Adhesivity and Rigidity of Erythrocyte Membrane in Relation to Wheat Germ Agglutinin Binding

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ABSTRACT Binding of the plant lectin wheat germ agglutinin (WGA) to erythrocyte membranes causes membrane rigidification. One of our objectives has been to directly measure the effects of WGA binding on membrane rigidity and to relate rigidification to the kinetics and levels of WGA binding. Our other objective has been to measure the strength of adhesion and mechanics of cell separation for erythrocytes bound together by WGA. The erythrocyte membrane rigidity was measured on single cells by micropipette aspiration. The slope of the suction pressure-length data for entry into the pipette provided the measure of the membrane extensional modulus. Data were collected for cells equilibrated with WGA solutions in the range of concentrations of 0.01–10 μg/ml. Erythrocyte-erythrocyte adherence properties were studied by micropipette separation of two-cell aggregates. First, a "test" cell was selected from a WGA solution by aspiration into a small micropipette, then transferred to a separate chamber that contained erythrocytes in WGA-free buffer. Here, a second cell was aspirated with another pipette and maneuvered into close proximity of the test cell surface, and adhesive contact was produced. The flaccid cell was separated from the test cell surface in steps at which the force of attachment was derived from the pipette suction pressure and cell geometry. In addition, we measured the time-dependent binding and release of fluorescently labeled WGA to single erythrocytes with a laser microfluorometry system. The results showed that the stiffening of the erythrocyte membrane and binding of fluorescently labeled WGA to the membrane surface followed the same concentration and time dependencies. The threshold concentration for membrane stiffening was at ~0.1 μg/ml where the time course to reach equilibrium was close to 1 h. The maximal stiffening (almost 30-fold over the normal membrane elastic modulus) occurred in concentrations >2 μg/ml where the time to reach equilibrium took <1 min. The WGA binding also altered the normal elastic membrane behavior into an inelastic, plastic-like response which indicated that mechanical extension of the membrane caused an increase in cross-linking within the surface plane. Similar to the stiffening effect, we observed that the membrane adhesivity of cells equilibrated with WGA solutions greatly increased with concentration >0.1 μg/ml. We found that the work of separation for unit change in area of the adhesive contact correlated well with a parameter that represented accumulation of WGA cross-bridges as the cells were separated. Values as large as 1 erg/cm^2 were measured for the work required to separate adherent cells per unit change in contact area for cells equilibrated with 0.4 μg/ml WGA.

The binding characteristics of plant lectins to cell surface sugars have been studied for over ten years (1, 2) and these properties have been useful in blood group serology (3). Recently, it has been demonstrated that lectin binding to erythrocytes alters the physical behavior of erythrocytes in suspension (4) and interferes with the ability of erythrocytes to undergo shape changes that normally occur when they are exposed to various chemical agents (2, 5). Lovrien and Anderson (2) demonstrated rigidification of the erythrocyte by wheat germ agglutinin (WGA)[1] in opposition to the action of an echinocytic (crenating) agent, SDS detergent. In addition,

[1] *Abbreviations used in this paper:* F-WGA, fluorescein isothiocyanate-labeled WGA; WGA, wheat germ agglutinin.

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these authors correlated the rigidification (which they called "protection") with binding measurements and deduced that the membrane stiffening occurred at a threshold binding level of about one WGA molecule per erythrocyte membrane glycophorin monomer. Total rigidification in opposition to SDS crenation required an order of magnitude higher level of binding. Even though physical-chemical models have been developed (6, 7) that can be used to evaluate the chemical work done on the membrane by the addition of echinocytic agents, the analysis is extremely difficult and requires that the membrane elastic properties not be altered by the agent. Thus, no quantitative measure of membrane rigidification can be derived from the competitive binding and shape observations of Lovrien and Anderson.

The only direct mechanical tests on erythrocyte membranes in WGA solutions were performed by Smith and Hochmuth (8) at concentrations $<10^{-8}$ M (below the threshold level at which Lovrien and Anderson observed "protection" against crenation); these experiments showed that the erythrocyte membrane elasticity was essentially unchanged at low WGA concentrations whereas the surface viscosity was appreciably altered. However, the surface viscosity only represents resistance to the rate of cell deformation or shape change but does not characterize static resistance to alteration of cell shape (i.e., rigidity). It is clear from the experiments of Lovrien and Anderson that the static rigidity of the erythrocyte membrane has been altered by the binding of WGA. Thus, one of our objectives has been to directly measure the effects of WGA binding on the membrane rigidity and to relate the results to the kinetics and levels of WGA binding to the cell membrane. In addition, we set out to evaluate the nature of alterations in membrane material behavior, that is, deviation from the normal elastic membrane response.

For deformations of normal erythrocytes that do not require the cells to become spherical, static shape changes are only resisted by the extensional and bending rigidities of the membrane (6). Furthermore, since the membrane is such a thin structure, the resistance to in-plane extension is the dominant stiffness for large deformations of the erythrocyte (such as those associated with discocyte-echinocyte transformations and whole cell extension in the microcirculation). This is in contrast to the behavior of a lipid bilayer membrane which has no resistance to in-plane extension since the bilayer is a "surface liquid." For normal erythrocytes, it has been shown that the extensional modulus, μ, is an elastic property of the membrane. In other words, extensions of the cell membrane follow the same force versus extension curve in the "loading phase" as in the "unloading phase" provided that the extensions take place slowly. Consequently, normal cells return to their original shape within a fraction of a second after the forces of deformation have been removed. As we will show for the erythrocyte with bound WGA, the membrane extensional modulus is significantly altered by the bound WGA and the elastic recoverability of the membrane is greatly impaired.

