Detachment of agglutinin-bonded red blood cells

II. Mechanical energies to separate large contact areas

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ABSTRACT As detailed in a companion paper (Berk, D., and E. Evans. 1991. *Biophys. J.* 59:861–872), a method was developed to quantitate the strength of adhesion between agglutinin-bonded membranes without ambiguity due to mechanical compliance of the cell body. The experimental method and analysis were formulated around controlled assembly and detachment of a pair of macroscopically smooth red blood cell surfaces. The approach provides precise measurement of the membrane tension applied at the perimeter of an adhesive contact and the contact angle θ_c between membrane surfaces which defines the mechanical leverage factor $(1 - \cos \theta_c)$ important in the definition of the work to separate a unit area of contact. Here, the method was applied to adhesion and detachment of red cells bound together by different monoclonal antibodies to red cell membrane glycophorin and the snail-helix pomatia-lectin. For these tests, one of the two red cells was chemically prefixed in the form of a smooth sphere then equilibrated with the agglutinin before the adhesion-detachment procedure. The other cell was not exposed to the agglutinin until it was forced into contact with the rigid cell surface by mechanical impingement. Large regions of agglutinin bonding were produced by impingement but no spontaneous spreading was observed beyond the forced contact. Measurements of suction force to detach the deformable cell yielded consistent behavior for all of the agglutinins: i.e., the strength of adhesion increased progressively with reduction in contact diameter throughout detachment. This tension-contact diameter behavior was not altered over a ten-fold range of separation rates. In special cases, contacts separated smoothly after critical tensions were reached; these were the highest values attained for tension. Based on measurements reported in another paper (Evans et al. 1991. *Biophys. J.* 59:838–848) of the forces required to rupture molecular-point attachments, the density of cross-bridges was estimated with the assumption that the tension was proportional to the discrete rupture force x the number of attachments per unit length. These estimates showed that only a small fraction of agglutinin formed cross-bridges at initial assembly and increased progressively with separation. When critical tension levels were reached, it appeared that nearly all local agglutinin was involved as cross-bridges. Because one cell surface was chemically fixed, receptor accumulation was unlikely; thus, microscopic "roughness" and steric repulsion probably modulated formation of cross-bridges on initial contact. To counter the steric repulsion, adhesive contacts were exposed to solutions of a high molecular weight polymer to draw the surfaces together by osmotic dehydration of the adhesion gap. These stresses exceeded initial mechanical assembly stresses by up to three orders of magnitude. As expected, the strength of adhesion was greatly enhanced by the added impingement stress.

INTRODUCTION

Recognition and adhesion between cell surfaces are important aspects of biological function. In general, these processes are controlled by specialized macromolecules to produce adhesion between cell surfaces and substrates. Conceptually, molecules are thought to form physical "cross-bridges" between the two cell surfaces. Such molecules can be native membrane-associated proteins that bind directly to the other surface (1) or multivalent proteins in solution that bind to sites on both surfaces (2). Although there is considerable interest in identification and characterization of cell adhesion molecules, most investigations of cell adhesion have been more descriptive than quantitative. Microscopic physical properties of cell–cell adhesion remain to be determined mainly because experimental techniques have not been adequately designed to directly measure these properties. Adhesion strength is usually evaluated on a relative scale, based on the degree of cell aggregation or on the level of force required to disaggregate a multicellular assembly (3). Such measurements are only qualitative because aggregation and disaggregation, although driven by adhesion, involve other factors, e.g., cell deformability, surface topography, the conditions of cell assembly, and separation.

Presumably, adhesion strength can be related to properties of discrete cross-bridges. A number of theoretical models have represented cross-bridges as elastic "springs" with a characteristic energy, force, or displacement at which failure occurs (4–6). In other treatments (7–10), cross-bridge formation and breakage were treated as chemical-thermodynamic reactions that depended on displacement through a postulated potential of mean

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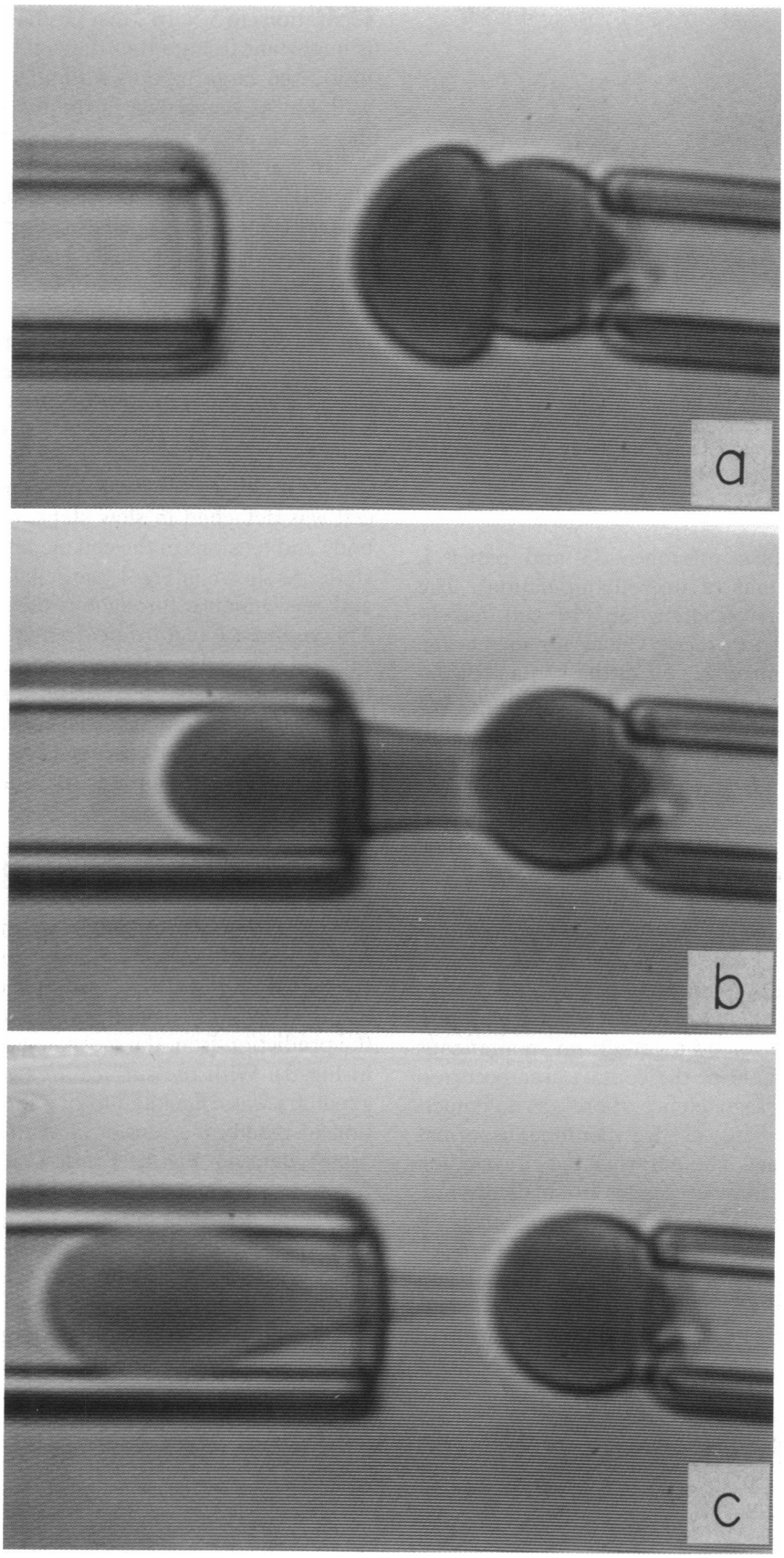
force. A more phenomenological approach has been to introduce a "fracture energy density" to describe cell adhesion (11), a concept developed in the study of fracture of materials (12). Fracture energy density is the work (mechanical energy) per unit area required to separate surfaces. From this observable quantity, the fracture energy per cross-bridge can be estimated if the surface density of cross-bridges (molecules per area) can be determined. Precise measurement of the mechanical work required to separate adhesive contacts yields an unequivocal physical property; however, distribution of the work as a true energy per cross-bridge involves an implicit assumption that the cross-bridges are sufficiently dense to be treated as a continuum (5). In the limit of sparse-point attachments between surfaces, the fracture energy approaches the force required to rupture a single cross-bridge x the number of attachments per unit length along the contact perimeter (6). Identification of the microscopic determinants of cross-bridge strength as energy- or force-based also depends on kinetic features of bond formation/release and surface mobility as well as surface density. Thus, in some cases, "fracture energy" is related to the energies required to dissociate cross-bridges whereas, in others, it depends on forces to rupture molecular attachments. In *all* cases, separation of contacts may or may not involve the bond between receptor and agglutinin molecule. The separation may cause other structural failures such as extraction of receptors from the membrane (evidence clearly supports this type of failure in our cellular detachment studies; 13).

In the idealized-classical view, fracture energy density w_f is treated as a reversible-thermodynamic quantity which is assumed equal to the "chemical affinity" w_a between surfaces. The affinity induces stresses in cells as a contact spreads; these stresses can often be determined from the shapes of cell aggregates (11, 14–16). On the other hand, measurement of w_f requires the application of a membrane "peeling" force to reduce the contact area. In studies of controlled adhesion/separation of artificial lipid-bilayer vesicles, it was demonstrated that adhesion is mechanically reversible ($w_a = w_f$) in concentrated solutions of large polymers and simple electrolyte buffers (17–19); but the vesicle–vesicle adhesion did not involve molecular attachments (18–19). In tests of adhesion which involved specific agglutinins, it has not been possible to characterize contact formation and separation with a single surface energy density. For example, in red blood cell aggregation produced by the lectin wheat germ agglutinin (20), adhesion was strongly irreversible ($w_f \gg w_a$). Similar observations (9) were made in studies of adhesion between cytotoxic T-lymphocytes and target cells. However, these experiments (and others) could not be accurately analyzed to obtain precise measurements of the membrane tension and contact angle at the contact perimeter which are needed to define w_a and w_f. Also, it appeared that cell surface receptor cross-bridges were "dragged" along the surface to accumulate at the contact perimeter. In experiments with more complicated types of cells like white blood cells (9, 21), analysis becomes even more difficult because the membrane surface is extremely irregular with folds, projections, and wrinkles which lead to tremendous redundancy of membrane between molecular attachment sites.

To quantitate the strength of membrane adhesion produced by agglutinins and other adhesion molecules, we have recently designed an experiment based on rigorous analysis that yields the mechanical properties intrinsic to formation and separation of large contact areas (22). Here, we have used this approach to study the mechanics of red cell contact formation and separation mediated by specific agglutinins. The results provide the first unambiguous measurements of "fracture energy density" for separation of agglutinin-bonded membranes. We compare a multivalent lectin (HPA from the snail helix pomatia) with two bivalent monoclonal antibodies (MAb) to glycophorin A, the major sialoglycoprotein of the red blood cell. We also examined the role of nonspecific repulsion between membrane surfaces in two ways: (*a*) MAb's were chosen with binding sites at two different locations on the same surface receptor. (*b*) We examined the ability of impingement stress to overcome nonspecific repulsion with the use of an "osmotic press" technique where a dehydration stress was applied to the membranes in the contact zone by exclusion of large polymers from the gap.

MICROMECHANICAL TEST PROCEDURE AND METHOD OF ANALYSIS

The following is an outline of the procedure used for assembly of two membrane capsules to form large adhesive contacts and for subsequent detachment by controlled force. In the test procedure, one set of red cells was chemically fixed in the form of smooth spheres to establish rigid surface matrices of receptors (see paper I for details of preparation, reference 13). Only cells from this set were bound with agglutinin before the adhesion-detachment tests. (Note: based on microfluorometric assay with labeled agglutinins, no appreciable reduction in bound agglutinin was detectable on time scales required for the adhesion experiments.) Assembly and detachment of a normal (deformable) cell to/from a rigid spherical cell was performed with a dual micropi-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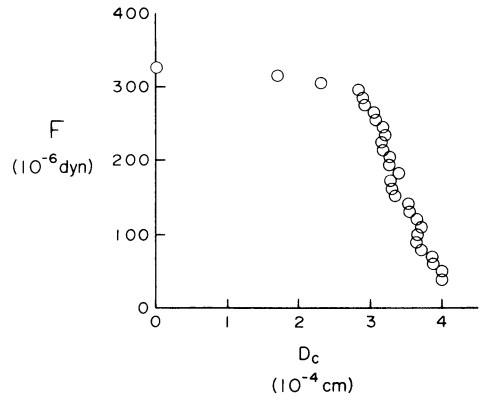

FIGURE 2 Measurements of detachment force from a single experiment. With each increase in suction force, the diameter of the contact region was reduced by a small increment.

pette arrangement (see reference 33 and paper I, reference 13, for details of micromanipulation). The measurements of pipette suction force vs. cell detachment were converted to precise values for membrane tension and contact angle vs. size of the contact region following the analysis outlined in reference 22. Fig. 1 is a sequence of video micrographs that show the mechanical assembly of the normal and rigid red cells to form a contact followed by detachment of the deformable cell by pipette suction. As shown, the rigid (fixed) cell bound with agglutinin was held at fixed position. The normal cell was forced to make contact with the rigid cell surface over a large region by positive pipette pressure on the order of 200 dyn/cm^2 (mechanical impingement by "blowing" the cell onto the rigid substrate). Initial diameters of the adhesive contacts were 3–5 $\times$ 10^{-4} cm. Adhesive contact was only produced over the area where the cell surfaces were forced together; *no* spontaneous spreading or enlargement of the contact area occurred beyond the initial coverage. Hence, there was no detectable affinity ($w_a < 10^{-3}$ erg/cm^2) for spontaneous formation of adhesive contact. The deformable cell was then detached from the adhesive contact in small discrete steps established by stepwise increase in suction pressure.

Measurements of suction pressure vs. contact diameter provided the "raw" data for each detachment test as illustrated in Fig. 2. The suction forces required to detach cells ranged from 10^{-5} dyn (at the onset of separation) to 5 $\times$ 10^{-4} dyn (at the end of separation). It is important to note that the cell body freely translated inside the large pipette without adhesion to the glass wall; but as was shown in the theoretical analysis (22), the membrane was pushed against the pipette wall by internal pressurization of the cell to form a seal. Hence, all of the suction pressure acted on the cell body. The normal (deformable) cells were preswollen by osmotic hydration in lower tonicity buffer to ensure complete pressurization of the membrane; thus, the surface did not fold or buckle. (The slow flow of water in the thin lubricating layer between membrane and glass wall does not appreciably alter the pressure field ahead/behind the cell in the pipette. Brownian motions of small particles ahead of the cell in the tube appeared unaffected by this small flow unless a major leak of fluid occurred between the cell and pipette wall.) Since the cell was detached in slow steps, the forces on the cell body and tensions in the cell membrane were essentially static. As shown in Fig. 1, the cell body remained smooth and axisymmetric throughout the detachment process. This geometric feature made rigorous analysis possible so that the suction pressure-contact diameter data could be converted to the membrane tension τ_m and contact angle θ_c (the included angle between surfaces) at the perimeter of the contact zone (22). An auxiliary piece of information was required in the analysis, i.e., cell surface area/volume ratio. Since it is well established that the red cell area and volume remain constant under these pressure conditions (23), the mechanical analysis predicted a unique relation for length of the deformed cell body as a function of contact diameter. Therefore, the surface area/volume ratio was determined in a self-consistent manner by correlation of the observed cell length vs. contact diameter (data shown in Fig. 3) with the prediction from the mechanical analysis (solid curve in Fig. 3). With the surface area/volume ratio, the cell geometry was calculated to provide relations for derivation of membrane tension and contact angle from the "raw" data in Fig. 2. Tension and contact angle as functions of contact diameter are plotted in Fig. 4 for the sample test data in Fig. 2. Based on the analysis, the full range of separation forces represented tensions at the contact perimeter from 0.02–2.0 dyn/cm.

Tension and contact angle measurements combine to specify the work (mechanical energy) to separate a unit area of adhesive contact through the classical Young-

FIGURE 1 Videomicrograph sequence from a typical assembly/detachment experiment. (*a*) An assembled pair of human red blood cells: the cell on the right was chemically fixed in the form of a rigid sphere and prebound with agglutinin. The deformable cell on the left was forced to form adhesive contact with the rigid test surface in a separate chamber without agglutinin; (*b*) and (*c*) Pipette suction was applied to the deformable cell to separate the cell surfaces. Time and suction pressure were recorded with the video image. Each discrete step of peeling appeared to be in static mechanical equilibrium.

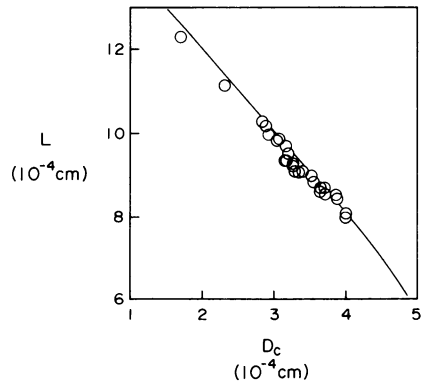

FIGURE 3 The end-to-end extension of the red cell was measured as function of contact diameter. The points are data from the experiment shown in Fig. 2. Mechanical analysis predicts a unique relation that depends on cell surface area, volume, and pipette diameter. The prediction for $R_p = 2.3$ μm, $A = 135$ μm^2, and $V = 115$ μm^3 is the solid curve.

Dupre equation,

$$w_f = \tau_m (1 - \cos \theta_c)$$

when higher order terms due to changes in membrane bending energy at the contact perimeter are negligible. As indicated by the sharp membrane bend at the perimeter of the contact, bending effects contribute $< 1\%$ to the mechanical energies measured here (15, 24). In the experimental tests, "fracture energies" were directly equal to the mechanical energies for cell separation because dissipation in the cell body and surrounding fluids was negligible. Because of the geometric proportions and method of detachment, the mechanical leverage factor $(1 - \cos \theta_c)$ only varied over the range 1.2–1.6 in these tests as seen in Fig. 4. Thus, the membrane tension applied at the perimeter of a contact was a close measure of the "fracture energy density" throughout at each detachment step.

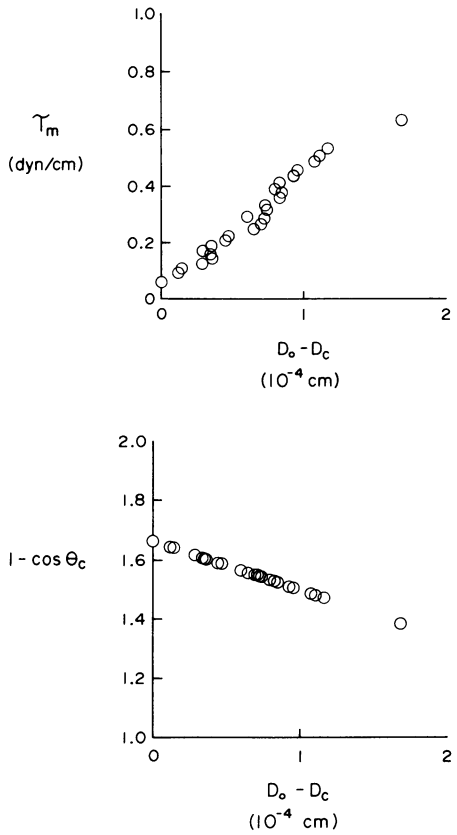

FIGURE 4 (*a*) Peeling tension at the perimeter of the contact vs. reduction in the contact diameter calculated from the data in Figs. 2 and 3 by mechanical analysis of the cell deformation. (*b*) Mechanical leverage factor $(1 - \cos \theta_c)$ calculated with values of contact angle θ_c at each step of separation (derived from the data in Figs. 2 and 3 by mechanical analysis of cell deformation).

MATERIALS AND OTHER METHODS

Rigid red cell substrates

Rigid spherical test surfaces were created by chemical fixation of swollen red blood cells with use of an established procedure (25) described in paper I (13).

Agglutinins

For adhesion molecules, we chose two monoclonal antibodies (MAb) that bind to the major sialoglycoprotein of the red blood cell membrane, glycophorin A, and the lectin (HPA) from edible snail-helix pomatia (obtained from Sigma Chemical Co.) which agglutinates type A red cells. B14 MAb (full name NBTS/BRIC 14; reference 26) is thought to bind near the hydrophobic bilayer-spanning portion of glycophorin (between residues 55 and 70; reference 26). R10 MAb (full name LICR LON R10; reference 27) binds to a more superficial region of the molecule between residues 25 and 40 (28, 29). HPA lectin has six binding sites for D-GalNAc sugar residues (30). Adhesive surfaces were prepared by incubation of the fixed red cell spheres in a buffered solution that contained the antibody or lectin. The surface density of bound agglutinin was estimated by a microfluorometric technique described previously (20) or from published data. In the microfluorometric technique, test surfaces were incubated in antibody or lectin conjugated with fluorescein isothiocyanate (FITC). An individual sphere was transferred to a fluorescence-free chamber under a fluorescence microscope, and the surface fluorescence was quantitated by analysis of the video image intensity profile of the fluorescent boundary. A binding curve and Scatchard plot were constructed from the data for many spheres incubated at different concentrations of antibody/lectin. For the antibody, saturation was taken as the surface density of glycophorin A ($0.5–1.0 \times 10^6$ per cell or $3–7 \times 10^{11}$/cm^2; references 31 and 31*a*). For HPA, saturation has been reported to be 2.8×10^{11}/cm^2 (3.8×10^5 per cell; reference 32); but our microfluorometric tests yielded order of magnitude higher values $2–3 \times 10^6$ per cell. To calculate molar concentrations, molecular weights were taken as 150,000 daltons for the MAb's and 79,000 daltons for the lectin.

Rigid-spherical test surfaces were selected from a chamber that contained buffer solution with antibody or lectin and transferred to an adjacent chamber that contained normal red blood cells in buffer but no agglutinin molecules. Thus, assembly of normal cells with the test surfaces was expected to provide the maximum cross-bridge formation and strength of adhesion. If both surfaces had been coated with adhesive molecules, agglutination would have been reduced because fewer sites would have been available for cross-bridge formation. The buffer solution contained human serum albumin (1 g/100 ml) to prevent adhesion of the normal cell membrane to the glass micropipette.

Osmotic dehydration of adhesive contacts

Dextran (Pharmacia, Sweden) with a weight-averaged molecular weight of 147,000 was added to the phosphate-buffered saline to obtain concentrations in a range from 0.5 to 10.0 g/100 ml. This solution was injected into a third chamber on the microscope stage. In one set of tests, a rigid spherical cell was first transferred to the antibody/lectin-free chamber and an adhesive contact was made with a normal erythrocyte. Next, the adherent pair was transferred to the chamber that contained dextran solution, held for 1 min, and then transferred back to the saline chamber for the separation test. In other tests, normal cells were assembled with rigid spherical cells directly in dextran solution then separated. No difference in results was observed between these two sets of tests; thus, it was concluded that dextran was excluded from the gap between surfaces and hence acted to dehydrate the gap. As such, the surfaces were forced together with a normal force equal to the difference between osmotic pressures in the gap and the bulk solution (19). Based on the first and second virial coefficients determined for dextran by osmometry (19), the osmotic stress was calculated from the following equation:

$$P_{osm} = (2.58 \times 10^5)c + (3.63 \times 10^7)c^2 \text{ dyn/cm}^2,$$

where c is the dextran concentration in grams per cubic centimeter.

RESULTS

Strengthening of adhesion by separation

In nearly 100 separation tests with the three agglutinins, the same type of relation was observed between tension and reduction in contact diameter: i.e., the tension required to separate a contact began at low values between 10^{-2}–10^{-1} dyn/cm, increased linearly with reduction in contact diameter (over most of the change in contact area), and reached ultimate values at least an order of magnitude higher (-1 dyn/cm or more). These observations were in marked contrast to the behavior predicted for separation of an "ideal" adhesive contact: i.e., the tension required to separate a contact should remain constant throughout detachment provided the contact angle is fixed. As noted in the previous section, variation of contact angle in these experiments contributed only a maximum of 30% change to the mechanical leverage factor and thus were not sufficient to account for the tension increase with separation. Because the initial linear increase in tension was common to all tests, it was apparent that strength of adhesion could be quantitated by a phenomenological "adhesion energy gradient" K_f *defined by* the increase in tension divided by decrease in contact diameter (or perimeter length if normalized by π). In exceptional situations, the contact separated in nearly ideal fashion when high levels of tension were reached (i.e., at this "critical" tension, the contact separated smoothly without further increase in tension). Thus, the critical tension was also an important characteristic of the strength of adhesion. However, this level of tension was achieved in only a few special cases (to be described).

Effect of rate of detachment

The unexpected necessity to increase the "peeling" tension suggested that microscopic dissipation and kinetic effects may have been important factors. However, no obvious motion ("creep") of the membrane was observed after each incremental reduction of contact size in respone to increase in suction force. Because of this quasi-static behavior, large-scale time-dependent processes were not significant. To examine possible microscopic effects, the rate of separation was changed by increase or decrease in the ramp rate of application of the suction force. For this purpose, the pipette suction was increased at fixed rates in the range from 1 to 20 dyn/cm^2/s (force rates ~ 0.2–4.0×10^{-6} dyn/s). For this range of rates of force application, complete separation occurred in time intervals from as short as 2 min up to as long as 30 min. The effects of separation rate were studied for adhesive contacts produced only by the lectin (HPA) because separation of adhesive contacts bonded by the other agglutinins exhibited the same mechanical behavior (as will be shown). In Fig. 5, data are plotted from individual tests with three force application rates that cover the full range. As shown, the tension vs. reduction in contact diameter did not depend on rate of separation. Table 1 presents the cumulated values of "adhesion energy gradients" K_f measured in all of the tests; again, there is no apparent dependence on rate of separation. When the separation rate was very large, greater variations in the gradient were observed. If the separation was very slow (over 20 min), other practical problems were encountered, e.g., evaporation of water from the microscope chamber caused shrinkage of the adherent cell. Consequently, the decision was to increase pressure at a rate of 8 dyn/cm^2/s (force rate $\sim 1.4 \times 10^{-6}$ dyn/s) in all subsequent experiments. For this rate, detachment times varied from $\sim$30 s for

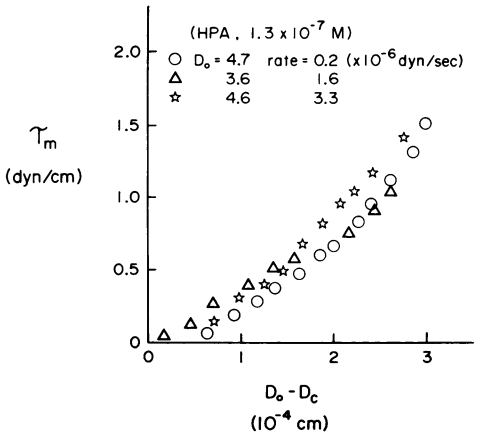

FIGURE 5 Peeling tension vs. reduction in contact diameter for three adherent cell pairs agglutinated with HPA. Rigid test cell surfaces were incubated in 1.3×10^{-7} M HPA. For detachment, separation force was applied at average rates of 0.2×10^{-6} dyn/s, 1.6×10^{-6} dyn/s, and 3.3×10^{-6} dyn/s. Initial diameters D_0 (micrometer) of the contacts are listed in the figure.

weakly adherent cells to over 6 min for strongly adherent cells.

Effects of agglutinin

As noted, the stiffening response to detachment of red cells from large contact areas was the same for cells bonded with all of the agglutinins. However, important differences were observed in the magnitudes of the adhesion energy gradient K_f in each case. Thus, the results are examined in the context of the agglutinin properties: i.e., B14 MAb binds deep in the surface glycocalyx close to the lipid bilayer; R10 MAb binds to the same receptor but more superficially (~30 peptides or ~45 Å further out); and HPA lectin binds to sugars presumably on other membrane receptors but at unknown depths. In the first set of tests, adhesive contacts were initiated simply by mechanical impingement. Within the contact region, mechanical impingement imparted compressive stresses (through an initial positive pipette pressure) which were only on the order of 10^2 dyn/cm^2. It was not surprising, therefore, that adhesive contacts between normal cells and test surfaces bound with B14 MAb were very weak. Indeed, it was not possible to form large contact areas and total detachment of adherent cells occurred at suction forces $< 20 \times 10^{-5}$ dyn. The test surfaces were incubated in 7×10^{-9} M and 3×10^{-8} M solutions of the B14 MAb. Based on an estimated association constant of $\sim 3 \times 10^5$ M^{-1}, between 20–50% of the glycophorin A molecules were likely bound with antibody, yet the level of adhesion was insignificant. (Note: insufficient amounts of B14 MAb were available for labeling and use in microfluorometric assay of binding.) On the other hand, for cells exposed to R10 MAb at similar solution concentrations, significant levels of adhesion and large contact areas were produced by mechanical impingement. Fig. 6 *a* shows measurements from a typical separation experiment plus the total range of results obtained with rigid cells incubated at low R10 MAb concentration (7×10^{-9} M). As shown, separation commenced when tensions of approximately 10^{-2} dyn/cm were applied at the perimeter of adhesive contacts. Each reduction in contact diameter required an increase in tension until the cell was detached from the rigid test cell. At cell detachment, the tension level reached 0.15–0.2 dyn/cm, an order of magnitude greater than the initial tension. Over the first few micron reduction in contact diameter (which began in the range of $3–5 \times 10^{-4}$ cm), the dependence of tension on contact diameter was always approximately linear.

