USE OF CELL CONTOUR ANALYSIS TO EVALUATE THE AFFINITY BETWEEN MACROPHAGES AND GLUTARALDEHYDE-TREATED ERYTHROCYTES

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ABSTRACT Recently, several authors evaluated the affinity between lipid bilayers or erythrocyte membranes by analyzing the deformation of cells or vesicles they brought into close contact using micromanipulators. In the present report, we extend this approach in a study of the adhesive properties of rough nucleated cells. Rat peritoneal macrophages were made to bind human red cells modified with glutaraldehyde or glutaraldehyde and polylysine. Conjugates were examined with electron microscopy, and photomicrographs were digitized for quantification of cell surface roughness in and out of adhesion areas. Also, macrophages were subjected to micropipette aspiration to find a relationship between apparent surface tension and area increase. Assuming that this increase was a direct consequence of a smoothing of the cell surface on the submicrometer scale, the actual affinity between macrophages and erythrocytes was estimated. The obtained values ranged between 8.4×10^{-5} and 18.2×10^{-5} J/m^2. It is concluded that cell surface roughness may be an important parameter of cell adhesion and perhaps deformation. This is made amenable to experimental study by the present approach.

INTRODUCTION

The interaction between biological surfaces is influenced by many physical effects such as electrostatic repulsion, steric stabilization, diffusion of membrane molecules, and formation of intermolecular bonds (1, 2). Measuring the affinity between model or actual cell surfaces may help unravel these complex phenomena and shed some new light on cell adhesion.

The experimental determination of the interaction potential between solid surfaces in the presence of macromolecules was achieved by several authors (3, 4) who studied the deformation of transparent mica cylinders that were brought into close contact. Also, the interaction potential between phospholipid bilayers was derived from x-ray diffraction experiments (5). However, these methods were not applied to surfaces more akin to biological membranes.

Recently, Evans developed a new method of addressing this problem: using micromanipulators. He brought into close contact phospholipid vesicles (6) or erythrocytes (7) made adhesive with dextran, and he measured the deformation induced by intermolecular forces. Comparing these deformations with those induced by known mechanical forces (8), he was able to derive a figure of the affinity between interacting surfaces.

However, these experiments did not provide a complete understanding of adhesions involving nucleated cells, since these largely differ from red cells with respect to the lateral mobility of membrane molecules and the presence of numerous surface asperities, such as microvilli, ruffles, or lamellipodia. Indeed, the actual contact area between two nucleated cells may be very different from the "apparent" contact area measured by conventional microscopic examination. Further, the mechanical properties of the tip of a microvillus may be very different from the material parameters characterizing the bulk cell surface.

In the present paper, we describe a new way of analyzing the interaction between a nucleated cell and a smooth surface. Rat macrophages were made to bind human erythrocytes modified with chemical treatments. Conjugates were then studied with electron microscopy. The cell contours were digitized and macrophage "roughness" was quantitated both inside and outside contact areas. The distribution of apparent distances between macrophage and erythrocyte membranes was also studied. Finally, macrophage deformability was assayed with micropipette aspiration techniques. The obtained results were used to derive an empirical relationship between cell surface smoothing and tensile forces, which allowed an estimate of the affinity between macrophages and modified erythrocytes.

MATERIALS AND METHODS

Cells and Particles

Macrophages were obtained by washing the peritoneal cavities of unstimulated Wistar rats with 10 ml of RPMI 1640 medium (Gibco, Glasgow, Scotland) as previously described (9).

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Human O^- red cells were incubated overnight with 0.25% glutaraldehyde (Sigma Chemical Co., St. Louis, MO) in pH 7.2 phosphate buffer, which made them bound and then ingested by phagocytic cells, possibly through hydrophobic interactions (10, 11).

A batch of erythrocytes was exposed to a mixture of glutaraldehyde and poly-L-lysine (Sigma type V, 20,000 mol wt, 200 μg/ml). Polylysine treatment resulted in a dramatic decrease of electrophoretic mobility, and treated particles were much more efficiently bound by macrophages than glutaraldehyde-treated erythrocytes, possibly due to the lowering of the electrostatic repulsive barrier (9).

Electron Microscopic Study of Binding

5×10^6 rat peritoneal cells were mixed with 10^7 treated erythrocytes in 1 ml of RPMI 1640 medium, then centrifuged (2 min, 450 *g*) at room temperature and resuspended 10 min later. Cells were then fixed for 60 min on a rotating agitator in 0.1 M pH 7.2 cacodylate buffer containing 1% glutaraldehyde, then incubated overnight at 4°C in fresh cacodylate solution. They were incubated for 1 h at 4°C in 1% osmium tetroxide, then stained with 1% uranyl acetate before being dehydrated in acetone and embedded in Epon (9).

Study of Cell Deformability

Our method is an adaptation of the micropipette aspiration technique of Rand and Burton (12) and of the techniques described in a previous paper (13). Briefly, the tip of a glass micropipette (2–3.5-μm inside diam) was settled in a 30-μl droplet of RPMI medium deposited on a glass coverslip, on the stage of an inverted Olympus IMT2 microscope. 3 μl of macrophage suspension (2.5×10^6 cells/ml, RPMI) was then added, and cells were aspirated with the micropipette before any adhesion to the glass. The length of the protrusion was measured with an eyepiece micrometer (Fig. 1). The pressure used ranged between 200 and 2,500 Pa, and the protrusion did not increase by more than 10–20% when the aspiration was maintained for 30–45 s after the first measurement.

The obtained data were used to derive an effective membrane tension by modeling cells as liquid droplets surrounded by a tensile membrane, as proposed by Evans (14). The effective tension T was calculated with Laplace law (15):

$$\Delta P = 2 \times T \times (1/a - 1/R), \quad (1)$$

where ΔP is the suction pressure, a is the pipette inner radius, and R is the radius of the spherical part of the deformed cell (Fig. 2). R was deduced from the radius R_o of the undeformed cell by assuming constant volume during aspiration, which yielded (using elementary geometrical formulae)

$$4\pi R_o^3/3 = \pi R^3(8 + 9\cos\theta - \cos 3\theta)/12 + \pi a^2(L - a) + 2\pi a^3/3 \quad (2)$$

$$\theta = \sin^{-1}(a/R). \quad (3)$$

The surface area A of the deformed cell was calculated as

$$A = 2\pi R^2(1 + \cos\theta) + 2\pi a(L - a) + 2\pi a^2, \quad (4)$$

and the relative area increase, I, was calculated as

$$I = A/4\pi R_o^2 - 1.$$

This was considered an apparent area increase, since it is assumed that cell deformation involved smoothing of the cell surface with conservation of the actual membrane amount (14, 16, 17).

Contour Analysis

The analysis was performed on micrographs of 28,000 magnification. Our technique was fully described and validated in a separate paper (18). Briefly, cell contours were digitized with a digitizer (model 2000; Calcomp, CA) connected to a Commodore 64 computer. The mean distance between two consecutive points was 0.06 μm (i.e., 1.7 mm on the photomicrograph). The analysis was done on 17-point segments of digitized contours, by defining as a "reference line" the circular arc enclosing the same area as the actual contour with the chord joining its ends (Fig. 3). Two roughness parameters were used. (*a*) The relative length excess:

$$E = (\text{length of cell contour})/(\text{length of reference arc}) - 1.$$

(This definition is at variance with that of reference 18 due to the factor 1, since it appeared reasonable that the length excess of a straight line be zero.) Parameter E represents the membrane excess that is associated with cell surface asperities whose linear dimensions range between ~0.06 and 1 μm. (*b*) The standard deviation of deviation, D, is the standard deviation of the deviation d, i.e., the distance between the midpoint of the

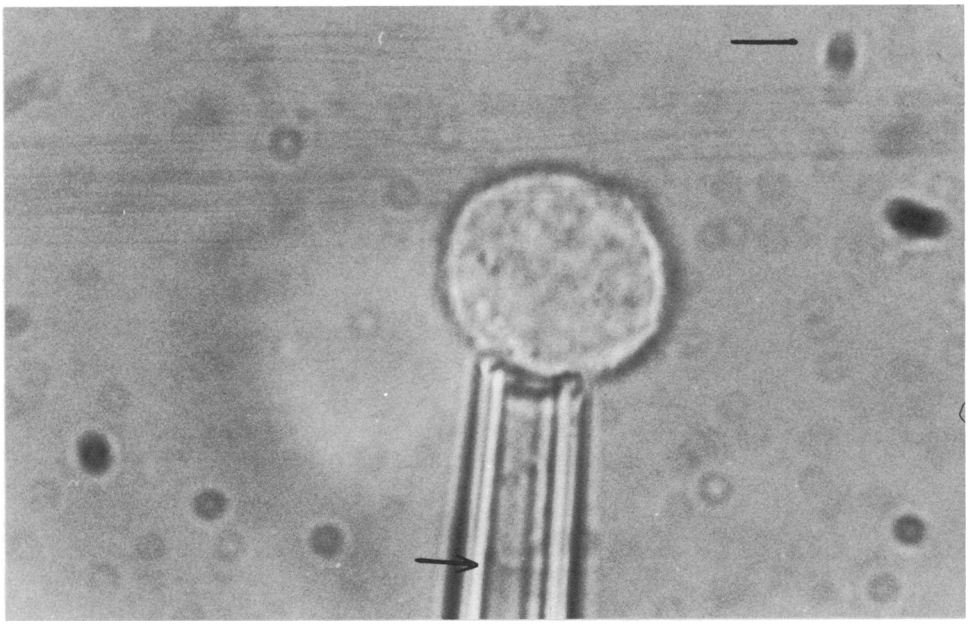

FIGURE 1 Study of macrophage deformability. A cell was sucked into a micropipette, and the distance between the tip of the protrusion (*arrow*) and the pipette mouth was measured with an eyepiece micrometer, under 1,000-fold enlargement. Bar, 5 μm.

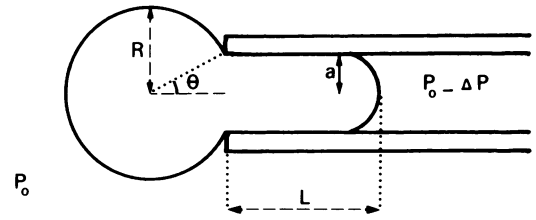

FIGURE 2 Geometrical parameters of deformed cells. Pipette aspirated cells were modeled as truncated spheres of radius R bearing a cylindrical protrusion (radius a, length $L - a$) ending into a half sphere (radius a).

digitized cell contour (i.e., M_9 on Fig. 3) and the intersection of the reference line with the straight line joining the former point and the center of the reference circle (i.e., point O on Fig. 3). A similar parameter was used in several studies made on adhesion between rough solid bodies (19, 20).

The choice of a value of 17 for the number of points per analyzed contour segment was a compromise between the requirement to take account of a sufficient fraction of studied contours (use of [2k + 1] − point segments resulted in neglect of $2k$ points per contour) and the impossibility to detect cell asperities that were larger than the test contour segment (the length of which was ~1 μm) (18).

The extent of the apposition between a macrophage and an erythrocyte was quantified by calculating the distance between each point of the digitized macrophage contour and the erythrocyte boundary, approximated as a set of straight segments. The reproducibility of these determinations was tested by performing five sequential digitizations of a portion of a macrophage contour that was bound to an erythrocyte and calculating the distances between the 92 points of the erythrocyte digitized boundary and the five digitized macrophage contours (Fig. 4). The mean value of the standard deviation of these 92 parameters was 0.26 mm, corresponding to a value of 93 Å units in actual size cell samples.

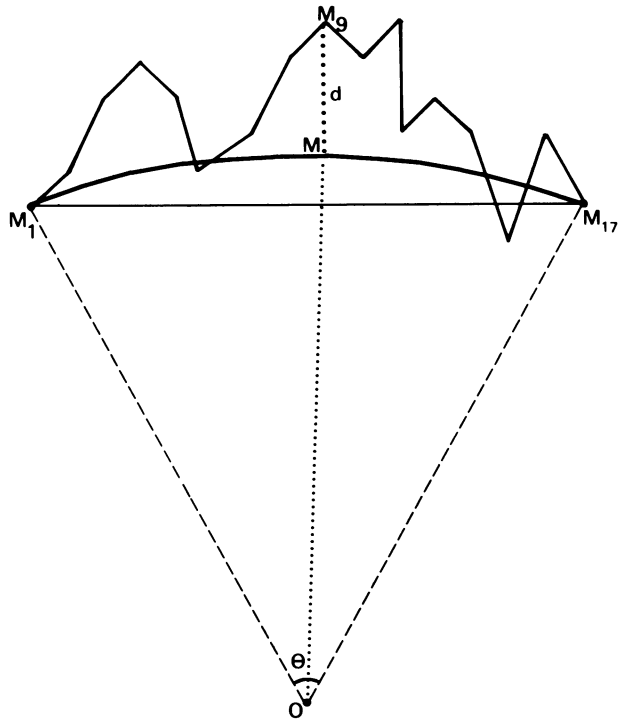

FIGURE 3 Contour analysis. A portion of digitized contour made of 16 segments (M_1M_2, M_2M_3 . . . $M_{16}M_{17}$) was compared with a circular line enclosing the same area with the straight segment M_1M_{17}. The relative length excess is the ratio between the contour length and the length of the reference line minus one. The deviation d is the distance between the middle point of the contour (M_9) and the middle of the reference line (M).

RESULTS

Deformability Measurements

22 individual cells were sucked into a glass micropipette with four sequential pressures of 200, 500, 1,000, and 2,500 Pa. The length of the cell protrusion was measured 15 s after the establishment of a given pressure. Our results are shown on Table I. In each experiment, the diameter of undeformed cells was measured, and this allowed proper determination of the relative area increase I (Eqs. 4 and 5). The effective membrane tension T was also calculated with Eq. 1 and the mean values of T and I corresponding to a suction pressure of 200, 500, 1,000, and 2,500 Pa were used to construct a "mean curve" by least-square determination of a second order polynoma with zero constant term, as shown in Fig. 5.

Contour Analysis

Macrophages were made to bind glutaraldehyde-treated erythrocytes and conjugates were processed for electron microscopy. A series of photomicrographs were digitized for numerical determination of surface roughness and distance between interacting surfaces. The sharpness of the digitization procedure is exemplified in Fig. 4. As shown on Table II, the erythrocyte-bound regions of the macrophage membranes were smoother than their free surface, since their relative excess length and standard deviation of deviation were ~50% lower ($P < 0.001$). Further, as expected, the roughness parameters of the erythrocytes were about 10-fold lower than those of macrophages.

It was tempting to speculate that the smoothing of the macrophage surface near the erythrocyte membrane was driven by the adhesive interaction between both surfaces. This point was addressed by studying the apparent distance between each point of the digitized contours of a series of macrophages and the surface of bound erythrocytes (approximated as a set of straight segments). 19 macrophage–red cell adhesions were studied. The total length of the macrophage contour segments that were separated from bound erythrocytes by more than 200, 500, 1,000, and 2,500 Å units (i.e., 0.56, 1.4, 2.8, and 7 mm on the photomicrograph) was determined in each case. The correlation between these parameters and the relative length excess E was calculated. As shown in Table III, the fraction of the macrophage contours separated from the erythrocytes by <500 or 1,000 Å units was significantly ($P < 0.01$) and negatively correlated to E.

It was of interest to know whether the parameters we measured were sensitive to the degree of affinity between macrophages and erythrocytes. Our experiments were therefore repeated with erythrocytes that were treated with