As the name implies, WGA strongly agglutinates erythrocytes. However, there have been no measurements of the strength of adhesion between WGA-bound erythrocytes as a function of the WGA binding. Agglutination assays can be misleading because the membrane stiffening opposes the forces of adhesion between cells: i.e., the erythrocytes stiffened by WGA are less capable of deforming to form adhesive contact even when the forces of adhesion are strong. Thus, as part of this study, we have measured the adhesivity between WGA "coated" erythrocytes by directly measuring the force required to separate adherent cells. Also, we have investigated the details of the contact separation in order to evaluate the mechanical properties of the adhesive contact. As with the mechanical stiffening of the erythrocyte membrane by WGA, we will show that the adhesivity between erythrocyte membranes mediated by the bound WGA is strongly correlated with the kinetics and levels of WGA binding.

Because of the avidity and slow kinetics of binding of WGA to the erythrocyte membrane at extremely low concentrations (on the order of 10^{-8} M), the number of cells in the suspension and the history of exposure to the ligand must be carefully controlled. Hence, we developed a method to measure the time-dependent binding of fluorescently labeled WGA to a single erythrocyte. By using a single cell, we were able to avoid the competitive binding effects between cells and also to observe the time-dependent levels of binding to the cell, followed by the time course of release of WGA from the same cell when transferred to a WGA-free medium.

MATERIALS AND METHODS

Erythrocytes were obtained by finger prick from the same donor (blood type A^+) for all studies. The cells were suspended in PBS; pH was adjusted to 7.4 and osmolarity to 270–290 mOsmol. WGA was obtained from two sources, Miles Laboratories Inc. (Elkhart, IN) and Sigma Chemical Co. (St. Louis, MO). No obvious differences in the two sources of this material could be detected. The WGA concentrations ranged from .01 to 10 μg/ml. To minimize adhesion of cells to the small glass micropipettes used in the mechanical and adhesion experiments, the solutions also contained human serum albumin at a concentration of 0.5 g/100 ml. It was found in the fluorescence studies that the presence of albumin did not effect the binding of WGA to the erythrocyte surface; however, the presence of albumin essentially eliminated the adsorption or binding of WGA to the glass surfaces. As required, erythrocytes were equilibrated with the WGA solutions by one of two methods: (*1*) Single erythrocytes were selected from a suspension in PBS-albumin in one chamber on the microscope stage; the cell was transferred to a separate chamber that contained the WGA in solution but no erythrocytes. The single cell was then left for various periods of time up to 60 min to equilibrate with the solution. (*2*) Cells were suspended directly in the WGA solution at extremely low hematocrits, $<10^4$ cells/ml; this suspension was agitated for a specific period of time and then placed in the chamber on the microscope stage.

Measurements of the membrane extensional rigidity and cell-cell adhesion were performed with a multi-micromanipulator microscope system. The system is centered around a Leitz inverted microscope with up to four small micromanipulators mounted directly on the microscope stage. For each test, the cell suspension was injected into one microchamber on the microscope stage and the WGA solution was placed in the adjacent but separate microchamber. Single cells were aspirated and/or maneuvered by small glass suction pipettes attached to the micromanipulators. The pipettes are produced from 1-mm glass tubes pulled to a needlepoint and then broken by quick fracture to obtain flat tips in the desired range from 0.5 to 50×10^{-4} cm. For the small pipettes ($1–2 \times 10^{-4}$ cm), the pipette inner diameter was measured from the insertion depth of a tapered microneedle which had been calibrated by scanning by electron microscopy. The pipettes were coupled by continuous water systems to micrometer positioned water monometers for zero pressure adjustment. Negative pressures were measured through the continuous water system connected to digital pressure transducers with a resolution of micro-atmospheres. All experiments were carried out at room temperature.

The microfluorometry system utilized a similar microscope-micromanipulator arrangement. The system is illuminated by an argon-ion laser source. The laser beam enters the microscope to epi-illuminate (through the objective) the cell surface. Fluorescence from labeled molecules on the cell surface was sampled by an optical fiber connected to a photomultiplier tube. The fluorescence intensity was quantitated with the use of a photon discriminator-counter system and fed directly into the memory of a microprocessor.

Measurement of Membrane Extensional Rigidity: The erythrocyte membrane rigidity was measured on single cells that had been equilibrated by the procedures described above. The experimental approach involved aspiration of a single cell along its axis of symmetry, and the length of the cell aspiration in the micropipette was measured as a function of aspiration pressure

both entering and exiting the pipette. Each pressure-length measurement was made at static conditions where the cell projection was not moving in the pipette. A video micrograph of a single cell aspiration test is shown in Fig. 1. Since the pressure loading and unloading curves were not the same for cells equilibrated with WGA solutions, it was not possible to define a single membrane modulus. Thus, by definition, we have derived the membrane modulus of rigidity from the loading phase of the aspiration experiment. Analysis of this experiment has shown that the extensional modulus, μ, is directly proportional to the derivative of the suction pressure, ΔP, with respect to aspiration length, L (6, 7),

$$\mu \sim R_p^2 \cdot \frac{dP}{dL}$$

where R_p is the pipette inner radius. For normal erythrocytes this modulus, which is an elastic property, is on the order of 7×10^{-3} dyn/cm (9); it is zero for a lipid bilayer membrane. To measure the extensional modulus, we used micropipettes with dimensions of $\sim 1.5 \times 10^{-4}$ cm and applied aspiration pressure in increments of 100 dyn/cm^2.

