Interaction Energies in Lectin-induced Erythrocyte Aggregation

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ABSTRACT Two *N*-acetylgalactosamine-reactive lectins, *Helix pomatia* (HPA) and *Dolichos biflorus* (DBA), were used to study the energies involved in cell-cell interactions through the specific binding of these lectins to their membrane receptors on genotype AO human erythrocytes (red blood cells) (RBCs). The energy required to dissociate a unit of aggregated membrane area (γ_d) of two RBCs bridged by lectin molecules was determined from the shear force needed to dissociate two-cell aggregates in a flow channel. When HPA were used as bridging molecules, γ_d (0.4×10^{-4} to 3.8×10^{-4} dyn/cm) was proportional to the density (D = 175 to 1,060 molecules/μm^2) of HPA molecules bound on the RBC membrane. A similar γ_d/D ratio was also obtained for DBA. These results indicate that the number of lectin molecules bound on the interface plays an important role in determining the energy required for cell-cell dissociation.

The aggregation energy per unit membrane area (γ_a) in lectin-induced aggregates was calculated from the degree of encapsulation of a lectin-bound, heat-sphered human RBC by a normal discoid RBC. A minimum of ~1,800 HPA molecules/μm^2 on the spheres was required to form stable aggregates with the RBC. By using spheres having a surface HPA density of 1,830 to 2,540 molecules/μm^2, or $1.1-1.5 \times 10^{12}$ combining sites/cm^2, the γ_a value for HPA-induced aggregation was found to be 2.2×10^{-3} dyn/cm. This higher value of γ_a than γ_d has been explained on the basis of several differences in aggregation and disaggregation processes. The γ_a value for DBA-induced aggregation was not obtainable by the sphere encapsulation method because of the relative low D values. A comparison of the present results with the published value of the free energy change of 5 kcal/mol for the interactions of HPA and DBA with their ligands suggests that only a small fraction of the lectin molecules bound to RBC surface participate in the bridging of adjacent cells.

The measurements of the force or energy involved in cell-cell interaction have significant implications in biological and medical sciences (1, 2). There have been recent advances in making quantitative determinations on the intercellular adhesion energy, especially in reference to human erythrocyte (red blood cell) (RBC)[1] aggregates induced by macromolecules (3–5). Use of RBCs is preferred because the biochemical and biophysical properties of these cells and their membranes are well characterized (6–9), their geometry is well established (10), they are relatively easy to obtain, they are mainly dispersed in the normal circulation but can be aggregated by plasma proteins and other macromolecules at low flow states (11, 12), and they may also be agglutinated specifically by anti-blood group antibodies, lectins, and viruses due to the presence of specific sugar residues on the cell membrane (13, 14).

The formation of rouleaux of human RBCs in dextran, a neutral polymer, and in fibrinogen has been studied experimentally and theoretically (15–17). RBC aggregation by fibrinogen or dextran is due to the nonspecific adsorption of these bridging macromolecules to the surface membrane (12, 17). In contrast, RBC agglutination by antibodies or lectins is

[1] *Abbreviations used in this paper:* DBA, *Dolichos biflorus* agglutinin or lectin; GalNAc, *N*-acetylgalactosamine; HPA, *Helix pomatia* agglutinin or lectin; PBS, phosphate-buffered saline, 0.001 M, pH 7.2; RBC, red blood cell of human genotype AO, unless stated otherwise; TSA, Tris-buffered saline containing 2.5 mg/ml of bovine serum albumin, pH 7.4; WGA, wheat germ agglutinin.

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a consequence of the specific fitting of membrane antigens or oligosaccharides to the combining sites of these multivalent macromolecules (18, 19). Such molecular complementarity enables noncovalent forces to act effectively at close range and results in specific and relative stable agglutination.

In this investigation, newly developed biophysical techniques were used to study the forces involved in the agglutination of human genotype AO RBCs by two blood group A–reactive lectins, namely those isolated from the albumin gland of the garden snail, *Helix pomatia* (HPA) (20, 21), and from the seed of horse gram, *Dolichos biflorus* (DBA) (22, 23). HPA (molecular weight ≃ 79,000) contains six homologous combining sites for carbohydrates; DBA (≃110,000) consists of four subunits, with two of them capable of binding carbohydrates. Both lectins precipitate blood group A substances and agglutinate group A RBCs specifically, with DBA being able to distinguish subgroup A_1 from other subgroups (24). Together with the experiments quantitating the binding of these HPA and DBA to their receptors on RBC membranes (25), the present investigations provide an experimental model system for studying the energies involved in cell–cell interactions through the mechanism of specific ligand–receptor recognition.

MATERIALS AND METHODS

Materials

HPA, ferritin-conjugated HPA, and DBA were purchased from E.Y. Laboratory (San Mateo, CA).

Flow Channel Technique

The flow channel technique used to determine the forces required to separate RBC rouleaux has been described in detail previously (3). A geometrically defined channel was created by sandwiching a 100-μm thick teflon sheet, with a cut-out of 0.9 × 5.5 cm, between a polyvinyl base and a cover glass (Fig. 1). The polyvinyl base had two apertures 5.0 cm apart, as inlet and outlet for the channel. The inlet was connected to a 20-ml syringe which was mounted on a servo-controlled infusion pump (Model 990, Harvard Apparatus Co., S. Natick, MA) with a continuously variable speed control. The pressure gradient (ΔP, in dyn/cm^2) between the inlet and the outlet was measured with a Sanborn differential pressure transducer (Hewlett-Packard Co., Palo Alto, CA) while the fluid was pumped through the channel. The shear stress (σ, in dyn/cm^2) acting on the aggregates that had settled on the base of the channel was calculated from the pressure gradient and the channel geometry (height [h] and length [L]) by the use of the following equation:

$$\sigma = (\Delta P)h/2L. \quad (1)$$

The shear stress may also be calculated from the flow rate (Q, in cm^3/s):

$$\sigma = 6\eta_0 Q/h^2 W, \quad (2)$$

in which η_0 is the viscosity of the fluid in Poise (dyn-s/cm^2), and W is the width of the channel in cm.

The height of the channel can be obtained by measuring the thickness of the teflon sheet with a caliper and may also be calculated by the following equation, derived from Eq. 1 and 2:

$$h = [12\eta_0 QL/W(\Delta P)]^{1/3} \quad (3)$$

These two methods of measuring h yielded results in excellent agreement. A 2% RBC suspension (0.5 ml) was mixed with 1.5 ml of the lectin solution (HPA or DBA) at a known concentration, and the mixture was kept at 4°C for 1 h before being introduced into the flow channel. 15 min were allowed for the RBCs to settle to the floor of the flow channel. A cell-free Tris-buffered saline solution containing 2.5 mg/ml of bovine serum albumin, pH 7.4 (TSA), was then pumped through the channel at a constant flow rate. The flow rate was increased in a stepwise fashion to achieve a shear stress range of 0.02–2 dyn/cm^2. Two-cell aggregates in the channel were observed under the microscope and photographed at each level of shear stress. The degree of disaggregation in response to shear flow was correlated with the shear stress acting on the aggregates.

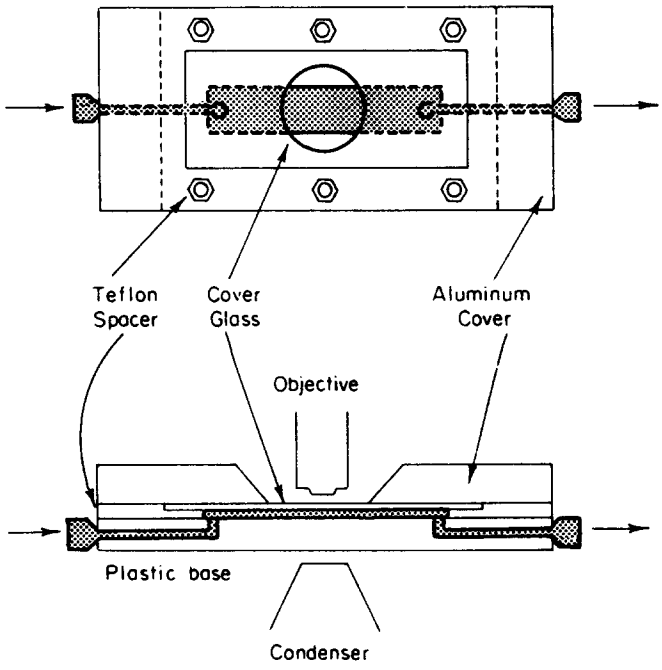

FIGURE 1 Schematic drawing of the flow channel for RBC disaggregation and deformability studies.

The same flow channel technique was also used to determine the membrane rheological properties of individual RBCs in the absence of aggregation. The RBC suspension, after having been reacted with a known amount of HPA, was introduced into the flow channel. A microscopic field covering ~10 single cells was chosen. Five levels of shear stress (0.65, 1.75, 3.0, 5.25, and 7.5 dyn/cm^2) were applied sequentially by increasing the pump rate in a stepwise fashion. Photographs of these single cells were taken at no flow and at each shear stress level. Each single cell was identified in the series of consecutive pictures. The length of the cell parallel to the shear direction was measured either manually or by using an electric caliper which is connected to a PDP 11/10 minicomputer for computational analysis. The extension ratio of the single cell was calculated as the ratio of the cell length under shear to that at basal (no flow) condition.

Preparation of RBCs and RBC Spheres for Sphere Encapsulation Studies

Fresh human genotype AO RBCs were washed three times with TSA solution. 2% RBC suspensions in polycarbonate test tubes (5 ml/tube) were heated at 53.5°C in a water bath for 5 min with constant agitation. The suspension, after the heat treatment, contained erythrocyte spheres of various sizes which can be examined under a light microscope as well as a scanning electron microscope. Samples were fixed in 1% glutaraldehyde in phosphate buffer, pH 7.4, and washed three times with distilled water. The air-dried samples were then coated with gold and palladium in a Hammer-I sputterer (Anatech Ltd., Alexandria, VA) and examined under a JEOL scanning electron microscope.

Determination of Lectin Binding to Heat-generated RBC Spheres

The binding of lectins on the erythrocyte spheres was studied by the quantitation of binding of tritiated lectins and the visualization of ferritin-conjugated lectins on membrane surface by transmission electron microscopy, and the results were compared with those on normal RBCs.

BINDING OF TRITIATED LECTIN: 50 μl of heat-treated (37, 45, 48, 49, 50, and 53.5°C) or control (25°C) RBC suspensions at 2% hematocrit were added to solutions containing 10 μl of ^{3}H-labeled HPA (17.3 μg/ml, specific activity = 8.5 × 10^4 cpm/μg) and 190 μl of phosphate-buffered saline (PBS) (0.001 M, pH 7.2). The detailed procedure of the binding assay is described in the previous paper (25). The mixture was kept at 4°C for 1 h with constant rotation, and then centrifuged. The pellets were washed twice with PBS,

dissolved in Soluene 350 and toluene-based scintillation fluid, and counted in a liquid scintillation counter (Siemens Gammasonics Inc., Des Plaines, IL).

