

Measurement of CD2 Expression Levels of IFN- α -Treated Fibrosarcomas Using Cell Tracking Velocimetry

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Methods: A methodology and a mathematical relationship have been developed that allow quantitation of the expression levels of cellular surface antigens, in terms of antibody binding capacities (ABC). This methodology uses immunomagnetically labeled cells and calibration microbeads combined with cell tracking velocimetry (CTV) technology to measure magnetophoretic mobilities corresponding to cellular ABC. The mobility measurements were accomplished by microscopically recording and calculating the velocity of immunomagnetically labeled QSC microbeads and cells in a nearly constant magnetic energy gradient.

Results: Transformed fibrosarcoma cells were given controlled treatments of interferon- α in order to manipulate CD2 antigen expression levels. These cells were then immunomagnetically labeled with anti-CD2 FITC antibodies

and anti-FITC MACS paramagnetic nanoparticles. Measured magnetophoretic mobilities were used to calculate ABC for these cells, corresponding to CD2 expression levels.

Conclusion: The results from CTV and flow cytometry (FCM) qualitatively verify that these fibrosarcoma cells express elevated levels of CD2 molecules with increasing interferon- α treatment from 0 to 24 h. The mean basal CD2 expression level, in terms of ABC, was calculated to be 27,000 from CTV analysis, whereas FCM indicates a comparable ABC value of 33,000. Cytometry 44:137-147, 2001. © 2001 Wiley-Liss, Inc.

Key terms: antigen density; quantitative flow cytometry; antibody binding capacity; magnetic cell separation; magnetophoretic mobility; particle tracking velocimetry

Quantitation of cell surface antigens describes calculating the total number of specific antigen molecules expressed on a given cell or cell population. In addition to the phenotypic significance of the expression of a specific antigen on a cell, the expression level (typically measured as antibody binding capacity [ABC]) of specific cell surface antigens is also becoming increasingly important in understanding cell function and the prognosis and diagnosis of disease (1-12). For example, the relative levels of CD34 surface expression in CD34+ bone marrow cells correlate with the maturity of hematopoietic progenitor cells. The CD34^{bright} cell populations contain the majority of immature hematopoietic cells, whereas the CD34^{dim} cell populations contain more lineage-committed progenitors (12). Regular cell-associated antigen expression levels are also either overexpressed or underexpressed in many pathological conditions. For example, researchers have found that CD38 membrane antigen expression on cytotoxic (CD8+) T cells is elevated with increased activation of these cytotoxic T cells, correlating to the progression of disease in human immunodeficiency virus-positive (HIV+) patients (6,9,10). Also, CD10 expression of B-lineage acute lymphoblastic leukemia cells is 33-45%

greater when compared with normal fetal bone marrow cells (4), the elevation of CD64 on neutrophils is believed to be an early indication of sepsis (11), and the p75 chain of the IL-2 receptor on T cells (also called IL-2Rp75 or CD122) appears to be down-regulated for patients with rheumatic disease (5).

The most common technique to obtain quantitative information about cell surface molecule expression levels is to use microbead standards combined with flow cytometry (FCM) analysis (13-15). Quantum Simply Cellular (QSC) microbeads (Flow Cytometry Standards, San Juan, Puerto Rico, recently purchased by Bangs Laboratories, Fishers, IN) are one example of such calibration microbeads used for FCM analysis. These microbeads have been designed to bind a specific number of monoclonal mouse antibodies (13). Under appropriate conditions, fluorochrome-antibody conjugates bind to specifically tar-

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ged surface receptors and the measured fluorescent intensity (FI) from these fluorochrome molecules is directly proportional to the number of receptors (antigen molecules) on that microbead. Consequently, when immunofluorescently labeled cells and calibration microbeads are analyzed using the same antibodies, it is possible to quantitate the number of specific antigens on a cell's surface.

In addition to using antigen quantitation to discretely differentiate a cell population, antigen expression level is also an important factor in continuous immunomagnetic separation (16–20). In order to optimize the design of immunomagnetic cell separators, it is desirable to know the degree to which a cell is immunomagnetically labeled. A technique to determine the magnetophoretic mobility, correlating to the antigen expression level, of an immunomagnetically labeled cell or particle has been developed by our laboratories (16,21). This technique involves videotaping the movement of immunomagnetically labeled cells through a medium of known viscosity in a well-defined magnetic energy gradient. The velocity of each cell along with its location within the magnetic energy gradient is recorded. From this information, the magnetic susceptibility of each cell is obtained. This technique, referred to as cell tracking velocimetry (CTV), now includes semiautomated computer video imaging and tracking analysis (22,23). Also, the CTV magnet has a region of nearly constant magnetic energy gradient, which greatly simplifies the analysis (24). The magnetic energy gradient of this magnet has been demonstrated to be stable over 3 months, and presumably indefinitely stable, due to the use of permanent magnets (25).

An analogy exists between FCM and CTV. In an FCM system, the measured parameter is the FI of an immunofluorescently labeled cell or microbead when excited by a laser beam of the appropriate wavelength. In the CTV system, the measured parameter is the velocity of an immunomagnetically labeled cell or microbead induced by the magnetic energy gradient in which the cells are placed. However, the induced velocity (magnetophoretic mobility) in the CTV system is reported on an absolute scale, $\text{mm}^3/\text{T}\cdot\text{A}\cdot\text{s}$ (millimeter cubed per tesla-ampere-second). In FCM, the FI signal is an amplified and relative value that requires significant standardization and control (13,26).

A mathematical model has been developed for magnetic CTV technology that describes all aspects contributing to the movement of an immunomagnetically labeled cell or particle placed in a magnetic energy gradient (16). McCloskey et al. (16) presented a methodology that describes the use of paramagnetically labeled microbeads and CTV technology for antigen quantitation. We have applied the proposed methodology to quantitate the expression levels of CD2 cellular surface antigens on a transformed fibrosarcoma cell line.

Mathematical Model of Magnetophoresis

The more complete derivation of the mathematical theory has been presented by McCloskey et al. (16). We will

summarize the main points of the theoretical development.

The paramagnetic force, F_m , acting on an immunomagnetically labeled cell or microbead and using a two-step labeling protocol, can be represented as:

$$F_m = (n_1\theta_1\lambda_1)(n_2\theta_2\lambda_2)n_3F_b \quad (1)$$

where subscripts 1 and 2 refer to the primary and secondary labeling steps, respectively, n_i is the number of binding sites per cell, including specific and nonspecific binding sites ($n_s + n_{ns}$), θ_i is the fraction of binding sites on the surface bound by the primary antibody, the parameter λ_i represents the valence of the primary antibody binding, n_3 is the number of paramagnetic nanoparticles conjugated to the secondary antibody, and F_b refers to the magnetic force acting on one, paramagnetic nanoparticle.

The combined term, $n_1\theta_1\lambda_1$, is equivalent to the ABC of a cell population (2). ABC is a measure of the number of primary antibodies binding to a cell or microbead. This value includes not only the number of antigen molecules per cell, but also variables such as valence of antibody binding, steric hindrance, binding affinities, and nonspecific binding. Thus, we have:

$$ABC = n_1\theta_1\lambda_1 \quad (2)$$

The same sequence of parameters is repeated for the binding of the secondary antibody to sites on the primary antibody. In this case, n_2 is the number of binding sites on the primary antibody recognized by the secondary antibody, θ_2 is the fraction of binding sites on the primary antibodies that are bound by the secondary antibodies, and λ_2 represents the valence of the secondary antibody binding. The parameters $n_2\theta_2\lambda_2n_3$ may be combined into one overall term, β . In this case, β represents the number of magnetic nanoparticles bound to each primary antibody on a cell or microbead. The lumped term, $ABC\beta$, describes the number of magnetic nanoparticles bound to each cell or microbead and is referred to as the magnetic nanoparticle binding capacity of a cell or microbead.

The magnetic force acting on one paramagnetic nanoparticle in the direction of the magnetic energy gradient, F_b , is described by:

$$F_b = \frac{1}{2\mu_0} \Delta\chi V_m \nabla B^2 \quad (3)$$

where μ_0 is the magnetic permeability of free space, $\Delta\chi$ is the difference in magnetic susceptibility between the magnetic material, χ_b , and the surrounding medium, χ_f . V_m is the volume of paramagnetic material per paramagnetic nanoparticle and B is the magnetic flux density.

These equations can be combined in a force balance where the magnetically induced and drag forces are in opposite directions. Assuming that this magnetically induced force operates in a direction that is perpendicular to gravity (which is the case in the experimental apparatus

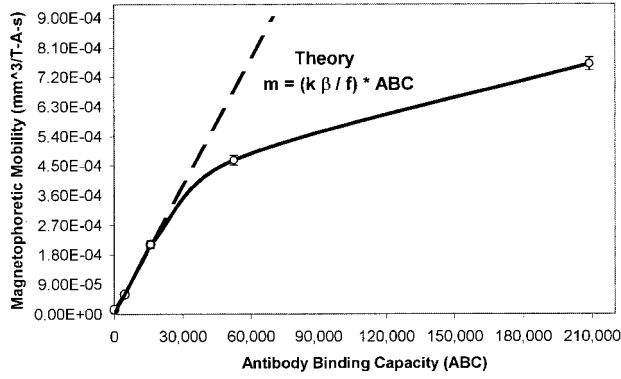


Fig. 1. Linear plot of magnetophoretic mobilities versus ABC for QSC immunomagnetically labeled microbeads. Reprinted from McCloskey et al. (16), with permission.

used in this study), a relationship for the induced velocity, v_c , of the moving paramagnetized cell or microbead is obtained:

$$v_c = \frac{(n_1 \theta_1 \lambda_1)(n_2 \theta_2 \lambda_2) n_3 |F_b|}{3\pi\eta D_c} = \frac{ABC\beta |F_b|}{f} \quad (4)$$

where v_c is the velocity of the cell moving through the fluid, D_c is the diameter of the cell or microbead, η is the viscosity of the fluid, and f is the friction coefficient of the moving cell or microbead. The magnetophoretic mobility, m , a "normalized" parameter analogous to electrophoretic mobility, is obtained by dividing the velocity term by the magnetic energy gradient.

$$m = \frac{v_c}{\left| \frac{\nabla B^2}{2\mu_0} \right|} = \frac{\Delta\chi V_m \beta}{3\pi\eta D_c} ABC = \frac{k\beta}{f} ABC \quad (5)$$

Note that the magnetophoretic mobility is independent of magnetic field strength. Also, $k = \Delta\chi V_m$ is a constant representing the magnetic property of a single magnetic nanoparticle. Again, the lumped term $ABC\beta$ represents the magnetic particle binding capacity of a cell or microbead.

It was experimentally demonstrated that the magnetophoretic mobility is a function of ABC for QSC microbeads up to approximately 210,000 and is linearly proportional to ABC up to 30,000 (16). Figure 1 is a plot of the measured magnetophoretic mobility versus ABC . We present the methodology and experimental results in which we attempt to approximate the ABC of a genetically engineered fibrosarcoma cell line using the linear portion of the calibration curve in Figure 1. We also outline the current assumptions, limitations, and potential solutions involved in obtaining accurate ABC measurements.

EXPERIMENTAL METHODS

Fibrosarcoma Cell Line and Culturing Conditions

The cells used for antigen quantification experiments consisted of a human sarcoma 2C4 cell line. This cell line was derived by stable cotransfection of human fibrosarcoma HT-1080 cells with pDW 9-27 CD2 and pTK-Neo vectors (thymidine kinase-neomycin; 27). Upon treatment of 2C4 cells with an interferon cytokine, these cells send signals via the Jak/STAT pathway to start production and express the CD2 protein molecule on their surface (normally expressed on T cells). This immortal cell line was kindly provided by George R. Stark, Ph.D., The Lerner Research Institute/NC11, Cleveland Clinic Foundation in October 1997 and again in January 2000.

Fibrosarcoma cells were grown to confluence in T-75 tissue culture flasks and split as soon as they grew to confluence. When split, the reseeding culture concentration was one half of the concentration of a confluent flask. The cells were washed with calcium and magnesium-free phosphate-buffered solution (PBS). Trypsin-EDTA (Life Technologies, Grand Island, NY) was used as the trypsinizing agent. Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS) and $1 \times$ penicillin-streptomycin (all three from Life Technologies) served as the culture medium.