Erythrocyte-Erythrocyte Affinity and Adherence Properties: The procedure for formation of two cell aggregates was to select two cells with small micropipettes and to maneuver these cells into position for contact. Since WGA binding to cells stiffens the membrane and interferes with cell-cell adhesion, we used the following test procedure to circumvent this problem: First a cell (referred to as the "test" cell) was selected from a WGA solution and aspirated into the small micropipette with sufficient suction pressure so that the residual portion of the cell outside the micropipette formed a tight spherical segment (~3–4 μm diam). This cell was then transferred to a separate chamber that contained erythrocytes in WGA-free medium. Here, a second cell was aspirated with another pipette at low suction pressure in order to leave a flaccid disk-like portion exterior to the pipette; the flaccid cell was then maneuvered close to the test cell surface to allow adhesive contact without forcing the cells together. This configuration is shown in Fig. 2, top panel. As we have established previously, deformation of the flaccid cell as it conforms to the other cell surface is a direct measure of the chemical affinity between the membrane surfaces (7, 10). However, it was observed that the chemical attraction (affinity) between the uncoated, flaccid erythrocyte and the WGA-coated test surface was minimal even though we established that the level of WGA bound to the test cell surface remained essentially constant after the cell was transferred into the WGA-free medium (see next section on the fluorescence studies). Next, the flaccid cell was forced to make contact with the spherical test surface which resulted in cell-cell adhesion. Finally, to measure the strength of attachment between the adherent cell surfaces, the flaccid cell was then separated from the test surface in steps at which the force of attachment was derived from the pipette suction pressure and cell geometry (Fig. 2, middle and bottom panels). The mechanical relation for the axial force is given by

$$F_p \cong \pi \cdot R_p^2 \cdot \Delta P \cdot \sin\theta_p$$

where ΔP is the pipette suction pressure and θ_p is the angle formed between

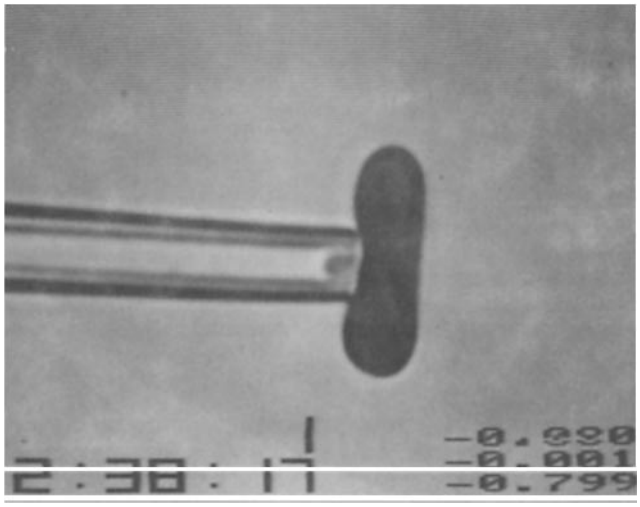

FIGURE 1 Videomicrograph of a single erythrocyte aspiration experiment. The cell shown here was aspirated with a pressure of ~400 dyn/cm^2; the pipette inner diameter is ~10^{-4} cm.

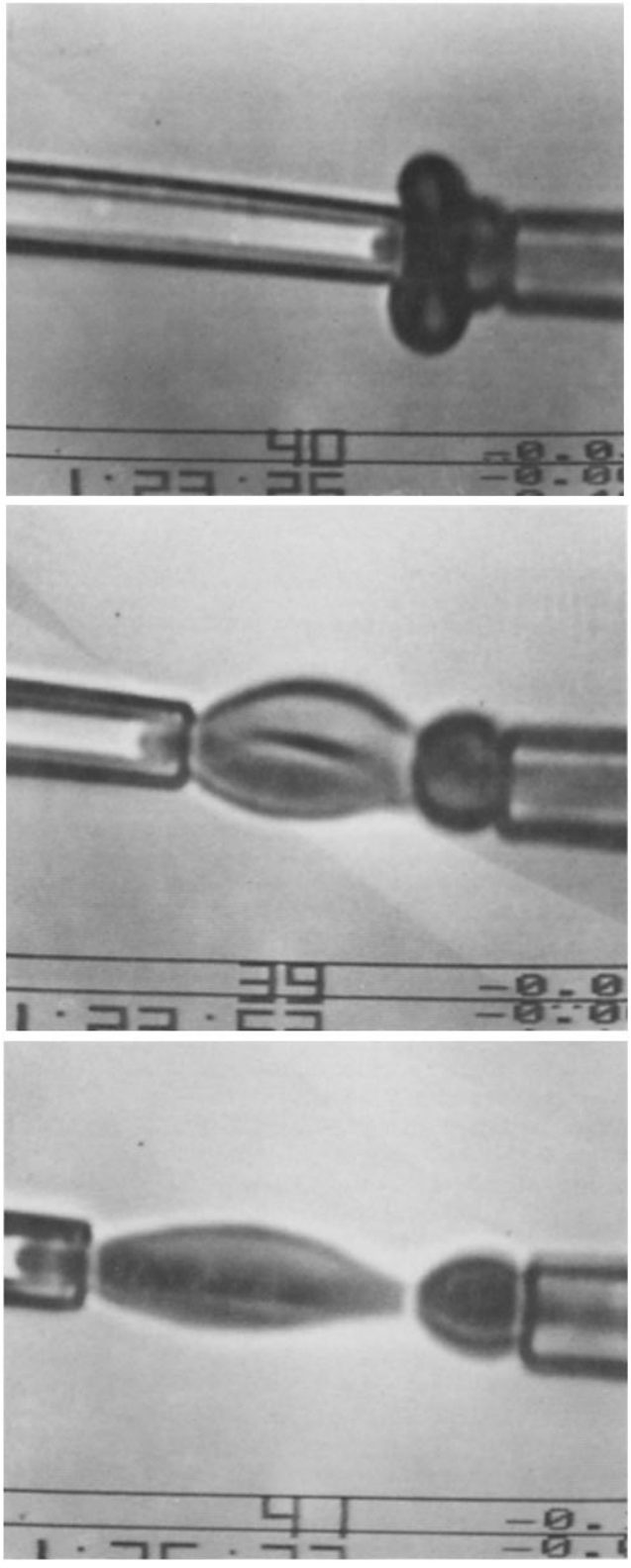

FIGURE 2 Videomicrographs of the stepwise separation of erythrocytes that have adhered via WGA cross-bridges. One cell was first coated with WGA, aspirated into a rigid spherical surface, and then transferred to the chamber that contained only erythrocytes in WGA-free buffer. The second cell (uncoated) was then maneuvered into position and adhesive contact was made as shown in the top panel. The flaccid (uncoated) cell was then separated in steps at which the force of attachment was measured from the pipette suction pressure and cell geometry as shown in the middle & bottom panels.

the flaccid cell membrane and the entrance to the micropipette (as illustrated in Fig. 3). The basis of the mechanical relation is discussed in the Appendix.

As described in the Appendix, the work of membrane separation per unit change in area of contact is proportional to the tension, T_m, which acts in the flaccid cell membrane proximal to the rim of the cell contact zone. This tension is given approximately by

$$T_m \cong F_p/(\pi \cdot D \cdot \sin\theta_m)$$

where θ_m is the angle formed between the outward surface normal to the membrane and the axis of symmetry and D is the diameter of the contact zone. The work of separation per unit change in contact area, w_a, is approximated by the relation,

$$w_a = T_m(1 - \cos\theta_a)$$

where θ_a is the contact angle between the cell membrane and the test surface. Since θ_a was close to 90° in the experiments, the work of separation per unit change in contact area was essentially equal to the membrane tension.

Fluorescence Binding Measurements: The binding of fluorescein isothiocyanate-labeled WGA (F-WGA) to the erythrocyte surface was measured at concentrations over the range of 0.01–10 μg/ml. The F-WGA was obtained from Miles Laboratories Inc. The procedure was to transfer a single erythrocyte from one chamber on the microscope stage that did not contain WGA to a second chamber that contained the fluorescently labeled material without other cells to compete in the binding process. This was facilitated by aspiration of a cell with a small micropipette and insertion into the bore of a larger transfer pipette. Next, the microscope stage was translated and the cell was withdrawn from the shelter of the large pipette. Binding was then determined from the differential fluorescence between the cell and background (obtained simply by moving the cell out of the illuminated region and without changing the microscope focus). Next the cell was transferred back to the chamber that did not contain the lectin and the subsequent release of labeled lectin was followed as a function of time. After an initial slight decrease in bound WGA, the level remained constant for >1 h. Thus, the WGA-coated cell could be used as a test surface in the adhesion measurements with the confidence that the WGA remained bound to the cell membrane surface. We also carried out tests of the effect of F-WGA on the erythrocyte membrane rigidity. We found that both the fluorescently labeled and unlabeled WGA produced the same membrane stiffening, which demonstrated that the label did not interfere with the process.

RESULTS

As noted in the previous paragraph, we measured the kinetics of binding and release of F-WGA to single erythrocytes without competition for the lectin from other cells. The results showed that for concentrations >1 μg/ml, the level of binding reached equilibrium in <1 min. However, for low concentrations, ≤0.1 μg/ml, the time-course to equilibrium took ≥1 h as shown in Fig. 4. At all concentrations, release (wash-off) of the lectin from the cell surface in a WGA-free medium was the same: an initial, short-term wash-off of 10–15% of the material, followed by no apparent change for up to 2 h. When the competitive sugar (*n*-acetyl glucosamine at 10 mM) was added to the second chamber, release of the bound lectin was "instantaneous," i.e., too fast to measure. We measured the extensional rigidity of erythrocytes equilibrated with WGA solutions from the pressure "loading" phase of the pipette aspiration experiment. Fig. 5 shows the pressure loading and unloading characteristics of two single cell measurements: one

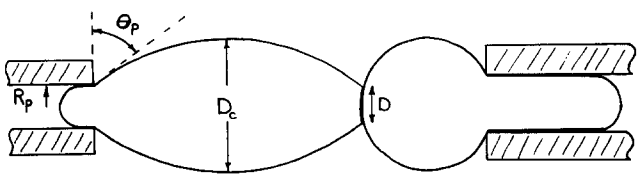

FIGURE 3 Schematic illustration of the geometry for the cell-cell separation experiment.

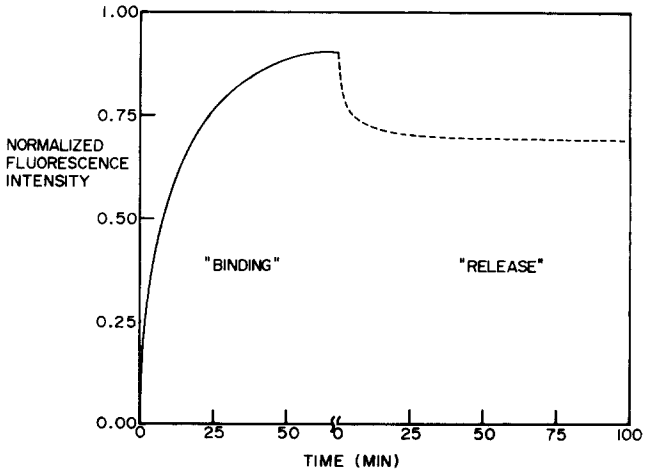

FIGURE 4 The kinetics of binding and release of fluorescently labeled WGA to-and-from a single erythrocyte surface at a concentration of 0.1 μg/ml. Note from the release sequence that the lectin appears to be bound essentially permanently after an initial short term "wash-off".

measurement is for a cell in 0.1 μg/ml WGA solution and the other is for a different cell in a 1.0 μg/ml WGA solution. For cells in WGA solution <0.1 μg/ml concentration, the pressure loading and unloading characteristics, as shown in Fig. 5, essentially follow the same curve with normal extensional rigidity; this lack of hysteresis is characteristic of an elastic material. On the other hand, cells in WGA solutions of greater concentration exhibited hysteresis or plastic-like behavior shown in Fig. 5.

