Specific adhesion of glycophorin liposomes to a lectin surface in shear flow

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ABSTRACT The adhesion of cells to other cells or to surfaces by receptor-ligand binding in a shear field is an important aspect of many different biological processes and various cell separation techniques. The purpose of this study was to observe the adhesion of model cells with receptor molecules embedded in their surfaces to a ligand-coated surface under well-defined flow conditions in a parallel plate flow chamber. Liposomes containing glycophorin were used as the model cells to permit a variation in the adhesion parameters and then to observe the effect on adhesion. A mathematical model for cell sedimentation was created to predict the deposition time and the velocity preceding adhesion for the selection of experimental operating conditions and the methods useful for data analysis. The likelihood of cell attachment was represented by a quantity called the sticking probability which was defined as the inverse of the number of times a liposome made contact with the surface before attachment occurred. The sticking probability decreased as the cell receptor concentration was lowered from $\sim 10^4$ to 10^2 receptors per 4-μm diam liposome and as the shear rate increased from 5 to 22 s^{-1}. The effect of the wall shear rate and particle diameter on detachment of liposomes from a surface was also observed.

INTRODUCTION

Receptor-mediated adhesion of cells in a fluid stream to specific ligand-binding surfaces plays an important role in many biological processes. For example, lymphocyte "homing" during the immune response (Woodruff et al., 1987) and tumor metastasis (Nicholson, 1984) both depend on selective adhesion of endothelial cells to blood vessel walls. The specific adhesion of cells to a surface in a flow field can also be used to develop cell separation methods for medical and biotechnological applications by techniques such as cell affinity chromatography (Sharma and Mahendroo, 1980). Yet, though cell adhesion in flow fields has been observed in many different systems and used in several applications, the relationships between fundamental parameters governing the process have not been well quantified or incorporated into a comprehensive predictive model. The aim of our work, therefore, has been to conduct fundamental experiments with a well-defined system of model receptor-bearing cells (liposomes) interacting with a ligand-coated surface under flow conditions. Results from these and other experiments should increase our understanding of the basic mechanisms involved in receptor-mediated cell adhesion.

Previous workers have demonstrated experimentally some of the critical parameters necessary for the specific adhesion of cells to other cells or surfaces. Rutishauser and Sachs (1975) measured the binding of cells to lectin-coated fibers and noted that the binding increased with increasing surface density of lectin on a fiber, with increasing density of cell receptors, and with increased mobility of cell receptors. In micro-manipulation experiments, binding has also been found to be a function of the rate of receptor diffusion to the binding region and of the contact time between the two surfaces (McCloskey and Poo, 1986). Other investigators have studied the process of cell adhesion to and detachment from surfaces during fluid flow in tubes and channels. The critical shear stress for cell detachment and the fraction of cells remaining attached in flow chamber experiments varied depending on whether the surface was glass, polymeric, or protein (Kowalczynska et al., 1982; Mohandas et al., 1974) and whether plasma proteins and cations were present in the perfusing solution (Forrester and Lackie, 1984). Doroszewski (1980) investigated the behavior of individual cells near a glass surface and measured a distribution of adhesion times on the surface, which ranged from short to infinite. At low shear rates, cells were observed to stop and stick, whereas at intermediate shear rates, cells frequently attached to and detached from a surface several times (Duszyk and Doroszewski, 1982). At high shear rates, a minimum critical shear stress, above which no cells adhere, has been observed (Mohandas et al., 1974). Mege and co-workers (1986) also showed that the number of cells adhering to the inside walls of capillary tubes, after they had settled and then had been exposed to a particular shear flow, increased with incubation time. This result suggests that cell-surface contact time affects the strength of adhesion. In summary, the receptor density, receptor mobility, ligand density on the surface,

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receptor-ligand binding strength, cell-surface contact time, contact area, and shear force due to fluid motion have been identified as parameters critical in adhesion.

We have selected a model adhesion system consisting of liposomes with glycophorin embedded in their surface lipid layers flowing past a wheat germ agglutinin (WGA)-coated surface. Glycophorin liposomes have been prepared previously for use as model cells in binding experiments (MacDonald and MacDonald, 1975; Ketis and Grant, 1983). An advantage of using liposomes is that the model receptor (glycophorin) concentration and lipid composition in the membrane can be varied to see the effect of various receptor surface densities and mobilities on adhesion. Furthermore, there are no cytoskeletal contributions because there is no intracellular matrix within the liposomes. Glycophorin was chosen as a model receptor because it is a well-characterized membrane glycoprotein which is known to bind specifically to WGA (Bhavanandan and Katlic, 1979). A microscope slide with covalently bound WGA was chosen as the second binding surface to have mobile binding molecules present on only one of the two interacting surfaces.

Our adhesion experiments were done in a parallel plate flow chamber. The advantages of this chamber are that the liposome approach to, attachment to, and detachment from a surface can be directly observed and recorded on video tape, and that a well-defined flow field and constant shear field can be generated. In our work, data were collected on individual liposomes as opposed to average measurements on cell populations to permit direct analysis of the process without the additional variability due to liposome nonuniformity. Previous work with cell adhesion in flow chambers has been focused on measuring the total number of cells adhering to the surface under different operating conditions. A collection efficiency was defined (Forrester and Lackie, 1984; Doroszewski, 1980), correlating the number of cells bound with the velocity before attachment (Doroszewski et al., 1979), the number of cells bound at different shear rates (Hochmuth et al., 1972), or the number remaining after an adhesion experiment and fixation process (Mege et al., 1986). However, as the flow rate changes, the rate of delivery of cells to the surface for binding changes. Thus, a more fundamental approach to adhesion studies involves the observation of single cells near the surface and then measuring the number of successful adhesion events as they occur.

