RED BLOOD CELLS EXPERIENCE ELECTROSTATIC REPULSION BUT MAKE MOLECULAR ADHESIONS WITH GLASS

ANNETTE TROMMLER,* DAVID GINGELL,‡ AND H. WOLF*
‡*Department of Anatomy and Biology Applied to Medicine, Middlesex Hospital Medical School, Cleveland Street, London W1P 6DB, United Kingdom; and * Biomaterials Research Unit, School of Medicine (Charite) Humboldt University, Schumannstrasse, Berlin 1040, German Democratic Republic*

ABSTRACT We have studied the detachment of unfixed red cells from glass coverslips under unit gravity and by centrifugation in buffered isotonic solutions over a range of ionic strengths. Cell-glass contact areas and separation distances were measured by quantitative interference reflection microscopy. Detachment under unit gravity is highly dependent on ionic strength: dilution increases electrostatic repulsion and greatly reduces the proportion of adherent cells. However, even at 1.5 mM some cells stick. Over the range 3–110 mM such adherent cells are progressively removed by increasing centrifugal forces, but in a manner virtually independent of ionic strength. This fact, together with the irreversibility of pre-adherent cells as ionic strength is progressively reduced, as well as the resistance of cells to lateral shearing forces, provide evidence sufficient to reject the notion of secondary minimum adhesion for unfixed cells at any ionic strength down to 1.5 mM. We conclude that all unfixed cells that stick at ionic strengths from 157 to 1.5 mM make molecular contacts with glass. Comparison with long range force calculations suggests that to penetrate the electrostatic repulsion barrier the contact regions are unlikely to have average surface properties. A new method that compares frequency distributions of contact areas with responses to detachment forces shows that detachment forces are not linearly related to contact areas. This lack of relationship is less clearly evident for rigid glutaraldehyde-fixed cells and may therefore depend on the degree of cellular deformability.

INTRODUCTION

The adhesion of red blood cells has been studied for several reasons. They are easy to get and need no disaggregative procedures, which inevitably complicate matters. Red cells have been studied in relation to the biophysics of adhesion because of their relative structural simplicity, lack of active spreading and locomotion, and because more is known about the molecular composition of their surfaces than any other cell type. George et al. (1971), using a centrifugation method, showed that unfixed red cells adhere rapidly and strongly to clean glass in physiological saline and also that adhesion is greatly reduced in the presence of plasma proteins. Using a fluid–shear-stress method to detach cells from siliconized, polymer coated, and clean glass, Mohandas et al. (1974) showed that above a critical value of shear stress all cells can be removed if given sufficient time. The critical stress value was found to be a function of initial cell-glass contact time as well as the nature of the substratum. Corry and Defendi (1981) also stressed the influence of time on the centrifugal detachment of cells from glass.

There have been several studies of red cell interaction with highly defined interfaces to see whether adhesion could be interpreted in terms of electrostatic and electrodynamic forces. Gingell and Todd (1975, 1980) studied the interaction of red cells with a hydrocarbon oil/saline interface, chosen because of its structural homogeneity and surface smoothness. Glutaraldehyde-treated cells were used to obviate protein adsorption at the liquid interface. Another study using aldehyde-treated cells by Gingell and Fornés (1976) used a chemically polished polarizable electrode, and adhesion was observed as a function of surface charge density at very low ionic strength. Analysis of these experiments (Parsegian and Gingell, 1980) showed that adhesion at very low ionic strength is consistent with an electrodynamic force balance in a secondary minimum energy configuration with an attractive force constant of $3–8 \times 10^{-14}$ erg, but that the existence of macromolecular cell-surface links or fine membranous protrusions beyond the resolution of the interference microscope could not be ruled out. Whereas fixed cells have a strictly limited ability to respond mechanically or biochemically to an interface, the same is not true of unfixed cells. Wolf and Gingell (1983) studied the effect of dilution on unfixed red cells pre-adherent to glass in isotonic saline solutions and got interferometric evidence for spatially uneven separation on dilution. Cells that settle at low ionic strength make smaller and more uniform contacts with glass than do cells that initially settle at higher ionic strength and are then subjected to dilution.

These results sounded a clear note of caution about equating the adhesive behavior of unfixed and fixed cells. The present report describes the adhesion of unfixed red cells to clean glass studied by simple methods of settling and inversion, followed by centrifugation of the cells that remain stuck.

MATERIALS AND METHODS

Human red blood cells from healthy O Rh+ males were obtained from the blood bank within 1–3 d of donation. Cells were separated from plasma by centrifugation at 500 *g* for 5 min and then washed twice in physiological phosphate buffered saline (PBS) pH 7.4 ± 0.1 by centrifugation at 2,000 *g* for 10 min. After incubation at 37°C at a volume concentration of 45% for 1 h the cells were resuspended in PBS solutions of various ionic strengths from 1.5 to 157 mM, pH 7.4, at a final volume concentration of ~0.015 % (1.5×10^6 cells/cc). Solutions of reduced ionic strengths were adjusted with sucrose to a constant osmotic pressure of 290 mOsm. In the text we refer for simplicity to millimolar solutions.

Glutaraldehyde-fixed red cells were prepared by washing cells 10 times in PBS at 2,000 *g* for 5 min then treating with 3.3 % glutaraldehyde in PBS at 4°C for 18–20 h followed by subsequent exhaustive washing by centrifugation in PBS.

Much of the cell adhesion work was done with a polymethylmethacrylate chamber using Thoma hemocytometer coverslips as the substratum. Experiments were repeated and extended using chambers made from EN58J saline resistant stainless steel, passified once prior to initial use by immersion for 10 min in 65 % nitric acid. Number 3 circular glass coverslips (Chance) were used in these chambers. All coverslips were cleaned by brief immersion in a mixture of 4% hydrofluoric acid in 50% nitric acid, then rinsed 10 times in distilled water and left in static distilled water for 20 h to standardize the degree of hydration of the glass surface.

Washed cells, unfixed or fixed, were allowed to settle for 50 min onto the glass surface in the measuring chamber at room temperature. The chamber was then carefully inverted and then left 30 min for detachment to occur under gravity. From photographs taken before and after detachment the number of cells remaining stuck to the glass was determined and the proportion of adherent cells was calculated. This was done for 10 random fields in each preparation. All experiments were repeated with blood from several individuals.

In parallel measurements, cells were allowed to settle onto glass as described, but were then subjected to detaching centrifugal forces acting perpendicular to the glass surface, corresponding to a range of accelerations from 75 to 1,500 *g* (1,000–4,500 rpm at 6.9 cm radius). After running at a constant speed for 5 min the centrifuge was allowed to decelerate and the number of attached cells was recorded, the procedure was repeated in stepwise increments until the maximum acceleration was reached. In one series of experiments each cell population was accelerated directly to one final value. In this way all cells in all preparations experience similar residence times at the glass surface, in contrast to the first protocol. In one series of experiments the centrifuge was run for 15 min instead of 5 min to ensure that no further detachment occurred.

Reversibility of adhesion was assessed by two methods. In the first, cells were allowed to settle and attach to glass in an open polymethylmethacrylate chamber. The supernatant was then carefully exchanged using a pipette for a medium of reduced ionic strength. Following this procedure adhesiveness under centrifugation was measured as described. In the second method electrolyte exchange was conducted in a flow chamber of EN58J stainless steel. The latter procedure was better for weakly attached cells and was therefore used to assess adhesion under gravity at all reduced ionic strengths, but it precluded subsequent centrifugation.

Quantitative interference reflection microscopy was performed as described by Gingell and Todd (1979) and Gingell et al. (1983) using parameters discussed by Wolf and Gingell (1983).

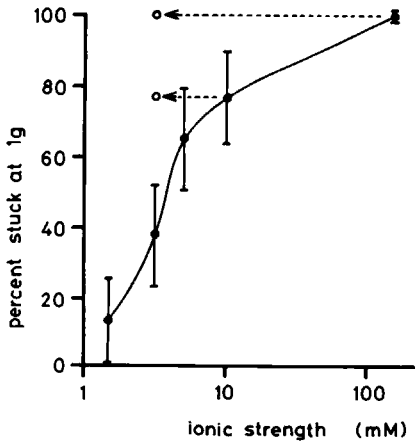

FIGURE 1 Detachment of cells from glass by gravity in isotonic buffered saline solutions as a function of ionic strength. Standard deviations shown. The numbers of independent measurements vary from 23 to 34. Blood from seven to ten different donors was used. ●: cells settled at single ionic strengths. ○ ←● : cells settled at the higher ionic strength followed by dilution to the lower value after inversion.

RESULTS

The gravitational results are shown in Fig. 1 on a logarithmic scale. On a linear scale it would be apparent that attachment was only modestly curtailed by dilution until ~10 mM, after which the proportion of attached cells fell rapidly to ~10% at 1.5 mM, the lowest ionic strength used. At 157 mM unfixed cells were nearly 100% adherent at 1 *g* while fixed cells showed a reduction to 74.5 ± 2.8% (SD).

A comparison between the two types of glass used in this study gave the following results. At 1.5 mM 12.8 ± 8.6 % of cells remained attached to Thoma glass, compared with 16.0 ± 4.2% on Chance glass coverslips. At 10 mM the respective values are 76.8 ± 13.6% and 77.4 ± 9.8%. There is therefore no difference in attachment to the two types of glass at the 5% significance level (Wilcoxon rank sum test).

In another series of experiments the reversibility of adhesion was investigated by allowing cells to attach in a higher ionic strength of saline and then carefully exchanging the electrolyte in the chamber until the desired reduced ionic strength was reached. The proportions of cells stuck under 1 *g* were assessed by photography before and after dilution (Fig. 1). As a control for cell removal by viscous shearing forces an equivalent volume of electrolyte was exchanged without dilution. Exchange from 157 to 3 mM and from 10 to 3 mM failed to remove any cells (200 cells in each field).

Experiments to investigate the influence of contact time on detachment by gravity indicate that the proportion of unfixed cells remaining stuck after inversion is increased when the settling time in low ionic strength solutions is prolonged (see Table I). No effect was found with fixed cells.

Figs. 2 *a*, *b* show the centrifugation results. The normalized curves for stepwise centrifugation in 3, 5, 10, and 157 mM [curves (v), (iii), (iv), (i)] are all virtually linear up to

TABLE I
INFLUENCE OF CONTACT TIME ON DETACHMENT BY GRAVITY

Ionic strength (in millimoles per liter)	Mean ± SD % adhesion at settling times (minutes)	
	50	150
1.5	16.0 ± 4.2	31.9 ± 5.0
10	77.4 ± 9.8	92.9 ± 7.2
1.5 (fixed cells)	0	0
10 (fixed cells)	3.5 ± 1.6	1.4 ± 0.6

250 to 350 cells per field counted.

the maximum experimental acceleration of 1,500 g (= 1.2×10^{-5} dyne per cell, assuming a mean buoyant cell mass of 9×10^{-12} gm). It should be emphasized that normalization scales all percentages of cells remaining attached at 1 g to 100% for all ionic strengths. This procedure facilitates comparison between those portions of the populations that do adhere at 1 g, and in particular emphasizes similarities. Unlike the closely grouped low ionic strength curves that show 60–75% detachment by 1,500 g, that for 157 mM shows only 20% detachment. Cells attached in 157 mM and then diluted to 1.5 mM behaved under centrifugation like cells in 157 mM curves [(i), (ii)]. Fig. 2 *a* also shows the strikingly different adhesive behavior of glutaraldehyde-fixed cells. Compared with unfixed cells in 157 mM, fixed cells at the same ionic strength are far easier to remove and show a markedly nonlinear detachment curve. At 10 mM no significant difference in detachment of unfixed cells by stepwise or direct centrifugation was found at the 5% level using the Wilcoxon rank sum test (Fig. 2 *b*).

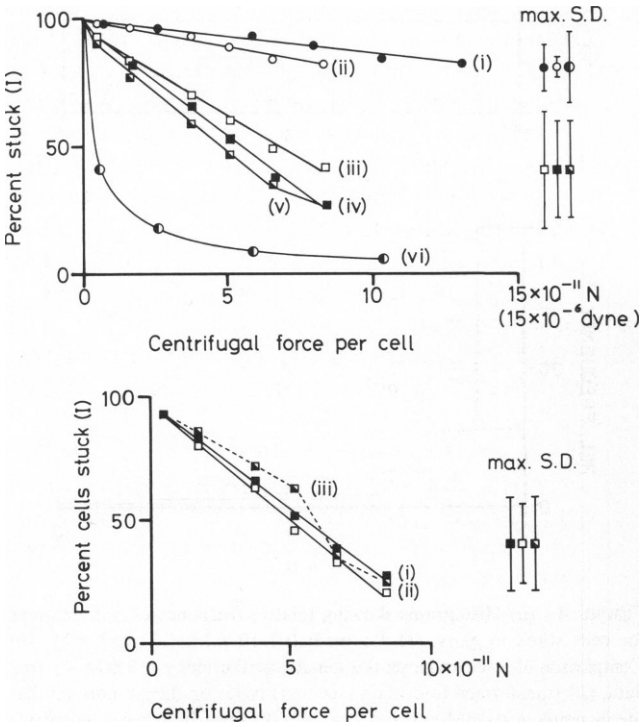

FIGURE 2 Detachment of cells (initially stuck under gravity) by centrifugal force. Curves are normalized so that percentage of cells remaining attached at 1 g after inversion is 100. Since the force acting on a cell at a given acceleration depends on the density of the suspending medium, the range of the curves for reduced ionic strength solutions containing sucrose is less than that for cells in physiological saline. Each point represents the mean of ~20 independent measurements. For each curve the greatest SD is given. (*a*) curve (*i*) 157 mM; (*ii*) cells settled in 157 mM then centrifuged in 1.5 mM; (*iii*) 5 mM; (*iv*) 10 mM; (*v*) 3 mM; and (*vi*) fixed cells in 157 mM. (*b*) curve (*i*) 10 mM stepwise centrifugation (5 min); (*ii*) 10 mM direct centrifugation (5 min); and (*iii*) 10 mM direct centrifugation (15 min). Forces calculated per mean cell effective mass.

