Biophysical Journal Volume 66 April 1994 1222–1230

Flow-Induced Detachment of Red Blood Cells Adhering to Surfaces by Specific Antigen-Antibody Bonds

Zheming Xia,* Harry L. Goldsmith,‡ and Theo G. M. van de Ven*
*Paprican and Departments of *Chemistry and ‡Medicine, Pulp and Paper Research Center, McGill University, Montreal, Canada H3A 2A7

ABSTRACT Fixed spherical swollen human red blood cells of blood type B adhering on a glass surface through antigen-antibody bonds to monoclonal mouse antihuman IgM, adsorbed or covalently linked on the surface, were detached by known hydrodynamic forces created in an impinging jet. The dynamic process of detachment of the specifically bound cells was recorded and analyzed. The fraction of adherent cells remaining on the surface decreased with increasing hydrodynamic force. For an IgM coverage of 0.26%, a tangential force on the order of 100 pN was able to detach almost all of the cells from the surface within 20 min. After a given time of exposure to hydrodynamic force, the fraction of adherent cells remaining increased with time, reflecting an increase in adhesion strength. The characteristic time for effective aging was approximately 4 h. Results from experiments in which the adsorbed antibody molecules were immobilized through covalent coupling and from evanescent wave light scattering of adherent cells, imply that deformation of red cells at the contact area was the principal cause for aging, rather than local clustering of the antibody through surface diffusion. Experiments with latex beads specifically bound to red blood cells suggest that, instead of breaking the antigen-antibody bonds, antigen molecules were extracted from the cell membrane during detachment.

INTRODUCTION

Detaching specifically adherent cells from a substrate through hydrodynamic or mechanical forces is a frequently used approach to evaluate the strength of adhesion (Weiss, 1961; Mège and Capo, 1986). The results often appear to be straightforward. It is usually assumed that the cells that survive a certain external force have a bond strength greater than the exerted force. Yet, there are many complications in the interpretation of detachment studies. In fact, a collection of cells that all have the same bond strength does not detach simultaneously. Instead, the percentage of the remaining cells decreases exponentially, due to the probability of the cells escaping from the energy minimum in which they are captured. In general, in the presence of an applied force, the lifetime of the bond, τ (average time for the cell to escape from the surface), can be written as

$$\tau = \tau(f)e^{E_o/kT} \tag{1}$$

where $\tau(f)$ is a function of the applied force per bond, f (in the present work, represented by shear stress at the surface), E_o is the bond energy, k is the Boltzmann constant, and T is the absolute temperature. A simple form of this expression, considered by Bell (1978), is:

$$\tau(f) = \tau_o e^{-\gamma f/kT}, \tag{2}$$

which yields

$$\tau = \tau_o e^{(E_o - \gamma f)/kT} \tag{3}$$

where τ_o is the reciprocal of a natural frequency of oscillation of atoms in solids, $\sim 10^{-13}$ s (Zhurkov, 1965), and γ determines the extent of reduction of the energy minimum with applied force. As the applied force increases to the "critical" value, ($f_c = E_o/\gamma$), the average time required to break-up the bond decreases nonlinearly to a value on the order of τ_o. This value of f_c can usefully be identified with the "strength" of a single bond. In the experiments described below, cells are probably attached by more than one bond. The applied force is distributed among n bonds; if bond breakage is sequential, then over the course of detachment, the force is distributed over a progressively smaller number of attachments, and thus f (Eq. 2) increases with time. It should not be taken for granted, therefore, that the force required to detach a cell is equivalent to the bond strength. Hence, information about the number of bonds per contact area is required. An inaccurate estimate of the bond density leads to improbable bond energies (Curtis, 1988).

A measure of adhesion (bond) strength, can also be inferred from the force dependence of the time course of the detachment of a population of cells. However, cells undergo morphological changes such as pseudopod formation and spreading (Pena and Hughes, 1978; Milton and Frojmovic, 1984), which tend to strengthen the initial binding, and thus adhesion strength can also be time-dependent, especially for living cells.

Moreover, unlike nonspecifically adherent cells, a fundamental question to be answered about the ligand-receptor-mediated detachment is where the rupture takes place along the ligand-receptor bridge. It may require several independent approaches to pinpoint the weakest link in the bridge.

In recent years, a substantial effort has been made to try to understand receptor-mediated cell adhesion through cell-cell and cell-surface detachment studies, both theoretically and experimentally (Tha et al., 1986; Cozens-Roberts et al.,

Received for publication 14 January 1993 and in final form 1 February 1994.

Address reprint requests to Dr. Theo G. M. van de Ven, Paprican and Department of Chemistry, Pulp and Paper Research Center, 3420 University St., McGill University, Montreal, Quebec, Canada H3A 2A7.

0006-3495/94/04/1222/09 $2.00

1990a; Evans et al., 1991; Berk and Evans, 1991). The techniques used in these studies varied from shear-induced break-up of doublets in bulk flow (Tha et al., 1986) to mechanical micromanipulation of individual cells (Evans et al., 1991) to testing a collection of model cells in a radial flow chamber (Cozens-Roberts et al., 1990a). The subject is complex, and despite a better understanding of some key parameters such as fracture forces, detachment rate, the effect of contact area, ligand density, and cell rigidity, two outstanding problems remain open for discussion. The first concerns the phenomenon of strengthening of adhesion with time, which has long been observed. However, no quantitative evaluation has been made, nor has the mechanism of aging been thoroughly explored. The second concerns the nature of the bond failure. It was recently suggested (Evans et al., 1991) that, rather than breaking the ligand-receptor bond, receptors in the cell membrane could be extracted during detachment.

The objectives of this study were to investigate the force and time dependence of detachment of a population of specifically adherent cells under well defined hydrodynamic conditions, in order to increase our understanding of adhesion strength. In addition, we wished to confirm the hypothesis that bond failure occurs through extraction of the antigen by measuring the size of antibody-adsorbed latex particles before and after interacting with antigens in the red cell membrane.

EXPERIMENTAL

Antibody coating of glass

Covalent coupling of antibody

Glass cover slips were washed with concentrated nitric acid for 2 h and with hydrochloric acid for 30 min, followed by thorough rinsing with distilled deionized water and drying in an oven at 100°C for 1 h. The glass slips were then subjected to a silanization procedure, similar to that described by Weetall (1972) and Cozens-Roberts et al. (1990). Essentially, clean slips were held vertically and parallel to each other by a specially designed pyrex slip holder in freshly prepared 2% (v/v) 3-aminopropyltriethoxysilane (Aldrich Chemical, madison, WI) in acetone at room temperature. This coating liquid was evaporated under vacuum until the liquid level was below the glass slips. The silanized slips were allowed to cure in an oven at 115°C for 2 h and were stored for later covalent coupling.