EXPERIMENTAL METHODS

Liposome preparation

Liposomes were prepared by a modification of technbiques due to Ketis and Grant (1982) and Alving and Swartz (1984). Different volumes of a glycoprotein solution (human type B-glycophorin G-9511; Sigma Chemical Co., St. Louis, MO; made up to a concentration of 1 μg/ml with distilled, deionized, and 0.2 μm Nucleopore filtered water) were mixed with 10 μg of lipid (dipalmitoylphosphatidylcholine, DPPC, P-0763; Sigma Chemical Co.) in weight ratios of 0.013:1, 0.0004:1, or 0.0001:1 and then were placed in a 75-ml pear-shaped flask with 3 ml of a chloroform/methanol solution (4:1 volume ratio). A protein/lipid film was dried on the inside of the flask by rotary evaporation under a gentle stream of N_2 at 45° C, and the flask was placed in an oven at 100°C under vacuum overnight. The next day, a buffered Ficoll solution (0.103 M Na_2HPO_4, pH 7.4, 0.02% sodium azide, with 0.151 g/ml of Ficoll type 400; Sigma Chemical Co., to create a density of 1.053 g/cc) was prepared and added to the flask while it was immersed in a hot water bath (40°C). The flask was shaken gently by hand to remove the lipid from the wall of the flask, and vesicles formed spontaneously in the solution.

The final step of the procedure was a density separation for isolation of a liposome fraction with a density of ~1.04 g/ml. The liposome-suspending solution was diluted to a density of 1.043 g/cc (0.118 g/ml of Ficoll) with the Na_2HPO_4 buffer, and the sample was centrifuged at 1,000 *g* for 10–15 min to pellet lipid fragments and debris. Then the supernatant was removed and diluted with buffer again to achieve a density of 1.037 g/cc (0.0943 g/ml Ficoll). This sample was centrifuged at the same conditions to pellet liposomes with a density between 1.037 and 1.043 g/cc. Finally, the pellet was collected and washed in buffer several times by suspension and centrifugation at 1,000 *g* for 10 min to remove Ficoll. The liposome preparation contained a variety of vesicle shapes and sizes, but a substantial fraction was spherical with a diameter of 3–10 μm. The binding activity of the glycophorin was confirmed by the fact that the glycophorin liposomes agglutinated in a WGA solution whereas plain liposomes did not.

Lectin surface preparation

WGA was covalently bound to a glass microscope slide in several steps. First, three or four slides were cleaned in a boiling detergent solution (Alconox) followed by a rinse in boiling water. Then they were soaked in cleaning solution for ~20 min (1:1 volume ratio of water and chromic-sulfuric acid Cleaning Solution, SO-C-88; Fisher Scientific Co., Pittsburgh, PA), rinsed well with distilled water, soaked in 3 mM NaOH for ~20 min, rinsed well again, and finally dried in an oven (110–125°C) overnight. The next day, the dry slides were placed in a covered 100 ml Petri dish containing 50 ml toluene plus 2 ml of 3-aminopropyltriethoxysilane (Kodak Chemical Co.). About 15–20 min, the slides were rinsed in toluene and placed in the oven for drying at 110–125°C overnight. The slides were then stored for future use.

The lectin was coupled to the slide by using the difunctional reagent glutaraldehyde which reacted with the aminopropyl groups on the surface and the amine groups on the lectin. Dry silanized slides were placed in a solution of 3% glutaraldehyce (Kodak Chemical Co.) for 2 h. The slides were then rinsed well with distilled deionized water and patted dry with paper towel, and a lectin solution was placed on a rectangular area of ~6.5 cm^2 in the center of the slide for 2 h. Three different concentrations of lectin were used, either 96, 43, or 5 μg of WGA (wheat germ agglutinin, L-9640; Sigma Chemical Co.) in 400 μl of the Na_2HPO_4 buffer. The lectin solution was rinsed from the slides with the Na_2HPO_4 buffer, and the slides were placed in 15 mM glycine-Na_2HPO_4 buffer solution for 2 h to saturate any unreacted aldehyde groups. Finally, the slides were rinsed in the Na_2HPO_4 buffer. The slides were stored in the Na_2HPO_4 buffer solution and used the same day.

Flow chamber

Fig. 1 gives a detailed schematic of the parallel plate flow chamber. The chamber was constructed from Plexiglas. The microscope slide fit into a

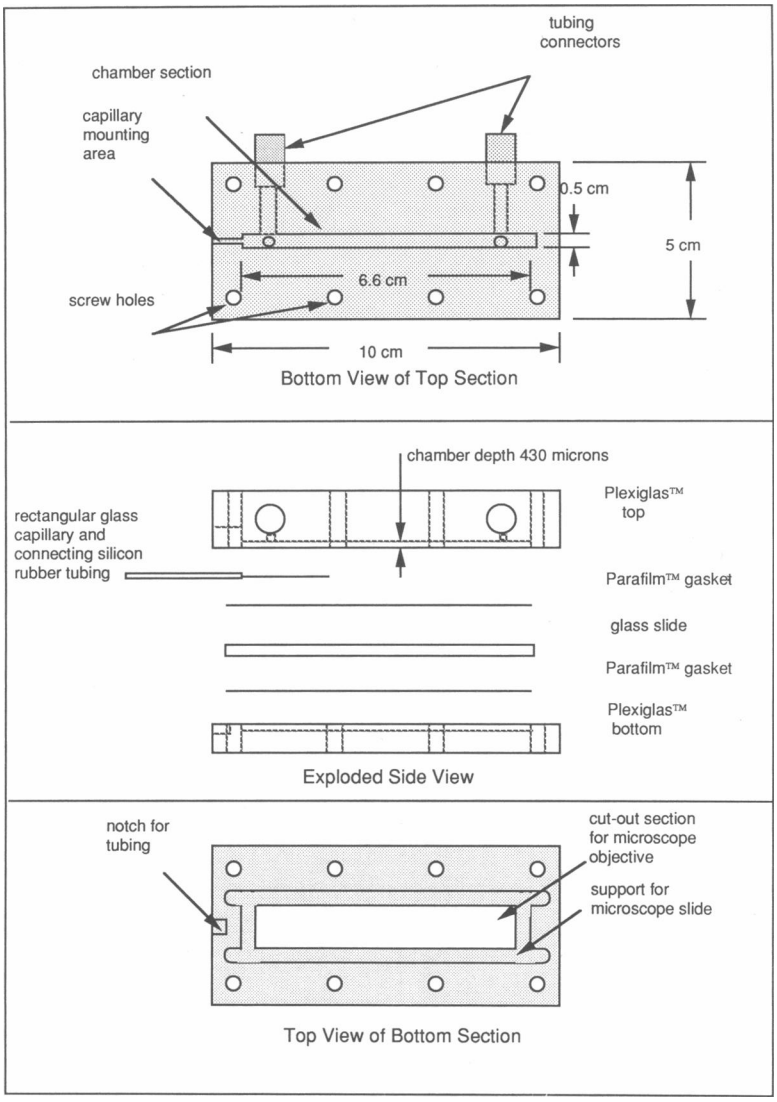

FIGURE 1 Flow chamber.

groove cut into the bottom piece. The walls and top surface of the chamber were formed by a channel 0.5 cm wide, 6.6 cm long, and 430 μm deep cut into the top piece of Plexiglas. The major modification of our chamber over previous designs is that a rectangular glass capillary tube (Microslides, No. 5005; Vitro Dynamics, Rockaway, NJ) was used to deliver the liposomes to the chamber. The capillary was used so that a small stream of liposomes could be introduced into a well-developed flow field at a particular height above the lectin surface. The tube had internal dimensions of 500 μm by 100 μm (width and depth, respectively), and it was placed in the chamber so that the mouth of the capillary was several chamber widths downstream of the buffer inlet port. The capillary was mounted and sealed into position with a gasket made from silicone adhesive. The distance between the mouth of the capillary and the chamber exit was ~4 cm. Parafilm gaskets were used on either side of the lectin slide to ensure a good seal. The Parafilm gasket placed between the slide and the chamber top increased the total depth of the chamber from 430 to 580 μm. The two halves of the chamber were held together by eight machine screws.

An illustration of the assembled flow chamber for the adhesion experiments appears in Fig. 2. The liposomes were delivered to the chamber by the glass capillary tube which was positioned midway between the two side walls of the chamber and ~20–40 μm above the lectin surface. Two Sage syringe pumps with Hamilton syringes were used to pump the buffer and liposomes separately through the chamber. The syringe size and the pump flow rate controller determined the volumetric flow rate delivered to the chamber. The sizes of the syringes used for the liposome and buffer fluids were 0.5 ml (pump 1) and 5 ml (pump 2), respectively, for wall shear rates of 22 s^{-1} and 5 s^{-1} or 1 ml and 10 ml for a wall shear rate of 10 s^{-1}. Liposome adhesion was observed with an inverted Zeiss microscope focused on the top surface of the lectin slide, and the data was recorded with a Dage-MTI 67M video camera which was connected to the microscope. A JVC video cassette recorder (BR-9000U) was used to record the data, and a Panasonic black and white monitor (WV-5410) was used both to observe the experiment in real time and to analyze the data collected on the videotape. The experiments were run at temperatures of 22–25°C.

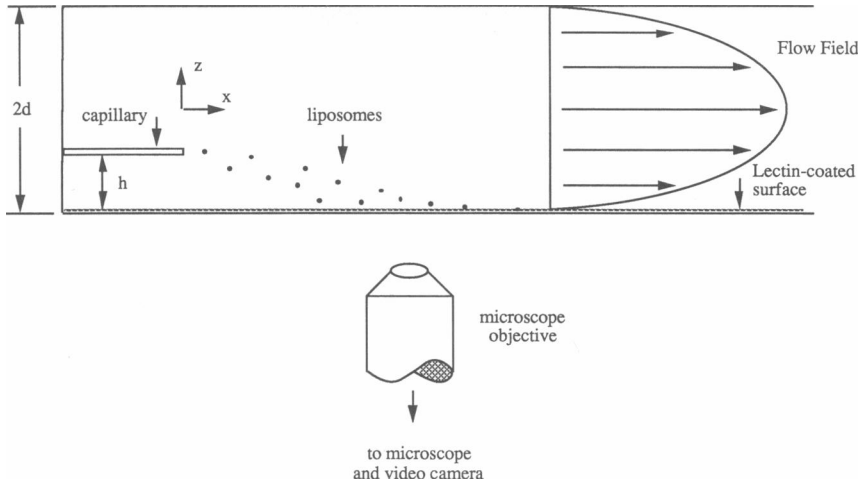

FIGURE 2 Side view schematic of chamber and variables used in glycophorin liposome/wheat germ agglutinin adhesion experiments under flow conditions. The coordinate system used in the model is shown, h represents the height of the lower edge of the capillary above the bottom surface, and $2d$ represents the depth of the chamber.

Attachment experiments

The attachment experiments were conducted to observe the behavior of a liposome just before and after deposition at wall shear rates of 5, 10, and 22 s^{-1}. The classical equation for calculating the wall shear rate (S, s^{-1}) between parallel plates is given by the following expression,

$$S = \frac{V}{40Wd^2}, \quad (1)$$

where V is the volumetric flow rate in milliliters/minute, W is the channel width in centimeters, and d is the half-channel depth in cm (Bird et al., 1960).

It was important to prevent the liposomes from settling in the syringe and tubing before they entered the chamber to assure that a low but uniform concentration of liposomes entered the chamber for a period of ~30 min. A low concentration was desirable for eliminating particle–particle interactions. To do this, we removed the silicone tubing connecting the capillary to the syringe pump near the chamber entrance and deposited the liposomes in the tubing with a Hamilton Co. (Reno, NV) 25-μl syringe. The buffer flow (created with pump 2) through the chamber was then allowed to stabilize (backlash in the pump gears was eliminated) for several minutes. Immediately thereafter, the silicone tubing was reconnected to pump 1 and liposomes were pumped into the chamber. The total volumetric flow rate varied from 0.085 ml/min to 0.37 ml/min for 5 and 22 s^{-1} shear rates, respectively, and the liposome flow rate was 3% of the total flow rate in all cases to match velocity with the surrounding flow. There was some inherent variability in liposome entry elevation due to the finite capillary inner diameter, but no way to eliminate this problem (e.g., by drawing down the capillary) was found which did not further complicate the matter by disturbing the flow patterns. The bottom surface of the chamber was observed at 125× magnification until a well-formed spherical liposome with a diameter of 3–10 μm moved into the field of view. The movement of this liposome across the field of vision was then recorded. The microscope stage was manually advanced as many times as necessary to keep the liposome in view until the liposome stuck permanently, exited the chamber, or encountered an obstruction. The data for a given liposome were discarded if debris or other liposomes directly contacted or obviously interfered with the trajectory of the target liposome. After this liposome stopped on the surface, the microscope objective was switched to provide 215× magnification and the diameter of the lipsome was estimated by comparing it with a calibrated grid in the microscope eyepiece. Later, the video tape was analyzed to calculate the liposome translational velocity at 1-s intervals from the change in position between successive frames on the videotape. The videotaping system automatically recorded time and date information on each frame. A stuck liposome was defined as a liposome that did not change position in two video frames, a time interval of 1/30 of a second.

THEORY

Particle trajectory model

Liposome adhesion to the lectin surface occurs in two steps. First, the particles must sediment to within a critical interaction distance of the lectin surface, and second, glycophorin and WGA must form bonds which arrest the particle. It has been estimated that cell-surface interactions become important at gap distances of 20–30 nm assuming that the glycophorin and lectin each extend above their respective surfaces ~7–10 nm (Bongrand and Bell, 1984). The model for the particle trajectories presented here includes only fluid mechanical forces, so it is valid only when the liposomes are >30 nm from the wall. Other colloidal forces should be negligible beyond this point.

The particles in the chamber have two velocity components, a translational velocity $u(z)$ down the length of the chamber and a sedimentation velocity $v(z)$ toward the bottom surface. There is no appreciable velocity component toward either side wall in the chamber because the liposomes are introduced in the center of the chamber and

there is no velocity gradient across the capillary mouth relative to the liposome diameter. Because the chamber Reynolds number is ~1 and the particle Reynolds number is $\sim 10^{-5}$, the translational velocity of the particles in the chamber is assumed to be the same as the fluid velocity at the midpoint of the particle. For the flow chamber, the Reynolds numbers based on the chamber and particle respectively are defined by

$$Re_c = \frac{V\rho}{\mu} \frac{2}{(W + 2d)} \tag{2}$$

$$Re_p = \frac{2u_p R\rho}{\mu}, \tag{3}$$

where V is the volumetric flow rate, ρ is the solution density, μ is the solution viscosity, W is the chamber width, d is half the channel depth, R is the particle radius, and u_p is the particle velocity relative to that of the fluid. A reasonable upper bound estimate for u_p is the Stokes law sedimentation velocity of the particle. The analysis is simplified because the Navier-Stokes equations and the boundary conditions are linear for small Reynolds numbers, allowing the overall particle movement to be broken down into two separate linear problems. The first problem is that of the translation of a neutrally buoyant sphere in a shear flow, and the second is that of a sedimenting sphere in a stagnant fluid. The velocity equations for each component may be derived separately, and the actual particle velocity is calculated by adding the results of the two solutions.

Because we are operating at a low Reynolds number, the velocity equation for a neutrally buoyant particle between two plates is given by the parabolic flow profile and the sedimentation velocity for a particle in a still fluid can be calculated from Stokes law. However, due to the presence of the wall, the particle velocity will be retarded as shown by Goldman and co-workers (1967) for the translational component and by Brenner (1961) for the sedimentation component. The correction factor derived by Goldman and co-workers for the translational velocity component of a rigid sphere near a wall in Couette flow can be applied in our system if we make the assumption that the correction factor is the same for a particle in Couette flow as in a Poiseuille flow profile. This is a reasonable assumption because the model is applied to the behavior of liposomes in the region near the wall where the fluid velocity gradient becomes linear. Also, because the length scale ratio of the channel depth to particle diameter is >50, the difference between a linear shear flow and a parabolic shear flow experienced by a particle at any particular height in the chamber is small. In our calculations, the shear rate at the midpoint of the particle is used to estimate the average value of the shear gradient on the particle for evaluation of the correction factor derived by Goldman and co-workers.

The second assumption made in applying the previous work on particle behavior in shear fields to this system is that the liposomes can be treated as rigid spheres during the sedimentation process. The validity of this assumption cannot be rigorously proven because little previous work has been done on the modeling of cell and liposome behavior in flow fields. The liposome is, in principle, deformable, and the membrane may distort in the presence of the shear gradient. However, we can refer to previous work by Bentley and Leal (1986) on drop deformation in steady, two-dimensional linear flows to estimate an upper bound for the extent of vesicle deformation in a particular shear field. Their experimental and theoretical work described drop deformation in terms of the difference between the longest and shortest semi-axes of the drop cross-section divided by the sum of the two dimensions for different liquid and droplet viscosity ratios, capillary numbers, and flow types. The capillary number, C, which is a dimensionless ratio of shear to surface tension forces defined as

$$C = \frac{S\mu R}{\sigma}, \tag{4}$$

where σ is the interfacial tension between the two fluids in dynes/cm. For cells or liposomes, σ represents the elastic extensional modulus of a membrane as described in work by Evans and Parsegian (1983). For a 4-μm diam particle in a shear field of 22 s^{-1}, the capillary number is $\sim 8 \times 10^{-3}$, using an estimate of 10^{-2} dynes/cm for σ as suggested by Evans and Parsegian (1983). At this low capillary number, Bentley and Leal predicted a deformation of less than a few percent which confirms our assumption of negligible deformation due to the shear field.

Thus, the solution for the translational velocity of a neutrally bouyant sphere in a parabolic flow field with the appropriate wall correction factor is

$$\frac{dx}{dt} = f_1(z)U_{max}\left\{1 - \frac{z^2}{d^2}\right\}, \tag{5}$$

where $f_1(z)$ represents the correction factors determined numerically and tabulated by Goldman and co-workers, U_{max} is the centerline velocity in the chamber, x is the distance from the mouth of the capillary, and z is the distance between the chamber centerline and the particle midpoint. The sedimentation velocity for a sphere settling in a still fluid as it approaches a wall is governed by

$$\frac{dz}{dt} = \frac{V_{sed}}{f_2(z)} \tag{6}$$

with

$$f_2(z) = \frac{4}{3}\sinh\alpha \sum_{n=1}^{\infty} \frac{n(n+1)}{(2n-1)(2n+3)} \cdot \left\{ \frac{2\sinh(2n+1)\alpha + (2n+1)\sinh 2\alpha}{4\sinh^2(n+1/2)\alpha - (2n+1)^2\sinh^2\alpha} - 1 \right\}, \quad (7)$$

where $f_2(z)$ is the correction factor derived by Brenner, α is a parameter defined in the solution of the equations which goes to zero as the particle approaches the wall, and V_{sed} is the sedimentation velocity of the particle in an unbounded fluid. Stokes law was used to estimate the liposome sedimentation velocity,

$$V_{sed} = \frac{2}{9}\frac{R^2(\rho_p - \rho)g}{\mu}, \quad (8)$$

where ρ_p is the particle density and g is the gravitational constant.

The solution for the trajectory of a liposome which is translating and sedimenting in the chamber was found by numerical integration of Eqs. 5 and 6 for specific cases corresponding to actual experimental conditions. The initial conditions used were the x and z coordinates of the particles at the initial time. The program was written in Fortran, and calculation was carried out on a Microvax II computer. A description and copy of the program may be found elsewhere (Reagan, 1988). When comparing the calculated model trajectory with the experimental data, we chose the initial time to correspond to the time at which the videotaping of a particular liposome was begun. The instantaneous translational and sedimentation velocity components were also solved for at each integration step in the program. To compare experimental and predicted particle trajectories at different flow rates, we defined a dimensionless velocity as the particle velocity divided by the chamber centerline velocity, u/u_{max}. u_{max} was calculated from the equation for fluid flow between parallel plates as a function of the volumetric flow rate, and the volumetric flow rates were measured experimentally.

A comparison of the calculations for sedimentation time and distance traveled before reaching the surface with and without the correction factors shown in Fig. 3 illustrates the importance of the corrections. The calculations are for a 4-μm diam liposome with a density of 1.040 g/ml, a fluid density of 1.009 g/ml, a fluid viscosity of 0.8904 cp, a wall shear rate of 22 s^{-1}, and an initial height of 65 μm above the surface. When the correction factors have been taken into account, a liposome should sediment in ~4.2 min at 3.5 cm from the entry point. When the corrections are neglected, the sedimentation time is 3.5 min and the distance traveled before sedimentation is 8.3 cm. The important difference between the two calculations is that, in the uncorrected solution, the particle translational velocity is the same as the fluid velocity, but in the corrected case, it is less than the fluid velocity as the particle approaches the wall. Thus, for the same time period, the travel distance is much longer for the uncorrected case than for the corrected case. However, a slightly longer sedimentation time is calculated for the corrected case because the sedimentation velocity decreases toward zero as the particle approaches the wall.

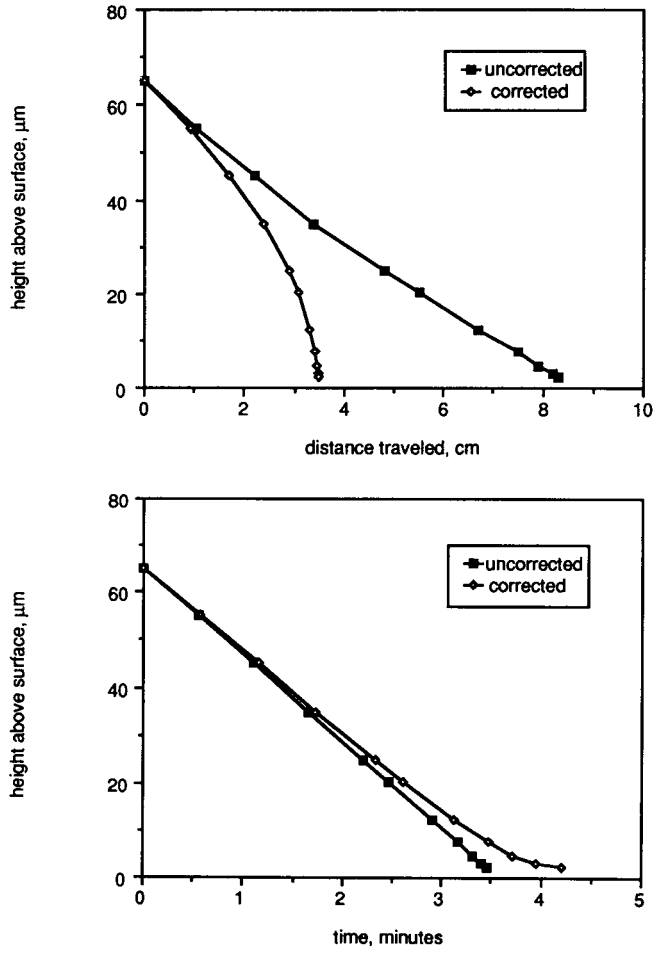

FIGURE 3 Effect of correction factors on model prediction of distance traveled and settling time before deposition. The example shown is for a 4-μm diam liposome with a density of 1.04 and a wall shear rate of 22 s^{-1} at an initial height of 65 μm above the surface. The dark squares represent the uncorrected cases and the open diamonds represent the corrected cases.

RESULTS AND DISCUSSION

Qualitative observations

Experiments with red blood cells (RBC) verified that specific adhesion of cells to the lectin surface could be

observed. RBCs were used for the initial adhesion experiments because they contain glycophorin molecules in their surface lipid layer and outdated cells were easily obtained from a local Red Cross blood bank.

The trajectories of RBC approaching plain glass, and aminopropyl, WGA, and glycine-coated glass surfaces were observed at different wall shear rates, and several types of behavior were seen (see Table 1). On the aminopropyl surface at all shear rates observed, the RBC did not stop or stick. They appeared to sediment and travel down the length of the chamber without approaching the surface close enough for the cell to be arrested and stop. A different behavior was observed both on the uncoated glass and on the glycine coated surfaces. At low shear rates, the cells would stop and stick, and at the higher shear rates the cells would stick and release intermittently. In contrast to these two previous types of behavior, cells on the WGA surface stuck very abruptly without detaching at all shear rates studied. The abrupt and permanent adhesion to the WGA surface as compared with no adhesion on the aminopropyl surface and weak adhesion on glass and glycine surfaces at high shear rates indicates that the WGA surface does have a strong specific affinity for the glycophorin-bearing RBC.

Liposome deposition and adhesion experiments

Glycophorin liposomes were prepared with three different ratios of glycoprotein to lipid to observe the effect of glycophorin concentration on adhesion. For the highest glycophorin/lipid ratio of 0.013:1, the liposomes adhered permanently upon first contact at all three shear rates observed in the same manner as the RBC. However, at lower glycophorin/lipid ratios, the liposomes repeatedly detached from and reattached themselves to the surface provided that the shear rate was high enough. The effect of glycophorin concentration on binding indicates the importance of specific receptor-ligand binding in permanent adhesion. Plain liposomes on a WGA surface were observed to detach repeatedly from and reattach to a WGA surface at wall shear rates of 10 and 22 s^{-1}, further indicating that, in the absence of glycophorin, the probability of permanent adhesion on first contact is reduced.

The glycophorin liposomes settled onto the WGA surface before exiting the chamber at low rates expected from the model calculations. A comparison between the experimental and predicted particle trajectories appears in Fig. 4. The agreement is quite good, suggesting that the model assumptions were appropriate. Most of the trajectory deviations from expected values could be attributed to other particles and debris interacting with the observed liposome. An increase in particle velocity could be observed whenever a liposome traveling at a higher velocity passed by a slower liposome or whenever a liposome traveled over an attached liposome or a piece of debris already on the surface of the slide. Such problems were prevented by keeping the liposome concentration in the chamber low to minimize the particle–particle interactions, and by doing the experiments for short periods of time so that the surface coverage by attached liposomes was low. Whenever the concentration on the surface became large enough that liposomes began to collide with those previously attached, the experiment was terminated. A second factor which contributed to the difficulty in eliminating particle interactions was that the number of well-formed spherical liposomes with a diameter between 3 and 10 μm was a small fraction of the total

TABLE 1 Observations of red blood cell behavior near different surfaces.

Surface	Shear rate 1/s	Observations
Aminopropyl coated glass	2.2–38	No sticking
Glycine coated glass	2.2–3.8	Some stopping and starting, some sticking
	22–38	Little stopping or sticking
Glass	2.8–3.8	Stopping and starting, sticking
	22	No stopping or sticking
Wheat germ agglutinin coated glass	3.8–38	Abrupt sticking

Definition of observation terms: stopping and starting—stopped for several seconds and then continued moving; sticking—stopped for duration of experiment.

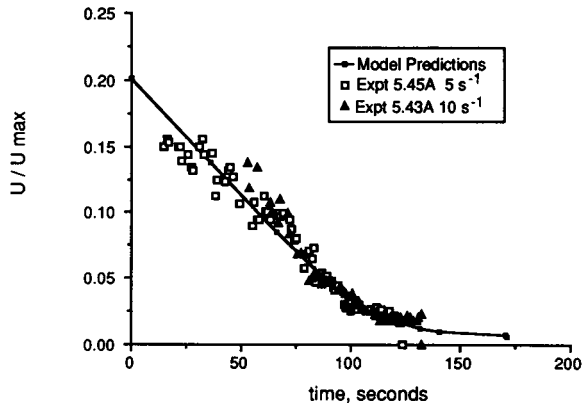

FIGURE 4 Comparison of experimentally observed liposome velocity with model calculations. Each set of data points represents the velocity of a single liposome as it travels down the length of the chamber. The curve represents the calculated model trajectory. The liposome diameter was 4 μm, the glycophorin/lipid ratio was 0.013:1, the surface lectin concentration was 96 μg, and the shear rate was 5 s^{-1} for Expt 5.45A and 10 s^{-1} for Expt 5.43A.

number of those in the preparation even after density fractionation. Discarding flawed data was preferable to using extremely low concentrations from the standpoint of gathering sufficient data in a reasonable period of time.

In nearly all of the results, the liposome trajectories follow the model trajectories near the surface before adhering abruptly within less than a few hundredths of a second. Thus, the liposomes were not gradually slowed by a few bonds forming and breaking as they rolled near the surface. Within our observational ability, it appears that the attractive force between the liposome and surface acts in an "all or none" manner; that is, either the cell travels according to the trajectory expected solely from fluid mechanical forces, or it adheres abruptly to the surface due to intermolecular forces. This behavior indicates that even for the lowest glycophorin concentration in the liposome membrane and the highest shear rate conditions, there were enough bonds formed upon initial contact to arrest the particle very quickly, even if it eventually detached a few seconds or tenths of a second later.

The experimental and predicted separation distances between the liposome and surface before adhesion were calculated to estimate the distance at which an attractive force may become significant. Fig. 5 is a plot of the separation distance between the liposome membrane and the surface calculated from the results in Fig. 4 and the theoretical predictions from the model. The distance which corresponds to a given experimental velocity measurement is determined by interpolation from a table of particle velocities at different distances above the surface constructed from the model calculations. The error bars shown in the plot are calculated from the experimental uncertainty in the velocity measurement and the resulting propagated uncertainty in the liposome height above the surface. The data do follow the predicted trends well to within ~1 or 2 μm from the surface. At this distance from the surface, however, the experimental error from the uncertainty in our measurement is of the same order of magnitude as the separation distance itself, so the actual distance over which adhesion occurs cannot be determined accurately. A simple calculation of the expected average displacement due to Brownian motion for a 4-μm diam sphere with a Stokes-Einstein diffusion coefficient of 1.1×10^{-9} cm^2/s in 1/30 s (frame-to-frame time interval) yields an estimate of 0.086 μm. Whereas some of the variability in position may be due to Brownian motion, it cannot completely account for the rapid attachment to the surface either. However, the need for further work is clearly indicated to identify the exact mechanism of particle-surface contact. An important implication of our observations is that our current experimental system can be used to examine the events following contact between particle and surface, but not the effect of colloidal forces on the initiation of contact. A more refined study will require an ability to make observations on a smaller length scale, such as that offered by the technique of Prieve and Alexander (1986).

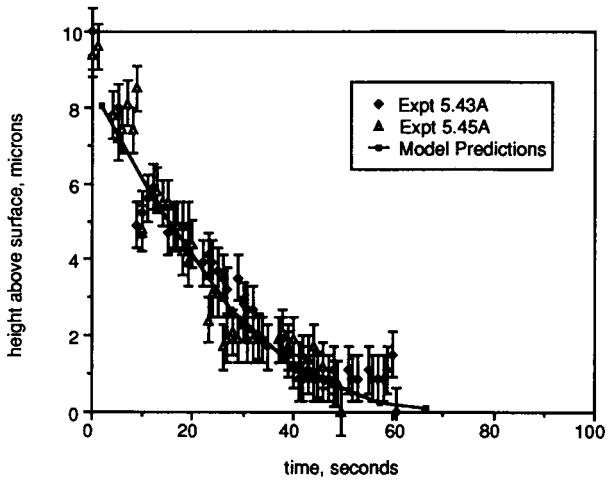

FIGURE 5 Decrease in separation distance with time traveled near surface. The data points represent results from two different experiments, and the curve represents the model prediction. The liposomes were 4 μm in diam, the glycophorin/lipid ratio was 0.013:1, the lectin concentration was 96 μg, and the shear rate was 5 s^{-1} for Expt 5.45A and 10 s^{-1} for Expt 5.43A. The error bars were calculated from the error in the velocity measurement.

Effect of receptor density and fluid force on attachment

We investigated the effect of the liposome membrane glycophorin concentration and the fluid force on the attachment process. The glycophorin concentration was varied by changing the ratio of lipid to glycophorin used in preparing the liposomes. Assuming that the cross-sectional areas of glycophorin and DPPC in the bilayer are 1 and 0.5 nm^2 (Goodwin et al., 1982) and that 50% of the glycophorin has sugar binding groups on the outside of the liposome, we can estimate the number of receptors on a 4-μm diam liposome for the three protein/lipid weight ratios of 0.013:1, 0.0004:1, and 0.0001:1 to be 3×10^4, 1×10^3, and 2.4×10^2, respectively. The fluid force was a function of the flow rate and the liposome diameter as defined in Eq. 9. After the density fractionation, the liposome diameters still varied from 3 to 10 μm in all samples. Thus, because the fluid force is proportional to the square of the particle radius, the variation in diameter allowed a greater range of fluid forces to be studied than if only the flow rate determined the fluid force.

Four general types of attachment behavior were observed in the experiments as the flow rate, glycophorin

concentration, and particle diameter changed. Case I behavior was abrupt permanent sticking to the surface upon first contact. Fig. 4 shows an example of this case. For the highest glycophorin membrane concentration prepared, all of the liposomes were observed to permanently adhere on first contact with the surface. Some of the smaller liposomes with the medium and low concentration of glycophorin also stuck on first contact at the lower flow rates. In case II (shown in Fig. 6) the liposomes would stick and release a few times before sticking permanently after a short period of travel near the surface. The conditions for case II were generally intermediate shear rates, medium glycophorin concentration, and larger liposomes. In case III, the liposomes stuck and released repeatedly from the surface as seen in Fig. 6. The case III results were observed mainly for low glycophorin concentrations at medium and high shear rates. In comparing cases II and III, we see that for case II the cells are near the surface for 10–20 s before adhering whereas in case III they are near the surface 100–150 s before permanent adhesion occurs. In case IV shown in Fig. 7, the cells stick and release many times while traveling down the surface and exit the chamber without ever

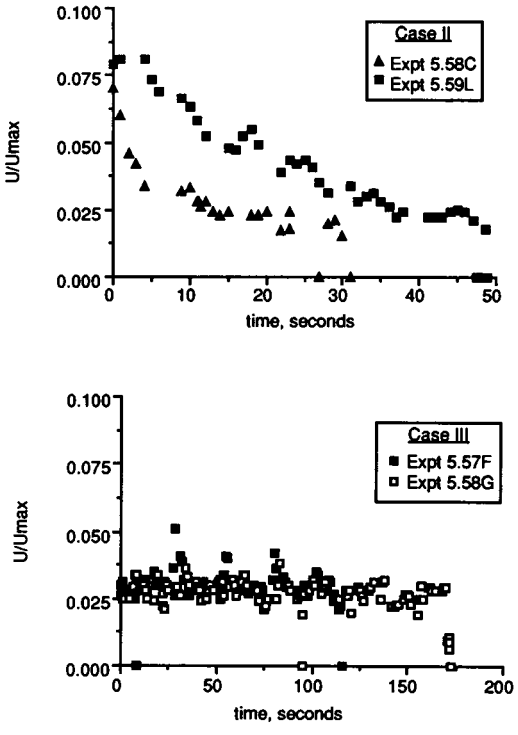

FIGURE 6 Liposome behavior on WGA surfaces, glycophorin/lipid ratio 0.0004:1, cases II and III. Experimental parameters – liposome diameter, lectin concentration, and shear rate: 5.58 C – 7 μm, 96 μg, and 22 s^{-1}; 5.59 L – 5 μm, 43 μg, and 10 s^{-1}; 5.57 F – 8 μm, 5 μg, and 22 s^{-1}; 5.58 G – 6–7 μm, 43 μg, and 10 s^{-1}.

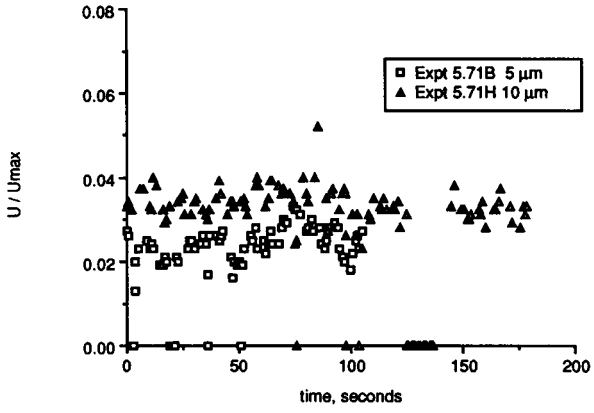

FIGURE 7 Liposome behavior on WGA surfaces, glycophorin/lipid ratio 0.0001:1, case IV. Experimental parameters – liposome diameter, lectin concentration, and shear rate: 5.71 B – 5 μm, 96 μg, and 22 s^{-1}; 5.71 H – 10 μm, 5 μg, and 22 s^{-1}.

permanently attaching. The conditions necessary for case IV behavior to be observed were generally high shear rates, low glycophorin concentration, and large liposomes. A relationship between the lectin surface density on the glass slide and which of the four cases a result fell into was not noticeable over the range we studied.

Multiple adhesive events can be described mechanistically as a temporary attachment during a time period when the cell-surface bonds are not fully stressed, but the cell will eventually detach from the surface because there were not enough bonds formed to resist the shear force. Temporary adhesion may occur briefly before the bonds are fully extended and stressed by the distracting force of the fluid. In the Hammer and Lauffenburger model, temporary cell adhesion corresponds to cell adhesion during a short time period when the bonds are not stressed followed by cell detachment at a time when the bonds are fully stressed. At intermediate shear rates, permanent adhesion may follow temporary adhesion because the receptor density may vary from region to region on the cell surface, and the number of bonds formed in the contact time will depend on the local receptor density of the area in contact with the surface. At higher shear rates, even the area with the highest receptor density may not form enough bonds to keep the cell permanently adhered to the surface, so the cell may repeatedly attach to and detach from the surface and never permanently adhere.

The four types of sticking behavior can be summarized graphically after we define a quantity called the sticking probability, which is the inverse of the number of adhesive contacts a liposome makes before it permanently adheres to the surface. A liposome which adheres on first contact has a sticking probability of 1, whereas for a liposome which never adheres it is 0. The sticking probability is

plotted as a function of the wall shear rate and the membrane glycophorin concentration in Fig. 8. The data for the three different lectin surface concentrations were averaged together. Only the data for liposomes with diameters between 3 and 6 μm were used in this plot because larger liposomes tended to detach and reattach many more times than the smaller ones. For example, at the highest shear rate and intermediate glycophorin concentration, 9- and 10-μm diam liposomes were seen to attach and detach 8 and 13 times, respectively. The average particle diameter for the data plotted was 4–5 μm. The glycophorin liposomes with the highest glycophorin concentration always adhered upon first contact, so the sticking probability is 1 at all three wall shear rates. For the intermediate glycophorin membrane concentration, the sticking probability is 1 at the lowest shear rate, but it decreases as the shear rate increases. For the lowest glycophorin membrane concentration, the sticking probability is <1 even at the lowest shear rate and it decreases further as the shear rate increases. This plot shows clearly the dependence of the probability of adhesion in a given encounter on membrane receptor concentration and wall shear rate. As the membrane receptor concentration decreases and the shear rate increases, the probability of permanent adhesion per contact decreases, and it is more likely that the liposome will not adhere permanently before exiting the chamber. The results obtained at this point should be understood to represent general trends and not exact quantitative results due to the small number of observations and the uncounted liposomes which exited the chamber before permanently sticking. