PE-microbeads antibody reagent, was achieved through magnetophoretic mobility measurements using cell tracking velocimetry.

**Results:** From the results of these studies, two different labeling protocols were used to separate CD56+ cells from human, peripheral blood by a Miltenyi Biotech MiniMACS cell separation system. The first of these two labeling protocols was based on company recommendations,

whereas the second was based on the results of the saturation studies. The results from these studies demonstrate that the magnetophoretic mobility is a function of both primary and secondary antibody concentrations and that mobility does have an effect on the performance of the separation system.

**Conclusions:** As the mobility increased due to an increase in bound antibodies, the positive cells were almost completely eliminated from the negative eluent. However, with an increase in bound antibodies, and thus mobility, the total amount of positive cells recovered decreases. It is speculated that these cells are irreversibly retained in the column. These results demonstrate the complexity of immunomagnetic cell separation and the need to further optimize the cell separation process. *Cytometry* 45: 285–293, 2001. © 2001 Wiley-Liss, Inc.

**Key terms:** natural killer cells; MiniMACS separation column; immunomagnetic label; magnetic cell separation; cell tracking velocimetry

The separation of a heterogeneous cell population based on cell surface markers is a fundamental practice in many biological and clinical laboratories. A number of commercial, immunologically based cell separations exist, one of the more popular being magnetically based (1).

One widely used commercial immunomagnetically based system is the MiniMACS magnetic isolation system (Miltenyi Biotech, Bergisch Gladbach, Germany). The MiniMACS isolation system is a batch magnetic separator in which magnetically labeled cells are retained in a steel microsphere packed column while nonlabeled cells pass through (high-gradient magnetic separation [HGMS]). Upon removal of the column from the magnetic field, the

magnetically retained cells are then eluted by forcing carrier buffer through the column. This batch mode of separation is used currently to isolate a variety of antigen-defined cells. In general, there are three primary measures of the overall performance of such a system: (1) the fractional (or percent) recovery of the target cell in the final product with respect to the total number of target cells in the original feed, (2) the fractional (or percent)

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purity of the target cells in the final product, and (3) the throughput (number of cells per second) the system can separate.

Each of these three measures of performance is affected by the operation of the cell retention device itself. As discussed by Melnik et al. (2), the actual process within the cell retention device can be thought of as two steps: (1) the attraction and subsequent movement of the immunomagnetically labeled cells to the retention surface (steel microspheres) and (2) the immobilization (or retention) of the immunomagnetically labeled cells on the retention surface.

The attraction and movement,  $v$ , of immunomagnetically labeled cells is the product of the magnetophoretic mobility,  $m$ , of an immunomagnetically labeled cell and the magnetic force field strength,  $S_m$ , of the separation device. It has been suggested that the magnitude of the magnetophoretic mobility of the cell using a two-step antibody labeling protocol is mathematically defined by:

$$m = \frac{v}{S_m} = \frac{(n_1\theta_1\lambda_1)(n_2\theta_2\lambda_2)n_3k}{3\pi\eta D_c} \quad (1)$$

where  $n_1$  is the number of antigen molecule sites per cell,  $\theta_1$  is the fraction of antigen molecules on the cell surface bound by the primary antibody,  $\lambda_1$  is the valence of the primary antibody,  $n_2$  is the number of secondary antibody epitopes on the primary antibody,  $\theta_2$  is the fraction of epitopes on the primary antibodies that are bound by the secondary antibodies,  $\lambda_2$  is the valence of the secondary antibody,  $n_3$  is the number of magnetic nanoparticles conjugated to the secondary antibody,  $k$  is the particle-field interaction coefficient equal to the volume of the magnetite in the colloid multiplied by the difference in magnetic susceptibilities of the colloid and the suspension medium,  $\eta$  is the viscosity of the fluid, and  $D_c$  is the diameter of the cell (3).

The magnetic force on a single magnetic bead,  $F_b$ , is equal to:

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

where  $\nabla B^2$  is the magnetic energy gradient,  $\mu_o$  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 (4).

An instrument has been developed, known as the cell tracking velocimeter (CTV), that records and analyzes the movement of cells in a constant magnetic energy density gradient,  $S_m$ . The images are analyzed using a computer algorithm to calculate the magnetophoretic mobility of each cell (5).

In the experiments presented in this study, human natural killer (NK) cells were chosen for selection and enrichment. NK cells are innate immune lymphocytes that have the ability to lyse tumor and virally infected cells

without prior sensitization (6,7). They are bone marrow-derived cells and are distinguished by the presence of the CD56 cell surface antigen and the lack of CD3. Human NK cells make up about 10% of all peripheral blood lymphocytes and can be divided into two distinct subsets on the basis of cell surface density of CD56 (i.e., CD56<sup>bright</sup> and CD56<sup>dim</sup>; 8). About 90% of NK cells are CD56<sup>dim</sup> and have a high expression of the FcγRIII (CD16), which mediates antibody-dependent cellular cytotoxicity. In contrast, the CD56<sup>bright</sup> subset makes up only about 10% of all NK cells and is CD16<sup>dim/neg</sup>, but recently these cells have been shown to be high cytokine producers compared with the CD56<sup>dim</sup> subset (9). NK cell-derived cytokines are critical for host defense against numerous bacterial and intracellular pathogens. Because of their ability to lyse tumor cells and secrete immunoregulatory cytokines, NK cells have been the topic of research for many laboratories and the ability to enrich these cells is an essential step in the investigation process.

