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Correction for Label Leakage in Fluorimetric Assays of Cell Adhesion

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ABSTRACT

Current fluorescence-based adhesion assays that use a 96-well plate format rely on the assumption that the fluorescent label does not significantly leak from the cells. Thus, we evaluated a calcein-based, in vitro adhesion assay in 96-well plates using five different types of leukocytes (HL60 cells, human neutrophils, rat neutrophils, mouse progenitor T cells and EL4 cells). Each cell type leaked calcein at a different rate, with the highest rates found for rat neutrophils and progenitor T cells, which lost as much as 20%–40% of the label within 90 min, the time required to complete the assay. Thus, we developed a procedure to measure the dye leakage rate during the assay in order to obtain a correction factor, which was then used to calculate the “true” number of adherent cells. Data for the adhesion of FTF1 cells to endothelial monolayers, after correction for calcein leakage, deviated less than 10% of adhesion data obtained with a well-established ^{51}Cr -based assay.

INTRODUCTION

The investigation of molecular interactions mediating the adhesion of cells to extracellular matrix proteins or other cells is a very active field of research, especially with the current heightened interest in developing therapeutic agents that block or modulate inflammatory and immune responses in vivo. The availability of several nontoxic chromophores that can be used to label cells has led to the development of safe and inexpensive fluorescence and colorimetric adhesion assays, which can be performed in 96-well plates (1–3, 12). Although the latter do not use a uniform detachment force to remove nonadherent cells from the surface (unlike centrifugal and parallel plate flow assays), their simplicity and ability to screen a large number of conditions in a short time makes them ideal for many

applications.

In colorimetric and fluorescence adhesion assays, cells are labeled with the chromophore, and it is assumed that the absorbance or fluorescence signal in each well is directly proportional to the number of adherent cells. The main assumptions that enables one to correlate fluorescence signal to cell number during the assay are that (i) the label is quantitatively retained by the cells during the assay and that (ii) the fluorescence intensity distribution is the same in the adherent cell subpopulation as it is in the overall population. Among fluorescent labels, calcein acetoxymethyl (calcein-AM) ester is commonly used because it has no deleterious effects on cell function, is a good viability indicator and its fluorescence properties are suitable for both fluorescence microscopy and flow cytometry (13–15). In addition, it is generally better retained by cells than bis-carboxyethyl carboxyfluorescein, carboxyfluorescein diacetate and carboxycalcein blue (6). Nevertheless, we have observed that significant leakage of cell-associated label was observed with certain cell types. Thus, further improvements to current methods were necessary to obtain reliable and quantitative data in such cases.

In the present report, we evaluated a calcein-based, in vitro adhesion assay in 96-well plates using five different types of leukocytes. A detailed characterization of the rate of fluorescent dye leakage was performed, and we developed a procedure to correct for the effect of dye leakage in order to obtain the “true” number of adherent cells.

MATERIALS AND METHODS

Reagents

Basal culture media, fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin, nonessential amino acids, sodium pyruvate and gentamicin were obtained from Life Technologies (Gaithersburg, MD, USA). Human recombinant tumor necrosis factor (TNF)- α (2×10^7 U/mg), 2% gelatin in water, and Hank's balanced salt solution (HBSS) were obtained from Sigma Chemical (St. Louis, MO, USA). Cal-

cein-AM was obtained from Molecular Probes (Eugene, OR, USA). $\text{Na}_2^{51}\text{CrO}_4$ was obtained from Amersham (Arlington Heights, IL, USA).

Cell Procurement and Culture

Human neutrophils were isolated from fresh blood of healthy volunteers by centrifugation on Ficoll-Hypaque® (Pharmacia Biotech, Piscataway, NJ, USA) followed by dextran sedimentation. Remaining red blood cells were lysed in hypotonic ammonium chloride (4). Rat neutrophils were isolated from fresh blood of rats transfused with 6% hetastarch in saline (McGaw, Irvine, CA, USA) by centrifugation on a discontinuous Percoll™ gradient as described elsewhere (8). Both human and rat neutrophil isolation procedures yielded >95% pure neutrophils and were immediately used in subsequent procedures. The murine progenitor T cell line FTF1 was provided by Dr. R. Palacios (MD Anderson Cancer Center, Houston, TX, USA) and derived from the thymuses of murine 14-day-old embryos and cultured as described previously (10). HL60 and EL4 cells were obtained from ATCC (Rockville, MD,

USA) and cultured as described previously (5,7,11). Primary bovine aortic endothelial cells were harvested by scraping bovine aorta using a sterile scalpel blade (9) and cloned to yield a bovine aortic endothelial cell strain. Endothelial cells were then cultured in gelatin-coated tissue culture flasks using RPMI 1640 supplemented with 20% FBS, 100 U/mL of penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine and 10 µg/mL gentamicin.