Interference microscopy of cells that remain adherent after inversion at 1 g gives information about the area of contact and the distance separating the lipid bilayer of the plasmalemma from the glass. Representative photographs taken at 546 nm with a microscope illuminating numerical aperture of 1.18 are shown in Fig. 3. There are several significant features to note in these photographs. Consider first the area of contact: the criterion we have adopted for defining the area of contact may err on the generous side and is based on experience in assessing cell shapes from interference fringes. Where cells flatten in contact with glass the fringes spread and the contact area is delimited by a more or less sudden transition from a broad zero-order fringe to narrower higher-order fringes. The zero-order contact may show different intensities, and characteristic colors in white light, according to the cell-glass separation. Often the separation is smaller near the center of the widely spread zero-order fringe than at its periphery; this

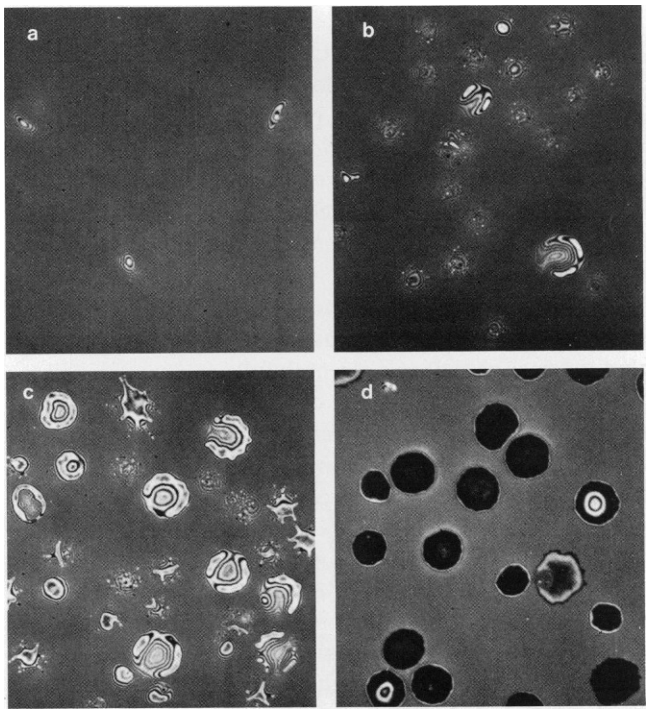

FIGURE 3 Interference reflection images of cells attached to glass under 1 g: (*a*) 1.5 mM; (*b*) 5 mM; (*c*) 15 mM; and (*d*) 157 mM.

can be seen very clearly in the images of several cells in 15 mM (Fig. 3 *c*). Moving radially from the contact zone the sudden transition to closely spaced fringes shows that the cell is curving very steeply away from the glass. At still lower ionic strengths the contact zone is reduced (Fig. 3 *a*, *b*) whereas at 157 mM (Fig. 3 *d*) the contact area occupies almost the entire image.

Contact areas were measured from photographs using a Kontron IBAS 1 interactive computer-based image analysis system and some of the results are shown as histograms in Fig. 4 *a*. Median contact areas are shown in Table II. Separations were computed from photometric data by the technique of finite aperture microscope interferometry (Gingell and Todd, 1979; Gingell et al., 1983). The results are model dependent insofar as the cell surface is considered to be a series of zones whose refractive indices and thicknesses are required. We obtained distances of closest separations as shown in Table II. The white light colors seen at the various ionic strengths under our experimental conditions correspond approximately to the characteristic zero-order white light colors for similar thicknesses. In monochromatic light, 546 nm, the closest contacts look dark (Fig. 3 *d*) and increasing separation lightens them (Fig. 3 *a*).

Between 5–15 mM a high proportion of cells make multiple punctate contacts. These images come from rounded cells attached to the glass by tethers or filopodia. They are referred to below as echinocytes, but we do not imply that they are the same as classical echinocytes seen in physiological ionic strength under certain conditions.

DISCUSSION

The main experimental results of our study are:

(1) If unfixed cells are allowed to settle onto glass and the system is then inverted, a proportion of the cells fall off under gravity. The proportion that fall increases with decreasing ionic strength.