We also evaluated the effect of exposure to the competitive sugar after equilibration with WGA solutions. The same effect was observed for the WGA-stiffened erythrocyte as in the fluorescence study: that is, the erythrocytes would remain stiff indefinitely when transferred to the WGA-free medium, but became soft immediately if the competitive sugar was present. Likewise, the time course for stiffening of the cell membrane was similar to that for binding of the lectin to the surface. Fig. 6 shows the ratio of membrane rigidities measured for WGA-bound cells to the normal value as a function of equilibration time at two concentrations, 0.4 and 0.1 μg/ml. For concentrations >1 μg/ml, equilibration time was too rapid to measure. No increase in extensional rigidity above normal levels was observed for concentrations <0.1 μg/ml. Previous reports (8) have stated that no stiffening occurred for concentrations <0.2 μg/ml; however, as is shown in Fig. 6, the equilibration time must be very long at low concentrations in order for the effect to become apparent. Fig. 7 shows the membrane rigidity ratio measured for cells equilibrated in WGA solutions for 30 min in comparison with the level of binding of F-WGA to the cell membrane after the same exposure time. It is impressive that the two curves exhibit essentially the same concentration dependence. A significant observation was that WGA binding to the cell membrane not only stiffened the surface but caused the membrane to become progressively inelastic, i.e., deformation of the cell by pipette aspiration did not follow the same course for pressure loading and unloading; also, persistent projections remained after release from the pipette. Coupled with the feature that the extensional modulus was enormously increased as shown in Fig. 7, the plastic-like behavior indicates that mechanical extension of the cell membrane increases the extent of WGA cross-linking within the

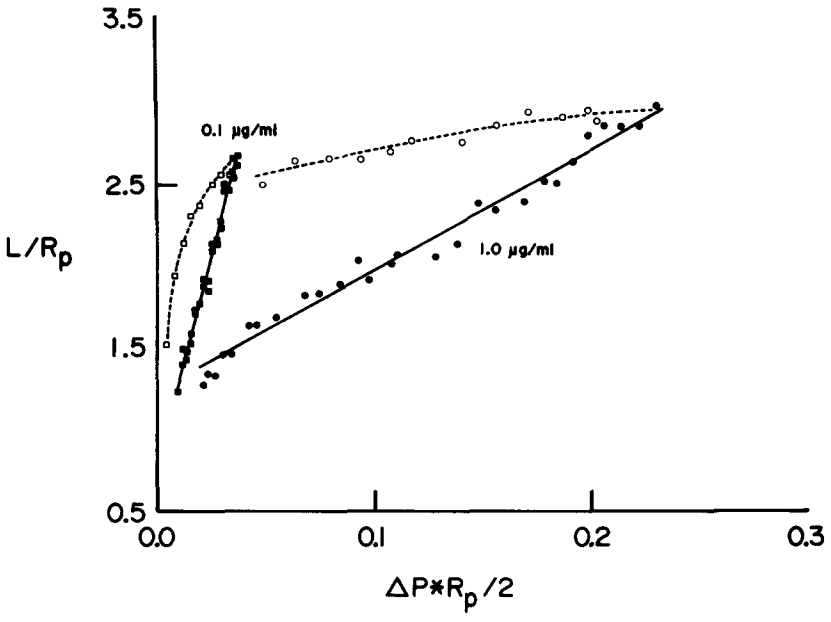

FIGURE 5 Suction pressure versus aspiration length data from two single cell aspiration experiments. The solid and open circles represent the data for a single erythrocyte equilibrated 30 min in 1 μg/ml WGA solution; the solid circles are the pressure loading (aspiration) phase and the open circles are the unloading (pressure release) phase. The solid and open squares represent data for a single erythrocyte equilibrated 30 min in a 0.1 μg/ml of solution; again, the closed squares are the aspiration phase and the open squares are the pressure reduction phase. The area encompassed by the loading and unloading characteristics represents hysteresis or inelastic membrane behavior.

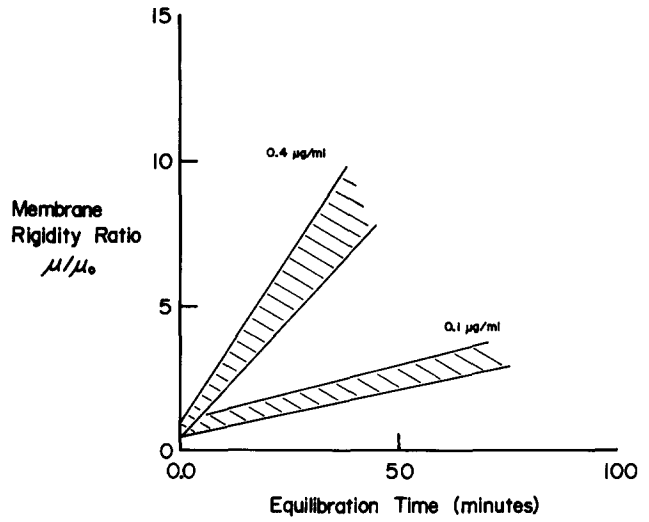

FIGURE 6 Plot of the membrane extensional rigidities measured for WGA-bound cells as a function of equilibration time at two concentrations: 0.4 and 0.1 μg/ml. For concentrations >1 μg/ml, equilibration time was too rapid to measure. The data have been normalized by the value for membrane extensional rigidity in WGA-free buffer, 7×10^{-3} dyn/cm. The cross-hatched area represents the limits on the measurements from more than 50 cells for each WGA concentration.