Cell Labeling and Characterization

A stock solution containing 150 µM of calcein-AM in dimethyl sulfoxide (DMSO) was prepared and stored at -20°C. This stock solution was added to a cell suspension (ca. 10^7 cells in 1 mL of RPMI 1640) to produce a final concentration of 5.25 µM of calcein-AM. This cell suspension was incubated for 30 min at 37°C, washed 3 times with cold HBSS and resuspended in HBSS with 10% FBS to a final concentration of 5×10^5 cells/mL. To measure the rate of calcein leakage from the cells, the labeled cell suspension was incubated at different temperatures (i.e., 4°, 23° and 37°C) for up to 3 h. At various

times during incubation, aliquots were placed in a U-shaped bottom 96-well plate (200 µL, 1×10^5 cells/well) and centrifuged at $250 \times g$ for 10 min. Following centrifugation, 100 µL of supernatant from each well were transferred to a separate empty well in the same 96-well plate. The fluorescence in each well was quantitated using a Cyto-Fluor™ 2350 Fluorescence Plate Reader (Millipore, Bedford, MA, USA) with the sensitivity setting at 4 and the filter settings at 485 and 530 nm for excitation and emission, respectively. No significant photobleaching occurred during fluorescence measurement, since repeated measurements gave similar data within 5%. Fluorescence of blank wells containing HBSS with 10% FBS (no cells) was subtracted from the raw fluorescence data. The fraction of retained cell-associated fluorescence (x_c) was calculated from Equation 1:

$$x_c = \frac{F_{\text{pellet}} - F_{\text{supernatant}}}{F_{\text{pellet}} + F_{\text{supernatant}}} \times 100 \quad [\text{Eq. 1}]$$

where F_{pellet} is the fluorescence in the well containing the cell pellet, and $F_{\text{supernatant}}$ is the fluorescence of the corresponding well containing super-

Figure 1. Calcein retention by leukocytes. Calcein-labeled cell suspensions were incubated at 37°C in HBSS with 10% FBS for up to 3 h. The fluorescence in the supernatant and that in the cells was determined as described in the Materials and Methods. Data shown are the fraction of total fluorescence remaining in the cells (x_c) and are the mean $\pm$ standard error of the mean (SEM) of three separate experiments (triplicate wells per experiment).

Figure 2. Calcein retention of FTF1 cells as a function of temperature. Cell suspensions of calcein-FTF1 cells were incubated in HBSS with 10% FBS at 37°C, room temperature (ca. 23°C) and 4°C for up to 3 h. In addition, aliquots were incubated in a 96-well plate and subjected to the standard washing procedure to remove nonadherent cells used in the adhesion protocol. The fraction of total fluorescence remaining in the cells (x_c) was determined as a function of time in each case. Data shown are the mean $\pm$ SEM for three experiments (triplicate wells per experiment), except for the 96-well plate data, which are the mean $\pm$ SEM of four experiments (triplicate wells per experiment).

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natant only.

Comparison between fluorescence- and radioactivity-based methodologies was performed using FTF1 cells. FTF1 cells were labeled with ^{51}Cr by adding 100 μCi of $\text{Na}_2^{51}\text{CrO}_4$ to approximately 10^7 cells in 1.5 mL of IMDM with 10% FBS and incubating at 37°C for 90 min. ^{51}Cr cells were then washed 3 times with HBSS and were subsequently labeled with calcein-AM and processed as above for fluorescence leakage determination. The leakage of ^{51}Cr from doubly labeled cells was determined by transferring the contents of each well into separate vials and measuring the radioactivity in a Gamma 5500 B Counter (Beckman Instruments, Fullerton, CA, USA). The fraction of retained radioactivity by the cells was found to be at least 95% after a 90-min incubation at 37°C .

Adhesion Assay

Flat-bottom, 96-well tissue-culture plates were treated overnight with 0.2% gelatin in HBSS, and 2×10^4 endothelial cells were seeded in 200 μL of RPMI 1640 with 20% FBS and allowed to proliferate until confluency (approx-

mately 24 h). The endothelial monolayers were then stimulated with $\text{TNF-}\alpha$ at concentrations ranging from 0 to 10^{-9} M and for 12 h. The monolayers were washed 3 times with warm HBSS, and aliquots of the cell suspension of calcein-labeled leukocytes (10^5 cells in 200 μL of HBSS with 10% FBS) were added to the wells. After incubation for up to 1 h, the wells were rinsed three times with HBSS at 37°C using a Bio-mek® 1000 Automated Laboratory Workstation (Beckman Instruments). The instrument was fitted with a multipipetting tool holding eight 200- μL pipet tips. The distance between the pipet tip and the bottom of the 96-well plate was adjusted to 0.18 in. Each rinse consisted of dispensing warm HBSS per well followed by mixing of the well contents at four equilaterally spaced locations on the perimeter of the well and removal of the added liquid. The exchange volume of liquid was 200 μL dispensed at the slowest rate setting (rate 1). This procedure did not affect the integrity of the endothelial monolayers as determined by phase-contrast microscopy. Nonadherent calcein labeled-cells from the initial re-

moval and subsequent three rinses with HBSS were collected in separate 96-well plates for fluorescence measurement. Thus, the initial number of labeled cells was divided into adherent cells in the original 96-well plate and nonadherent cells in four additional 96-well plates. The fluorescence of adherent and nonadherent cells was measured using the CytoFluor 2350 Fluorescence Plate Reader. Background fluorescence in blanks wells containing 200 μL of HBSS with 10% FBS with no calcein-labeled cells was subtracted from the fluorescence data of other wells. When calcein- and ^{51}Cr -labeled cells were used, contents of individual wells were transferred to vials, and the radioactivity in each well was measured in the gamma-counter.

Concurrent to the adhesion assay, an aliquot of the calcein-labeled cells was kept at 37°C , and the fraction of retained cell-associated calcein was determined as described in the previous section. The percentage of adherent cells was determined from Equation 2:

$$\% \text{ adherent cells} = \frac{F_{ac}/\bar{x}_c}{F_{nc} + F_{ac}} \times 100 \quad [\text{Eq. 2}]$$

Figure 3. Effect of incubation time of leukocytes on endothelial monolayers on the fraction of adherent cells. Endothelial cells were pretreated with 10^{-10} M $\text{TNF-}\alpha$ for 12 h at 37°C . Calcein-FTF1 cells were then incubated on top of endothelial cells for up to 1 h, and the nonadherent cells were removed using the washing protocol described in the text. Data shown are the mean $\pm$ SEM of two separate experiments (triplicate wells per experiment).

Figure 4. Comparison of calcein and ^{51}Cr adhesion data. Adhesion assays using FTF1 cells that were double-labeled with calcein and ^{51}Cr were conducted on endothelial cells pretreated with 10^{-10} M $\text{TNF-}\alpha$ as described in the text. Percentage of adherent cells was calculated by three methods based on: (i) uncorrected fluorescence data ($\square$), (ii) fluorescence data corrected for leakage ($\blacksquare$) and (iii) radioactivity data ($\bullet$). The results shown are the mean $\pm$ SEM of four experiments (3–5 replicate wells for each experimental condition).

where F_{nc} is the sum of fluorescence of all wells containing the nonadherent cells, F_{ac} is the fluorescence of the well containing the adherent cells and x_c is the fraction of retained cell-associated fluorescence from the fluorescence leakage determination.

RESULTS AND DISCUSSION

The experimental conditions (5.25 μ M calcein-AM for 30 min at 37°C) for cell labeling resulted in greater than 95% labeling of the leukocytes. Fluorescence per well correlated linearly ($r^2 > 0.99$) with the number of cells added in the range of 0 to 10^5 cells per well (data not shown), and the fluorescence of 1000 cells was readily distinguishable from background (ca. 300 U vs. ca. 230 U for the background). Thus, the sensitivity and linearity of the response are equivalent to previous reports using this fluorescent-labeling technique and a 96-well plate format (2,12).

Before studying adhesion to endothelial monolayers, calcein was used to fluorescently label five different cell lines. This compound is intracellularly retained by viable cells for longer periods of time than most other cytoplasmic fluorescent dyes. However, the different cell types studied exhibited strikingly different rates of calcein leakage. Leakage of fluorescence from labeled leukocytes was monitored at 37°C for up to 3 h post-labeling. Calcein-FTF1 cells and rat neutrophils showed the most significant loss of fluorescence (Figure 1). This leakage, if not taken into account during an adhesion assay, would introduce a systematic error in the determination of the number of adherent cells.

To develop a method that would account for this leakage, we examined the effect of temperature on the leakage rate. The fluorescence leakage from calcein-FTF1 cells was strongly dependent on incubation temperature (Figure 2). To determine the appropriate leakage profile describing the cells in the 96-well adhesion plate, calcein-FTF1 cells (10^5 cells/well) were placed in a separate 96-well plate and subjected to incubations and washes identical to that to be used in the adhesion assay. The

results showed that the cell-associated fluorescence of the calcein-FTF1 cells at the end the cell-removal procedure (90-min post labeling) was very similar to that of calcein-FTF1 cells maintained at 37°C for 90 min (Figure 3). Thus, one can save an aliquot of the cell suspension and incubate it at 37°C to estimate the amount of fluorescence lost by the cells during the assay in the 96-well plate.

Previous adhesion assays in 96-well plates have used a manual procedure to remove nonadherent leukocytes. To minimize potential variations from assay to assay and user to user, we standardized the cell detachment procedure using a programmable laboratory workstation (Biomek 1000). The distance between the pipet tip and the bottom of the well, the speed of the rinsing fluid and the number of mixes per rinse can be adjusted at will, which provides a means of controlling the detaching

force applied to the cells. However, because the mechanical washing procedure to remove nonadherent cells takes 7 min from one end of the plate to the other, we needed to design the assay so that this difference would not introduce a variation in the number of adherent cells across the plate. Thus, we determined the time necessary for adhesion between FTF1 cells and TNF- α -treated endothelial cells to reach steady state by varying the incubation time of FTF1 cells with endothelial monolayers. The results show that a 20-min incubation time was required to reach maximum adhesion of FTF1 cells to endothelial cells, after which the percentage of adherent leukocytes remained constant (Figure 3). Thus, by using a 60-min incubation time before removing nonadherent cells, the time taken to wash the plate would not introduce a significant variation in the number of adherent cells across the plate.

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The adhesion of FTF1 cells to TNF- α -treated endothelial monolayers was characterized using FTF1 cells labeled with both ^{51}Cr and calcein-AM (Figure 4). The results clearly indicate that failure to correct for fluorescence leakage underestimates the number of adherent FTF1 cells by as much as approximately 50%. However, incorporation of the correction for fluorescence leakage dramatically improved agreement with ^{51}Cr -based adhesion results. Since ^{51}Cr did not significantly leak from the cells, we concluded that the corrected fluorescence values reflect the "true" number of adherent cells. One can establish a criterion for determining whether correction for leakage is necessary or not by assuming that the deviation introduced by the leakage must be less than the standard deviation of the measurements. In the current studies, the standard deviation approximates 10% bound cells, thus correcting for label leakage when it is less than 10% after 90 min is not necessary. This is the case for HL60 cells, which retained 94% of

the fluorescence. Conversely, all the other cells tested here leaked more than 10%, and data accuracy would be significantly reduced without correcting for label leakage.

In summary, we have described an improved method to quantitate cell adhesion based on fluorescence labeling using calcein. We showed that it is necessary to characterize the leakage rate of the fluorophore. In cases where the leakage rate is significant, the procedures described herein can be used to correct the data and determine the "true" number of adherent cells. Thus, it is relatively simple to determine the fluorescence leakage rate at 37°C and to correct the measured fluorescence for adherent cells accordingly. The rate of dye leakage, being determined from a suspension of labeled cells in a separate tube throughout the duration of the adhesion experiment, does not increase the time required to perform the assay. Thus, it is possible to use fluorescent labeling methods to label any cell, provided that the leakage rate is determined. These improvements in 96-well plate adhesion assays will result in more accurate methods for efficient quantitation of cell adhesion.

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