(2) Adhesion exhibits lack of reversibility since cells settled at either 157 or 10 mM cannot be removed by dilution to 3 mM.

(3) Cells that settle and remain stuck under unit gravity show interferometrically measured separations from glass that increase as the ionic strength falls.

(4) After exposure to 1 *g*, centrifugation removes more cells but there is little difference between the removal curves over the 3–10 mM range, in distinction to the very large effect that ionic strength has on gravitational removal over the same range.

(5) Cells in 157 mM cannot be removed by 1 *g* and loss by maximal centrifugation (1,500 *g*) is only ~20%. Furthermore, cells stuck in 157 mM and then centrifuged in 1.5 mM behave like cells centrifuged without dilution.

(6) Cells pre-fixed with glutaraldehyde before settling are far more easily removed from glass by centrifugation than are unfixed cells at 157 mM.

(7) The proportion of cells that stick to glass under unit gravity at low ionic strength (1.5, 10 mM) is a function of contact time in the range 50–150 min.

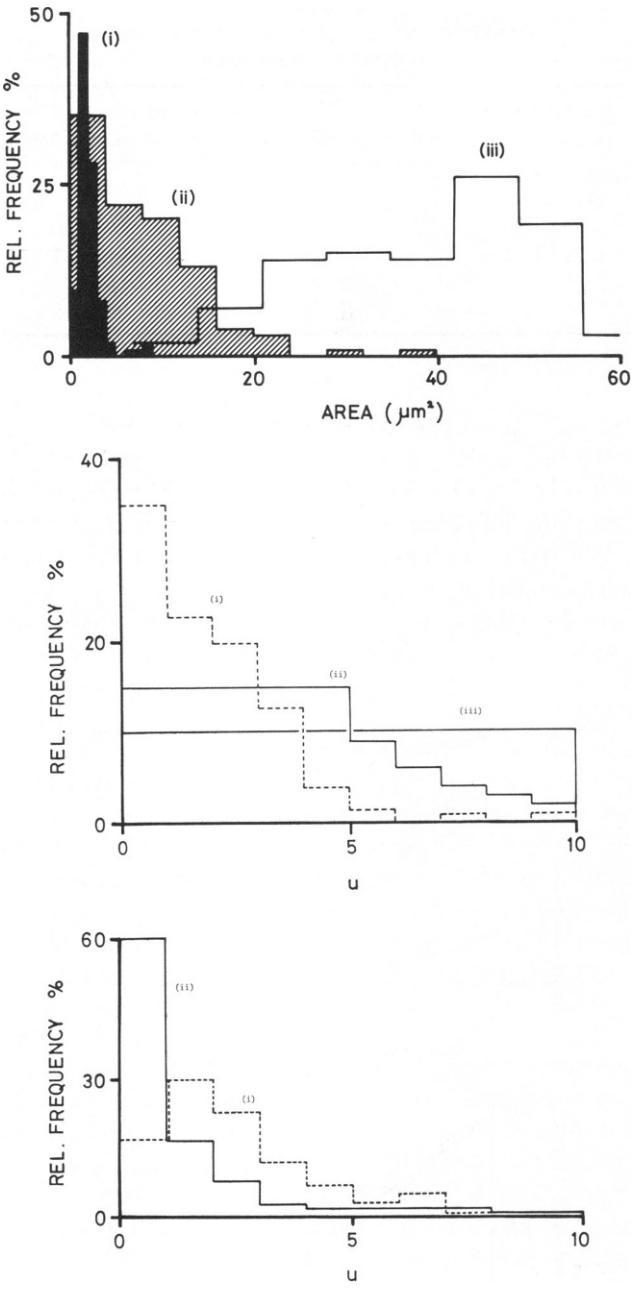

FIGURE 4 (*a*) Histograms showing relative frequency of contact areas for cells stuck to glass. (*i*) 1.5 mM; (*ii*) 10 mM; (*iii*) 157 mM. (*b*) Comparison of force and area frequency distributions at 10 mM (*i*) area data, (*ii*) transformed force data (see text) based on the assumption that the incomplete 10 mM curve in Fig. 2 *a* falls linearly to zero, and (*iii*) is based on the opposite extreme assumption that there is a smooth tail to the 10 mM curve that falls to zero at 18×10^{-6} dyne. (*c*) Similar to *a* but for fixed cells at 157 mM. (*i*) area data; (*ii*) transformed force data.

From these results, what conclusions can be drawn about cell-glass interaction?

There arise two distinct questions: (*a*) regarding the unit gravity experiment, we can ask whether the prevention of adhesion is due to increased electrostatic repulsion at

TABLE II
CONTACT AREA AND SEPARATION DATA

	Ionic strength (in millimoles per liter)						
	1.5	5	10	15	70	157	157 (fixed)
median contact area (in micrometers squared)	1.5	5	7	9	15	42	7
closest separation (in nanometers)	140	64	42	36	16	12	—

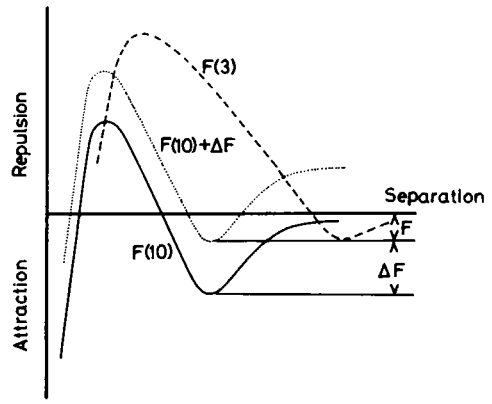

FIGURE 5 Schematic summed electrodynamic and electrostatic forces of interaction for cells in secondary minima interacting with glass in 10 mM electrolyte [curve $F(10)$] and 3 mM [curve $F(3)$]. The adhesion force in $F(3)$ is F and the difference in removal force between these two cases is ΔF. In the dotted curve a constant centrifugal force $m'a = \Delta F$ is added to $F(10)$ resulting in an adhesive force F.

low ionic concentrations; and (*b*) regarding cells that do stick under unit gravity, we can ask how they stick. The answers we shall find are (*a*) results are consistent with this explanation; and (*b*) molecular contact occurs at all ionic strengths.

Modeling the RBC-glass Interaction at 3–10 mM

We make the provisional hypothesis that cell-substratum interaction is entirely due to long-range electrostatic repulsive and electrodynamic attractive forces. There are then two possible modes of adhesion, molecular contact with a net attractive force and energy (primary energy minimum) and a force balance at a distance, without molecular contact (secondary minimum). In making an order of magnitude assessment of cell-glass interactions we assume that cells have uniform surface properties as determined by electrophoretic zeta potentials. The analysis is outlined in the Appendix and only the results follow.

We find that the modeled interaction curves have secondary minima. Utilizing our measured areas of adhesion the forces necessary to remove the cells can be compared with gravity and the adhesive energies can be compared with thermal energy (kT) to get an idea of stability. In 3 mM we find a weak adhesive energy, $< 2\,kT$, and the adhesive force is similar to gravity. This result corresponds with 60% of cells detaching under gravity (Fig. 1). In 10 mM the adhesive energy is larger ($< 15\,kT$) and the force of adhesion is ~25 *g*. This result also fits with the experimental result that only 20% fall under gravity.

While these calculations give a satisfactory electrostatic explanation for the decrease in the proportion of cells able to settle and stick at a series of low ionic strengths (Fig. 1), we shall next show [points (*i*) to (*v*)] that the cells that *do* stick are far too strongly stuck for the secondary-minimum idea to be right.

(*i*) In contrast to the force calculations, which show that most cells at low ionic strengths should be removed by <25 *g*, only ~50% of the cells could be detached by maximal centrifugation at 1,500 *g*.

(*ii*) If detachment is simply the result of overcoming long range attraction in a secondary minimum, either by increasing electrostatic repulsion or by increasing *g* force, it should be possible to show quantitative equivalence between the two modes of removal (see Fig. 5). The analysis of this situation in the Appendix suggests that this is not the case and that in consequence primary molecular contact is implicated.

(*iii*) After allowing cells to settle at 10 mM, diluting to 3 mM and then exposing to shearing flow sufficient to cause visible deformation, > 80% remained stuck. Further observations (unpublished) show that cells that initially settle and stick on a Langmuir-Blodget film of barium arachidate at ionic strengths from 1.5 to 157 mM cannot slide under gravity when the interface is made vertical. Since secondary minimum contacts would have no resistance to shear we conclude that adhesion involves molecular contacts. These probably occupy a small fraction of the more-or-less parallel area of apposition, and are maintained on dilution but can be ruptured by sufficient centrifugal force.

(*iv*) The experimental results (Fig. 2 *a*) show that the 3, 5, and 10 mM adhesion curves do not fall in an electrostatically determined sequence; the curves are closely grouped and within this range it is clear that once stuck their adhesions are virtually independent of ionic strength. The increase in cell-glass separation seen on dilution is due to electrostatic repulsion, which probably causes the unfolding of surface polyanions. It does not in this case imply a secondary minimum force balance. Little is known about changes in the surface structure of glass in response to changes in ionic strength (Voigt et al., 1983) so we cannot judge whether such changes play any part in ionic-strength–dependent adhesion.

(*v*) There is a final fact that clinches the molecular contact conclusion. From Fig. 1 it can be seen that the difference in the proportion of cells stuck after settling directly from 10 and 3 mM is 40%, whereas the transition from 10 to 3 mM by dilution removes none. This is not

consistent with secondary minimum contact at 10 mM and the same argument can be applied for cells in 157 mM.

Removal Force is Not Linearly Proportional to Contact Area

We can exclude a simple linear relationship between removal force and area of contact. From our IRM area measurements we obtain the area distributions for the different populations (Fig. 4 *a*). The histograms of areas at reduced ionic strength are strongly skewed. If force were proportional to area (as in the simple force balance model) we could replace the abscissa with force/cell and compare this distribution with that needed to generate the centrifugation result. The key point in making this comparison is that the inverse of the centrifugal fall curve is itself a cumulative frequency distribution, and that the population distribution (histogram) can be obtained from it by graphical differentiation. The two types of curve can be drawn on a common abscissa if we assume that they represent the same basic process and therefore have equal population spreads.

For the 10 mM case we obtain the results superimposed on the histogram in Fig. 4 *b*. It is clear that the force and area distributions for unfixed cells reflect completely different populations. With fixed cells in 157 mM (Fig. 4 *c*) a somewhat closer degree of coincidence is seen except at the smallest contact areas, which shows that fixed cells behave more as if force and area are linearly related. We have also treated the fixed cells as if they are elastic solids, following Johnson et al. (1971), but the fit is not improved. If cells peel under centrifugation, perimeter might be more significant than area; however, under this assumption the fit is also made worse. In the above considerations we have excluded echinocytic cells, but their inclusion also worsens the correspondence between the curves. Force/area behavior may be influenced by the existence of punctate adhesions within areas of contact and gross deformation of cells under applied force, as indicated by dilution and liquid-flow experiments in progress.

We considered the possibility that the wide range of accelerations (Fig. 2 *a*, *b*) needed to remove cells from glass might be due to the population spread in mass, rather than area of contact. No values for mass variation appear to be available, but from density (Danon and Marikovsky, 1964) and volume data (Canham and Burton, 1968) we obtain a maximum estimate for the coefficient of variation for the mass equal to 0.16. Since volume and density are symmetrically distributed mass must be similar. However, the removal force (Fig. 4 *b*) is not normally distributed and shows a much larger coefficient of variation. Mass variation is therefore considered unlikely to cause the centrifugal behavior.

Time Dependence of Adhesion

Table I shows that there is a clear effect of settling time on adhesion for unfixed cells in dilute solutions over the period 50–150 min. This means that adhesion may increase during centrifugation. Because centrifugation is performed discontinuously, cells removed early under low acceleration remain in contact a shorter time than those that survive to experience higher accelerations. The latter cells may increase their adhesiveness during this time. But experiments in which cells in 10 mM were centrifuged directly (not stepwise) over equal times (5 and 15 min) to a series of different final accelerations did not indicate the reduced adhesion that might be expected, particularly at higher accelerations. Thus the linearity of detachment curves in the stepwise centrifugation mode cannot be explained by progressive consolidation of adhesions with time.

Time-dependent adhesion of cells that neither locomote nor actively spread may be due to statistical jumping over the electrostatic repulsion barrier. There is no evidence of a liquid drainage problem since red cells in the absence of added proteins can adhere within one second of settling onto clean glass in 157 mM buffered saline.

Corry and Defendi (1981) state that when red cells in physiological saline stuck to clean glass were subjected to 3,900 *g* the proportion attached fell from 50% after 1 min to 25% after 10 min (no graphs given). Over a lower acceleration range (max = 1,500 *g*) we found that in 10 mM there was no change in detachment between 5 and 15 min (Fig. 2 *b*) so that we can rule out error from inadequate centrifugation time in our work.

CONCLUSIONS

The behavior of unfixed red cells rules out secondary minimum adhesion. This conclusion is based on the following: (*a*) Extreme irreversibility of adhesion under 1 *g* in response to changes in ionic strength in the 3–157 mM range. (*b*) Cells adherent in 157 mM then exposed to 1.5 mM and centrifuged adhere as if in 157 mM. (*c*) Adhesion under centrifugation does not follow electrostatic predictions at reduced ionic strength. (*d*) Cells that attach in 3 mM are strongly stuck, in contrast to force balance predictions. (*e*) Adherent cells cannot slide parallel to the interface.