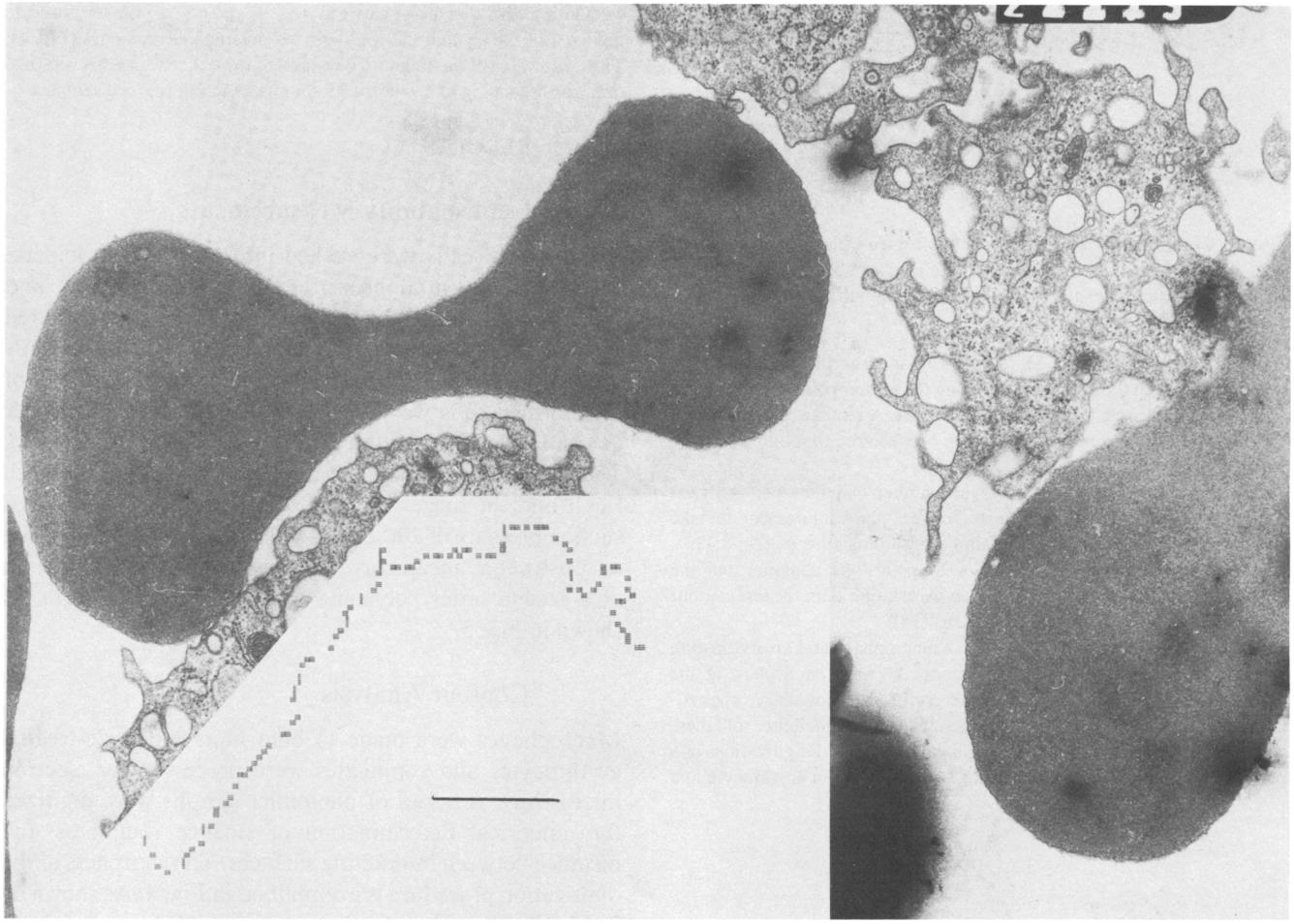

FIGURE 4 Digitization of cell contours. A representative macrophage–glutaraldehyde-treated erythrocyte interaction area is shown together with the digitized contour. Bar, 1 μm.

polylysine in addition to glutaraldehyde, to increase adhesion. Results are shown on Tables IV and V. A highly significant ($P < 0.01$) negative correlation was found between the percent length of bound macrophage contours that was <500 or 1,000 Å apart from attached erythrocytes and the relative length excess (Table V). Also, the relative length excess was significantly ($P < 0.01$) higher in free macrophage contours than in bound regions (Table IV).

Finally, as demonstrated by comparing Tables III and V, the average length of the portion of macrophage contours that was <2,500 Å apart from the erythrocyte surface was significantly higher when erythrocytes were coated with polylysine (5.2 μm versus 2.8 μm, $P < 0.01$ according to Student's t test).

TABLE I
DEFORMABILITY OF RAT PERITONEAL MACROPHAGES

	Length of the protrusion obtained after 15-s aspiration with a negative pressure of		
	200 Pa	500 Pa	1,000 Pa
Mean	2.9 μm	6.7 μm	9.2 μm
Standard error	0.4	0.4	0.7

19 rat peritoneal cells were aspirated into a micropipette of 3.5-μm inside diam with increasing pressure. 15 s after the establishment of a given pressure, the length of the cell protrusion was measured. Mean values are shown together with standard errors. The mean cell diameter was 10.2 ± 1.8 μm (SD). The same pipette was used throughout the series of measurements.

DISCUSSION

The quantitative results described in the present work may, in principle, yield some information concerning the affinity between the membranes of macrophages and glutaraldehyde-treated erythrocytes as well as the mechanism of macrophage shape control. However, extracting this information requires specific assumptions that we shall discuss before interpreting our experimental data.

Basic Assumptions

(*a*) We assume that the establishment of adhesive bonds between a macrophage and an erythrocyte membrane results in a free energy decrease that is the product of a

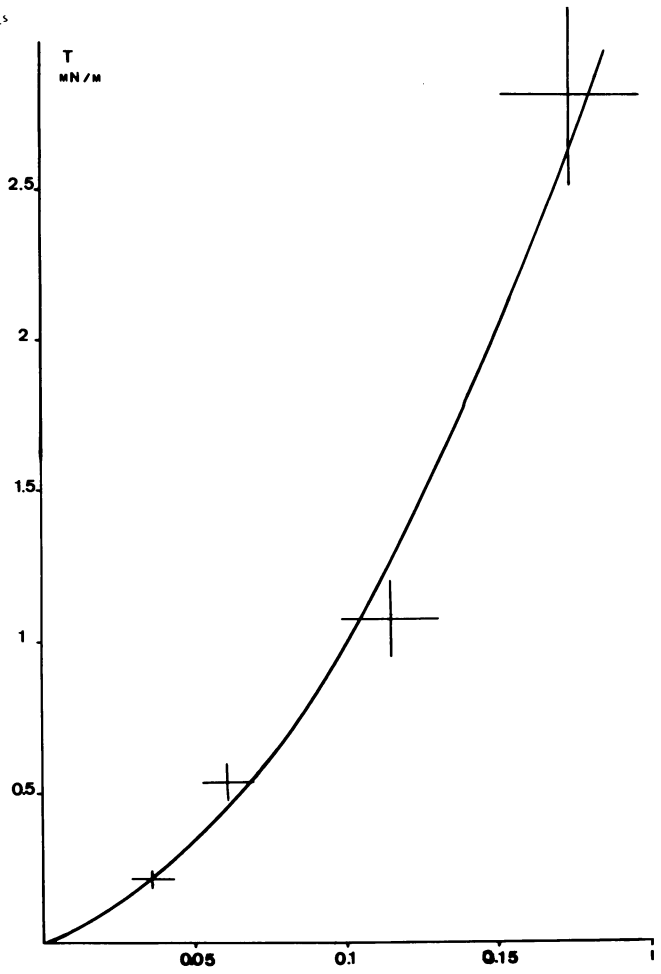

FIGURE 5 Relationship between membrane effective tension and apparent cell area. The diameter of individual cells was measured before aspiration into a micropipette with calibrated pressure. The length of induced protrusions was then determined and data were used to calculate the effective membrane tension (T) and the relative area increase of deformed cells (I). Each point represents the mean values obtained by pooling the data found with aspiration at 200, 500, 1,000, and 2,500 Pa, respectively, in a study using 22 cells. The full line is a least-square fit of experimental points with a second order polynoma with zero constant coefficient. The vertical and horizontal bar lengths represent twice the standard errors.

constant γ (representing the work of adhesion) and the real adhesive contact area A^r_{rc} (see Fig. 6 for notations). Although it is reasonable to assume that cell surfaces are bound when they are less than a few hundred angstroms apart (1, 2), there is no rigorous way of delineating the adhesive contact area on electron micrographs. Plotting the frequency distribution of distances between macrophage and erythrocyte membranes did not reveal any obvious "cutoff" value, allowing objective discrimination between adhering and nonadhering regions. Indeed, the equilibrium distance between actually interacting surfaces may display some local variations if the glycocalyx is heterogeneously distributed. Tentatively, we defined the adhesive contact area on a macrophage membrane as the set of points that were <500 Å apart from an erythrocyte surface.

The hypothesis of constant work of adhesion was not compatible with a dependence of γ on the contact area, in contrast with a previous report emphasizing the possibility that the work of adhesion might progressively decrease when the contact area increased, if adhesion was mediated by a limited amount of mobile ligand molecules (21). Clearly, this possibility may be less important in the present experimental model of fixed erythrocytes, since lateral diffusion is low on one of both interacting surfaces. Further, lack of experimental data prevented us from incorporating this possibility in our discussion.

(*b*) We assume that the contact area between a macrophage and a foreign surface is entirely determined by the balance between adhesive forces and cell resistance to deformation. The reality of such a mechanism is strongly supported by elegant experiments from Folkman and Moscona (22). When cells were plated on a series of substrates coated with varying amounts of the anti-adhesive polymer polyhydroxyethylmethacrylate, cell spreading increased when the thickness of the polymer layer decreased.

(*c*) We assume that the energy required to produce a given cell deformation is some function of the concomitant increase of the apparent cell area A^a, i.e., the area calculated after neglecting electron microscopical details (as

TABLE II
MORPHOLOGICAL PROPERTIES OF CONJUGATES MADE WITH MACROPHAGES AND GLUTARALDEHYDE-TREATED ERYTHROCYTES

	Macrophage						Erythrocyte		
	Free membrane			Bound area			Bound area		
	$L(\mu m)$	E	D	$L(\mu m)$	E	D	$L(\mu m)$	E	D
Mean value	8.7	0.33	0.078	3.1	0.147	0.035	3.6	0.01	0.01
Standard error	1.4	0.03	0.008	0.56	0.03	0.006	0.6	0.002	0.001
No. of determinations		11			19			7	

Macrophages binding glutaraldehyde-treated erythrocytes were studied with electron microscopy. Free (macrophages and erythrocytes) and bound (macrophages) regions of the cell membranes were analyzed. Only contours of length higher than 1 μm were retained. The length L of each contour, the relative length excess (E), and standard deviation of deviation (D) were calculated. Mean values and standard errors are shown. The mean length of bound erythrocyte contours was 2.1 μm.

TABLE III
CLOSENESS OF INTERACTION BETWEEN MACROPHAGES AND GLUTARALDEHYDE-TREATED ERYTHROCYTES

	Length of the portion of the macrophage contour separated from bound erythrocyte by a distance lower than							
	200 Å		500 Å		1,000 Å		2,500 Å	
Mean value	0.37 μm	(28%)	1.3 μm	(51%)	2.1 μm	(78%)	2.8 μm	(100%)
Standard error	0.12	(11)	0.29	(6)	0.38	(4)	0.53	
No. of measurements	19		19		19		19	
Correlation with E	−0.20	0.18	−0.35	−0.65	−0.15	−0.79	0.29	
	NS	NS	NS	$P < 0.01$	NS	$P < 0.01$	NS	

19 regions of interaction between a macrophage and a glutaraldehyde-treated erythrocyte were studied on 10 random photomicrographs. The cell contours were digitized and the distance between each point of the macrophage digitized contour and the erythrocyte boundary (approximated as a series of straight segments) was calculated. The total length of the segments of macrophage contours that were separate from bound erythrocytes by a distance lower than 200, 500, 1,000, or 2,500 Å was determinated, and results were expressed as micrometers or percent of the total contour length up to 2,500-Å separation. Means and standard errors are shown. Also, the correlation coefficients between these parameters and the relative length excess of the macrophage contour were calculated. NS means, not significant at the 0.05 confidence level.

shown on Figs. 1 and 2). This assumption is supported by recent micropipette aspiration experiments reported by Evans (14), who proposed a representation of human leukocytes as liquid drops surrounded by a "contractile surface carpet" (14). An apparent area increase may be managed by smoothing the cell surface, with microvilli acting as sources of reserve membrane, in accordance with quantitative electron microscopic analyses (16, 17). The energetics of cell deformation may thus be studied by considering cells as surrounded with an elastic membrane corresponding to their apparent (or light microscopic) surface and with an effective tension T. T is a function of the membrane "stretching" that is related to the ratio between the real membrane area A^r (that can be estimated with electron microscopy) and the apparent membrane area A^a (estimated on light micrographs).