The focus of this study was to determine if the magnetophoretic mobility of immunomagnetically labeled NK cells affects the performance of a commercial, immunomagnetic cell isolation system, Miltenyi Biotech MiniMACS. It will be shown that mobility is a function of both primary and secondary antibody concentrations, which are assumed to be directly proportional to the terms  $\theta_1$  and  $\theta_2$  in Equation [1]. It will also be shown that magnetophoretic mobility does have an effect on the performance of the MiniMACS separation system, but in a manner more complex than initially anticipated.

## MATERIALS AND METHODS

### Cell Preparation

Peripheral blood source leukocytes (PBL) from apparently healthy donors were purchased from the American Red Cross, Central Ohio Region, prior to each day of experiments. A Ficoll-Hypaque (Accurate Chemical and Scientific Corporation, Westbury, NY) density gradient centrifugation was used to obtain the mononuclear cell layer. In addition, monocytes/macrophages were removed by allowing the mononuclear cells to adhere to plastic for 2 h at 37°C. The nonadherent cells were then washed and collected in calcium and magnesium-free phosphate buffer solution containing 0.1% bovine serum albumin (BSA) and 2 mM EDTA. Red blood cells were removed by the addition of 25 ml of lysis buffer (154 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM EDTA), followed by a 5-min incubation at room temperature. The cells were washed and resuspended in the buffer solution. Size distributions and concentrations of the PBL mixture were then determined using a Coulter Counter Multisizer II (Beckman-Coulter, Miami, FL).

### Flow Cytometry Analysis

The flow cytometry studies were performed on a Beckman-Coulter EPICS Elite II flow cytometer. The device is equipped with a 488-nm, 15-mW air-cooled Argon laser. The optical laser was aligned using Beckman-Coulter's Flow-Check alignment fluorosphere beads.

In order to distinguish PBL cells from red blood cells and platelets, a forward scattering and a side scattering analysis were used. A gate was then set to exclude red blood cells and platelets. In addition, an unlabeled blood mixture was analyzed first to determine autofluorescence levels.

#### Primary Antibody Studies Using Flow Cytometry

Saturation studies were conducted to ensure that the concentration of primary mouse (isotype IgG1) anti-CD56-phycoerythrin (PE) antibody conjugate (Immunotech, Marseille, France) used for immunolabeling was sufficient. Prior to examining the saturation of the primary antibody, several experiments using IgG blocker were conducted. It was determined that IgG blocker was not necessary to prevent nonspecific binding provided that the monocytes had been depleted from the blood sample. To check for primary antibody saturation, seven different concentrations of antibody reagent were analyzed on the flow cytometer using the same, original PBL sample. This saturation study was performed in triplicate using three different PBL blood samples.

The flow cytometry histograms from each sample were further analyzed using Peakfit (SPSS, Chicago, IL). The software uses a residual method for finding hidden peaks to differentiate between unlabeled and CD56+ cells. A residual is the difference in y-value between a data point and the sum of component peaks evaluated at the data point and the sum of component peaks evaluated at the data point's x-value. The sum of the area of each of the revealed peaks is equal to the total area of the data.

#### CTV Analysis

CTV was used to measure the velocity of the paramagnetically labeled cells in this study. It is a unique technique that enables true magnetic separation optimization. The complete descriptions of the CTV technique have been presented previously (5,10). The cell suspension is pumped in to a 1-mm<sup>2</sup> glass channel that passes through a constant magnetic energy gradient containing a microscope objective. The images of the cells are then recorded, converted to digital data, and analyzed to produce a histogram of the magnetophoretic mobility in millimeters<sup>3</sup> per Tesla-Ampere-second (mm<sup>3</sup>/T-A-s).

#### Secondary Antibody Studies

In order to achieve a separation, magnetophoretic mobilities that are distinguishably higher than the unlabeled cell magnetophoretic mobilities are required. Therefore, various concentrations of secondary mouse (isotype IgG1) anti-PE (50 nm)-microbeads antibody reagent (Miltenyi Biotech) were used to determine which concentration would provide the appropriate magnetophoretic mobility. The manufacturer does not provide information with respect to the number of micrograms of antibody in solution, therefore, all antibody amounts in this study are reported in microliters. Microbeads are submicroscopic and, therefore, do not affect the light scattering of labeled cells when analyzing on flow cytometry.

In order to analyze samples using the CTV technique, an enriched population of NK cells must first be obtained. Rosettesep Antibody Cocktail (Stemcell Technologies, Vancouver, Canada) for human NK cells was used to obtain approximately 80% CD56+ enrichments. The enrichment cocktail was added (50 µl/ml of blood) and incubated for 20 min at room temperature prior to the Ficoll-Hypaque density gradient centrifugation. Unwanted cells are cross-linked to red blood cells using Rosettesep tetrameric antibody complex reagent. After the centrifugation, the unwanted cells are pelleted with the red blood cells while the desired CD56+ cells are enriched at the Ficoll-plasma interface.

To ensure saturation of the primary antibody reagent, 250 µl of mouse anti-CD56-PE per 10<sup>6</sup> cells was added to each sample aliquot. After an incubation time of 15 min at 4°C, the cells were washed using calcium and magnesium-free phosphate buffer solution containing 0.1% BSA and 2 mM EDTA. Five different amounts of secondary mouse anti-PE-microbeads antibody reagent (1, 10, 50, 100, and 250 µl per 10<sup>6</sup> cells) were added to the CD56+ enriched PBL. Additional studies were conducted to establish the extent of nonspecific binding at different concentrations.