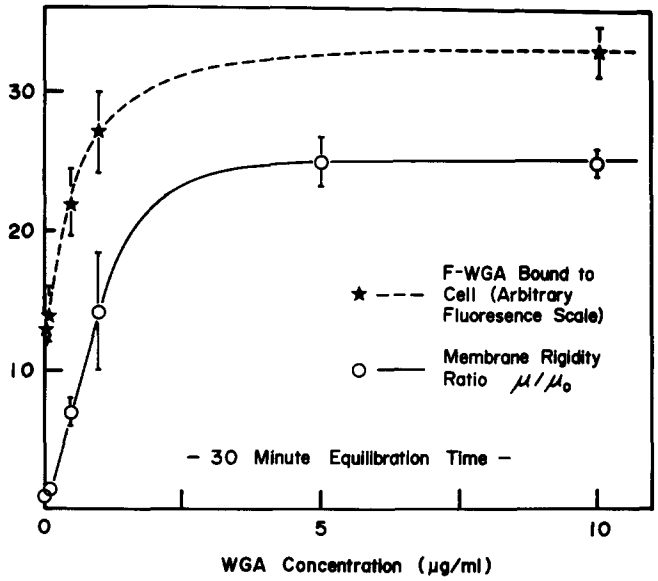

FIGURE 7 Plots of the membrane extensional rigidity ratio measured for cells equilibrated in WGA solutions for 30 min and the levels of binding of fluorescent WGA to the cell membrane after the same exposure time versus concentration of WGA in solution. The error bars represent the range of measurements on about 10 cells at each condition.

plane of the membrane surface. This cross-linking could not have been due to increased binding of WGA from the solution because we obtained the same results for cells that had first been equilibrated with a WGA solution, then transferred to a WGA-free medium where the mechanical test was performed.

As described in Materials and Methods, we measured the force required to separate two erythrocytes that were bound together by WGA. Fig. 8 shows examples of the force required to separate two adherent cells, one curve for a test cell equilibrated for 60 min in 0.4 μg/ml WGA and the other curve for a test cell equilibrated the same length of time in 0.1 μg/ml WGA. The contact dimension could only be measured down to 0.5×10^{-4} cm because of optical diffraction limitations. It was observed that the contact dimensions became stationary at each level of separation force, i.e., there was no continuous rate of separation. In the procedure, we used a WGA-coated cell as a test surface and an uncoated, flaccid cell as an "elastic coupler" to the suction pipette to measure the force of separation. The use of the flaccid cell also permitted us to evaluate the level of chemical affinity (attraction) between the surfaces. Since the flaccid cell did not deform and spread on the spherical test surface, there was not sufficient long range attraction to promote adhesion. The attraction has to be greater than the elastic rigidity of the cell. (For erythrocytes, we have shown that the threshold free energy per unit area required to deform the cell to make adhesive contact is $\sim 5 \times 10^{-4}$ erg/cm^2 [7, 10].) By forcing the cells together, strong subsequent bonds were formed by the WGA

molecules between opposite cell surfaces. From the separation force versus contact dimension (illustrated in Fig. 8), we derived the membrane tension at the edge of the contact zone as a function of contact size. Fig. 9 shows the tension calculated from the two separation measurements plotted in Fig. 8. The separation force and tension were strong functions of the levels of WGA bound to the surface; also, no adhesion between cells could be produced for test cells that had been equilibrated with concentrations <0.075 μg/ml. What was striking was that the tension increased in inverse proportion to the contact dimension. This was not expected since for simple adhesives, one observes that the tension must first be increased to a threshold or yield level which is then followed by continuous separation at constant tension (i.e., a simple plastic-like behavior). As pointed out, the contact dimension versus force measurements were stationary observations with no discernable flow-like behavior.

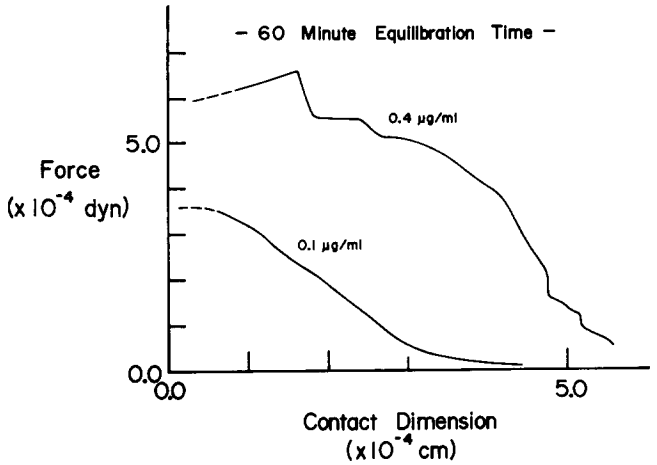

FIGURE 8 Examples of the force required to separate erythrocytes that had adhered via WGA cross-bridges versus contact dimension for two different WGA concentrations; in each case, the test cell was equilibrated in the WGA solution for 60 min prior to the adhesion test.