As shown in Fig. 5, cells agglutinated with the lectin HPA exhibited the same detachment behavior as the R10 MAb-agglutinated cells. For comparison, results for detachment of a cell from a test surface bound with low levels of HPA are plotted in Fig. 6 *b* along with results from a detachment test for R10-MAb. These data appear virtually identical. However, when test surfaces were incubated in higher concentrations of HPA, much larger tensions were needed to achieve comparable separations. Fig. 6 *c* shows the range of results for separation of cells bound at low HPA (7×10^{-10} M) and high HPA (1.3×10^{-7} M) concentrations. For high HPA concentrations, tensions were as large as 4 dyn/cm! At low HPA surface binding, the increase of tension over the first 2 μm reduction in contact diameter (~75% of the contact area) was characterized by a gradient K_f of $\sim 2 \times 10^{-2}$ dyn/cm/μm (s.d. 5×10^{-3} dyn/cm/μm for five tests). At high HPA surface binding, the gradient K_f parameter was a factor of 20 larger, 0.4 dyn/cm/μm, over the initial stage of separation (s.d. 9×10^{-2} dyn/cm/μm for 22 tests). Based on an association constant of 10^7 M^{-1}, the surface density at the higher concentration was estimated to be almost 100 times that at the lower concentration.

TABLE 1 Effect of separation rate on the strengthening of adhesion for hpa agglutinated cells

Rate of force application	K_f Adhesion energy gradient	± S.D.	*n*
10^{-6} *dyn/s*	*dyn/cm*/10^{-4} *cm*	*dyn/cm*/10^{-4} *cm*	
0.33	0.44	0.07	7
1.6	0.40	0.09	6
3.3	0.43	0.15	9

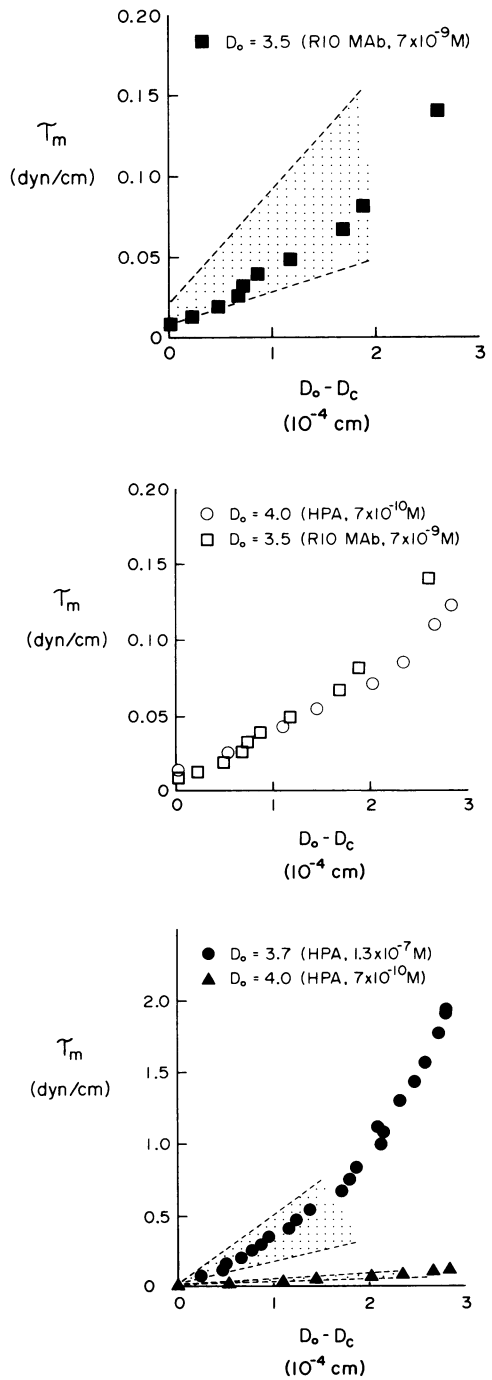

FIGURE 6 Peeling tension vs. reduction contact diameter for adherent cell pairs. Initial diameters D_o of the contact are listed in the figures. (*a*) Cells agglutinated with R10 MAb. A typical result from a single detachment test is plotted as points. Dotted lines show the range of results obtained from 22 R10 MAb separations. Rigid test cell surfaces were incubated in 7×10^{-9} M solution of R10 MAb. (*b*) Cells agglutinated by HPA and by R10 MAb. Rigid test cell surfaces were incubated in the agglutinin concentrations noted for each set of test results. (*c*) Cells agglutinated with HPA. Rigid test cell surfaces were incubated in either low HPA concentration (7×10^{-10} M) or high HPA concentration (1.3×10^{-7} M) as indicated. Dotted lines show the range of results.

Effect of impingement stress

As described, application of positive pipette pressure to initiate contacts only created stresses of 10^2 dyn/cm^2 to press the membranes together. Also, mechanical impingement only produced minimal adhesion for cells coated with the B14 MAb even though significant amounts of agglutinin were available to form cross-bridges. Thus, the "osmotic stress" technique was employed to augment the impingement stress. Two procedures were used to apply osmotic stress to the contact. In the first procedure, adhesive contacts were formed initially by mechanical impingement; next, the contact was exposed to osmotic dehydration by a polymer solution in a separate chamber; then the adherent cells were separated in the original chamber that contained only the buffer solution. In the second procedure, adhesive contacts were formed by mechanical impingement directly in the polymer solution and then the cells were separated. No difference was observed between the tensions required to separate contacts after either of these two procedures was followed. This result supported the view concluded previously (19) that dextran polymers in solution are excluded from the vicinity of cell surfaces to create a "depletion" effect.[1]