BINDING OF FERRITIN-CONJUGATED LECTIN: 200 μl of a 2% RBC suspension, normal or heat treated (53.5°C), were mixed with 40 μl of ferritin-conjugated HPA (protein concentration = 0.4 mg/ml). After 2 h at 4°C, the specimens were fixed with 1% glutaraldehyde in phosphate buffer (pH 7.4) at room temperature, washed three times with Tris-buffered saline (pH 7.4), and postfixed with OsO_4 (1% in distilled water) at 4°C for 1 h. The specimens were washed twice with distilled water to remove OsO_4 before dehydration with a graded series of ethanol with concentrations ranging from 30 to 100%. Each step involved centrifugation and resuspension of the specimens in the next desired solution. Absolute ethanol was replaced sequentially by propylene oxide, a propylene oxide:Epon mixture (1:2), and pure Epon. The beam capsules containing specimens embedded in Epon were kept in an oven at 60°C for 5 d. Sections were made by using an MT2-B Ultra-microtome (Sorvall, DuPont Co., Newtown, CT), stained with uranyl acetate, and examined under a Zeiss transmission electron microscope.

Encapsulation of Lectin-coated Spheres by Normal RBCs

100 μl of heat-treated (53.5°C) RBC suspension at 2% hematocrit were added to known amounts of lectin solution (HPA or DBA) in a total volume of 400 μl, and kept at 4°C for 1 h. An aliquot of this suspension containing lectin-coated spheres was diluted with TSA solution in a small round chamber (10-mm diameter and 8-mm height) located on the stage of a Nikon inverted microscope (Ehrenreich Photo-Optical Industries Inc., Garden City, NY). After the spheres had settled to the chamber floor, the suspending medium was gently replaced by the lectin-free TSA solution with a syringe, without disturbing the settled spheres. This procedure was done five times to remove all free lectins, and 5 μl of a 0.5% normal RBC (genotype AO) suspension were then added to the chamber.

Micropipettes, with diameters from 1.0 to 1.5 μm, were prepared with the use of a micropipette puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan). The micropipette was filled with saline, first from the tip by capillarity, and then from the wide end with a fine needle and a syringe. The filled micropipette was mounted on a micromanipulator (Narishige Scientific Instrument Laboratory) with the wide end of the pipette connected to a pressure regulation system as described previously (8) (Fig. 2).

The RBC spheres and normal RBCs were viewed through the bottom of the chamber with the use of an oil lens objective (100×) and an eye-piece (20×). The image was also monitored and recorded with the use of a video camera and tape recorder system (Panasonic Co., Div. of Matsushita Electric Corp. of America, Franklin Park, IL). The micropipette tip was manipulated to be near the membrane surface of an RBC sphere with a diameter of 2 to 3 μm. A negative pressure was applied via the micropipette by adjusting the relative heights of two reservoir bottles, thus aspirating the sphere and holding it steady at the tip of the micropipette. The micropipette was then manipulated to move the lectin-coated cell sphere toward the dimple region of an adjacent normal RBC. When the sphere was sufficiently close to the RBC, the negative pressure was removed to release the sphere, thus allowing its interaction with the dimple region of the normal RBC. The process of encapsulation of the 2–3 μm sphere by the normal RBC (8 μm in diameter) was observed on the video monitor and recorded on videotape. During the replay of the videotape, the image of the aggregate was subject to further analysis as described below.

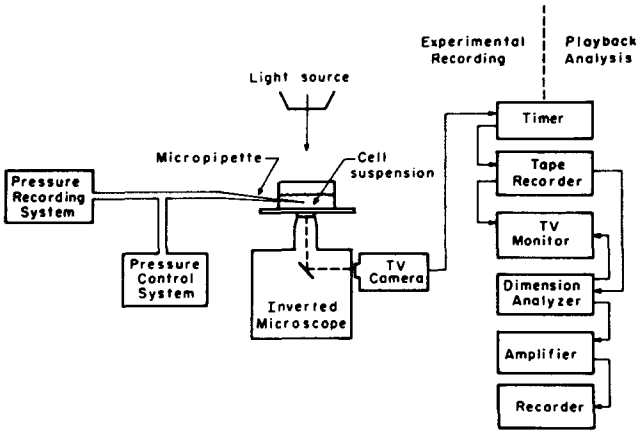

FIGURE 2 Schematic drawing depicts the set up for the micropipette experiment and for playback analysis.

RESULTS

Disaggregation Study with the Flow Channel Technique

Microscopic observations of human genotype AO RBC suspensions, treated with known amounts of HPA and introduced into the flow channel, revealed that the lectin-induced aggregates were of various sizes. Two-cell aggregates that stacked themselves in a top–bottom configuration, with the bottom cell adhered to the channel floor, were chosen for the disaggregation study. The transparency of this flow channel (height = 100 μm) made it possible to observe the process of disaggregation of these two-cell aggregates under shear. Upon applying the shear stress, the top RBC was pelled away from the bottom RBC, which remained stationary because of its adherence to the polyvinyl base forming the floor of the channel. As a result, a certain percentage of the initial interaction area (or agglutinated area) between the two cells was separated. With increasing shear stresses, the interaction area between the two cells progressively decreased as the area of separation increased. The relationship between the percentage of area separated and the shear stress is shown in Fig. 3.

Such stress–separation experiments were performed on aggregates having five different levels of lectin density on the RBC membrane, which was estimated from the binding study described in the previous paper (25). With the lectin concentrations used in the flow channel study, all the HPA molecules added were bound to the RBC membranes (25). The lectin

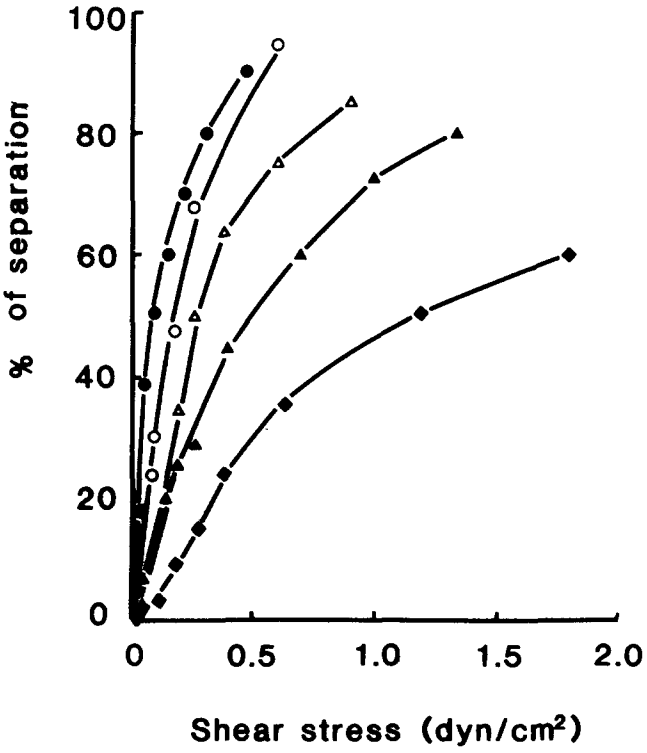

FIGURE 3 Separation of the two-cell aggregates in response to shear stress. The shear-separated area between two cells relative to the initial interaction area under no shear is expressed as percent of separation. Surface densities (molecules/μm²) of HPA molecules are as follows: ●, 175; ○, 350; △, 530; ▲, 720; and ◆, 1,060.

density (D, in molecules/μm^2) on the RBC surface can be estimated as follows:

$$D = [(B/mw) \times N]/(N_c \times A) \quad (4)$$

in which B is the total amount of lectin bound (in grams), mw is the molecular weight of the lectin (79,000 for HPA, and 110,000 for DBA), N is Avogadro's number (6.02×10^{23} molecules/mol), N_c is the total number of RBCs in the suspension, and A_c is the surface area of each RBC ($\simeq 150\ \mu m^2$).

As the surface density of the lectin molecules increased, there was a corresponding increase of the shear stress required to separate a certain interaction area of the aggregates. Fig. 4 shows the relationship between the lectin density on the cell surface and the shear stress required to separate 50% of the interaction area (σ_{50}).

The disaggregation energy (γ_d, dyn/cm), i.e., the energy required to disrupt a unit area of interaction between two cell membranes, can be derived from the work done by the shear stress as follows: the total disaggregation energy involved in separating 50% of the interaction area is equal to the disaggregation energy per unit area (γ_d) times the total area being separated (A_{50}). This total disaggregation energy is equivalent to the total work done. Work is measured as the product of the force (F) acting on the top cell and the distance through which the top cell moves (d). The force acting on the top cell is the force per unit area (shear stress, in dyn/cm^2) times the projected area of the top cell ($A \simeq 50\ \mu m^2$). At σ_{50}, the distance through which the top cell has moved is ~4 μm. The total work done is determined by integrating the area under the curve of the force applied ($\sigma_{50} \times A$) versus the distance moved (d_{50}). Since there is almost a linear relationship between the force ($\sigma \times A$) and d, the integral area under that curve would be close to one-half of the product of ($\sigma_{50} \times A$) and d_{50} (Fig. 5). Therefore,

$$\gamma_d \times A_{50} = \frac{(A \times \sigma_{50}) \times d_{50}}{2}. \quad (5)$$

Using the above mentioned values, Eq. 5 can be written as

$$\gamma_d(\text{dyn/cm}) \times 25\ \mu m^2 = \frac{50\ \mu m^2 \times \sigma_{50}(\text{dyn/cm}^2) \times 4\ \mu m}{2}, \quad (6)$$

or

$$\gamma_d(\text{dyn/cm}) = \sigma_{50}(\text{dyn/cm}^2) \times 4 \times 10^{-4}\ \text{cm}. \quad (7)$$

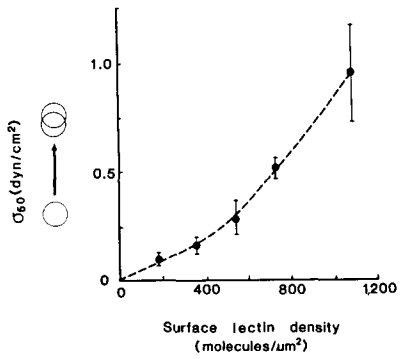

FIGURE 4 Relationship between the surface density of HPA molecules and σ_{50}, the shear stress required to separate 50% of the interaction area in two-cell aggregates.

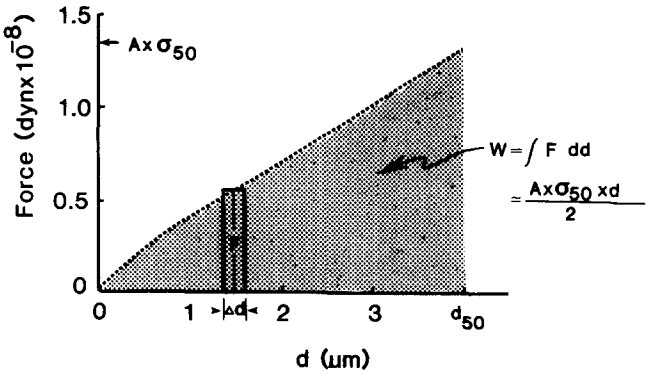

FIGURE 5 The work done by the shear stress (dyn/cm^2) between d = 0 and d_{50} is equal to the area bounded by the F-d curve from (0, 0) to (A × σ_{50}, d_{50}).

TABLE I. *The Disaggregation Energy (γ_d) and Aggregation Energy (γ_a) for HPA-induced Erythrocyte Aggregates*

Surface density	σ_{50}	γ_d	γ_a
molecules/μm^2	dyn/cm^2	dyn/cm	dyn/cm
175	0.10	0.4×10^{-4}	—
350	0.16	0.6×10^{-4}	—
530	0.27	1.1×10^{-4}	—
720	0.50	2.0×10^{-4}	—
1,060	0.95	3.8×10^{-4}	—
1,520	—	—	*
1,830	—	—	$2.4 \pm 0.2 \times 10^{-3}$
1,930	—	—	$1.4 \pm 0.9 \times 10^{-3}$
2,290	—	—	$2.8 \pm 1.6 \times 10^{-3}$
2,540‡	—	—	$2.1 \pm 0.6 \times 10^{-3}$

* Two out of three spheres studied did not agglutinate with normal RBC.
‡ Saturation level for HPA on human genotype AO RBC, equivalent to 1.8×10^{11} molecules/cm^2 or 1.1×10^{12} combining sites/cm^2.
—, cannot be determined by the method used.

The σ_{50} and γ_d values for HPA-induced aggregates with different surface lectin densities are listed in Table I. There is a direct correlation between γ_d and $D(r = 0.976)$ and the mean ratio of γ_d/D is 0.25 (SD = 0.07) $\times 10^{-14}$ dyn-cm/HPA molecule.

The shear stress–separation relationship for DBA-induced aggregates was also determined. A cell suspension containing 5×10^7 RBCs/ml was incubated with 0.18 μM of DBA at 4°C for 1 h before its loading into the channel. The surface density of DBA molecules on these cell membranes was estimated from the binding studies (25) and Eq. 4 was estimated to be 365 molecules/μm^2. The σ_{50} value was found to be 0.26 dyn/cm^2, corresponding to a σ_d value of 1.0×10^{-4} dyn/cm. The γ_d/D ratio is 0.29×10^{-14} dyn-cm/DBA molecule, which agrees well with the value for HPA.

The rheological properties of the membrane of individual RBCs with lectin moelcules bound on their surfaces was also examined by using the flow channel technique. Single RBCs adhered to the bottom of the channel were deformed to a teardrop shape by the action of shear stress during the fluid flow. The relationship between the extension ratio and the shear stress (Fig. 6) was used to assess the deformability of the RBCs (26). RBCs having 175 to 1,440 molecules/μm^2 of HPA bound on their surface exhibited the same deformability as the lectin-free normal RBCs. This indicates that the elastic property of the RBC membrane having bound HPA, with

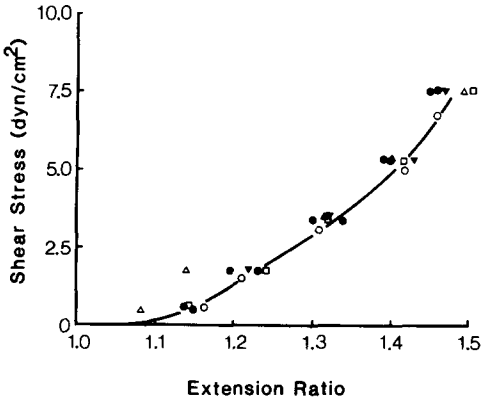

FIGURE 6 Relationship between the shear stress and the extension ratios of human RBCs with varying surface densities of HPA. O, control, no lectin bound; ●, 175 molecules/μm^2; □, 350 molecules/μm^2; △, 720 molecules/μm^2; ▼, 1,440 molecules/μm^2.

densities up to 1,440 molecules/μm^2, is not different from that of the normal RBC membrane.

Sphere Encapsulation Study with the Micropipette Technique

After heating an RBC suspension at 53.5°C for 5 min, a number of small membrane-bound spheres of various sizes spontaneously bud from the RBCs. The scanning electron micrograph in Fig. 7 shows some RBC spheres of 2 to 3 μm in diameter, as well as altered RBCs with other sizes and shape. The following experimental results indicate that the membrane of the heat-generated spheres retained the receptors for HPA present in the normal RBC membrane. First, the uptake of ^{3}H-labeled HPA by cell suspensions which had been heated at various temperatures, up to 53.5°C, was the same as that of the normal RBC suspension (Fig. 8). Second, transmission electron microscopy shows that the distributions of ferritin-conjugated HPA on the membranes of heat-treated RBCs, RBC spheres, and normal RBCs are similar (Fig. 9). Thus, the binding properties of normal RBC membranes for HPA as reported in the previous paper (25) are applicable to the sphere membranes.

A sphere of 2 to 3 μm in diameter, pretreated with a known amount of lectin, was aspirated with a micropipette and manipulated for agglutination with a normal RBC (Fig. 10). The receptors on the normal RBC, which had no lectin molecules present on the membrane, interacted with the lectin bound on the sphere and resulted in sphere–RBC agglutination.

The lectin density on the spheres appeared to be important in determining the formation of stable sphere–RBC aggregates. All spheres having 1,830 or more HPA molecules/μm^2 bound on their surface formed stable aggregates with normal RBCs. As the HPA density on the spheres was reduced to ~1,520 molecules/μm^2, two of the three spheres used did not form aggregates with normal RBCs.

Similar experiments were also carried out with DBA by mixing heat-treated RBC suspension with 2.7 μM of DBA, which resulted in the binding of DBA of ~1,600 molecules/μm^2 on the sphere membranes (25). Only three out of the ten attempts to bring such spheres into contact with individual normal RBCs resulted in the formation of stable aggregates; six of them failed to form an aggregate and one formed only a transient aggregate. No further experiment using higher concentration of DBA was carried out as 1,600 molecules/μm^2 is already very close to the saturation level of DBA on genotype AO RBCs (1,800 molecules/μm^2) (25).