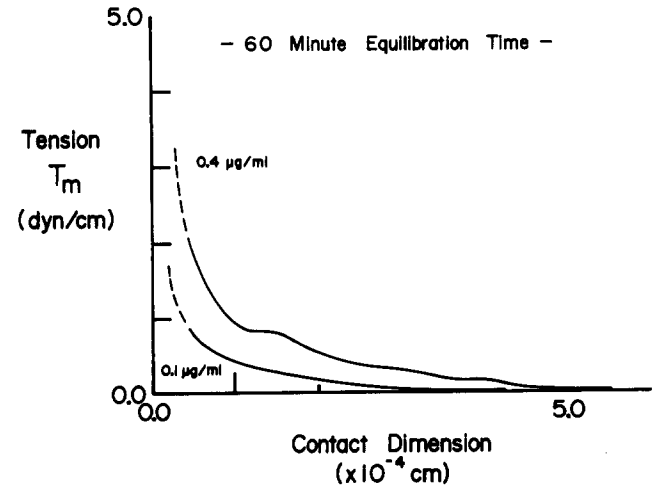

FIGURE 9 Membrane tension values calculated from the two separation force measurements plotted in Fig. 8 versus the contact zone dimension. The membrane tension, which acts in the flaccid cell membrane proximal to the rim of the contact zone, is essentially equal to the work of membrane separation per unit change in contact area.

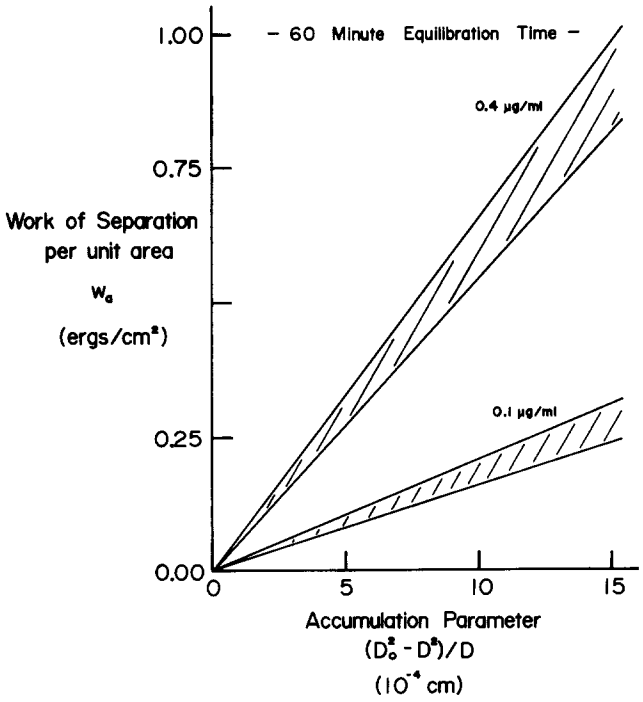

FIGURE 10 Data for the work of separation per unit change in contact area derived from the separation force measurements versus the geometric parameter which represents accumulation of adhesive cross-bridges at the periphery of the contact zone. The data ranges encompass all measurements for two concentrations of WGA and an equilibration time for the test cell of 1 h prior to the adhesion tests. The data range represents detailed separation measurements on about 5–10 cell pairs for each WGA concentration.

Another important observation was that the force build-up (and thus the tension) depended strongly on the initial area of the contact zone. This observation indicated that perhaps there was accumulation of WGA-bound sites at the edge of the contact zone when the cells were separated. Hence, we derived a parameter to represent the cumulation or build-up of WGA-receptor cross-bridges at the edge or seam of the contact zone. This parameter is based on the assumptions that the surface density, ρ, of WGA-receptor cross-bridges is essentially uniform over the initial contact zone and that the accumulation occurs at the circle that forms the edge of the contact zone; as such, the cumulation per unit length around the edge of the contact zone is given by

$$l = \frac{\rho}{4D}(D_o^2 - D^2)$$

where D_o and D are the initial and final sizes of the contact zone respectively. Thus, the appropriate geometric parameter is

$$(D_o^2 - D^2)/D.$$

When we used this parameter to represent the dimension of the contact zone, we found that all of the data could be reduced to an essentially linear form where the membrane tension or work of separation per unit change in the contact area was proportional to the cumulation parameter as shown in Fig. 10. The work of separation of the adhesive contact (i.e., the tension) ranged over values from 10^{-2} to 1 erg/cm^2. This illustrates that the work of separation of agglutinated cells can be orders of magnitude greater than the chemical

affinity between the cell membranes. Accumulation of WGA-receptor cross-bridges is not the only possible explanation for this correlation. Another could be that separation of the cells causes cross-bridges to be formed between WGA-bound sites that were not previously connected. In any case, the correlation is very good. We have observed (but not quantitated) fluorescence build-up at the edge of the contact zone as cells were separated which had been previously bound together with F-WGA. Also, we have observed that when cells have been partially separated they will not readhere over the separated region when forced together a second time. Thus, WGA has been removed from the separated region.

CONCLUSIONS

Both the stiffening of erythrocyte membrane and binding of fluorescently labeled WGA to the membrane surface exhibited similar concentration and time dependencies. The threshold concentration for membrane stiffening was at ~0.1 μg/ml where the time course to reach equilibrium was close to 1 h. The maximal stiffening almost 30-fold over normal values occurred for concentrations in >2 μg/ml where the time to reach equilibrium took <1 min. WGA stiffening of the membrane also altered the normal elastic membrane behavior into an inelastic plastic-like response which indicated that mechanical extension of the membrane caused an increase in cross-linking within the surface plane. Binding and stiffening effects persist indefinitely after the cells have been transferred to WGA-free medium; however, addition of the competitive sugar immediately removes the bound lectin and the cells recover their normal elastic behavior.