#### Labeling Protocol for Magnetic Cell Separations

The peripheral blood cells were suspended in calcium and magnesium-free phosphate buffer solution containing 0.1% BSA and 2 mM EDTA at an amount of 150 µl per 10<sup>6</sup> cells. A two-step labeling procedure was used in which a saturating amount of primary antibody was added and the cells were incubated at 4°C for 15 min. The cells were then washed in buffer with 10× the staining volume and resuspended at 150 µl per 10<sup>6</sup> cells. One half of the cells were labeled with the company recommended amount of secondary antibody whereas the other one half were labeled with an appropriate amount based on the saturation studies with the secondary antibody. Both were incubated for 15 min at 4°C and then washed two times in buffer with 10× the staining volume. Each sample having a concentration of approximately 9 × 10<sup>6</sup> cells was resuspended in 500 µl of buffer solution.

#### Immunomagnetic Separation

The MiniMACS magnetic isolation system using the MS+ column was used to positively select NK cells from PBL. First, 500 µl of degassed buffer was applied to the top of the column and allowed to run through. The cell suspension (~9 × 10<sup>6</sup> cells) in 500 µl was pipetted onto the column. The column was then washed with 1.5 ml of buffer and the total effluent was collected as the negative eluent. The column was removed from the magnet followed by the application of 1 ml of buffer to the reservoir of the column. Finally, a plunger was applied to the top of the column and firmly pushed to flush the cells into a new collection tube. The purged cell suspension was referred to as the positive eluent. The plunging step was repeated in some of the experiments to help increase cell recovery in the positive eluent. The concentration of both the negative and the positive eluents was calculated using a

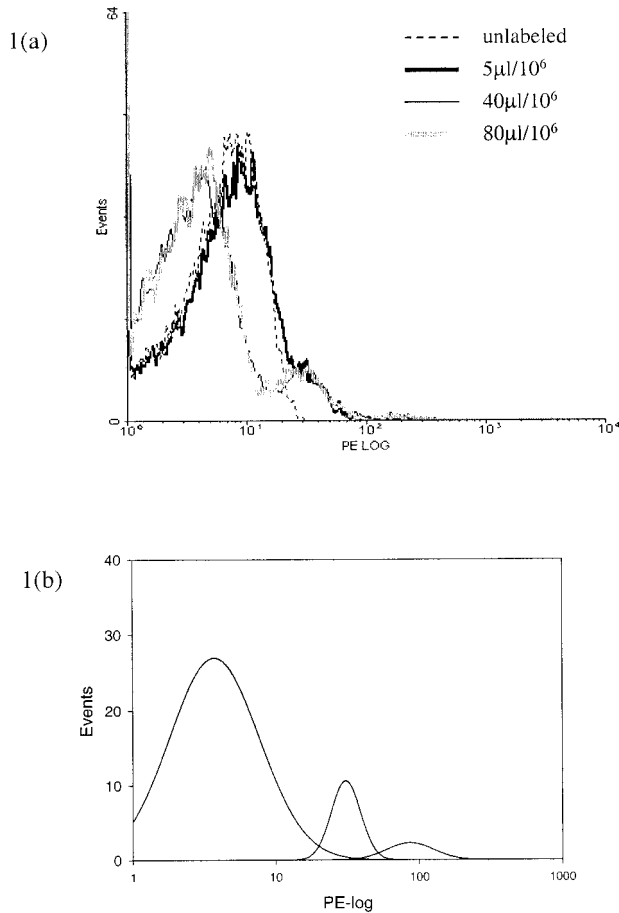


FIG. 1. **a:** Flow cytometry histograms of the FI distribution of unlabeled PBL and three different concentrations of anti-CD56-PE antibody. The sample used in the four histograms came from the same donor. **b:** Peakfit histogram of the sample from Figure 1a labeled with 40  $\mu\text{l}$  per  $10^6$  cells of anti-CD56-PE.

Coulter Counter Multisizer II. In addition, the eluents were analyzed by flow cytometry as described above.

## RESULTS

### Primary Antibody Studies

A saturation study using nine different concentrations was performed on three independent blood samples. Figure 1a shows the flow cytometry histograms for four different concentrations (unlabeled, 5, 40, and 80  $\mu\text{l}$  of anti-CD56-PE per  $10^6$  cells) for PBL sample 1. Figure 1b is the Peakfit histogram of the flow cytometry histogram for PBL sample 1 at a concentration of 40  $\mu\text{l}$  of anti-CD56-PE per  $10^6$  cells. Figure 2a presents the flow cytometry histograms for four different concentrations (unlabeled, 5, 40, and 80  $\mu\text{l}$  of anti-CD56-PE per  $10^6$  cells) for PBL sample 2. Figure 2b is the Peakfit histogram of the flow cytometry histogram for PBL sample 2 at a concentration of 40  $\mu\text{l}$  of anti-CD56-PE per  $10^6$  cells.

The results from sample 1 (Fig. 1) show the clear formation of a CD56+ peak with increasing amount of antibody. The results from sample 2 (Fig. 2), however, do not

have an obvious separation between CD56+ and unlabeled cells. These differences can potentially be attributed to blood sample variation. Regardless, Peakfit was able to distinguish between unlabeled and CD56+ populations for all of the blood samples. In Figure 1b, three peaks were fitted to the flow cytometry data. The first peak corresponds to unlabeled cells, the second to CD56<sup>dim</sup>, and the third to CD56<sup>bright</sup>. In Figure 2b, only two peaks were fitted to the flow cytometry data, possibly due to a low number of CD56<sup>bright</sup> in the blood sample or overlap of the fluorescence intensity (FI) in the flow cytometry histogram.