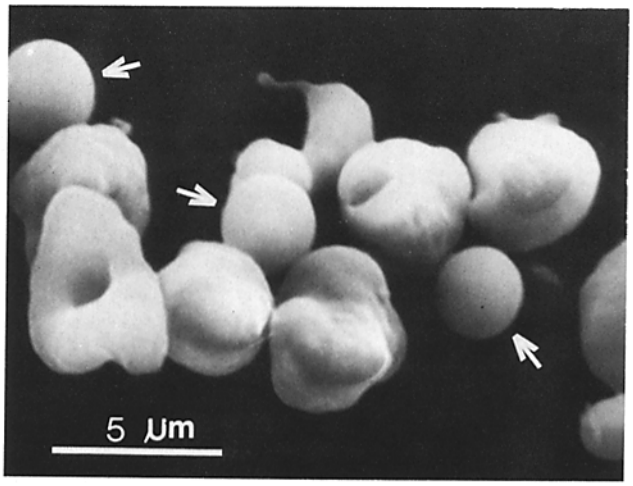

FIGURE 7 Scanning electron micrograph of RBC spheres (arrow) generated by heating normal RBC suspension at 53.5°C for 5 min.

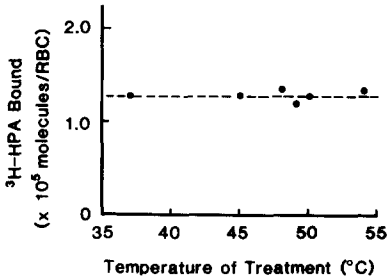

FIGURE 8 The binding of ^{3}H-labeled HPA on RBC surface membrane after heat treatment of a genotype AO RBC suspension at various temperatures. Dotted line indicates the control level of binding by cells without heat treatment.

It seems that a lectin density of ~1,800 molecules/μm^2 or more on a sphere would be needed to form stable aggregates with normal RBCs. The HPA molecules consist of six identical subunits, each containing a combining site for GalNAc or related structures. A surface density of 1,800 HPA molecules/μm^2 (or 1.8×10^{11} molecules/cm^2), which is equivalent to ~1.1×10^{12} combining sites/cm^2 for this hexavalent lectin, is the lower boundary for the formation of stable sphere–RBC aggregates and sphere encapsulation.

From the degree of encapsulation of the sphere by the normal RBC and the lectin density on the sphere, the energy involved in RBC aggregation induced by the lectin–receptor interaction can be calculated. This is based on the principle that the surface interaction in RBC aggregation involves a reduction of free energy per unit area of the interface (4). The equation derived by Evans and Buxbaum (4) to calculate the aggregation energy per unit membrane area (γ_a, or membrane affinity, in dyn/cm) is as follows:

$$\frac{\gamma_a}{\mu} = [x^2/(1 - x)] - x \ln(1 - x), \quad (8)$$

where μ is the elastic modulus of the normal RBC membrane (in dyn/cm); $x = Z_c/2R_s$ is the degree of encapsulation, in which Z_c is the height of the portion of the sphere embedded

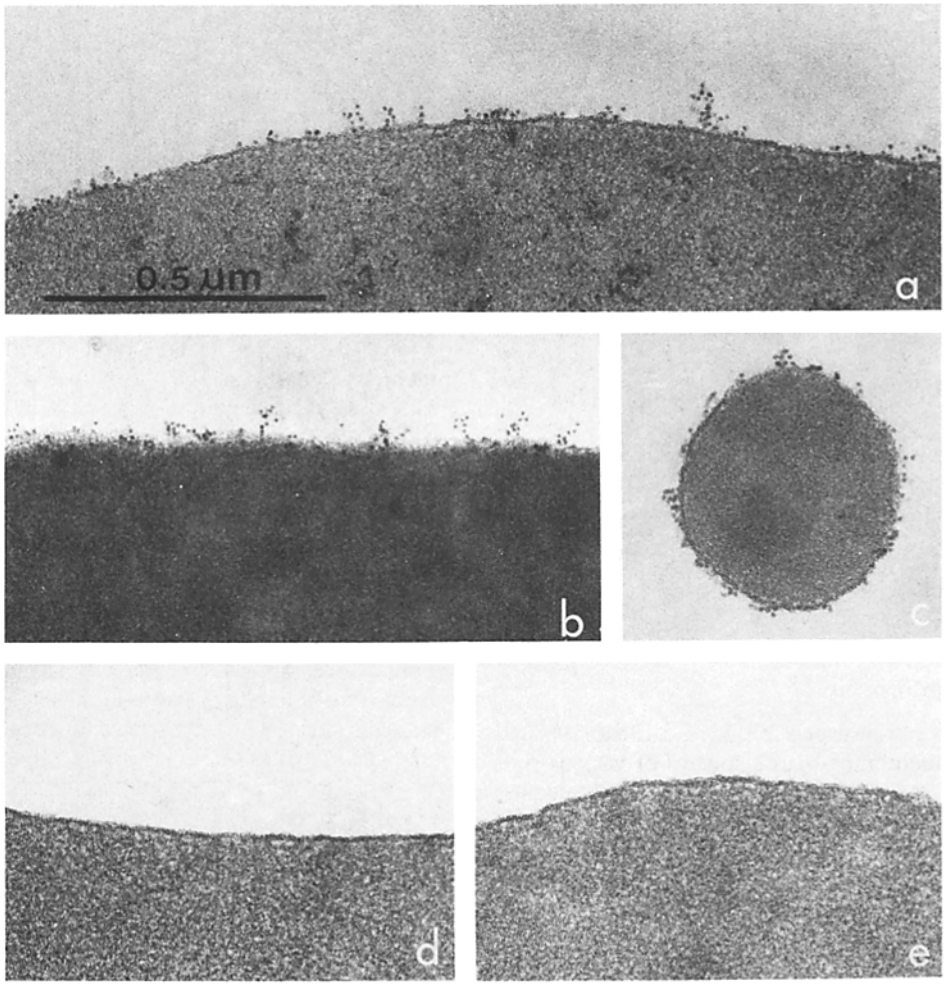

FIGURE 9 Transmission electron micrographs show the binding of ferritin-conjugated HPA on RBC membranes. (*a*) Normal genotype AO cell. (*b*) Heat-treated genotype AO cell. (c) Heat-generated genotype AO RBC sphere. (*d*) Normal OO cell. (*e*) Heat-treated OO cell. × 74,000.

in the normal RBC (in μm); and R_s is the radius of the sphere (in μm).

Based on this analysis, the degree of encapsulation, i.e., $Z_c/2R_s$, and the elastic modulus of the RBC membrane are the two parameters needed for the calculation of γ_a. $Z_c/2R_s$ can be obtained by tracing the image of the sphere–RBC aggregate on the video screen when the videotape is replayed for single frame analysis (Fig. 10). The elastic modulus of the normal RBC membrane has been determined with the use of the micropipette aspiration technique to be 4.2×10^{-3} dyn/cm (8) to 6.6×10^{-3} dyn/cm (9). The elastic modulus of the RBC membrane may also be calculated from the extension ratio of individuals RBCs found in the flow channel study (26). Calculation of γ_a values for HPA-induced aggregation from experiments using RBC spheres with surface densities greater than 1,800 molecules/μm^2 yielded values of 1.4×10^{-3} to 2.8×10^{-3} dyn/cm (Table I). These γ_a values do not show a correlation with the surface HPA density, probably because the surface densities used were in a very narrow range near the saturation level. The mean γ_a value of HPA-induced aggregation obtained from all these experiments is 2.2 (SD 0.6) $\times 10^{-3}$ dyn/cm, and the mean γ_a/D ratio is 1.02 (SD 0.29) $\times 10^{-14}$ dyn-cm/molecule.

DISCUSSION

The energy involved in cell–cell association and dissociation in an experimental system, i.e., human RBCs agglutinated by lectin molecules, was quantitated by using the flow channel and micropipette techniques. The shear stress needed to separate HPA-induced two-cell aggregates was found to be proportional to the surface density of the lectin molecules. Interestingly, when the surface densities of HPA and DBA were comparable, similar values of disaggregation energy (γ_d) were obtained for these two lectins; however, to achieve a similar surface density, the bulk concentration of DBA needs to be much higher than that of HPA (25). In the sphere encapsulation experiments using the micropipette technique, the formation of stable aggregates requires a surface lectin density of ~1,800 molecules/μm^2, and this threshold was about the same for both HPA and DBA. These findings suggest that the number of lectin molecules bound to the cell membrane surface plays an important role in determining the cellular aggregation and disaggregation energy.

The choice of using different ranges of HPA densities in the flow channel (175–1,060 molecules/μm^2) and the sphere encapsulation studies (1,830–2,540 molecules/μm^2) was dictated by methodological limitations. In the flow channel

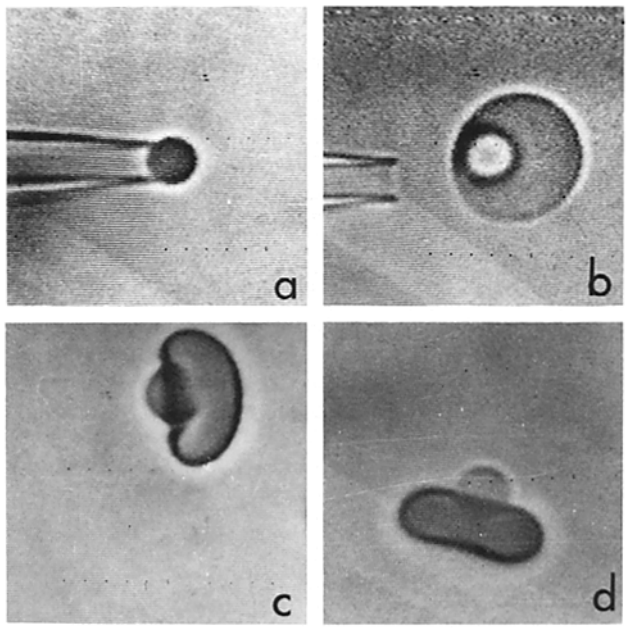

FIGURE 10 Encapsulation of a lectin-coated RBC sphere by a normal RBC. (*a*) An RBC sphere of 2–3 μm in diameter was aspirated by a negative pressure applied via the micropipette. (*b*) The sphere was brought close to the normal RBC, and the negative pressure was released to free the sphere which was allowed to agglutinate with the normal RBC. (*c* and *d*) The aggregates with different degrees of encapsulation.

technique, the large shear stresses required to disaggregate the agglutinates with high interaction energy would detach the bottom cell from the floor of the channel; hence this method is more suitable for studies at low levels of interaction energy, in the order of 10^{-5} to 10^{-4} dyn/cm. In the sphere encapsulation technique, in order to obtain a sphere-RBC aggregate, a higher level of interaction energy ($\sim 10^{-3}$ dyn/cm) is needed.