Similar to the stiffening effect, we observe that the membrane adhesivity of cells first equilibrated with WGA solutions greatly increased with concentration >0.1 μg/ml (with no detectable adhesivity for concentrations <0.075 μg/ml). Even though there is a significant level of WGA bound to the cell at 0.075 μg/ml (~5×10^5 per cell based on Lovrien and Anderson's work [2]), it appears that this WGA is not available to form cross-bridges, perhaps because it is bound near the plasma membrane surface and shielded by the more superficial carbohydrate groups. We found that the work of separation per unit change in area of the adhesive contact correlated well with a parameter that represented accumulation of WGA-receptor cross-bridges as the cells were separated. This correlation accounted for the strong dependence of the membrane tension on the initial area of the contact zone and the inverse dependence of the tension on the size of the contact.

Both the mechanical stiffening and adhesion data imply that it is the more superficially bound WGA (near the terminal ends of the carbohydrate "trees") that is involved in the membrane stiffening and adhesive processes through cross-linking reactions. However, other researchers (5) have concluded that the binding of WGA to glycophorin induces stiffening of the subsurface spectrin matrix (referred to as the "cytoskeleton"). The question of "inside" versus "outside" of the membrane cannot be resolved as yet. It is certain that membrane-membrane adhesion involves cross-linking of glycophorin molecules between surfaces. Our data shows that both the threshold levels for adhesion and stiffening plus the time dependencies for these phenomena are the same, which is circumstantial evidence in support of the same mechanism on the "outside" of the cell. Arguments against this are that there is insufficient WGA to form a uniform surface coat and the average distance between glycophorin monomers is larger than the WGA molecule. On the hand, WGA could form local external surface "bridges" between glycophorin molecules and/or other sialic acid residues in the surface plane. As such, these surface bridges would effectively act as cross-links from one local domain of the membrane to another (through association with the cytoskeleton).

APPENDIX

In the analysis of the mechanics of separation of adherent cells, the goal is to obtain physical properties that quantitate membrane-membrane adhesion, independent of the shape of the adherent cells. The appropriate parameter is the membrane tension proximal to the contact zone. This tension is a direct measure of the mechanical work required to separate a unit area of the contact. Thus, the tension is an intensive variable characteristic of the adhesion process, not of the cell geometry. The relationship between membrane tension at the edge of the contact zone and the work of separation per unit area is derived from a simple statement that the mechanical work required to displace the membrane force just balances the work of separation of the membrane from the substrate. The result is

$$w_a = T_m \cdot (1 - \cos\theta_a) \tag{A1}$$

where w_a is the work done in separation of a unit area of contact (erg/cm^2), T_m is the tension in the membrane at the rim of the contact zone, and θ_a is the angle between the membrane and the substrate at the contact. For the experiments reported here, the task is to derive a relation for the membrane tension at the edge of the adhesive contact in terms of the pipette suction pressure and the shape of the cell as it is separated from the rigid test cell surface. This will necessitate some approximations because of the cell conformation. First of all, the force at the pipette entrance, F_p, equals the force applied to the cell-cell contact, F_a. The force at the pipette entrance is given by

$$F_p = \pi \cdot R_p^2 \cdot (P_p \cdot \sin\theta_p - P_o) \tag{A2}$$

where P_p is the pressure across the cell membrane inside the pipette, P_o is the pressure difference across the cell membrane exterior to the pipette, and θ_p is the angle between the cell membrane and the entrance to the pipette (i.e., the angle between a normal to the membrane surface at the pipette entrance and the axis of symmetry). In terms of the pipette suction pressure, $\Delta P = P_p - P_o$, the force at the pipette entrance is

$$F_p = \pi \cdot R_p^2 \cdot \Delta P \cdot \sin\theta_p + \pi \cdot R_p^2 \cdot P_o \cdot (\sin\theta_p - 1). \tag{A3}$$

Similarly, the force applied to the adhesive contact is given by

$$F_a = \pi \cdot D \cdot T_m \cdot \sin\theta_m - P_o \cdot \pi \cdot D^2/4 \tag{A4}$$

where T_m is the membrane tension at the edge of the contact zone, D is the cross-sectional dimension of the contact zone, and θ_m is the angle formed between the outward normal to the membrane and the axis of symmetry. The equality of Eq. A3 and A4 specifies a relation for the membrane tension at the rim of the contact zone in terms of the pipette suction pressure and the cell internal pressure. A problem arises here in that it is difficult to ascertain the pressure, P_o, inside the

cell. The pressure inside an undeformed erythrocyte discocyte is essentially zero (6), but the cell becomes pressurized as membrane tension increases when the cell is extended. The level of internal pressure depends on the circumferential tension in the extended cell surface. The circumferential tension depends on the nature of the membrane material and the deformation. The maximum level of internal pressure would exist for a smooth, unfolded membrane (such as would be the case for a lipid bilayer vesicle) and the minimum level (essentially zero) would exist for a folded or creased membrane where the axial fold relieves the circumferential tension. Thus, the limits on the internal pressure are given by

$$0 \leq P_o \leq 2\Delta P \cdot R_p/(D_c - 2R_p) \qquad \text{(A5)}$$

where D_c is the cross-sectional dimension of the extended cell body. As we have observed in the cell separation experiments (e.g., Fig. 2), the cells exhibited axial creases or folds; hence, we have chosen the lower limit to approximate the cell internal pressure, i.e., $P_o = 0$. Deviations from this approximation would amount to <30% variation in the calculation of the membrane tension. Thus, with this approximation, we can specify the membrane tension at the contact zone in terms of the pipette suction pressure,

$$T_m \cong \pi \cdot R_p^2 \cdot \Delta P \cdot \sin\theta_p/(\pi \cdot D \cdot \sin\theta_m), \qquad \text{(A6)}$$

from which we have a measure for the work of separation per unit change in contact area.

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