The importance of impingement stress in potentiation of adhesion was dramatically demonstrated in experiments with the B14 MAb. With mechanical assembly, agglutination was very weak with no formation of large contact areas. Detachment occurred at suction forces of $< 2 \times 10^{-5}$ dyn. However, when cell aggregates were exposed to 5 g% polymer solution, much larger forces on the order of 4×10^{-4} dyn were required for cell detachment. The data presented in Fig. 7 *a* show that exposure to even 1 and 2.5 g% polymer solutions markedly enhanced the adhesion. With the latter levels of impingement stress (6×10^3 and 3×10^4 dyn/cm^2), separation was still characterized by a continuous increase of membrane tension throughout detachment. On the other hand, with very high impingement stresses (10^5 dyn/cm^2 in 5 g% polymer), the stiffening of adhesion occurred only over a limited range (~ 1 μm reduction in

[1]The only subtlety here is that the effective "excluded volume" (or concentration) of the surface carbohydrate groups plays an important role. This parameter regulates penetration of polymers into the interfacial layer (34). When the polymer concentration is below the concentration of surface groups, nearly all of the polymer molecules are excluded from the interfacial layer which creates the maximum depletion effect. However, when the polymer concentration exceeds the concentration of surface groups, polymer molecules are forced to penetrate the interfacial layer because of greater osmotic activity which reduces the depletion effect. For red blood cells, the cross-over in exclusion behavior most likely occurs at concentrations around 5–6% because the affinity w_a for contact formation has been shown to peak at concentrations of 5–6% (16).

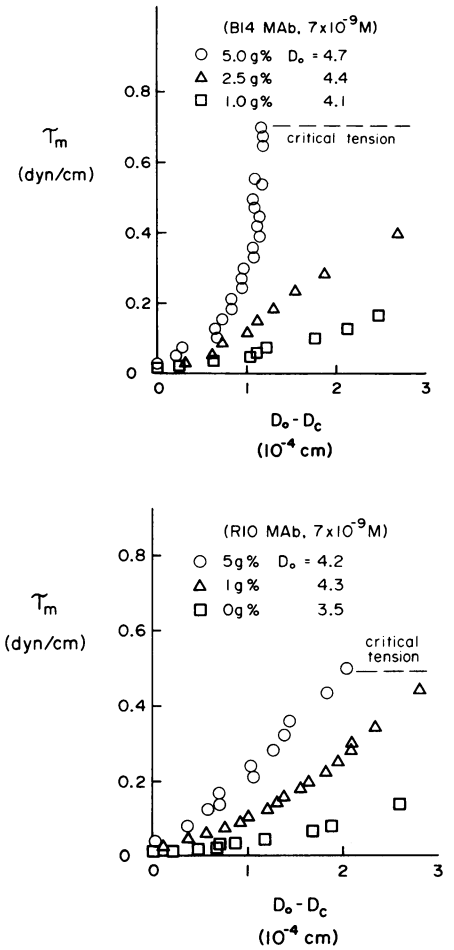

FIGURE 7 Peeling tension vs. reduction in contact diameter for three adherent cell pairs. After assembly, each adherent contact was exposed to osmotic dehydration in a dextran polymer solution (concentration listed above) then separated. Initial diameters D_o of contacts are given for each test. (*a*) Cells agglutinated with B14 MAb. Rigid test cell surfaces were incubated in 7×10^{-9} M solution of B14 MAb. (*b*) Cells agglutinated with R10 MAb. Rigid test cell surfaces were incubated in 7×10^{-9} M solution of R10 MAb.

contact diameter). Then, the tension reached a critical level ($\sim$0.7 dyn/cm) where a large reduction in contact diameter suddenly occurred and the cell detached. This behavior suggested that, after a small separation, the perimeter of the adhesive contact became saturated with cross-bridges to reach the ultimate adhesive strength. Apparently, direct application of the osmotic impingement stress was not sufficient to cause complete formation of cross-bridges. Although separation was initiated at low tensions, the gradient K_f of the adhesive contact was greatly increased by added impingement stress and led to the apparently "ideal" separation behavior at the critical tension. This type of behavior was never observed in separation of cells assembled by mechanical impingement alone.

The same dramatic augmentation in strength of adhesion was observed for R10 MAb-agglutinated cells that had been stressed by the polymer solutions as shown in Fig. 7 *b*. For cells assembled only by mechanical impingement, the tension increased up to a maximum value of 0.2 dyn/cm or less. By comparison, adherent cells exposed to 1 g% polymer required a much larger increase in tension for separation; at detachment, the maximum tension reached 0.6 dyn/cm in these tests. Adherent cells stressed by 5 g% polymer required even greater increases in tension for comparable separations, but the maximum tension remained at 0.6 dyn/cm. As in the B14 MAb studies, rapid detachment followed when the tension reached 0.6 dyn/cm (after 1–2 μm reduction in contact diameter). This critical tension was found in separation tests with R10 MAb agglutinated cells exposed to 1, 2.5, and 10 g% polymer solutions. The critical tensions appeared to be almost identical for cells agglutinated by either MAb to red cell glycophorin. Another common feature was that exposure to even low polymer concentrations (0.5 g% – 2×10^3 dyn/cm^2 stress) augmented adhesion. However, the stiffening of adhesive contacts after exposure to the polymer solutions was noticeably different for each MAb as shown in Fig. 7.

Exposure to polymer stress produced little effect on adhesion strength for lectin HPA-agglutinated cells. This result was very surprising since measurements with test surfaces incubated in either 7×10^{-9} M R10 MAb or 7×10^{-10} M HPA led to similar strengths of adhesion (Fig. 6 *b*); and exposure of the R10 MAb-agglutinated cells to polymer stress strengthened adhesion by as much as 600%. However, for HPA-agglutinated cells, stiffening of adhesion with separation was only 50% greater on the average for adherent cells exposed to polymer solutions than for cells assembled only by mechanical impingement. Another significant difference from the antibody tests was that a critical tension was never observed with HPA-agglutinated cells.

CONCLUSIONS AND DISCUSSION

These results clearly show that detachment of antibody and lectin-agglutinated red cells does not obey the ideal "fracture energy" law where surfaces separate continuously at a constant mechanical energy per unit area. In all separations of agglutinin-bonded red blood cells, a common amplification of adhesion was observed where the tension increased progressively with reduction in contact diameter (perimeter) usually over 75–80% of the contact area. The adhesion energy gradient depended strongly on the amount of agglutinin bound to

the test cell surface and the level of impingement stress applied to the initial contact. However, for fixed conditions (impingement stress and amount of agglutinin bound to the test surface), the tension measurements did not depend on initial size of the contact *or* the rate of separation. Thus, we conclude that time-dependent processes at the microscopic level were not affected by the rate of separation. (Based on the rates of "peeling" [$\sim 4 \times 10^{-2}$–4×10^{-3} μm/s], single molecular cross-bridges were stressed for periods of 1–10 s before rupture.) In special cases, cells separated smoothly at a critical tension without further increase in detachment force. The critical tension was reached only in tests of cells agglutinated with the monoclonal antibodies to glycophorin *and* where cell contacts had been subjected to compression by osmotic dehydration in polymer solutions. In tests with the HPA lectin, no critical tension was observed even though much higher levels of tension were required to separate contacts in some cases.

In previous studies (9, 20), it appeared that the strengthening of adhesion was due to accumulation of cross-bridges at the perimeter of the contact. Consistent with this concept, micromechanical analysis (6) has shown that cross-bridges at the perimeter of the contact are subject to a small force parallel to the surface as well as the dominant rupture force normal to the surface. Thus, if membrane receptors are mobile, cross-bridges could be dragged inward to produce accumulation and augmentation of the adhesive strength. Also, if receptor-agglutinin bonds are chemically labile, the lateral force could produce an energy gradient sufficient to drive agglutinin into the contact zone. Even though these effects may lead to cross-bridge accumulation in many cases, this mechanism did not seem likely in the antibody agglutination experiments reported here. The proteins in one of the adherent cell surfaces were rigidly cross-linked and showed no evidence of mobility. However, it was possible that HPA receptors remained mobile because it is known that HPA binds to glycolipids in the cell surface. Also, when large regions of adhesive contact were agglutinated by fluorescently labeled MAb molecules then separated, no bright ring was observed at the perimeter of the contact region. Further, other circumstantial evidence indicated that accumulation was not the major source for strengthening of the adhesive contact. For instance, no time-dependent changes were observed in contact formation (i.e., contacts did not spread) and the "fracture energies" did not depend on rate of separation; this implied that cross-bridges were locally restricted and sparsely distributed. As an alternative to cross-bridge accumulation, we propose that separation progressively increased the fraction of surface area in intimate contact and thereby induced additional agglutinin to form molecular attachments. The reasoning is as follows:

Although red cell surfaces appear smooth in the light microscope, they are actually rough and irregular on the mesoscopic scale below optical resolution. For example, scanning electron micrographs of the rigid test cell surfaces have revealed small asperities. Also, freeze-fracture micrographs of flexible red cell surfaces exhibit topological variations with amplitudes of a few hundred Angstroms over distances of 10^3 Å (E. Sackmann, personal communication). Even when surfaces are pressed together with a large impingement stress, molecular attachments probably lock in much of this roughness to frustrate continuous contact between surfaces. In fact, when cell suspensions are aggregated by uncontrolled agglutination, regions of intimate contact are interrupted in the adhesion zone by large intervals of wide gaps between the surfaces (35). Hence, initial formation of contact probably produces only a few molecular attachments even though many more agglutinin sites are present. Given this topographical "roughness," mechanical actions local to the contact perimeter may be important in increasing the fraction of smooth surface contact. The mechanism is predicted from analysis of separation of a flexible membrane that is bonded at discrete points to a substrate (6). When tension is applied to the membrane, bending stresses at the perimeter of the contact assert disjoining forces on local molecular cross-bridges which lead to rupture and separation. But as the membrane pulls-up on cross-bridges, the membrane region interior to an attachment site experiences a large compressive stress that pushes the surfaces together. Conceptually, the membrane acts as a lever: i.e., the tension-force is applied about a fulcrum (the perimeter cross-bridges) and pushes the membrane against the substrate interior to the attachment site. Both the magnitude of the compressive stress and the size of the membrane region affected by compression increase with the level of tension applied to the membrane which leads to the proportional increase the local density of molecular attachments as tension increases. In addition to pressing surfaces toward one another, the mechanical action of "peeling" also translates the membrane laterally to smooth out irregularities. Hence, the progressive increase in adhesion strength could represent proportional recruitment of bound agglutinin as cross-bridges. As such, values measured for critical tensions should correspond to complete participation of local agglutinin molecules as cross-bridges.

To examine the assumptions and predictions of this mechanism for amplification of the "fracture" strength, we have estimated the "apparent" surface densities of cross-bridges over the course of cell detachment. The "apparent" surface density can be deduced from the

force per unit perimeter length (tension required to separate the contact) *divided by* the force required to rupture a single molecular cross-bridge (this ratio is squared to obtain an estimate of surface density). In the first paper (13), values of the force to rupture single molecular attachments were obtained for the same antibodies and lectins. Surprisingly, the detachment force was found to be between 1–2 $\times$ 10^{-6} dyn for all of the agglutinin types (13). Thus, for low tensions required to commence separation (10^{-2} dyn/cm), attachment densities would be on the order of 10^8/cm^2, i.e., there would only be $\sim$10–15 attachment sites along the initial perimeter of the contact. By comparison, critical tensions for separation of the MAb agglutinated cells were 0.6–0.7 dyn/cm; the values imply a maximum surface density of $\sim$4–5 $\times$ 10^{11}/cm^2 for these antibodies (which are comparable to the values published for surface density of glycophorin A in red blood cell membranes, reference 31). On the other hand for the HPA lectin, critical tensions were never observed even though tensions were as large as 4 dyn/cm. Tensions of this magnitude indicate that the surface density of attachments would be very large on the order of 10^{13}/cm^2. Thus, there would only be $\sim$30 Å between sites along the contact perimeter which seems surprising unless lectin-receptor complexes were accumulated at the edge of the contact. Also, because of the high surface density of attachments, a significant number of cross-bridges interior to the contact perimeter were stressed and contributed to the strength of adhesion. Thus, the assumption that only attachments along the perimeter supported the membrane stress would no longer be valid.

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