There remains the question of how molecular contacts are made. What cell surface components are involved? Is it surprising that such contacts can be formed in the face of electrostatic repulsion at low ionic strength? Due to the high potentials near the surfaces at low ionic strength and the unknown distribution of charges within the red cell glycocalyx, which is certainly swollen at low ionic strength, we cannot make an accurate estimate of the repulsive barrier. However, it is possible to calculate the repulsion for a separation in excess of the probable extension of glycophorin molecules; the repulsion and the error in calculation will be less than at contact. The result may be expressed as a multiple (R) of the penetrating pressure due to gravity acting over a minimal effective area of, say, 0.1 μ m^2. Calculations for 30 nm separation in 3 mM gives $R \sim$ 50. It therefore appears that the areas that make molecular

contact cannot be treated as having average surface properties. Their nature remains to be determined.

APPENDIX

We neglect the polyvalent anions in the phosphate buffer solutions since the error is small where Na^+ is the only counterion. The electrostatic potential of glass is set equal to that of the cell surface, since the zeta potentials at low ionic strength are similar (Voigt et al., 1983; Schulze, 1984). At 10 mM buffer ionic strength the Debye length is ~3.8 nm and at 3 mM it is ~6.8 nm; since we will only be interested in the interaction at separations exceeding 10 Debye lengths we assume that the cell surface charges are coplanar and then use an approximate nonlinear solution of the Poisson-Boltzmann equation valid for high potentials and small interactions (Verwey and Overbeek, 1948, Eqs. 33 and 41). The repulsive force approximation was checked against the results of Ohshima (1975) and the tabulations of Devereux and De Bruyn (1963) and was found to be accurate to within a few percent. For the surface potentials of red cells at low ionic strengths we use the electrophoretic results of Donath and Gingell (1983) giving −37 mV at 10 mM and −44 mV at 3 mM. These values refer to glutaraldehyde-fixed cells, which are known to have zeta potentials ~10% higher than unfixed cells at 157 mM.

For modeling the electrodynamic interaction between a glass surface and a red cell we use the Lifshitz method in plane parallel multilayer geometry, as developed by Parsegian and Gingell (1973), but with the zero-frequency term screened out. We compute energies and obtain forces either by differentiation or more simply from the Hamaker functions, $A(1)$, where 1 is separation. These functions are constant to within 10% in the 30 to 90 nm range of separations ($1 \times 10^{-14} > A(1) > 0.9 \times 10^{-14}$). Forces were then calculated simply as $F = 1 \times 10^{-14}/6\pi\, 1^3$. This is quite accurate enough in view of the imperfections in the physical modeling.

We find that the interaction curves have secondary minima and we calculate the force (dyne/cm^2) needed to separate the surfaces from these minima as follows:

$$3\ \text{mM} : F_{\text{min}} = 0.3\ (110\ \text{nm})$$

$$10\ \text{mM} : F_{\text{min}} = 2.6\ (55\ \text{nm}).$$

The figures in parentheses give the separations at which the F_{min} values occur.

A decrease in ionic strength thus moves cells to a more distant equilibrium, with a weaker adhesion force. Assuming the existence of a long-range electrostatic/electrodynamic force balance, we can compute the change in adhesive force ΔF resulting from dilution Δc (Fig. 5) and measure the proportion of cells detached in the process, p_1%. (Note that we cannot speak of dilution applying a single value of force because the force is strongly distance dependent, unlike an applied g force, which is independent of distance). Since applying a centrifugal force adds a constant to the repulsive force, there must exist some value of the centrifugal force such that $m'a = \Delta F$ where m' is the effective cell mass. Having computed ΔF we thus find the acceleration (a) and then look up the corresponding p_2% for cells attached in Fig. 2 *a*. For a dilution from 10 mM to 3 mM, the preceding force and energy calculations show that $\Delta F = 2.3$ dyne/cm^2 and we see from Fig. 1 that $p_1 = 0$. From Fig. 2 *a*, we find that if cells in 10 mM at 1 g are exposed to a force of $m'a = 2.3$ dyne/cm^2 ($= 0.15 \times 10^{-6}$ dyne/cell contact in 10 mM) $p_2 \ll 5$%. The unique result that $p_1 \sim p_2 \sim 0$ is in general consistent with both primary and secondary minimum adhesion. However, a very strong indication that we are seeing primary (molecular) contact is given by the fact that $p_1 = 0$ (> 200 cells measured) for a change from 10 to 3 mM. We have argued that in 3 mM adhesion should be extremely weak, so the result that no cells fall in the 10 to 3 mM transition is improbable in the extreme.

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This work is dedicated to the memory of David Ubee whose enthusiasm and skill will be greatly missed.

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