Treating Cells With IFN- α

The recombinant leukocyte A interferon used for these experiments was kindly provided by Hoffman-La Roche, Nutley, NJ (for experimental use only). Before treating fibrosarcoma cells with IFN- α , the cells were grown to confluence in T-75 tissue culture flasks and split down to a one-half reseeding concentration 24 h prior to IFN treatment. The cells were allowed to grow to confluence, or close to confluence, for the next 24 h, at which time a sufficient quantity of the 5 million U/ml of IFN solution was added to 12 ml of culture media to obtain a final concentration of 1,500 U/ml. These cells were then incubated with the IFN-treated media for a predetermined number of hours at 37°C.

Cell Harvesting

Treated cells were washed with PBS and given Versene (PBS and 5 mM EDTA, Life Technologies) and incubated for 5-10 min at 37°C to lift cells off the bottom of the flask. After this incubation, FCS was added. These cells were then centrifuged, washed, and resuspended in PBS.

Cell Size Analysis

The cells were cultured, treated with IFN, and harvested according to the protocols described above. They were then analyzed with a Coulter Counter Multisizer II.

Antibody Saturation Studies

Experiments were conducted to ensure that the concentration of antibody reagent used in immunolabeling cells saturated all of the available antigen binding sites. To ensure this, the 24-h IFN-treated cell population (the cell

population believed to express the greatest number of antigen binding sites) was incubated with different volumes of FITC-conjugated antibody following the protocols below. Because the primary antibody was an antibody-FITC conjugate, FCM analysis was used for the saturation study of the primary antibody. The CD2 immunofluorescently tagged antigen sites were assumed to be saturated when the FI signal produced little increase in FI with increasing concentration of FITC-conjugated antibody reagent, as suggested in the literature (28).

To check for saturation of the primary antibody, five different volumes (100, 111, 140, 200, and 250 μl) of the primary mouse (isotype IgG2a) anti-CD2 FITC antibody reagent (CD2 Leu-5b, Becton Dickinson Immunocytometry Systems, San Jose, CA) were tested on the 24-h treated cells. The mean fluorescence intensities (MFI) of these cells were approximately equivalent, FI = 80–95, on a four-decade log scale (FCM was a Beckman Coulter Elite) with a mean autofluorescent intensity of FI = 0.4 for these cells.

Once saturation of the primary antibody was verified, the 24-h IFN-treated cells were immunofluorescently tagged with anti-CD2 FITC primary antibody followed by incubation with different volumes (60, 75, and 100 μl) of the secondary mouse (isotype IgG1) anti-FITC MACS antibody reagent (Miltenyi Biotec, Auburn, CA). The mean magnetophoretic mobilities, as measured by CTV analysis, of all three volumes were approximately equivalent ranging from 5.5×10^{-4} to 5.8×10^{-4} $\text{mm}^3/\text{T-A-s}$ whereas the unlabeled control cells exhibited a mean mobility equal to 1×10^{-5} $\text{mm}^3/\text{T-A-s}$.

Immunofluorescent Labeling the Fibrosarcoma Cells for FCM Analysis

The IFN-treated and harvested cells were placed in tubes of approximately 7 million cells each and adjusted to a concentration of 1.5 million cells per milliliter with PBS. Cells were blocked by adding 67 μl of 3 mg/ml of goat IgG (Sigma, St. Louis, MO) and allowed to incubate for 15 min at 7°C. After this incubation, 140 μl anti-CD2 FITC (lot 80523) was added to the tubes and allowed to incubate for 1 h at 7°C. Cells were then washed with 3–5 ml of PBS and subjected to FCM (Beckman Coulter Elite) analysis.

Immunomagnetic Labeling the Fibrosarcoma Cells for CTV Analysis

The IFN-treated harvested cells were labeled according to the protocols above. It was determined that the IgG blocker was not necessary for these magnetically labeled cells (data not shown), so the IgG blocker was omitted from the labeling procedure for CTV analysis. After the cells were incubated with the fluorescent antibody, they were resuspended in 5 ml of PBS and labeled with 80 μl of the secondary mouse anti-FITC MACS antibody. Cells treated with IFN for 0, 24, and 48 h were labeled with mouse anti-FITC MACS antibody reagent from lot NE5505. The cells treated with IFN for 12 and 36 h were labeled with mouse anti-FITC MACS antibody reagent from a lot

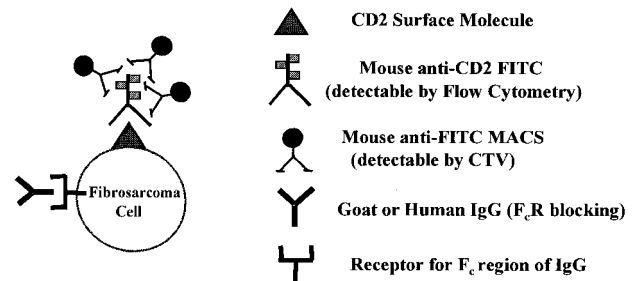


Fig. 2. Schematic of an immunomagnetically labeled fibrosarcoma cell.

NE5557. This incubation lasted 30 min at 7°C, after which the cells were washed twice with 3–5 ml of PBS. The cells, now ready for CTV analysis, were resuspended in PBS with 10% 20 mM EDTA solution so that a concentration of approximately 1–2 million cells per milliliter was obtained. Figure 2 depicts the fibrosarcoma cell with both primary and secondary antibody labels.

Immunofluorescent Labeling the Fibrosarcoma Cells for CD2 Quantitation Using FCM

This later FCM study was included to provide an independent calculation of the ABC for untreated fibrosarcoma cells. It should be noted that these FCM experiments were conducted many months after the initial experiments for CD2 quantitation. More importantly, the FCM used in this later study was a FACScan rather than the earlier Beckman Coulter Elite instrument. Because the FI values on these two instruments were very different, FI values ranged from 0.1 to 1,000 on the Beckman Coulter Elite, whereas the FI values on the FACScan ranged from 1 to 10,000. Thus, the FI values cannot be compared between the two instruments.

The cells were cultured and harvested in the same manner as described above. The labeling method for this later study included a human IgG blocker rather than the goat IgG blocker. Approximately 4 million of the harvested cells (without IFN treatment) were placed in a tube and adjusted to 2 million cells per milliliter with PBS. Cells were incubated with 25 μl of human IgG blocking reagent (Miltenyi Biotec, lot NE6846) and a saturating amount, 100 μl , of mouse (isotype IgG2a) anti-CD2 FITC antibody reagent (CD2 Leu-5b, lot 12609) was then added to the tubes and incubated for 30 min at 7°C with mixing every 15 min. Cells were then washed twice with PBS, resuspended in 300 μl of PBS, and subjected to FCM (FACScan, Becton Dickinson) analysis.

QSC Microbeads

The QSC microbeads used in this study were uniform, 8.0- μm polystyrene microbeads with calibrated numbers of goat anti-mouse antibodies (GAM) bound to their surfaces. Individual sets of these microbeads are coated with four distinct populations of GAM antibodies that bind the Fc region of IgG1, IgG2a, and IgG2b isotypes of mouse monoclonal antibodies and one blank microbead popula-

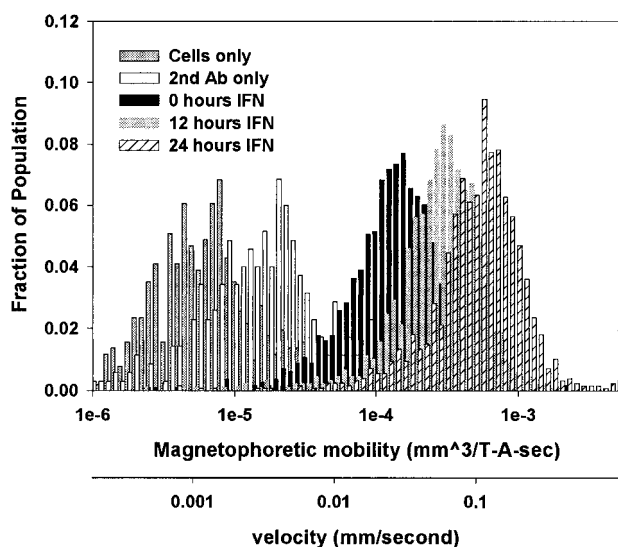


Fig. 3. Histograms of the magnetophoretic mobilities of the immunomagnetically labeled 0, 12, and 24-h IFN treatment cell populations along with the unlabeled cells and nonspecifically labeled cells.

tion for controls. The QSC microbeads were shipped in one vial (lot A050699) having uniform antibody binding populations of median ABC of approximately 0, 5,500, 18,000, 51,000, and 150,000.

Immunofluorescent Labeling the Microbeads for CD2 Quantitation Using FCM

100 μ l of the QSC microbead suspension and 100 μ l of PBS were mixed in a 15-ml centrifuge tube. Four volumes (20, 40, 80, 100 μ l) of the primary mouse (isotype IgG2a) anti-CD2 FITC (CD2 Leu-5b) antibody reagent were tested on the microbeads to ensure saturation. The MFIs of the 80 and 100- μ l volumes were approximately equivalent. Subsequently, the QSC calibration microbeads were labeled with 80 μ l of primary mouse (isotype IgG2a) anti-CD2 FITC antibodies (lot 12609) and incubated for 1 h at room temperature. The microbeads were then washed twice with 2 ml of PBS, resuspended in 300 μ l of PBS, and analyzed immediately.

The CTV Apparatus and Analysis

The CTV image analysis system was designed to measure the velocity of the paramagnetically labeled cells or other similarly sized particles in a well-defined magnetic energy gradient. A more thorough discussion of the apparatus can be found in previous publications (16,22–25).

RESULTS

Histogram composites of the magnetophoretic mobilities of five separately analyzed fibrosarcoma cell populations incubated with IFN for 0, 12, and 24 h, along with controls including unlabeled cells and cells labeled with only the secondary antibody nonspecific for the cellular antigen site, are presented in the semilog plot in Figure 3. The abscissa represents the magnetophoretic mobility values of individual cells. The ordinate represents the fraction of cells with that specific magnetophoretic mobility. Note that the histograms for each cell population treatment group are distinct and that the mean magnetophoretic mobility increases with increasing IFN incubation periods from 0 to 24 h. Note also that the untreated cells, 0 h of IFN treatment, exhibit a distinct mobility significantly greater than that of the controls. This indicates that the cells express CD2 antigen inherently, possibly due to the presence of IFN in the culture serum or a low level of constitutive expression.

Table 1 lists the means, medians, modes, SDs, and coefficients of variation (CV) for the magnetophoretic mobility measurements, as well as the number of cells analyzed for each experiment. The variance in the mobility measurements represents the inherent distribution in the numbers of the surface molecules per cell, as well as the inherent distribution of cell sizes within a given cell population (Equation 4). Additional sources of variation include immunolabeling techniques and CTV experimental analysis methods. The large SDs and CVs are common to both FCM and CTV analysis methods for biological systems. In order to be confident that the mean and median values are accurate, many cells from one population have been analyzed. Results from a pairwise multiple comparison Tukey test indicate that the differences in magnetophoretic mobilities of all fibrosarcoma treatment groups, including the unlabeled cells and the cells labeled with the

Table 1
Means, Medians, Modes, SD, and CV for the Magnetophoretic Mobility Measurements of Immunomagnetically Labeled Fibrosarcoma Populations*

IFN treatment (h)	m ($\text{mm}^3/\text{T-A-s}$)			SD	CV (%)	N
	Mean	Median	Mode			
Cells only	1.66E-05	5.70E-06	7.50E-06	4.94E-05	299	513
Second antibody only	4.42E-05	1.83E-05	2.11E-05	9.73E-05	230	350
0	1.61E-04	1.30E-04	1.50E-04	1.59E-04	99	1129
12	3.34E-04	2.98E-04	2.99E-04	2.16E-04	65	893
24	7.31E-04	5.88E-04	5.96E-04	7.15E-04	100	1279
36	5.65E-04	5.02E-04	5.31E-04	4.07E-04	77	1051
48	6.31E-04	4.66E-04	4.73E-04	6.63E-04	109	1663

*Also included are number of cells analyzed for each population group.

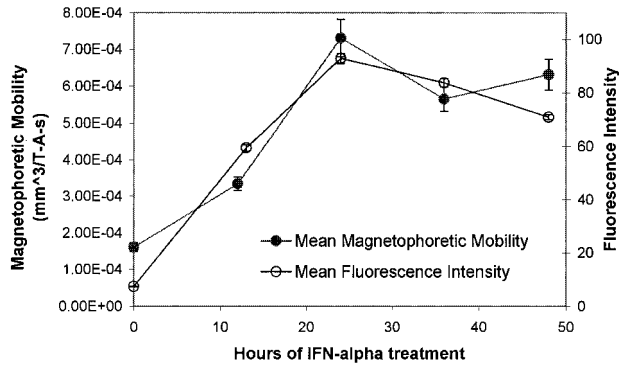


Fig. 4. Linear plot of the magnetophoretic mobilities, as measured by CTV, and the FI, as measured by FCM, for different IFN treatment populations of fibrosarcoma cells.

secondary antibody only, are statistically significant ($P < 0.05$).

Figure 4 is a linear plot of the mean magnetophoretic mobilities from CTV analysis of the 0, 12, 24, 36, and 48-h IFN-treated cell populations. It is also a plot of the mean linear FIs from FCM analysis (Beckman Coulter Elite) of the same 0, 12, 24, 36, and 48-h IFN-treated cell populations. The error bars around each data point are the 99% confidence intervals (CI) for the mean of each sample population. These CIs are small, even though the variance for the data is large, due to the large number of cells analyzed in each sample population (29). The CIs for the FI data are smaller than the CIs for the magnetophoretic mobility data. This is expected because FCM measures approximately 10,000 cells, whereas CTV measures approximately 1,000 cells. The plots in Figure 4 clearly show that FCM and CTV can measure relative changes in signal intensities related to CD2 expression levels and that the CD2 levels on fibrosarcoma cells increase with increasing hours of treatment with IFN to up to 24 h of treatment. Of particular interest is that the magnetophoretic mobilities measured from CTV have absolute units, whereas the signals from FCM are unitless values.

At first glance, one may note that the magnetophoretic mobility curve has pronounced inflection points at 12 and 36 h, unlike the curve depicting MFIs. After a more complete analysis of the experimental methods, it was noted that the lot of anti-FITC MACS secondary antibody reagent used for the 0, 24, and 48-h treatment populations differed from the lot of anti-FITC MACS antibody reagent used for the 12 and 36-h treatment populations. The same lot of antibody was used on all treatment groups for FCM analysis. Previous studies reported up to a 20% deviation in mobility measurements between two lots of anti-FITC MACS antibody reagent (16). Since the intensity of the fluorescence signal is directly proportional to the number of FITC molecules, a similar phenomena could possibly be observed in FCM if different lots of primary antibody had been used since the fluorescein-to-protein (F/P) ratio is also known to vary between antibody lots (13).

When a cell is immunomagnetically labeled, the size of that cell or microbead has a significant impact on the

resulting magnetophoretic mobility. There are two primary aspects in which cell size contributes to the resulting magnetophoretic mobility. The first aspect is more obvious and can be readily observed from the mathematical model (Equation 5). Because the drag force on the cell or microbead moving through viscous media is proportional to the diameter of the immunomagnetically labeled cell, the magnetophoretic mobility is inversely proportional to the cell diameter. The second, and less obvious aspect, is the potential limitation of the surface area available on the cell or microbead to bind high numbers of nano-sized magnetic particles (a steric hindrance limitation). For the above reasons, it is important to consider the particle diameters in magnetophoretic mobility evaluations. Figure 5a shows the size distribution of QSC microbeads as measured using a Coulter Multisizer II. The median QSC microbead diameter of 8.5 μm is chosen to be the most representative statistic over the mean value of 9.1 μm (SD 1.55 μm). This median diameter value, 8.5 μm , is also the value reported from the vendor (Flow Cytometry Standards) as the size of these microbeads for this specific lot of microbeads. The mean diameter for the fibrosarcoma cell populations ranged from approximately

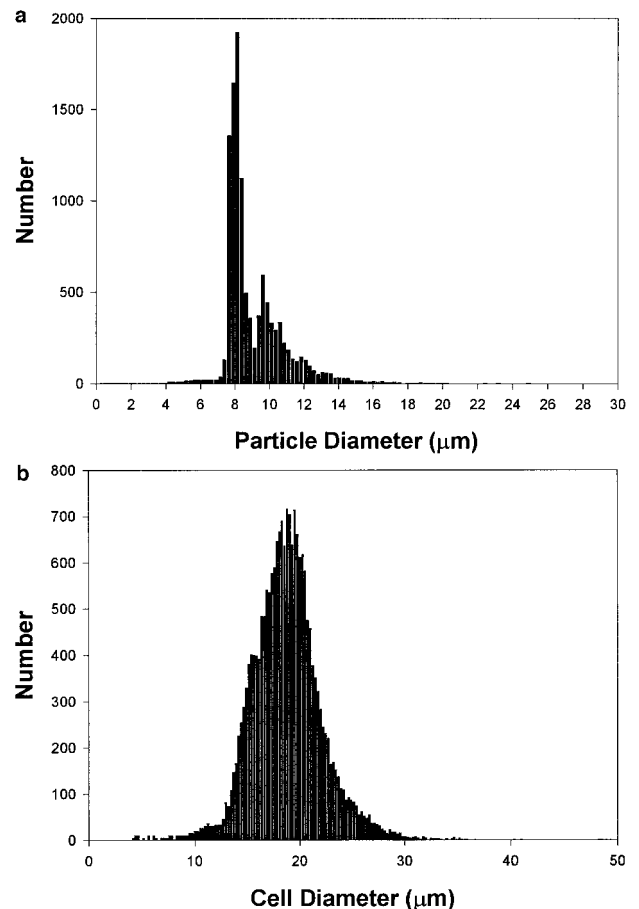


Fig. 5. a: Histogram of QSC microbead diameter measurements. b: Histogram of diameter measurements of untreated (0-h IFN treatment) fibrosarcoma cells.

16 to 18 μm as measured using a Coulter Multisizer II. Figure 5b shows the size distribution of the untreated cell population. The mean cell diameter of this population is 18.12 μm (SD 3.17 μm).