(*d*) An important approximation is to identify the relative length excess E measured on electron micrographs and the relative area excess calculated by comparing the real cell area to the apparent area, which yields

$$A^r = A^a \cdot (1 + E). \quad (6)$$

This approximation is based on classical results from stereology (23) that may be rewritten as follows. If we cut solid bodies enclosed in a given box with random planes, the mean area of the sections of a given body is proportional to its volume, and the mean perimeter of these sections is proportional to its surface area. As a consequence, the mean ratio between the digitized contour length and the smooth reference line shown on Fig. 3 is equal to the mean ratio between the cell surface area and the area of a smooth surface enclosing the same volume.

However, asperities smaller than the distance between two consecutive points of the digitized contour (i.e., ~0.06 μm) did not appear on the digitized contour. Also, "blebs" with a radius of curvature higher than 1 μm did not contribute to the relative length excess because they increased the length of both the digitized cell contour and the reference line. Hence, the relative length excess we measured (E) represented the contribution to the membrane excess area of the cell surface asperities with a characteristic size ranging between ~0.06 and 1 μm, as previously stated (18).

(*e*) A final assumption that seems a reasonable consequence of the previous ones together with geometrical constraints is that in the area of contact between a macrophage membrane and a smooth erythrocyte area the former membrane displays a local smoothing (monitored by a decrease of the relative length excess E). Further, the ratio $X = A^r_{rc}/A^a_c$ between the real adhesive contact area and the apparent contact area is a function of E that depends on the fine membrane structure and possibly the adhesive mechanism.

We shall now confront qualitative and quantitative predictions of our model with experimental results.

TABLE IV
MORPHOLOGICAL PROPERTIES OF CONJUGATES MADE WITH MACROPHAGES AND POLYLYSINE + GLUTARALDEHYDE-COATED ERYTHROCYTES

	Macrophage free membrane			Macrophage bound membrane		
	L (μm)	E	D	L (μm)	E	D
Mean value	4.1	0.296	0.055	6.7	0.101	0.041
Standard error	0.25	0.057	0.005	1.5	0.031	0.017
No. of measurements	16			19		

Macrophages binding polylysine + glutaraldehyde-treated erythrocytes were studied with electron microscopy. Free and bound regions of the macrophage membrane were analyzed. Only contours of length higher than 1 μm were retained. The length (L) of each contour, the relative length excess (E), and standard deviation of deviation (D) were calculated. Mean values and standard errors are shown. The mean length of bound erythrocyte contours was 5.0 μm.

TABLE V
CLOSENESS OF INTERACTION BETWEEN MACROPHAGES AND POLYLYSINE + GLUTARALDEHYDE-TREATED ERYTHROCYTES

	Length of the portion of the macrophage contour separated from bound erythrocytes by a distance lower than						
	200 Å		500 Å		1,000 Å		2,500 Å
Mean value	1.51 μm	(31%)	4.13 μm	(80%)	4.79 μm	(92%)	5.2 μm
Standard error	0.21	(4)	0.44	(3)	0.53	(2)	0.6
No. of measurements	19		19		19		19
Correlation with E	−0.12	−0.29	−0.16	−0.58	−0.21	−0.74	0.37
Significance of correlation	NS	NS	NS	$P < 0.01$	NS	$P < 0.01$	NS

19 regions of interaction between a macrophage and a polylysine + glutaraldehyde-treated erythrocyte were studied on photomicrographs. The cell contours were digitized, and the distance between each point of the digitized macrophage contour and the erythrocyte boundary (approximated as a series of straight segments) was calculated. The total length of the segments of macrophage contours that was separated from bound erythrocytes by a distance lower than 200, 500, 1,000, and 2,500 Å was determined, and results were expressed as micrometers or percent of the total contour length up to 2,500 Å separation. Mean values and standard errors are shown. The correlation between these parameters and the relative length excess of the macrophage contour was calculated. NS means, not significant at the 0.05 significance level.

Qualitative Discussion of Experimental Data

First, our observation that micropipette-aspirated cells yielded large and stable projections when they were subjected to high negative pressures, and the finding of a fairly elastic relationship between cortical tension and apparent cell area (Fig. 5) are clearly at variance with Evans' report on the behavior of human granulocytes (14), since he reported constant flow of the latter cells for prolonged periods of time (14). Clearly, it might be worthwhile to study the significance of these differences and compare the behavior of both cell types during aspiration with pipettes of different diameter under varying pressures. Indeed, we observed some examples of prolonged growing of macrophage projections when larger pipettes were used (i.e., more than 4–5-μm diam).

Second, concerning macrophage-particle interaction, an immediate prediction of assumption (*b*) would be that the interaction area between macrophages and glutaraldehyde-treated erythrocytes be smaller than that observed with glutaraldehyde + polylysine-treated red cells, since polylysine was demonstrated to enhance erythrocyte uptake by macrophages (9). This was clearly found, since the mean length of the real contact area section (A_c^r) was significantly ($P < 0.01$ according to Student's t test) smaller when erythrocytes were not coated with polylysine (Table II, $L = 2.8$ μm) than when the polymer was used (Table V, $L = 5.2$ μm).

A second prediction was that the relative length excess of the macrophage membrane be smaller in contact areas than in free areas. This was indeed found with both particle types (Tables II and IV) with a confidence level of 0.01, according to Student's t test.

Another consequence of assumption (*e*) would be the occurrence of a negative correlation between the relative length excess E_c of the macrophage membrane in contact areas and the fraction X of the membrane actually involved in adhesive interactions (i.e., A_{rc}^r/A_c^r). This was indeed found at the 0.01 confidence level (Tables III and V) if membranes were considered as adherent when they were <500 Å apart, as explained above.

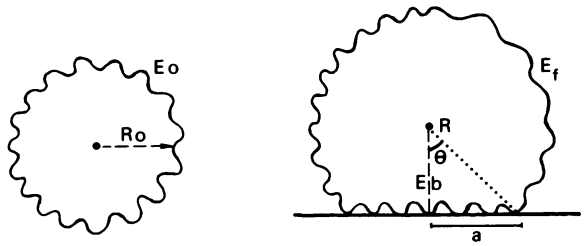

FIGURE 6 General model for cell deformation. Cells were modeled as spheres of radius R_o deforming with constant volume and real area. A_c^a is the apparent contact area (it is πa^2 for a flat disk of radius a). A_c^r is the real contact area, i.e., the electron microscopical area of the membrane portion located in the apparent contact zone. A_{rc}^r is the real adhesive contact area, i.e., the electron microscopical area of the possibly discontinuous patches of the macrophage membrane adhering to an erythrocyte. E is the relative length excess. Subscripts o and f refer to the surface of isolated cells and the free surface of erythrocyte-bound macrophages, respectively.

Quantitative Evaluation of the Work of Adhesion by Combination of Electron Microscopy and Micropipette Experiments

We follow the approach described by Evans (24) by considering a virtual increase dA_c^a of the apparent contact area: the potential decrease in free energy dF is just balanced against the potential increase in elastic energy dE_{el} due to the virtual deformation of the cell surface. However, the first term is the work of adhesion parameter γ multiplied by the real (not apparent) adhesive contact area A_{rc}^r (see Fig. 6), and the elastic energy E_{el} is the sum of (*a*) the energy required to stretch the membrane to man-

age an apparent area increase, and (*b*) the energy required to smooth the membrane in the binding area to achieve sufficient adhesive real contact area:

$$dF + dE_{el} = 0. \quad (7)$$

An evaluation of the above quantities remains. If the apparent contact area A_c^a increases by dA_c^a with constant relative length excess E_c, the real contact area A_c^r will increase by

$$dA_{rc}^r = X(E_c) \cdot dA_c^r = X(E_c) \cdot (1 + E_c)dA_c^a,$$

where $X(E_c)$ is the fraction of the real macrophage membrane area actually involved in adhesion. The free energy variation due to binding is therefore

$$dF = -\gamma \cdot X(E_c) \cdot (1 + E_c) \cdot dA_c^a. \quad (8)$$

As discussed above, X was calculated as the fraction of the real (i.e., electron microscopic) membrane area that was <500 Å apart from erythrocytes. A choice of 200 Å instead of 500 would have yielded about twofold lower values of X (Tables III and V).

The elastic energy may be calculated as follows. Let us consider a membrane region of real area A^r. In the absence of any mechanical force, the relative length excess is E_o, and the apparent area A_o^a is $A^r/(1 + E_o)$ in view of the aforementioned stereological results. The elastic energy may be defined as zero.

After stretching, the relative length excess is E and the apparent area A^a is

$$A^a = A^r/(1 + E).$$

Hence, the relative apparent area increase I is related to E by the formula

$$I = (A^a - A_o^a)/A_o^a = (E_o - E)/(1 + E). \quad (9)$$

Further, denoting as $T(I)$ the effective tension corresponding to the apparent area increase I, the elastic energy E_{el} is

$$E_{el} = \int_0^I T(I) \cdot dA^a(I) = A^a \cdot J(I), \quad (10)$$

where

$$J(I) = \left[\int_0^I T(I) \cdot dI\right]/(1 + I). \quad (11)$$

$J(I)$ may be evaluated by numerical integration of the function $T(I)$ shown in Fig. 5. The total elastic energy of a macrophage binding an erythrocyte is therefore (see Fig. 6 for notations)

$$E_{el} = J[(E_o - E_c)/(1 + E_c)] \cdot A_c^a + J[(E_o - E_f)/(1 + E_f)] \cdot A_f^a. \quad (12)$$

To complete our determination of γ with the balance equation, we need to evaluate dA_f^a/dA_c^a and dE_f/dA_c^a. The former parameter may be obtained with elementary geometrical calculations, modeling macrophages as truncated spheres deforming with constant volume. The latter derivative may then be obtained by assuming constant real area

$$d/dA_c^a[A_c^a \cdot (1 + E_c) + A_f^a \cdot (1 + E_f)] = 0. \quad (13)$$

Detailed calculations are described in the Appendix. The obtained work of adhesion is

$$\gamma = 8.4 \times 10^{-5} \pm 4.8 \times 10^{-5}\ J/m^2$$

(glutaraldehyde-treated erythrocytes)

$$\gamma = 18.2 \times 10^{-5} \pm 8.7 \times 10^{-5}\ J/m^2$$

(glutaraldehyde + polylysine-treated erythrocytes).