Figure 3a shows the normalized FI as calculated by flow cytometry versus the primary antibody concentration. The normalized FI is calculated by dividing the mean FI of the CD56+ population for that specific antibody concentration by the maximum measured mean FI of the CD56+ cell population for each PBL sample. The CD56+ population is determined by setting a gate based on the unlabeled control. Figure 3b shows the normalized FI as calculated by Peakfit versus the primary antibody concentration. In this case, the

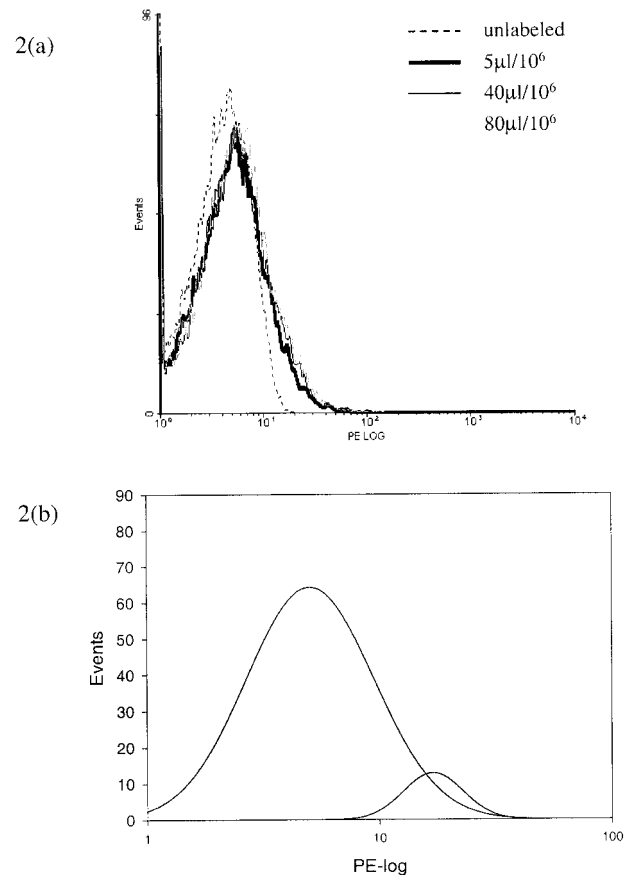
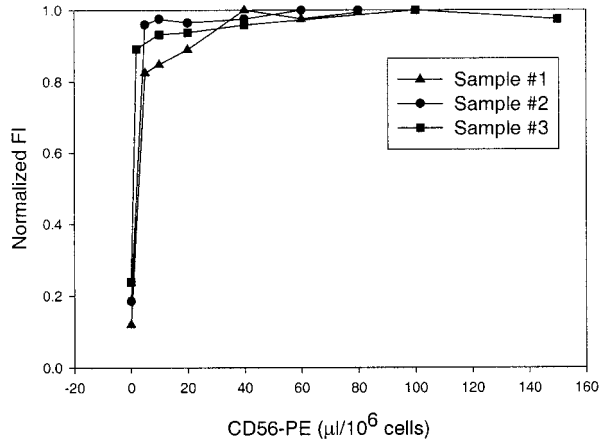


FIG. 2. **a:** Flow cytometry histograms of the FI distribution of unlabeled peripheral blood and three different concentrations of anti-CD56-PE antibody. The blood used for the four histograms came from the same donor, but is different from the sample presented in Figure 1. **b:** Peakfit histogram of the sample from Figure 2a labeled with 40  $\mu\text{l}$  per  $10^6$  cells of anti-CD56-PE.



3(b)

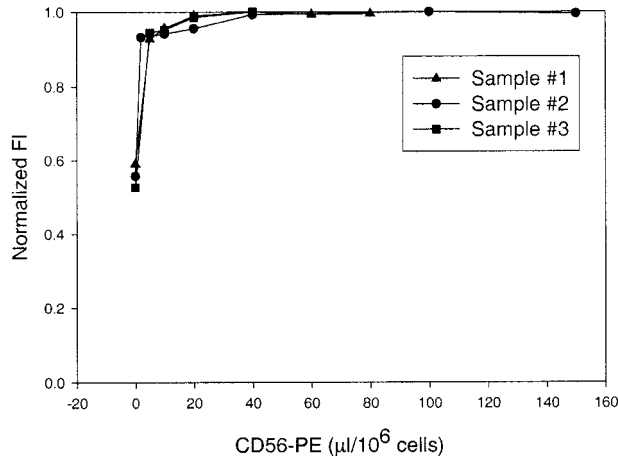


FIG. 3. **a:** Normalized FI as a function of antibody concentration. The data were normalized by dividing the mean FI at a given concentration by the maximum mean FI for the given donor. **b:** Normalized FI as a function of antibody concentration. In this case, the values used for means came from Peakfit analysis.

mean FI is equal to the mean of the fitted peak that corresponds to the CD56+ population, and the normalized FI is calculated as stated above. Because the normalized FI is the fraction of antigen sites bound by the primary antibody, it is assumed that the normalized FI is directly proportional to  $\theta_1$  (the fraction of antigen molecules on the cell surface bound by the primary antibody from Equation 1). Figure 3b indicates that nearly complete saturation of the CD56 binding sites ( $\theta_1 = 1$ ) occurs at approximately 40  $\mu\text{l}$  of primary antibody per  $10^6$  cells. Consequently, this concentration was chosen for a majority of the remaining studies.