The alkylamine groups on the silanized glass surface were reacted with glutaraldehyde to form aldehyde groups by soaking each slip in a petri-dish in freshly prepared 10 ml of 1% glutaraldehyde (electron microscopy grade; Fisher Scientific, Montreal, Canada) in saline for 30 min. Each glass slip was rinsed 5 times with saline to remove unbound glutaraldehyde before it was exposed to monoclonal mouse IgM, specific to human blood group type B antigen (Dako Corporation Dimension Labs, Mississauga, Canada), of desired concentration in physiological saline (pH 6.5–6.8) at 4°C for 2 h.

The antibody-coated glass slip was transferred to another petri-dish containing 10 ml of 0.1 M glycine (ICN Biomedicals Inc., Irvine, CA) in saline. It was incubated for 1 h for the glycine molecules to bind unreacted aldehyde groups, and was thoroughly washed with saline. The coupling process was performed within 24 hours prior to an experiment. To cover the area of the treated glass where no antibody was attached and to prevent nonspecific adhesion of antibody to the surface (Xia et al., 1993), the slip was incubated with 1% bovine serum albumin (BSA) (ICN) in saline for 2 h at room temperature immediately before each experiment.

Adsorption of antibody

Glass cover slips were incubated in an antibody solution for 10 h at 4°C. The excess antibody was washed off with saline, and the slides were incubated with 1% BSA for 2 h, rinsed with saline, and then used immediately.

Detachment of red blood cells from glass surfaces

Fixed spherical swollen human red blood cells (FSRC) of blood group type B, having a mean diameter of 6.1 μm, were prepared as previously described (Xia et al., 1993). The cells were suspended in physiological saline and allowed to adhere onto glass cover slips coated with the corresponding antibody in a stagnation point flow created in an impinging jet (Xia et al., 1993). The flow rate for deposition of the cells was constant throughout the experiments, 45 $\pm$ 5 μl s^{-1}, less than 30% of the lowest detachment flow rate. The geometry of the jet was identical to that used previously; the radius of the jet, R, was 1.0 mm, and the distance between the exit of the jet and the glass cover slip, h, was 1.8 mm. At the inlet of the jet, a three-way valve was attached that led to two reservoirs, one containing a cell suspension and another containing a saline solution. At the end of a 30 min adhesion experiment, the inlet fluid was switched to saline with a pre-adjusted flow rate. The flow rate was controlled by varying the pressure in the reservoir, which was connected to a nitrogen cylinder. The detachment of the adherent cells from the glass surface was recorded for further analysis by a video camera mounted above the surface on a high power microscope.

The tangential hydrodynamic force, F_{hydr}, on a sphere of radius a ($\bar{a}$ = 3.05 μm for the FSRC) located on the surface at a radial distance r from the stagnation point is given by (Goldman et al., 1967):

$$F_{hydr} = 1.7 \times 6\pi\eta\alpha_s r a^2 \tag{4}$$

where α_s is the strength of the stagnation point flow given by

$$\alpha_s = \frac{Re\nu}{R^3}(4.4\,Re^{1/2} - 8.24) \tag{5}$$

where ν is the kinematic viscosity of the medium and Re is the Reynolds number, defined as

$$Re = \frac{\bar{u}R\rho}{\eta}, \tag{6}$$

$\bar{u}$ being the mean velocity of the jet and ρ and η the density and viscosity of the medium, assumed to be that of water. From the value of $\bar{u}$, one can calculate Re and, hence, the strength of the stagnation point flow by means of Eq. 5.

For aging experiments, detachment was delayed for a time interval varying from 0.5 to 10 h after the completion of the adhesion period. During the delay, the adherent cells remained on the glass surface and in contact with the same suspension used during deposition. The microscope light was kept off during the adhesion and aging periods to avoid heating effects. It was only on when necessary to record during the detachment period, usually the first 30 min and for about 1 min every 30 to 60 min for the rest of the experiment.

Adsorption of antibody molecules to latex particles

5 μl of 10% w/w carboxylated polystyrene latex particles (Syradyn, Indianapolis) of diameter 0.134 μm (according to the supplier) were suspended in 30 ml of physiological saline, centrifuged at 500 rpm for 15 min to remove possible aggregates. 300 μl of monoclonal anti-B IgM antibody (0.14 mg/l) were added to 2.5 ml of the latex suspension. The suspension was mixed for 1 min by inverting the vial, before 0.2 ml of 1% BSA (by weight in saline) was added to cover the surface area on latex particles where no antibody was adsorbed. The suspension was incubated on an orbital shaker at room temperature for 1 h.

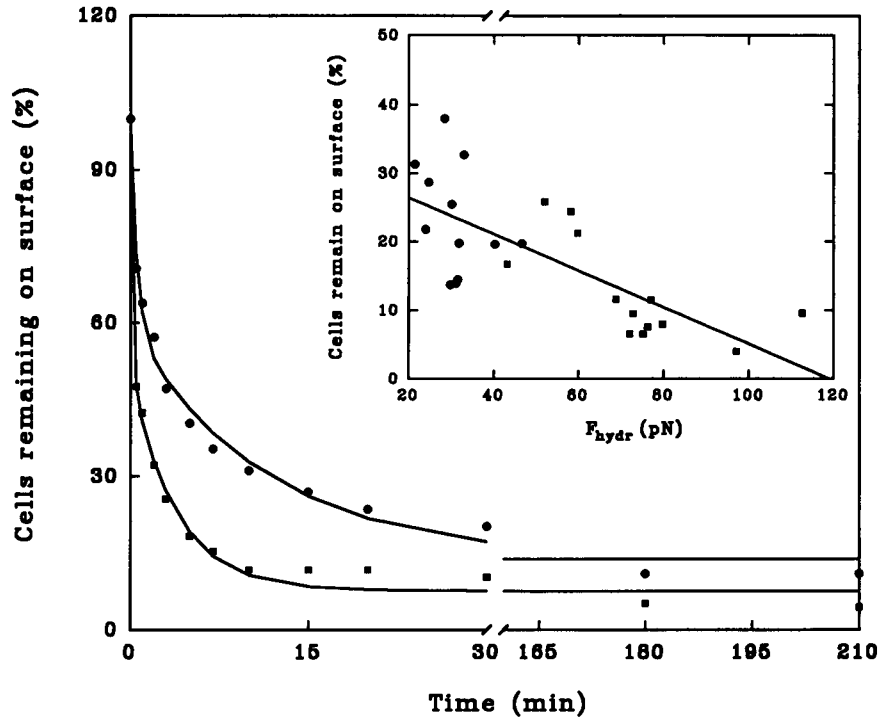

FIGURE 1 A typical example of shear-induced detachment of specifically adherent sphered red cells (FSRC, antigenic type B) from a glass surface covered with 0.26% adsorbed monoclonal IgM antibody to blood group B antigen: plot of the normalized surface density as a function of time. Average tangential hydrodynamic force, F_{hydr}, for the two regions, R1 (= 70 μm; ●) and R2 (= 180 μm; ■) are 31 and 75 pN, respectively. The points are experimental, and the lines drawn are for a biexponential fit of the data (Xia, 1992). (*Inset*) Plot of the number of cells remaining on the surface versus the average applied hydrodynamic force.

Albumin molecules are prolate spheroids with semi-major and semi-minor axes of 14 and 5 nm (Squire et al., 1968), respectively. For the final number concentration of the latex particles used, approximately 1.1×10^7 ml^{-1}, the surfaces of the latex particles would be completely covered if only 1% of the added albumin adsorbs. The ratio of latex particles to antibody molecules was 1:0.9.

The diameters of the carboxylated latex particles suspended in 0.15 M NaCl were constant for at least a week, measured by photon correlation spectroscopy (Brookhaven Instruments, Brookhaven, NJ) at an incident angle of 110°. This indicated that the latex particles did not coagulate in 0.15 M NaCl. The latex suspensions show a tendency to be unstable, however, when mixed with antibody solutions, due to bridging of the adsorbed antibody molecules between the latex particles. Adding BSA molecules shortly after the mixing of the antibody and latex particles effectively prevented the aggregation by creating steric repulsion between the latex particles. After coating with antibody and BSA, we found that the average diameter of the latex particles increased from 135.6 ± 0.5 nm to a mean value of 190.3 nm. The experimental error in the PCS measurements was ±3 nm for five runs. The hydrodynamic radius of the antibody-coated latex particles remained constant at 4°C for at least 24 h. Because the diameter of the antibody molecule is about 30 nm and, on average, about one antibody is adsorbed per particle, it is likely that a small number of doublets were formed during the antibody adsorption, before BSA addition. Stirring or vortexing did not accelerate the process of aggregation, because diffusion is more important than convection for submicron latex particles.

Adhesion and detachment of antibody-coated latex particles from red cells

0.2 ml of a suspension of FSRC in saline was added to 5.4 ml of the suspension of antibody-coated latex particles with a final concentration of 8.2×10^3 μl^{-1}. This mixture of FSRC, latex particles, antibody, and albumin molecules was incubated at room temperature on an orbital shaker for 40 min to allow antibody-coated latex particles to bind to the FSRC. The ratio of the total surface area of FSRC to the total cross-sectional area of latex particles in the final suspension was 6:4:1.

The suspension was then subjected to shear for 10 min in the annulus of a small Couette device consisting of an outer glass cylinder of diameter 29.5 mm and a concentric inner teflon cylinder of diameter 28 mm. The inner cylinder rotated at 100–3000 rpm, corresponding to shear rates of about 300–6000 s^{-1}. Due to shear, and to collisions between particles in the suspension, a fraction of latex particles was removed from the surface of the FSRC.

The FSRC were removed from the suspension by centrifugation at 500 rpm for 40 min. The diameter of the latex particles and the scattering intensity of the supernatant were measured in a photon correlation spectroscope (PCS). The size was compared to that of latex particles before and after coating with antibody and albumin.

Evanescent wave light scattering of red blood cells on quartz

Fixed sphered red cells in a suspension of concentration 1.2×10^5 μl^{-1} were allowed to adhere on a trapezoidal quartz prism by impinging a jet on its surface. The adherent red cells in the evanescent wave region, at the quartz/suspending medium interface, scatter light (Xia and van de Ven, 1992). After 30 min of adhesion, the flow was stopped and the intensity of the scattered light from the adherent red blood cells was measured by a photomultiplier to detect any changes in the intensity due to possible deformation of the scattering red cells. The penetration depth of the evanescent wave was 200 nm. Microscopic observation showed that approximately 600 red cells were in the evanescent wave region.

RESULTS AND DISCUSSION

Force of detaching specifically bound red blood cells

The time course of a typical detachment experiment is shown in Fig. 1 in a plot of the number of cells per unit area remaining on the surface (surface density) at time t normalized to the initial value. The observation area in the stagnation point region of the glass cover slip was divided into two regions, R1 and R2, R1 being circular with radius r = 0.14

mm and R2 being annular with $0.14 < r < 0.22$ mm. At a flow rate of 198 μl s^{-1}, the average wall shear rates $G(\bar{r})$ in R1 and R2 for this particular experiment were 124 and 290 s^{-1}, respectively, corresponding to average values of F_{hydr} of 31 and 75 pN, respectively.

The number of cells remaining of the surface at the beginning of the detachment was typically 100–200 in each region. As shown by the symbols in the figure, the surface density decreased with time, at a rate that is very high initially but gradually decayed to almost zero. At the end of an experiment lasting typically 3.5–4 h, a fraction of cells always survived the shear force and remained adhering to the surface, even at an average tangential force as high as 140 pN.

Fig. 1 (*inset*) shows a plot of cells finally remaining against the average tangential force given by Eq. 1. The scatter in the data can be attributed to several sources. First, the pre-set flow rate for the adhesion period varies from experiment to experiment, within a range of 47–55 μl/s, which could result in different surface distributions of the adherent cells. Second, the error in the flow rate in the detachment period is around 7%. Third, the surface roughness and heterogeneity in antibody density could also contribute to the error. Nevertheless, it is obvious that as the tangential force increases, the number of cells remaining decreases (Fig. 1, *inset*). Extrapolating the linear regression function to zero surface coverage results in a value of F_{hydr} of about 120 pN. In other words, under a tangential force greater than this value, all of the cells will be detached given enough time.

The diameter of the contact area of between a red cell and the surface in which antibody bridging is possible is only about 0.7 μm (Xia et al., 1993). For the measured average antibody surface density of 3.7 μm^{-2}, the average number of antibody molecules in this area is about 1.4, corresponding to 7 cross-links, assuming that the maximum valency of each IgM antibody is 5 (for the other 5 of the 10 valencies, it is geometrically impossible to form bonds). Therefore, the average rupture shear force per bond is 17 pN, provided that all the bonds are identical. However, it should be emphasized that local clusters of antibody molecules might conceivably increase the number of cross-links.