The values obtained for both erythrocyte samples are not significantly different, due to the low accuracy of the experimental determination of parameter E. These values are about 10-fold higher than the affinity between dextran-coated erythrocytes (7) and 100-fold higher than the affinity between erythrocytes coated with limiting amounts of lectins (25).

Admittedly, our approach is subject to several pieces of criticism. First, glutaraldehyde fixation might change intercellular distances and cell asperities. However, this possibility is not supported by the earlier finding that electron microscopic measurement of the distance between membranes bound by molecules of known size such as immunoglobulins (26) or dextran (27) displayed reasonable values after processing for electron microscopy. Also, glutaraldehyde treatment was reported not to modify membrane protrusions, as observed with phase-contrast microscopy (28).

Another important point is the possibility that the fine structure of the cell surface render untenable the comparison between large scale deformations induced by pipette aspiration and local deformation of microasperities. Indeed, the existence of a submembranal network with a mesh size of ~0.05 μm (29) would make it very dangerous to use similar material parameters to analyze cell deformations of, say 0.5- and 0.05-μm amplitude. Further experiments are required to prove that apparent cell surface area increases are mainly limited by local resistance to smoothing. We plan to address this point in our laboratory by comparing the effects of mechanical and adhesive forces on cell surface roughness.

Third, a problem might result from a low reversibility of the adhesive process. Indeed, when Evans and colleagues used micropipette experiments to study the agglutination of erythrocytes coated with wheat germ agglutinin, they found that adhesion was not a reversible process, since after contact occurred cell separation seemed to require greater work than that involved in bond formation (30). The contact areas we measured might thus be a consequence of centrifugation-induced cell deformations that would be maintained by adhesive forces. However, this

possibility is unlikely since: (*a*) Comparing the contact areas between co-centrifuged macrophages and particles consistently revealed a significant dependence of this area on the binding mechanism (9, 31, 32). (*b*) Studying the deformations induced by centrifuging cells on plane substrates (13) revealed that the minimal centrifugal force required to induce detectable cell flattening was about 10-fold higher than that used in the present experiments.

Finally, our main conclusion is that cell surface roughness is a potentially important parameter of cell adhesiveness and perhaps deformability. The quantitative approach we describe makes this parameter amenable to experimental study.

APPENDIX

Quantitative Determination of the Work of Adhesion

Calculation of the Elastic Energy. The function $J(I)$ defined in Eq. 11 was evaluated by numerical integration of the function $T(I)$ shown in Fig. 5. This was performed by using a least-square method to fit the experimental points with a second order polynoma passing through the origin, which yielded

$$T(I) = 0.0664 \cdot I^2 + 0.00355 \cdot \mathrm{I}\ (\mathrm{Newton/m}),$$

as shown in Fig. 5. The agreement between the obtained curve and experimental points was quite reasonable.

Now, using Eq. 12, the variation of the elastic energy reads

$$\begin{aligned} \mathrm{d}E_{el}/\mathrm{d}A_c^a &= J[(E_o - E_c)/(1 + E_c)] \\ &\quad + J[(E_o - E_f)/(1 + E_f)]\mathrm{d}A_f^a/\mathrm{d}A_c^a \\ &\quad - \ldots J'[(E_o - E_f)/(1 + E_f)]A_f^a \cdot (1 + E_o)/(1 + E_f)^2. \quad (14) \end{aligned}$$

There remains to obtain a numerical estimate for E_f, A_f^a, $\mathrm{d}E_f/\mathrm{d}A_c^a$, and $\mathrm{d}A_f^a/\mathrm{d}A_c^a$. This was done by using the truncated sphere model shown on Fig. 6. First, the apparent contact area A_c^a was evaluated by using the mean length L of the apparent contact region between macrophages and erythrocytes on photomicrographs (measured along the smoother erythrocyte surface). The radius a of the model contact disc was calculated with the formula

$$a = 2 \cdot L/\pi. \quad (15)$$

(This is the relationship between the radius of a circle and the length of a random chord [31].)

Using the values of 2.1 and 5 μm for T (Tables II and IV), the mean apparent contact areas with glutaraldehyde-treated and glutaraldehyde + polylysine-modified erythrocytes were 5.6 and 31.8 μm^2, respectively. Using elementary geometrical formulae and a computer-calculated table of A_c^a and A_f^a corresponding to different degrees of cell spreading, the respective apparent free areas were 321 and 299 μm^2. Further, the apparent total area increase of bound cells was not higher than 1% as compared with spheres of identical volume. Since A_f^a/A_c^a was much higher than 1, it is concluded that E_f was not very different from the relative length excess E_o of undeformed cells, and a tentative value of 0.31 seemed reasonable (Tables II and IV). $J[(E_o - E_f)/(1 + E_f)]$ and $J'[(E_o - E_f/1 + E_f)]$ were thus found to vanish, and Eqs. 8 and 14 yielded

$$\gamma = J[(E_o - E_c)/(1 + E_c)]/[X \cdot (1 + E_c)]. \quad (16)$$

This equation could be used to estimate the accuracy of affinity estimates, ascribing the major error to the limited accuracy of the determinations of X and E_c, which yielded

$$\delta\gamma = \{[(J + (1 + E_o)J')/(1 + E_c)]^2\delta E_c^2 + [(\gamma/X)\delta X]^2\}^{1/2}, \quad (17)$$

where J and J' are the numerical values of the function J and its derivative at point $(E_o - E_c)/(1 + E_c)$. The standard errors of E_c and X (Tables II–V) were used to obtain an error estimate $\delta\gamma$ for the affinity determination.

The electron microscopical work used in the present study was done in the laboratory of Dr. A. Ryter (Institut Pasteur, Paris) and qualitative results were described in a previous report (9).

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