The calibration plot (Fig. 1) depicting the magnetophoretic mobilities of QSC microbeads versus the ABC for each microbead population presents the possibility of using magnetophoretic mobility measurements as a method of quantifying the ABC of a specific cell population. The plot presents the opportunity to potentially quantitate the ABC for the fibrosarcoma cell populations presented in this paper because the same primary and secondary antibodies were used for immunomagnetic labeling the cells and the QSC microbeads. However, two complicating factors also exist, 1) the significantly larger size of the fibrosarcoma cells compared with the QSC microbeads and 2) the nonlinear nature of Figure 1 for ABC values greater than 30,000–50,000.

As previously published, the linear portion of the calibration curve shown in Figure 1 consists of the following slope:

$$\text{slope} = \frac{\Delta\chi V_m (n_2 \theta_2 \lambda_2) n_3}{3\pi\eta D_c} = \frac{k\beta}{f}$$

$$= 1.27 \times 10^{-8} [\text{mm}^3/\text{T} - \text{A} - \text{sec}] \quad (6)$$

where the variables contributing to the slope in this equation are the antibody reagents used to magnetically tag the microbeads, $k\beta$, the diameter of the cells or microbeads, D_c , and the viscosity, μ , of the suspending medium (16).

Correction for the differences in the drag force, due to differences in the size between the QSC microbeads and the fibrosarcoma cells, is relatively simple. Specifically, the slope (Equation 6) is multiplied by the ratio of the QSC microbead diameter, 8.5 μm , to the mean diameter of that fibrosarcoma cell population, e.g., 18.12 μm for the untreated fibrosarcoma cell population (Table 2). Using the ratio of diameters to correct for the differences between QSC microbeads and the fibrosarcoma cell sizes, new slope values are obtained (Table 2). The new slope values

Table 2

Mean Magnetophoretic Mobility Measurements, Mean Cell Diameters of the Fibrosarcoma Treatment Populations, the Slope, Adjusted for the Differences in Drag Force due to Differences in the Mean Cell Sizes, and the Calculated Mean ABC for Each Cell Treatment Population

Treatment (h)	Mean m ($\text{mm}^3/\text{T-A-s}$)	Cell diameter (μm)	Slope (adjusted for diameter)	Calculated ABC
0	1.61E-04	18.12	5.96E-09	27,000
12	3.34E-04	18.05	5.98E-09	56,000
24	7.31E-04	16.92	6.38E-09	110,000
36	5.65E-04	16.53	6.53E-09	87,000
48	6.31E-04	16.16	6.68E-09	94,000

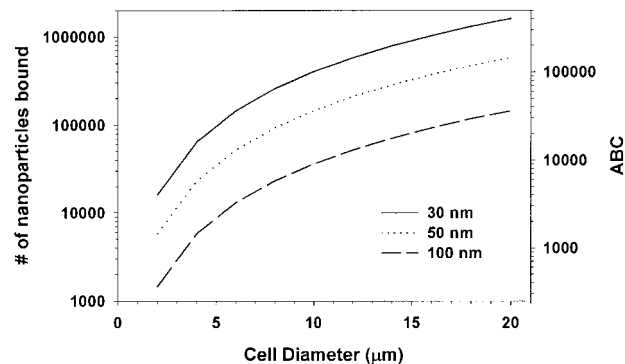


Fig. 6. Linear plot of the estimated maximum number of MACS nano-beads (approximately 30, 50, and 100 nm in diameter) and corresponding ABC, assuming $\beta = 4$, to a cell or microbead surface versus size of that cell or microbead.

account for the larger drag force from the larger fibrosarcoma cells.

The magnetophoretic mobility measurements for the IFN-treated fibrosarcoma cell populations can be used to obtain the predicted ABC for each of the cell treatment populations. The predicted ABC values are listed in Table 2. The calculated mean ABC for the untreated cell population is 27,000 and increases up to a maximum of 110,000 ABC for the 24-h treatment cell population.

The second complicating factor results from the departure from linearity of mobility versus ABC for $\text{ABC} > 30,000$ (Fig. 1). In a previous publication (16), we suggested that this departure was most likely caused by insufficient space on the surface of the QSC microbeads to allow all of the binding sites to be bound by the magnetic nanoparticles (i.e., a steric hindrance mechanism). The steric hindrance mechanism would limit the secondary antibody (with the magnetic nanoparticle conjugates) from binding to sites on the primary antibody ($\theta_2 < 1$), thus causing the deviation from linearity at high ABC values. This point of deviation from the normally linear relationship can be approximated as a function of the surface area of the cell or microbead, and correspondingly, cell diameter. Figure 6 is a semilog plot of the maximum number of magnetic nanoparticles that are potentially able to bind as a function of the diameter of the cell or microbead. Because the size of the magnetic nanoparticles used in this study has been reported to range from 20 to 100 nm in diameter (30), three nanoparticle sizes (30, 50, and 100 nm) were used in the calculations in Figure 6. Each line represents a different magnetic nanoparticle diameter. The assumptions used to make these calculations are that both the cell or microbead and nanoparticles behave like rigid bodies, the magnetic nanoparticles form a densely packed hexagonal lattice on the surface of the cell (90.7% coverage), only a single layer of nanoparticles forms on the cell or microbead surface, and the curvature of the cell or microbead may be neglected. The corresponding ABC values are also shown in Figure 6. The ABC values were obtained from the lumped term

βABC , which describes the number of magnetic nanoparticles bound to the cell or microbead, using an estimated value of $\beta = 4$ (16). Close inspection of Figure 6 indicates that for 50 nm-sized nanoparticles binding to an 8.5- μm microbead, we expect to reach an ABC of approximately 30,000 before steric hindrance effects start to dominate. For the fibrosarcoma cells, 16–18 μm in diameter, we expect to reach an approximate ABC between 93,000 and 120,000 before steric hindrance effects start to dominate.

Because quantitation of cell surface antigens using FCM analysis has become an acceptable technique to determine cell surface antigen molecule numbers, quantitative FCM was conducted on the untreated (0-h IFN treatment) cell population in order to compare the ABC value obtained from CTV analysis with the ABC value obtained from FCM analysis (13–15). The methodology for antigen quantitation using FCM analysis involves labeling the target cells and the calibration microbeads with a fluorescently conjugated antibody reagent. FIs of the calibration microbeads from FCM analysis are plotted against the ABC for each microbead population. Once this calibration plot is obtained, the FI of the target cell population is applied to calculate the mean ABC for that cell population.

The linear FIs of the immunofluorescently labeled QSC calibration microbeads are depicted in Figure 7a. Note that the MFI for each microbead population increases with increasing ABC. Figure 7b is a linear plot of the MFIs of these QSC calibration microbeads versus the median ABC for each microbead population. The regression line in this plot is obtained using only the first four data points because the FI for the cell population of interest lies within this region. Figure 7c is a semilog histogram depicting the unlabeled and labeled untreated (0-h IFN treatment) fibrosarcoma cell populations. Note that the median autofluorescence for these large, cultured fibrosarcoma cells is large, $\text{FI} = 28$, compared with the median autofluorescence, $\text{FI} = 3$, of the blank microbeads in Figure 7a. Compensation for the larger cellular autofluorescence was accomplished by subtracting the FI for the unlabeled cell population, $\text{FI} = 28$, from the FI for the labeled cell population, $\text{FI} = 154$ (31). This new value, $\text{FI} = 126$, was then used for ABC determination for this untreated cell population. Using the equation in Figure 7b, the calculated ABC for the untreated fibrosarcoma cell population is 33,000. This compares favorably with the calculated ABC value of 27,000 from CTV analysis. Note that the above study (including Figs. 7a–c), which aimed to determine the ABC for the untreated fibrosarcoma cell population using FCM analysis, was conducted at a later date and more importantly, the study was conducted on a separate FCM instrument than that used to obtain the FCM results reported in Figure 4. Therefore, it is not possible to compare the FI values as seen in Figures 4 and 7.

Table 3 lists the CVs for the microbead and cell populations used in this study for the two methods of data analysis, CTV and FCM. In general, the CVs from the two methods are similar. This indicates that the instrumentation noise introduced from CTV and FCM is approximately equal. The major contribution to signal variation in both

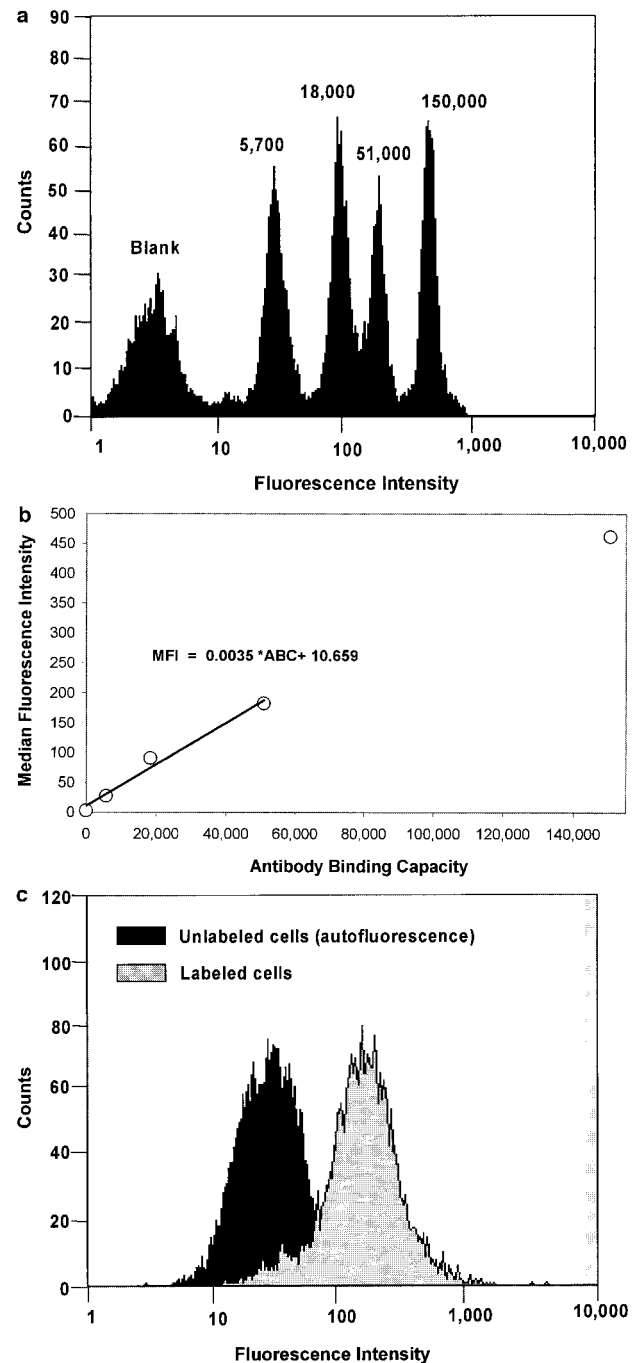


FIG. 7. **a**: Histograms of FI of the QSC microbeads. Each peak represents a different population of microbeads with discrete ABC. **b**: Linear plot of the median FI of the QSC microbead versus ABC. **c**: Histograms depicting the FI of the immunofluorescently labeled and unlabeled fibrosarcoma cells, untreated (0-h IFN treatment).

CTV and FCM is most likely the inherent spread in diameters and ABC (related to antigen expression) for these biological systems. Also, note that for the cells and microbeads with the lowest signal intensities, the CVs measured by CTV are significantly larger than the CVs for the analogous FCM data. The most likely explanation for the

Table 3
CVs for the Two Types of Analyses, FI Measurements From FCM and Magnetophoretic Mobility Measurements From CTV, for QSC Microbeads and Fibrosarcomas

ABC	QSC microbeads		Fibrosarcomas		
	CTV (%)	FCM (%)	IFN treatment (h)	CTV (%)	FCM (%)
0, no antibody	221	33	Cells only, no antibodies	299	82
0	140	39	0	99	98
4,500	59	20	13	65	92
16,000	36	17	24	100	100
52,000	32	15	36	77	99
210,000	22	13	48	109	100

larger CVs by CTV results from the definition of CV, SD divided by the mean. In CTV, as the mean mobility of a cell approaches zero, approximately 0.001 mm/s for the slowest or nonmoving particles, the CVs will approach infinity. This phenomenon is not seen in FCM. Because cells are inherently autofluorescent, the FCM signal does not approach zero; consequently, one does not “divide by zero” in calculating the CV of FCM data.

DISCUSSION

The major aim of this study was to demonstrate that, in addition to FCM analysis, CTV technology measures the magnetophoretic mobility of an immunomagnetically labeled cell and also has the potential to quantitate cellular ABC. This potential was demonstrated by calculating the mean ABC values for the untreated fibrosarcoma cell populations. These ABC values were calculated to equal 27,000 from CTV analysis and 33,000 using FCM analysis. In addition, both CTV mobility measurements and FCM FI measurements qualitatively indicated elevated ABC levels, correlating to CD2 expression levels, for the IFN-treated fibrosarcoma cells (Fig. 4).

However, at this stage of CTV development and immunomagnetic labeling methods, these results demonstrate only preliminary attempts to quantitatively correlate magnetophoretic mobility to antigen expression levels. To date, we have identified three major challenges in using CTV to quantitate cellular antigen expression levels. These challenges include (1) the possible steric hindrance of magnetic nanoparticles binding to the limited space available on the cellular surface, (2) the limited information available on the precise immunochemical behavior of the antibody reagents, and (3) the current lack of a direct calibration method correlating magnetophoretic mobility to ABC.

As a result of the first challenge, the potential steric hindrance effects due to magnetic nanoparticle binding, we are less confident in our calculations of the mean ABC values for the IFN-treated fibrosarcoma cell populations. These calculated ABC values for the IFN-treated fibrosarcomas (Table 2) assumed that $\beta = 4$ (16). However, this prediction was made using the linear portion of the calibration curve in Figure 1. Remembering that the term β was defined as the lumped parameters $n_2\theta_2\lambda_2n_3$, where θ_2 was defined as the fraction of binding sites on the primary antibodies that are bound by the secondary antibodies, θ_2

will decrease when steric hindrance becomes a factor, therefore β will also decrease. Within this sterically hindered region, the exact value for θ_2 , and thus β , is not known. Without a concrete value for β , the calculations for cellular ABCs become much more difficult. In future work, it may be possible to circumvent this problem of steric hindrance by mixing FITC-conjugated primary antibodies with pure, nonconjugated antibodies. The secondary antibody would have fewer binding sites, thus allowing a larger ABC range for linear calibration and analysis. However, this mixing of antibodies would significantly lower the magnetophoretic mobility of these labeled cells, therefore making them more difficult to separate from nonmagnetically labeled cells. For now, we have chosen to focus on the untreated cell population because these cells express a low enough ABC to escape problems associated with steric hindrance.

When attempting to quantitate antibody binding, it is important to understand the immunochemical behavior (binding affinities, disassociation constants, and nonspecific binding) of the antibody reagents. This information would allow accurate determination of the required amounts of antibody needed to guarantee antibody saturation of the specific binding sites while also minimizing nonspecific binding. In this study, only relatively primitive antibody saturation experiments were conducted according to a currently accepted technique. This involved adding increasing amounts of antibody reagent until the additional antibody reagent produced very little change in FI and, in our case, magnetophoretic mobility (28). However, this technique was designed for the qualitative studies typically conducted on FCM or for binary magnetic cell separations. These studies are not advanced enough when accurate quantitative results are desired. Therefore, more refined understanding of the functional relationships among the antibody, the specific antigen sites, and the nonspecific sites is desired. Current investigations in our laboratories are attempting to address these questions.

In addition to the optimization and evaluation of the antibody reagents, either all of the terms in Equation 5, or the values of lumped terms, such as k , β , and f , are also needed for ABC quantitation. Values for the lumped terms have been experimentally determined using QSC calibration microbeads (16). However, due to significant differences between QSC microbeads and cells, the values for these terms also need to be determined independently for

the cells. In this study, the ABC of the untreated fibrosarcoma cells was independently obtained using quantitative FCM analysis. Unfortunately, these fibrosarcoma cells have a high autofluorescence (Fig. 7c), which compromises our confidence in the calculations of ABC. In addition, because the use of QSC calibration microbeads with FCM is an indirect method for ABC quantitation, any limitations in FCM and QSC microbeads would be compounded with the limitations of CTV. Ultimately, it would be desirable to use radioisotopically labeled antibodies to directly quantify the ABC of the cells used in a CTV calibration study.

In other laboratories, a study comparing the sensitivity of immunofluorescence and immunomagnetic techniques has been conducted by Tchikov et al. (31). They used immunofluorescent and immunomagnetic antibody labeling techniques, changing the primary antibody dilution rate, and they compared "positive" cell fraction (binary, on/off type measurements rather than the more sophisticated analog mobility measurements conducted in our laboratories) achieved from both techniques. However, it appears as if these investigators used a 10-fold greater cell sample size for magnetophoretic analysis than for FCM analysis without changing antibody protocols accordingly. This would erroneously point toward FCM as the more sensitive measurement system. Also, the sensitivity of the measurement of a positive signal depends highly on the magnetic field strength, i.e., magnetic energy gradients, and the magnetic antibody labels. Thus, if the magnetic energy gradients were poorly designed, it would inaccurately appear as if magnetophoretic analysis is not as sensitive as FCM analysis.

From the results presented in Figures 3 and 4 and also in Table 3, it is apparent that CTV is capable of measuring CD2 antigen expression levels with comparable sensitivity to that of FCM. In addition, FCM measures only relative intensity signals, whereas CTV measures mobility signals with absolute units. Table 3 verifies that both techniques provide data with similar variance, indicative from the CVs reported from the two methods of analysis. Additionally, CTV does not have a significant intrinsic mobility signal as seen in FCM autofluorescence signals.

Other researchers have recognized the potential for using magnetophoresis velocity to quantitate numbers of labels per cell. However, they have chosen to focus on the ratio of magnetophoresis to sedimentation velocity, thus neglecting the dependence of this term on medium viscosity or on the hydrodynamic properties of cells (32-34). Our work also allows quantitation of the number of paramagnetic labels per cell. Furthermore, it includes investigations into ABC and cell diameter as they affect the number of paramagnetic labels that can be attached to a target cell. Because the ABC is related to the number of antigens expressed on a cell, this value may contain information about possible disease-related conditions (1-11). It also provides the structural framework for antibody paramagnetic nanoparticle binding required to create a magnetophoretic mobility. Current research in our laboratory continues to investigate ABC and other parameters involved in immunomagnetically labeling a target cell to

impart magnetophoretic mobility for magnetic separation processes. Also, because accurate ABC values are obtained only when correct concentrations of the labeling antibody reagents are used, our labs are currently investigating new and possibly better strategies for determining accurate antibody saturation conditions.

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