In practice, because cells are "peeled" off, the bond strength in the normal direction is expected to be higher. Furthermore, we cannot rule out the possibility that new bonds form downstream of the cells as the bonds upstream of the cell break, causing the cell to rotate along the surface, although no migration of the cells along the surface was observed under the microscope.

A typical critical shear stress necessary to detach, in a radial flow chamber (Cozens-Roberts et al., 1990b), all the 10 μm (diameter) latex particles covalently coated with rabbit anti-IgG from a surface covalently coated with rabbit IgG is 1.5 Nm^{-2}, equivalent to 3 nN, i.e., 30 times higher than the force found in our experiments. The density of receptors on the surface was reported to be 10^{14}-10^{16} m^{-2}, one to three orders of magnitude higher than the typical density of 3.7×10^{12} m^{-2} assumed in our experiments (if the difference in valency between IgG and IgM molecules is included). Moreover, as described below, in the present experiments detachment probably occurred through antigen being pulled out of the cell membrane, or in the case of the adsorbed antibody, possibly by antibody being peeled off the surface, either mode requiring less energy than breaking an antigen-antibody bond such as must have occurred in the experiments of Cozens-Roberts et al. (1990b). These effects explain the difference in the critical shear stress.

Fig. 2 shows the effect of antibody density on the final cell surface density under a constant hydrodynamic force of 46 pN. At higher antibody density, more cells remain on the surface for the same flow conditions, suggesting that the number of bonds per cell is increased. The curve is anticipated to level off when the number of cross-bridges per cell is optimum.

Effect of aging on detachment

When the adherent cells are allowed to remain on the surface for a given time, it is observed that fewer cells detach under the same hydrodynamic force (Fig. 3), reflecting an increase in adhesion strength. The increase in the fraction of cells remaining on the surface depends on the length of aging time: under an average tangential force of 111 ± 4 pN at $\bar{r}$ = 0.18 mm, the normalized final surface density increases from 14.5 to 83.2% as aging time increases from 0 to 10 h. The data can be fitted by a biexponential detachment rate (Xia, 1992). The detachment rate of the slowest time constant decays approximately exponentially with a characteristic time of ~4h.

Strengthening of specific adhesion could result from the formation of more bonds after the initial ligand-receptor bridging, i.e., by secondary bond formation, or by improving the conformational fitting of the originally formed antigen-antibody complexes. The affinity between large molecules

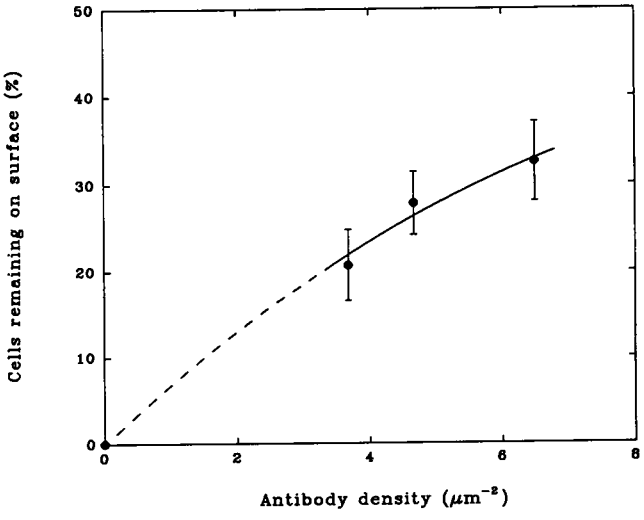

FIGURE 2 Effect of adsorbed antibody coating density on the final surface coverage of red cells. Average value of F_{hydr} = 46 ± 3 pN; range of F_{hydr} is from 0 to 92 pN.

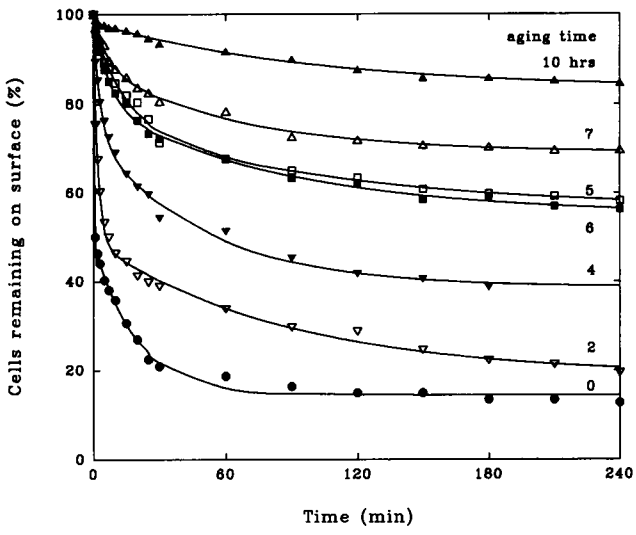

FIGURE 3 Effect of aging on shear-induced detachment of red cells from adsorbed antibody (0.26% IgM). Plot as in Fig. 1 of the surface density of FSRC as a function of time for increasing aging times. The points are experimental, and the lines drawn are for a biexponential fit of the data (Xia, 1992). Average value of F_{hydr} = 111.3 ± 3 pN; range of F_{hydr} is from 0 to 223 pN.

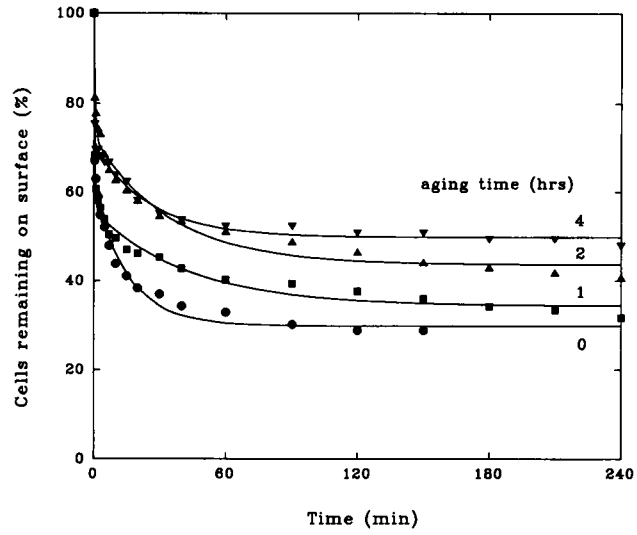

FIGURE 4 Plot as in Fig. 3 showing the effect of aging on shear-induced detachment of red cells from covalently bound antibody. The points are experimental, and the lines drawn are for a biexponential fit of the data (Xia, 1992). Concentration of IgM in the coating solution is 0.143 mg/l. Average value of F_{hydr} = 239 ± 7 pN; range of F_{hydr} is from 0 to 478 pN.

such as the antigen and antibody may be affected by their micro-environment as well as by the molecular point of the specific binding site (Benacerraf and Maurer, 1969). An increase in the number of bonds could result from migration of ligands and receptors into the contact area, or by an increase in the contact area between a cell and the surface for given receptor or ligand densities. If the strengthening of the bonds is due to the accumulation of antibody in the contact area, the characteristic aging time should be comparable to the time it takes for the antibody molecules to diffuse into the contact area. Because, however, the lateral diffusion constant of IgM molecules is unknown, we can only qualitatively verify this hypothesis by physically immobilizing the adsorbed antibody in order to attempt to eliminate the strengthening of the bonds. The approach we took to immobilize the antibody was to bind them covalently to the surface.

Fig. 4 shows the effect of aging for covalently bound antibody. It is evident that, although the mean hydrodynamic force, 239 ± 7 pN, was appreciably higher than that in the case of the adsorbed antibody molecules shown in Fig. 3 (111 ± 4.3 pN), the percentage of cells remaining on the surface after 2 h was twice as great in the case of the covalently bound antibody (30 vs. 15%). However, the changes in the fraction of cells remaining with increasing aging time was smaller than for the adsorbed antibody, implying that lateral diffusion of the antibody molecules might still play a role in aging, although not the only one. In the light of the antigen extraction experiments described below, there appear to be two possible explanations for the differences between adsorbed and covalently-bound antibody:

(i) In both cases, detachment occurs through antigen being pulled out of the membrane of the red cell. However, the surface density of antibody is greater in the case of the covalently-bound antibody, resulting in a higher average number of Ag-Ab bonds per adhering cell.

(ii) In the case of the adsorbed antibody, detachment occurs by the antibody being peeled off the surface. In the case of the covalently bound antibody, detachment occurs by pulling antigen out of the membrane; this may require more energy, perhaps in part because of the higher average number of Ag-Ab bonds.

We do not know whether the surface density of the covalently bound antibody is greater than that of the adsorbed antibody, although the coating concentrations in both preparations procedures were kept the same. It is impossible, therefore, to decide between the first and second explanations. The possibility that covalently bound antibody is more active than adsorbed antibody, i.e., more susceptible to changing conformation, can also not be ruled out. Hence, no conclusions can be drawn as to whether the adhesion strength is higher for the covalently bound antibody or the adsorbed one.

An alternative explanation of bond aging is the enlargement of the contact area by cell deformation. Although cells fixed with glutaraldehyde are relatively rigid, they might still undergo local morphological changes in the vicinity of the contact area. The evanescent wave light scattering technique was used to detect the possible flattening of cells at the interface. The penetration depth of the evanescent wave was kept at one-fifteenth of the diameter of a red cell. When an adherent cell deforms, the fraction of its volume within the penetration depth increases, resulting in a change in the amount of light the cell scatters. The fixed spherical red cells were allowed to adhere for 30 min on a quartz surface, preincubated with 0.42 mg/l of antibody and 1% BSA successively. The increase in the intensity of the scattered light,

recorded immediately after cessation of flow, is plotted against time in Fig. 5. The scattering intensity increased even after the flow was stopped and then began to decay, but it always remained higher than the intensity at the time of cessation of flow (2.5×10^6 min^{-1}). Cells adhering to bare quartz showed similar trends. The initial rise of scattering intensities cannot be due to continued adhesion in the absence of flow, as a result of diffusion, simply because it was never observed during the aging period for any of the experiments monitored under the microscope. In the absence of flow, cells might diffuse into the evanescent wave region, causing a transient increase in scattering. However, this increase will be overwhelmed by the effect of sedimentation. The rate of sedimentation is 1.6 μm/s, assuming the density of the fixed red cells is 1.08 g/ml. Hence, the change in the scattering intensity is most likely due to the deformation of the adherent cells. Cell deformation results in changes in both the volume of the cell in the evanescent wave region, and their contact angle between the cell and the surface. Both of these changes may affect the scattering intensity, although the exact relation between the intensity and these changes is not known. It is possible that the increase in cell volume in the evanescent wave regions results in a higher scattering intensity, whereas the increase in the contact angle causes a lower intensity due to multiple scattering between the cells and the surface. Fig. 5 shows that the cells deform for at least a period of 4 h.

Possibility of extracting antigen from cell membrane

When a cell adhering specifically through antigen-antibody bridging detaches, at least one of the following events takes place: (i) an antibody detaches from the surface; (ii) the "lock" and "key" engagement of an antibody and an antigen dissociates; (iii) the polysaccharide terminal of an antigen detaches from the lipid or protein molecule to which it is bound; and/or (iv) the entire glycolipid or glycoprotein is pulled out from the cell membrane. The rupture of polysaccharides from their lipid or protein bases is unlikely, because the energy required to break a covalent bond is typically ~400 kJ/mol (Weast and Astle, 1982). The reaction constant of antigen-antibody association/dissociation has been estimated to be around 35 kJ/mol (Duszyk et al., 1986), or 5×10^{-20} J per bond. For a bond strength of 100 pN, this energy corresponds to a stretching of the bond by 5 Å before it breaks. On the other hand, lipid molecules in a cell membrane and some embedded proteins, if not cross-linked with other proteins and polysaccharides through the cytoskeletal network or in the glycocalyx layer through fixation, are merely held in the membrane by intermolecular forces. It is possible for such weakly associated molecules to be pulled out of the membrane without breaking the cell envelope.

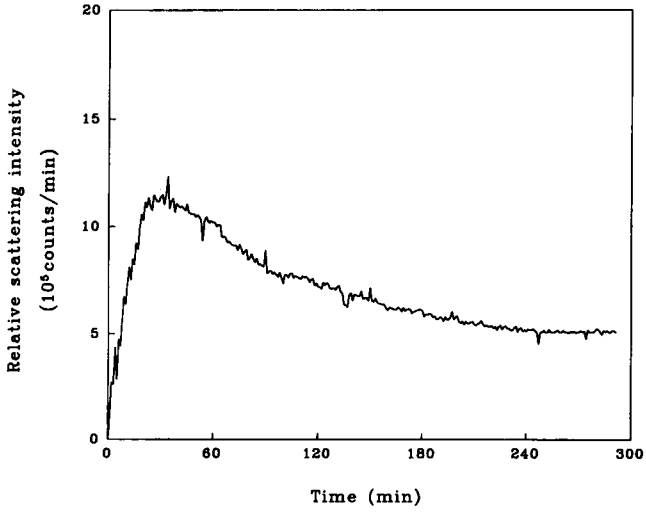

FIGURE 5 Changes in intensity of scattered light from FSRC (antigenic type B) adhering on a quartz surface, pre-incubated with monoclonal IgM antibody to blood group B antigen, in an evanescent wave region in the absence of flow. Time zero is the end point of a 30 min adhesion period. Penetration depth is 200 nm.

Experimentally, detachment forces in RBC were estimated (Berk and Evans, 1991) to be of the order of 10 pN, regardless of the type of receptor and agglutinin (e.g., lectin and anti-A immunoglobulin). This value happens to be the force required to extract a receptor from a cell membrane. Other results for the minimum rupture force from independent techniques (Tha et al., 1986) are in the same range. Most of the receptors in those studies are membrane proteins and membrane glycolipids.

In the present work, instead of studying individual Ag-Ag bridges, we investigated a collection of these bridges simultaneously, using PCS. We measured the hydrodynamic size of antibody-coated latex particles before and after the particles were bound to, and detached from, red blood cells carrying the corresponding antigen. Thus, an increase in latex diameter would indicate that some membrane material was extracted, whereas a decrease in diameter would suggest that antibody molecules were removed from the surface of the latex beads. Similarly, no change in diameter would mean that the antigen and antibody had been separated.

Latex particles coated with antibody molecules were allowed to adhere specifically to red cells, by incubating them with red cells of the corresponding antigen. The suspension of the red cells and the latex particles was then subjected to a linear shear field. The red cells were removed from the suspension by centrifugation. The supernatant was then used for further analysis in the PCS apparatus.

Although the tangential force exerted on a 136-nm sphere attached to a flat surface at the maximum applied shear rate (6000 s^{-1}) is only around 0.5 pN (Eq. 4), not sufficient to detach it, the force on the latex sphere due to collisions with other red cells could be a few orders of magnitude higher. The probability of having an effective collision between two red cells, i.e., a collision in which a latex sphere is in the gap between the colliding cells, is given by the ratio of the projected area of a latex sphere to the total surface area of a red

cell, approximately 1.2×10^{-4}. The number of collisions per FSRC in a suspension containing n_o cells per unit volume may be computed from the flux, J_s, for orthokinetic coagulation of equal sized spheres of radius a (Smoluchowski, 1917):

$$J_S = \frac{32}{3} n_o G a^3. \quad (7)$$

For the FSRC ($a = 3.05$ μm, $n_o = 8.2 \times 10^6$ ml^{-1}) at $G = 6 \times 10^3$ s^{-1}, $J_s = 14.9$ s^{-1}. Hence, the probability of detaching a particular latex sphere is about 100% in 10 min of shearing at $G = 6000$ s^{-1}, provided that a latex sphere is removed during each "effective" collision. For lower collision efficiencies the time needed to detach the particles is correspondingly larger.

Electron micrographs of B cells exposed to latex particles coated with antibody (specific to type B antigen) before (Fig. 6 *a*) and after (Fig. 6 *b*) shearing provide the evidence that latex beads bind specifically to red cells and that some latex beads initially bound to the cells were detached under shear. In contrast, no latex particles were observed on an A cell (Fig. 7).

The results from the electron micrographs are consistent with those from scattering intensity measurements (by PCS)

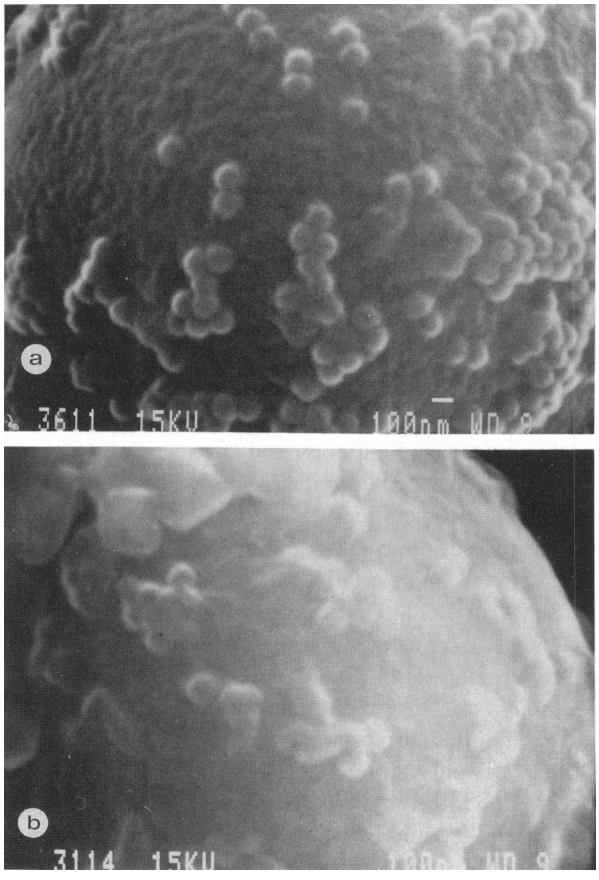

FIGURE 6 Latex particles coated with monoclonal IgM antibody to blood group B antigen adhering to sphered red blood cells of blood group type B: (*a*) before shear; (*b*) After shear (10 min, 6000 s^{-1}). The large feature on the upper part of the FSRC is a salt crystal.

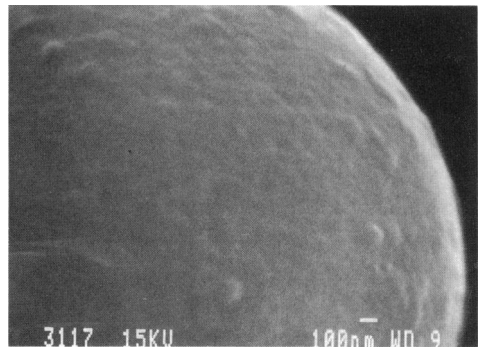

FIGURE 7 Surface of a sphered red blood cell of blood group type A after incubation with latex particles coated with monoclonal IgM antibody to blood group B antigen, in the absence of shear.

of the suspensions of antibody-coated latex particles that interacted with red cells and were then separated from the red cells by centrifugation. These results are shown in Fig. 8. Because the scattered light intensity of these suspensions is proportional to the number concentration of the suspension, if the particles are identical, the low scattering intensity of those samples that interacted with antigenic type B cells confirms that a certain number of latex particles were carried away by the B cells during centrifugation. As the shear rate increased, more latex particles were removed from the B cells, resulting in a higher particle concentration in the supernatant and, thus, a higher scattering intensity. In contrast, the light intensity of the samples that interacted with antigenic type A cells shows only a small increase with increasing shear rate. The increase is most probably due to the removal of small numbers of spheres nonspecifically bound to

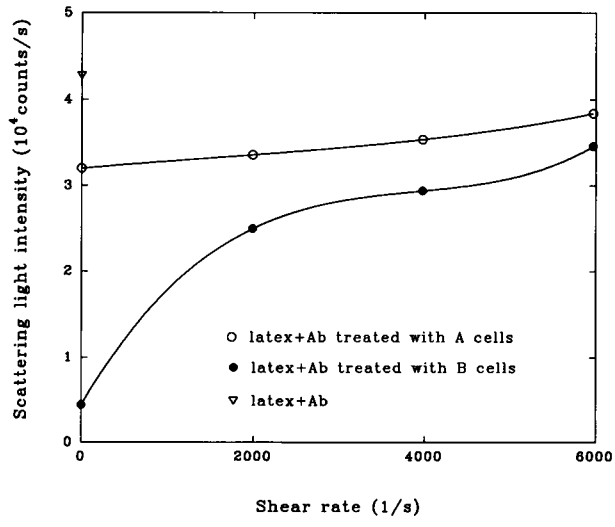

FIGURE 8 Intensity of the scattered light from suspensions of antibody-coated latex particles which had interacted with FSRC of antigenic type A or B, as a function of the shear rate applied to detach the spheres. After shearing, the spheres were separated from the FSRC by centrifugation. The triangle indicates the value obtained at zero shear rate for a suspension of antibody-coated latex particles that had not interacted with the FSRC.

the A cells. Such nonspecifically bound latex spheres could be removed from the FSRC by washing and shearing of the suspension.

Fig. 9 shows the hydrodynamic diameter of latex particles, measured by PCS before and after interacting with red blood cells at various shear rates. Fixed, sphered red cells of blood group type A were also used as control samples. After the latex particles adhered and detached from B cells, their size varied according to the shear rate applied during the detachment period. At zero shear rate, the diameter decreased by nearly 30 nm from the mean value of 190.3 nm found for latex spheres coated with antibody and albumin. The diameter then increased monotonically with shear rate from 155.6 nm at 0 s^{-1} to 196.8 nm at 6000 s^{-1}. In contrast, the diameter of spheres in the suspensions that interacted with A cells exhibited only a slight increase from 186.3 nm at 0 s^{-1} to 188.0 nm at 6000 s^{-1}. In the case of the B cells, the initial decrease in size of the latex spheres is due to the fact that most of the spheres having adsorbed antibody and, thus, a larger diameter than bare latex particles, were attached to the B cells and carried away during the centrifugation, resulting in a low average diameter of the latex suspension. As the shear rate increased, more antibody-coated latex particles were removed from the B cells and resuspended in the medium, causing an increase in the average size, reaching 180 nm at $G = 2{,}000\ s^{-1}$. This is in agreement with the light scattering measurements of the samples. In the case of the latex spheres that had interacted with A cells, the slight increase in diameter, although not statistically significant, corresponds to a small increase in the number of latex spheres (Fig. 8) due to shear-induced release of spheres nonspecifically bound to the FSRC.

At higher shear rates, the diameter of the latex particles that interacted with B cells became larger than the diameter of those that interacted with A cells by 5 and 9 nm at 4000 and 6000 s^{-1}, respectively. This represents an increase of 4 and 7 nm, respectively, over that of latex spheres coated with antibody and albumin. These increases differed by 3% for two independent experiments, and thus the difference of 7 nm in diameter is significant. This increase must have been due to material, other than antibody and BSA, attaching itself to the latex particles, because antibody-coated latex particles did not coagulate under the same treatment in the absence of red blood cells. The diameter of these particles, 189.6 nm, was independent of shear rate, as shown by the triangles in Fig. 9. If debris from lysed cells had adsorbed onto the latex particles, it should also have adsorbed nonspecifically to latex particles exposed to A cells. Moreover, the increase cannot be due to some of the red cells remaining in the suspension after centrifugation, because they, too, should be present in the control sample. Therefore, the difference between latex particles that have interacted with B cells and A cells likely resulted from the antigen molecules being extracted from the cell membrane.

It may appear that the cell-surface detachment process is different in the case of the latex sphere-FSRC interaction experiment than in the case of the impinging jet experiment. However, in both cases it is a tangential shear stress that results in dislodging the red cell or latex bead, with the probable result that antigen is pulled out of the cell. In the impinging jet experiment, there is a constant tangential stress exerted on the spherical red cell. In the case of the latex bead-coated spherical red cells, we surmise that the beads are detached during a collision between two FSRC. During such a collision, fluid between the approaching particles is rapidly expelled as the gap between surfaces decreases to nanometer values (but not zero), thereby creating locally high shear stresses on the surfaces.

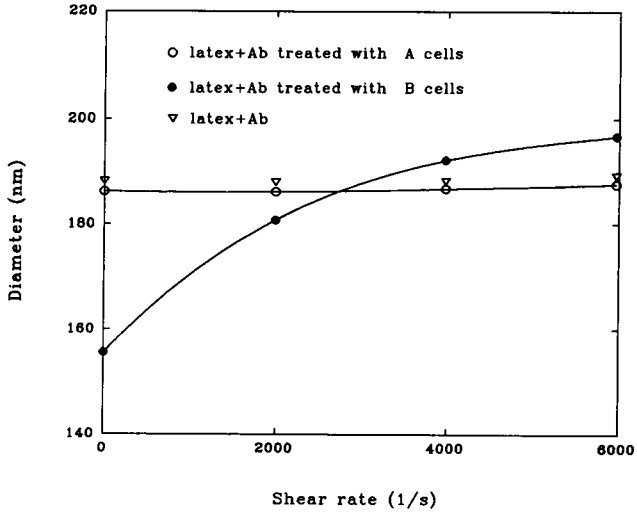

FIGURE 9 System as in Fig. 8, showing the average hydrodynamic diameter of latex spheres in suspensions of antibody-coated latex particles as a function of shear rate applied during detachment, obtained using photon correlation spectroscopy. The triangles indicate the values for suspensions of antibody-coated latex particles subjected to the same shear in the absence of red cells.