**Secondary Antibody Studies**

Figure 4 is a histogram showing the magnetophoretic mobility of cells labeled with 0, 1, 10, and 50  $\mu\text{l}$  of secondary antibody (anti-PE-microbeads) per  $10^6$  cells with a saturating amount of primary antibody (anti-CD56-

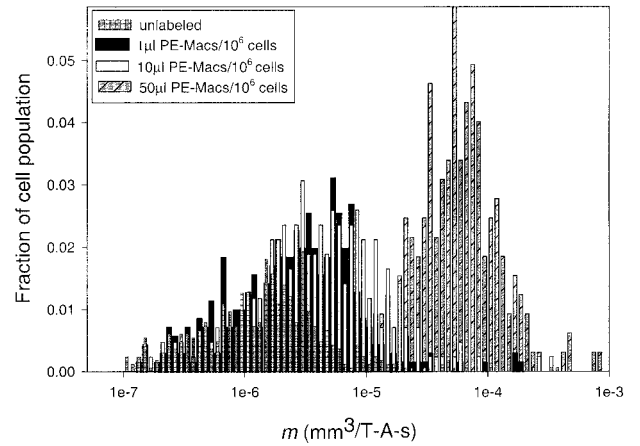


FIG. 4. Histograms of the magnetophoretic mobility of PBL labeled with 250  $\mu\text{l}$  per  $10^6$  cells of anti-CD56-PE and various concentrations of anti-PE-microbeads reagent.

PE). Note that the magnetophoretic mobility increases with increasing amounts of secondary antibody. Figure 5 presents the mean cell mobility of both the specific binding of secondary antibody (primary and secondary) and the nonspecific (secondary only) as a function of increasing concentration of secondary antibody.

**Effect of Primary Antibody Concentration on Mobility**

As stated earlier, a fundamental premise of Equation [1] is that the magnetophoretic mobility of the cells is proportional to the number of primary and secondary antibodies bound to a cell. It was demonstrated in this study that the FI is a function of the concentration of primary antibody and that the magnetophoretic mobility is a function of the concentration of the secondary antibody. It

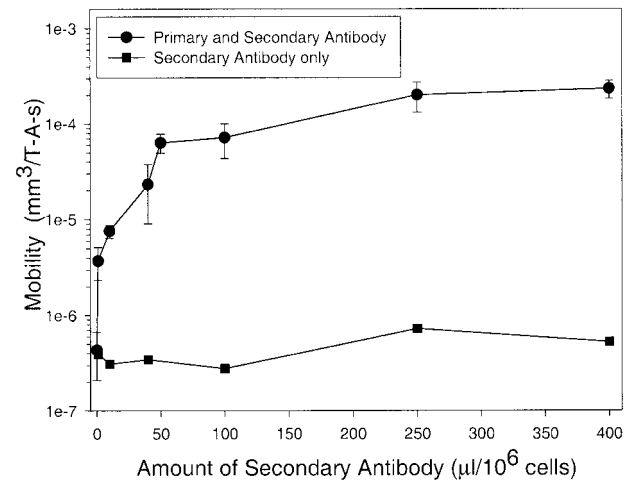


FIG. 5. A plot of the mean magnetophoretic mobility of PBL as a function of the concentration of anti-PE-microbeads reagent for cells labeled with 250  $\mu\text{l}$  per  $10^6$  cells of anti-CD56-PE primary antibody. Also included is a control without the primary antibody.

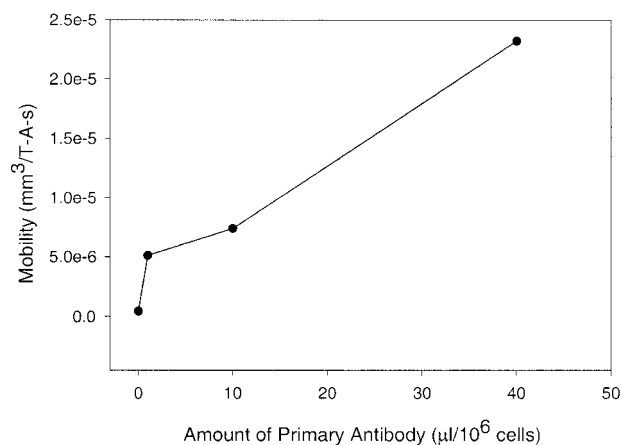


Fig. 6. A plot of the mean magnetophoretic mobility of PBL as a function of the concentration of anti-CD56-PE primary antibody and a constant, 40 µl per 10<sup>6</sup> cells of anti-PE-microbeads.

was not demonstrated, however, that for a constant amount of secondary antibody, the magnetophoretic mobility is also a function of the concentration of the primary antibody. To confirm that the concentration of primary antibody does affect the magnetophoretic mobility of labeled cells, studies were conducted in which various amounts of primary antibody were used with a constant, 40 µl per 10<sup>6</sup> cells of secondary antibody. The concentration 40 µl per 10<sup>6</sup> cells of secondary antibody was chosen because this was the same amount used as a comparison study in the immunomagnetic separations. Figure 6 presents the results of these studies. Specifically, samples of enriched NK cells were labeled with various concentrations of primary, anti-CD56-PE antibodies and then each sample was labeled with the secondary, anti-PE-microbeads reagent at a concentration of 40 µl per 10<sup>6</sup> cells. As shown, the mobility increases as the amount of primary antibody increases.