For HPA, the disaggregation γ_d/D ratio derived from the flow channel studies was 0.25×10^{-14} dyn-cm/molecule, whereas the aggregation γ_a/D ratio derived from the sphere encapsulation experiment was 1.02×10^{-14} dyn-cm/molecule. There are many possible reasons for this difference. One possible explanation is that the membrane strain energy stored as the cell changes its shape during aggregation is released during the shearing to help the disaggregation process (27), thus requiring less work done by the shear stress to separate the aggregates. The shear stress applied tangentially to the cell surface may also result in a mechanical advantage in disaggregation (27) and hence reduces the force requirement. Furthermore, the processes of aggregation and disaggregation may not be symmetrical. In addition, recent studies by Evans and Leung (28) have shown that the disaggregation of RBC agglutinates induced by wheat germ agglutinin (WGA) may involve the planar displacement of the bridging molecules. In our flow channel studies, the percent of area separation increases in proportion to the applied shear stress (Fig. 3). This is inconsistent with a constant adhesive energy density, in which case there should be no separation below a threshold level of shear stress and the top cell shall "fly" away above this level. Our finding of increasing work required to cause additional separation (see also reference 3) supports the notion that the bridging lectin molecules are collected at the leading edge of the separation (28). Finally, methodological differences such as the study of different ranges of surface concentration and the use of RBC spheres may also contribute to the discrepancy between γ_d/D and γ_a/D. Further studies are needed to more fully explain these findings.

The binding of lectins to the appropriate sugar residues on the membrane receptors is not covalent linkage and may involve the weaker noncovalent interactions such as ionic interaction, hydrogen bonding, hydrophobic interaction and Van der Waals interaction (18). For HPA, the free energy changes in its interaction with ARL 0.52[2] or GalNAc are both 5.04 kcal/mol (20, 21). For DBA, its K_a with GalNAc has been found to be 3×10^{-3} liters/mol (23), and theoretical calculation of the free energy change in the interaction between DBA and its ligand would also be ~5 kcal/mol. An interaction energy of 5 kcal/mol would correspond to 34×10^{-14} dyn-cm/molecule. This value is much higher than the ratio of the aggregation or disaggregation energies to the surface density of lectin molecules (γ_a/D or γ_d/D, respectively) found in the present study. These results suggest that probably only a small fraction (perhaps 3%) of the bound lectin molecules participate in bridging the two adjacent cell surfaces, whereas the majority of lectin molecules bind only to one cell surface. This is not impossible considering the restricted three-dimensional distribution of the lectin receptors on the glycoproteins or glycolipids on the cell membrane, which would reduce the probability of a lectin molecule bound on one cell surface to react with another lectin receptor on the adjacent cell. The fact that the receptors on human RBCs (genotype AO) for HPA or DBA may not necessarily all have the same structure as ARL 0.52[2] or GalNAc, with which the K_a values were determined, may also partially account for the relatively low values of γ_d and γ_a. In addition, it should be noted that the measured γ_a and γ_d values are the algebraic sum of the binding energy and the electrostatic repulsive energy exerted between the two adjacent membranes. Thus, it is reasonable to have a lower value of γ_d or γ_a than the free energy change derived from lectin–ligand interaction in the neutral solution. Since *N*-acetylneuraminic acid is the major negatively charged molecule on the surface membrane, further investigations on lectin-bound RBCs before and after neuraminidase treatment could provide additional insights into the energy balance in these lectin-induced cell–cell interactions.

The adhesive energy for RBC aggregation induced by WGA was reported to be as high as 1 dyn/cm (28) which is about three orders of magnitude greater than the γ_a or γ_d value found for HPA in this study. While WGA reacts with *N*-acetylneuraminic acid and glcNAc, HPA reacts with GalNAc and galactose on the cell surface. Therefore, their interaction energy may not necessarily be the same. It is interesting to note that the maximum number of WGA that could bind to RBCs may be as high as $1–2 \times 10^7$ molecules/RBC (29), which is nearly two orders of magnitude higher than the value of 3.8×10^5 molecules/RBC we found for HPA (25). Therefore, the ratio of adhesion energy to surface density may not differ that much between WGA and HPA; in this connection we should mention again that probably only a fraction of lectin molecules bound to the RBC membrane surface actually participate in bridging. In addition, the motion of the force applied in WGA and HPA experiments was different; in our shear separation experiment, the force applied was

[2] ARL 0.52 is a blood group A active pentasaccharide with a structure of α-D-GalNAc-(1→3)-[α-L-Fuc-(1→2)]-β-D-Gal-(1→4)-β-D-GNAc-(1→6)-3-hexenetetrol(s).

tangential to the interaction area, whereas in the work with WGA, the mechanical force applied to pull the RBC away from sphere was more normal to the interaction area.

In the present study, the genotype AO RBC aggregates induced by two blood group A-reactive lectins have been used as a model system to investigate cell–cell interaction through the mechanism of specific ligand–receptor recognition. By experimental measurements (flow channel and sphere encapsulation) and theoretical analysis, the energies involved in cell–dissociation and assocation have been assessed. Comparative results of HPA and DBA, which have similar sugar specificities but markedly different binding curves, demonstrate the importance of the surface density of the bridging molecules in determining the energy involved in cell–cell interaction. Thus, the combination of biochemical (25) and biophysical approaches has provided new information and raised new questions on the energies involved in intercellular association and dissociation. The approaches used in this RBC-lectin system may be extended, with proper modifications, to other cell systems involving other interacting molecules. Such information on the energy existing between cells may help to understand many important physiological and pathological phenomena, including tumor dissociation and metastasis, embryonic cell association and movement, and the recognition and the programming for lysis of target cells by the specific cytotoxic T lymphocytes (30).

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