CONCLUSION

Red blood cells of antigenic type B adhering to glass precoated with monoclonal anti-B IgM antibody in a stagnation point flow chamber can be detached by the action of a tangential hydrodynamic force. For a given time of exposure to shear stress, the percentage of the adherent cells remaining on the surface decreases with increasing hydrodynamic force. A measure of the adhesion strength was the finding that, at an IgM surface coverage of 0.26%, a tangential force of the order of 100 pN was required to detach all the cells immediately after the adhesion period. Under a given hydrodynamic force, the fraction of cells remaining on the surface (and presumably, the adhesion strength) increased with aging, with a characteristic time of about 4 h. A larger hydrodynamic force was required to detach cells bound to covalently bound, immobilized antibody on glass, possibly due to a higher coating density or a better conformation of the covalently bound antibody. There was no evidence of desorption of the adsorbed antibody by hydrodynamic forces. Immobilization of antibody failed to prevent the strengthening of bonds during aging, but the effect was smaller than

in the case of the adsorbed antibody. Light scattering measurements of adherent cells in an evanescent wave region revealed that deformation of red cells near the contact area, rather than local clustering of antibody molecules through lateral diffusion, appears to be the principal cause of aging. The hypothesis that, during detachment, antigen is extracted from the cell membrane was also tested by measuring the difference in the average size of antibody-coated latex particles before they were specifically bound to antigenic type B red cells and after shear-induced detachment from the cells. The average diameter of latex particles that had previously adhered to red cells through antigen-antibody bonds increased by about 7 nm after detachment of the particles from the cells. Because there was no such increase in the diameter of latex particles that had interacted with antigenic type A red cells, nor in the diameter of antibody-coated latex particles in the absence of interaction with red cells, the increase in size is attributed to antigen molecules, extracted from the cell membrane, attached to the antibody.

This interpretation of the results is in agreement with the finding of Evans et al. (1991a) using a micro-fluorometric technique, but with normal, not fixed, red cells. If, indeed, antigen is being extracted from the membrane of the glutaraldehyde-fixed cell, it suggests that lipid-linked antigenic sites are the ones involved in this process. Thus, it is possible that the latex spheres remaining on the FSRC surface after shear (Fig. 6 *b*) are the ones in which antibody is attached to glycoprotein-linked antigen or, alternatively, the efficiency of removing such spheres is low, and a fraction of beads was never removed during the detachment experiments.

REFERENCES

Bell, G. I. 1978. Models for the specific adhesion of cells to cells. *Science (Washington D. C.).* 200:618–627.

Benacerraf, B., and P. Maurer. 1969. Relationship between the net electrical charge of antigens and specific antibodies. *Israel J. Med. Sci.* 5:171–176.

Berk, D., and E. A. Evans. 1991. Detachment of agglutinin-bonded red blood cells III. Mechanical analysis for large contact areas. *Biophys. J.* 59:861–872.

Cozens-Roberts, C., D. A. Lauffenburger, and J. A. Quinn. 1990a. Receptor-mediated cell attachment and detachment kinetics I. Probabilistic model and analysis. *Biophys. J.* 58:841–856.

Cozens-Roberts, C., J. A. Quinn, and D. A. Lauffenburger. 1990b. Receptor-mediated cell attachment and detachment kinetics II. Experimental model studies with the radial-flow detachment assay. *Biophys. J.* 58:857–872.

Curtis, A. S. G. 1988. The data on cell adhesion, *In* Physical Basis of Cell-cell Adhesion. P. Bongrand, editor. CRC Press, Inc., Boca Raton, FL. p. 211.

Duszyk, M., M. Kamalec, and J. Doroszewski. 1986. Specific cell-to-cell adhesion under flow conditions. *Cell Biophys.* 8:119–130.

Evans, E. A., D. Berk, and A. Leung. 1991a. Detachment of agglutinin-bonded red blood cells. I. Forces to rupture molecular-point attachments. *Biophys. J.* 59:838–848.

Evans, E. A., D. Berk, A. Leung, and N. Mohandas. 1991b. Detachment of agglutinin-bonded red blood cells II. Mechanical energies to separate large contact areas. *Biophys. J.* 59:849–860.

Goldman, A. J., R. G. Cox, and H. Brenner. 1967. Slow viscous motion of a sphere parallel to a plane wall. I. Motion through a quiescent fluid. *Chem. Eng. Sci.* 22:637–652.

Mège, J. L., and C. Capo. 1986. Determination of binding strength and kinetics of binding initiation. A model study made on the adhesive properties of P388 macrophage-like cells. *Cell Biophys.* 8:141–160.

Milton, J. G., and M. M. Frojmovic. 1984. Adrenaline and adenosine diphosphate-induced platelet aggregation require shape change. *J. Lab. Clin. Med.* 104:805–815.

Pena, S. D. J., and R. C. Hughes. 1978. Fibronectin-plasma membrane interactions in the adhesion and spreading of hamster fibroblasts. *Nature (Lond.).* 276:80–83.

Smoluchowski, M. von. 1917. Versuch einer Mathematischen Theorie der Koagulationskinetik kolloider Lösungen. *Z. Physik. Chem.* 92:129–168.

Squire, P. G., P. Moser, and C. T. O'Konski. 1968. Hydrodynamic properties of bovine serum albumin. *Biochemistry.* 7:4261–4271.

Tha, S. P., J. Shuster, and H. L. Goldsmith. 1986. Interaction forces between red cells agglutinated by antibody. II. Measurement of hydrodynamic force of breakup. *Biophys. J.* 50:1117–1126.

Weast, R. C., and M. J. Astle. 1982. CRC Handbook of Chemistry and Physics. 62nd Ed. CRC Press, Boca Raton, FL.

Weetall, H. H. 1972. Covalent coupling methods for inorganic support materials. *The Chemistry of Biosurfaces* 2:134–149.

Weiss, L. 1961. Studies on cell adhesion in tissue culture. IV. The alteration of substrata by cell surfaces. *Exp. Cell Res.* 25:504–517.

Xia, Z. 1992. Kinetics of specific and non-specific adhesion of biological cells subjected to flow. Ph. D. Thesis, McGill University, Montreal, Quebec.

Xia, Z., and T. G. M. van de Ven. 1992. Adhesion kinetics of phosphatidyl choline liposomes by evanescent wave spectroscopy. *Langmuir* 8: 2938–2946.

Xia, Z., H. L. Goldsmith, and T. G. M. van de Ven. 1993. Flow-induced detachment of red blood cells adhering to surfaces by specific antigen-antibody bonds. *Biophys. J.* 65:1073–1083.

Zhurkov, S. N. 1965. Kinetic concept of the strength of solids. *Int. J. Fracture Mechanics.* 1:311–323.