### Immunomagnetic Separations

In these experiments, two concentrations of secondary antibody were chosen to test the concept that the mean mobility of the positively labeled cells affects the performance of a MiniMACS immunomagnetic cell separator: Miltenyi Biotech-recommended 1 µl of secondary antibody per 10<sup>6</sup> cells, and 40 µl of secondary antibody per 10<sup>6</sup> cells as determined by the saturation studies.

Twelve separations were conducted using the protocols described previously. All of the cells were labeled with the same amount of primary antibody: 40 µl of anti-CD56-PE per 10<sup>6</sup> cells. One half of the separations was performed with cells labeled with 1 µl of secondary antibody (PE-MACS) per 10<sup>6</sup> cells whereas the other one half was labeled with 40 µl per 10<sup>6</sup> cells. The mean mobility of the cells labeled with 40 µl of secondary antibody per 10<sup>6</sup> cells (2.32E-5 mm<sup>3</sup>/T-A-s) is higher than the mean mobility of the cells labeled with 1 µl of secondary antibody per 10<sup>6</sup> cells (3.74E-6 mm<sup>3</sup>/T-A-s).

The results of the separations are summarized in Table 1. The percent positive in the feed, the negative eluent, and the positive eluent were all calculated by flow cytometry as described in the Materials and Methods. Total cell recovery was calculated by dividing the sum of the total number of cells in each of the recovered eluents (negative and positive) by the total number of cells added to the system. The total positive cell recovery was calculated by dividing the sum of the positive cells as calculated by flow cytometry from both the positive and the negative eluent by the total positive cells in the feed.

A number of interesting trends and observations can be made from the data in Table 1. First, increasing the amount of secondary antibody from 1 to 40 µl per 10<sup>6</sup> cells decreases the percent positive CD56 cells in the negative eluent. Ideally, 100% of the positive cells would be retained in the column, resulting in 0% positive CD56 cells in the negative eluent. This trend is illustrated in Figure 7.

Table 1  
Summary of Results

Experiment	Primary antibody (µl/10 <sup>6</sup> cells)	Secondary antibody (µl/10 <sup>6</sup> cells)	Positive in feed (%)	Total cell recovery (%)	Positive in negative eluent (%)	Positive in positive eluent (%)	Total positive cell recovery (%)
1	40	1	21	88.3	22.9	83.8	98
2	40	40	20.3	78.9	3.8	89.7	44
3 <sup>a</sup>	40	1	12.9	87	14.1	69	100
4 <sup>a</sup>	40	40	12.9	88.1	3.1	81.6	84.3
5	40	1	20.6	79.6	18.9	64.8	107.7
6	40	40	20.6	66.9	2.9	51.7	33.8
7 <sup>a</sup>	40	1	20.6	96.7	19.6	70.9	95.6
8 <sup>a</sup>	40	40	20.6	89.9	3.1	68.7	63.2
9	40	1	6	83.6	4.7	49.3	71.3
10	40	40	6	52.1	1	37.6	45.4
11 <sup>a</sup>	40	1	6	75.1	5.7	53.7	79.7
12 <sup>a</sup>	40	40	6	72.5	0.7	42	59.4

<sup>a</sup>Cells were purged twice from the column with the plunger.

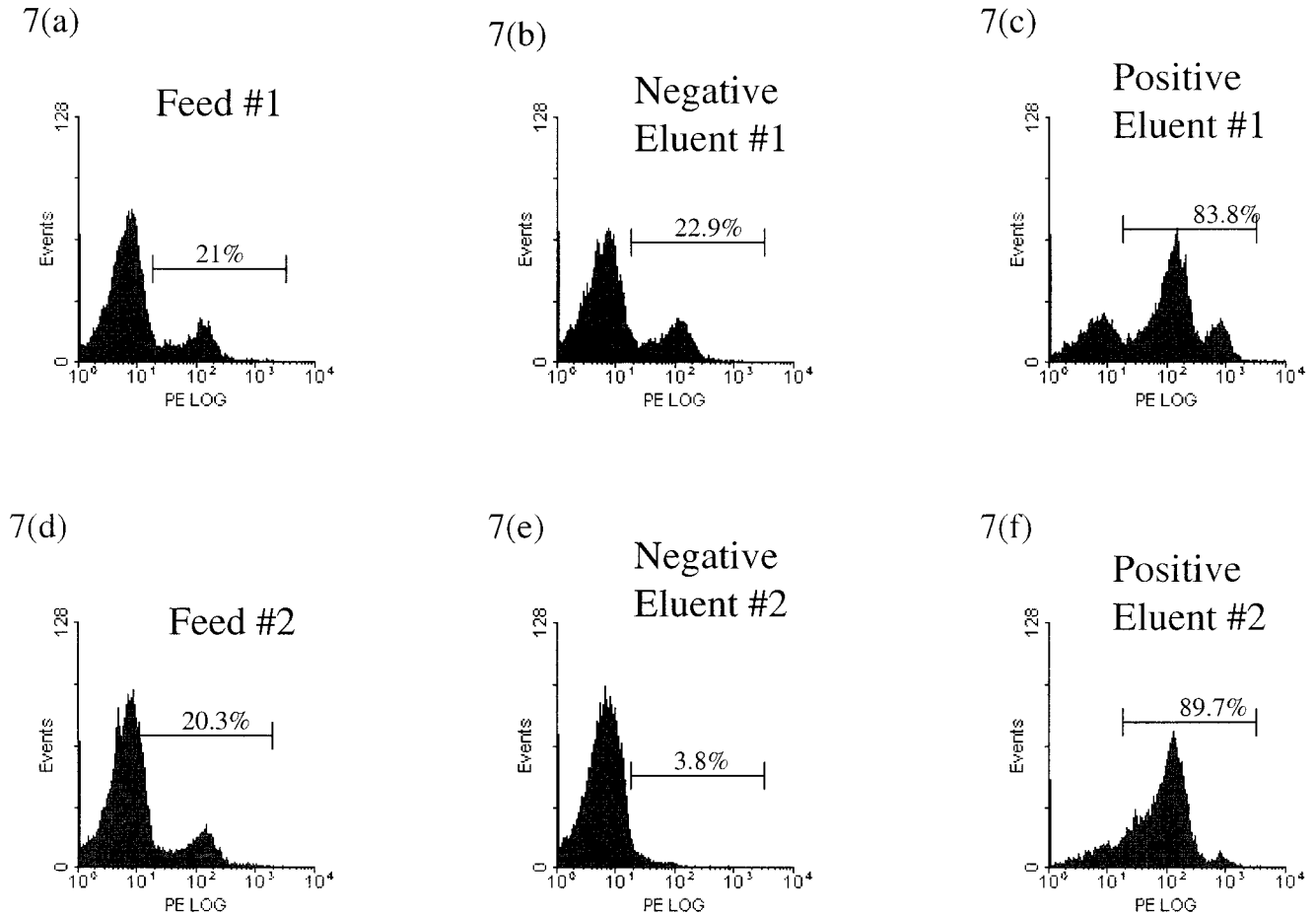


Fig. 7. Flow cytometry histograms of the results from experiments 1 and 2 listed in Table 1. a,d: Histograms of the feed. b,e: Negative eluent. c,f: Positive eluent from the MiniMACS separations.

Figures 7a-f are histograms of the flow cytometry results from representative experiments 1 and 2. Similar histograms were produced for all of the experiments (data not shown). A gate was set based on the unlabeled control (at a PE FI of 18) to determine the percent CD56+. All events to the left of this value are considered CD56- whereas all events to the right are CD56+. When considering the negative eluent of experiments 1 and 2 (Figs. 7b,e), significantly less CD56+ cells are present when the concentration of secondary antibody is  $40 \mu\text{l}$  per  $10^6$  cells (Fig. 7e). A similar trend can be observed in the histograms from all of the experiments (data not shown).

Second, the total cell recovery decreases as the amount of secondary antibody increases from 1 to  $40 \mu\text{l}$  per  $10^6$  cells in the experiments where only one purge (experiments 1 and 2, 5 and 6, and 9 and 10) was used to remove the positive cells. In contrast, when the cells were purged twice (experiments 3 and 4, 7 and 8, and 11 and 12) from the column with the plunger, a similar total cell recovery for both concentrations of antibody ( $1$  and  $40 \mu\text{l}$  per  $10^6$  cells) was obtained.

A similar trend can be observed in the total positive cell recovery, which decreases as the amount of secondary

antibody increases from 1 to  $40 \mu\text{l}$  per  $10^6$  cells. These decreases are more significant (compared with the total cell recovery) for the samples that were purged only once from the column. This trend is also demonstrated in Figures 7c,f. In the histogram of the positive eluent collected in experiment 1 (Fig. 7c), where only  $1 \mu\text{l}$  of secondary antibody per  $10^6$  cells was applied, two distinct peaks of NK cells can be observed: CD56<sup>dim</sup> and CD56<sup>bright</sup>. In the histogram of the positive eluent collected in experiment 2 (Fig. 7f), where  $40 \mu\text{l}$  of secondary antibody per  $10^6$  cells was applied, the number of events presented for the CD56<sup>bright</sup> population is significantly reduced. This is best demonstrated in Figure 8a, which overlays the positive eluents from experiments 1 and 2. Also notice that the first peak from experiment 1 (representing the presence of unlabeled cells in the eluent) is reduced significantly in experiment 2. This suggests that less unlabeled cells are collected in the positive fraction for experiment 2, i.e., a higher purity fraction is obtained.

Figure 8b presents the positive eluents from experiments 3 and 4 (two purges were used in both experiments). As in Figure 8a (experiments 1 and 2), both the CD56<sup>bright</sup> population and the unlabeled cells are reduced

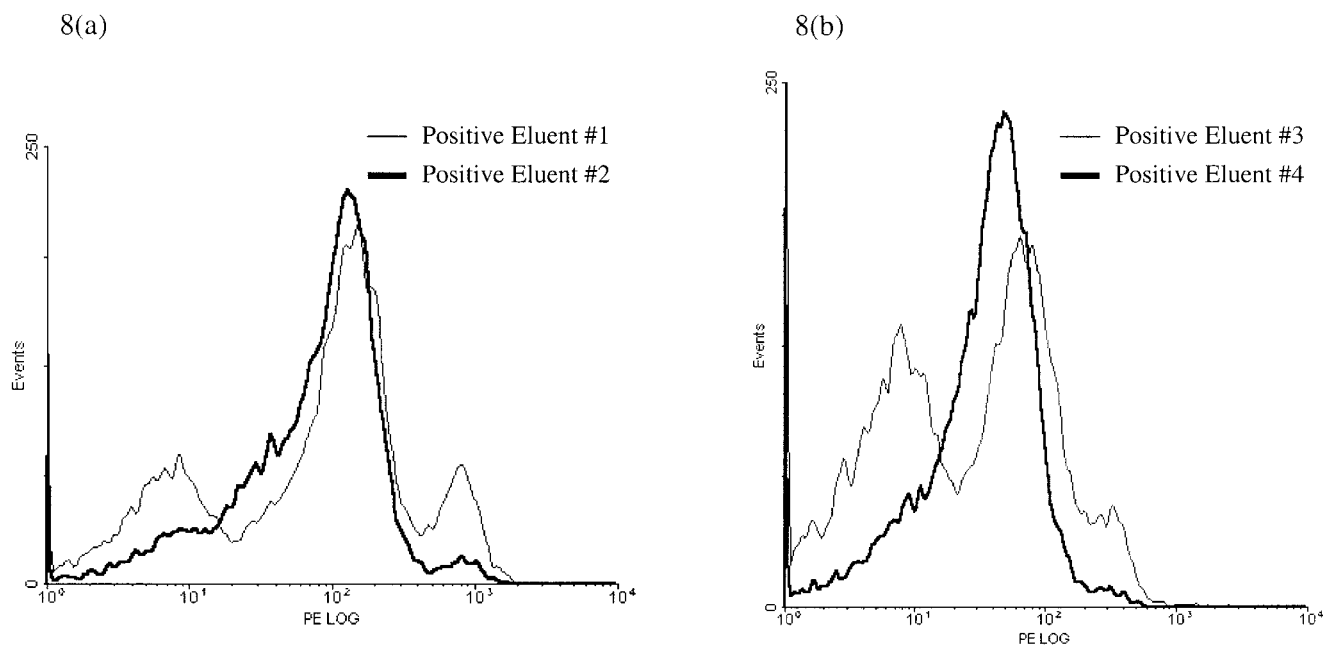


FIG. 8. **a:** Flow cytometry histograms of the positive eluents from experiments 1 and 2. **b:** Flow cytometry histograms of the positive eluents from experiments 3 and 4.

when 40  $\mu\text{l}$  of secondary antibody per  $10^6$  cells was applied. However, the total cell recovery and the total positive cell recovery for experiment 4 are significantly higher than experiment 2, possibly due to the use of a double plunge.

### DISCUSSION

The primary and secondary antibody saturation studies demonstrate that, as expected, the magnetophoretic mobility,  $m$ , as defined by Equation [1], can be altered by varying the concentration of antibody during the labeling process. Plots of both the FI and  $m$  as a function of antibody concentration (Figs. 3, 5, and 6) resemble classic saturation kinetics and further support the hypothesis that the terms  $\theta_1$  and  $\theta_2$  are correct in that they represent the fraction of binding sites bound with an antibody.

The results in Figure 4 demonstrate that cells labeled with 1  $\mu\text{l}$  of secondary antibody per  $10^6$  cells (according to company protocol) have a mobility that significantly overlaps the mobility of unlabeled cells. Interestingly, the MiniMACS isolation system is still able to achieve a reasonable level of separation using 1  $\mu\text{l}$  of secondary antibody per  $10^6$  cells. Theoretically, an optimal separation should occur when there is minimal overlap of the magnetophoretic mobility of the unlabeled and positively labeled cells, as seen at 40  $\mu\text{l}$  of secondary antibody per  $10^6$  cells. Although certain aspects of the separation at this higher concentration of secondary performed closer to this theoretical ideal, the separation is more complex than initially anticipated.

As expected, increasing the concentration of the secondary antibody (from 1 to 40  $\mu\text{l}$  per  $10^6$  cells) caused an

increase in the mean magnetophoretic mobility from  $3.74 \times 10^{-6} \text{ mm}^3/\text{T-A-s}$  to  $2.32 \times 10^{-5} \text{ mm}^3/\text{T-A-s}$ . This increase in mobility almost completely depleted the percent positive cells in the negative eluent (ranging from 0.7% to 3.8%) as calculated by flow cytometry. These values may even be lower given the difficulty in estimating the fraction of rare cells in a sample by flow cytometry (11,12). It is assumed that more positive cells are retained in the column with an increase in bound, secondary antibody resulting in a lower percent positive represented in the negative eluent. The ability to completely eliminate the positive cells from the negative eluent is a desirable characteristic in clinical applications such as bone marrow transplantation. Self-reactive lymphocytes can cause autoimmune diseases and therefore should be depleted completely from the recipient before transplantation (13).

Although increasing the mean magnetophoretic mobility of the positively labeled cells reduced the amount of positive cells in the negative eluent, it was also observed that the total amount of positive cells recovered was also reduced. Interestingly, the total positive cell recovery (when 40  $\mu\text{l}$  per  $10^6$  cells was used) was restored partially when the column was purged twice using the plunger to remove the positive cells. This suggests that the positive cells are retained in the column even after the magnetic field has been removed and that it may be a function of magnetophoretic mobility. These results are under investigation in our laboratory. The retention of positively labeled cells in the column is supported further by the poor recovery of the CD56<sup>bright</sup> population even after the cells were purged twice (Fig. 8b). A speculation can be made

that the more magnetically labeled cells are bound irreversibly to the column.

The results obtained indicate the complexity of immunomagnetic cell separation using a HGMS column and the need for further optimization. Although increasing the magnetophoretic mobility of the cells reduced the number of positive cells in the negative eluent, it also decreased the number of cells recovered from the column. Our future studies include establishing proper labeling protocols for optimal magnetic separations as well as possible solutions to this problem.

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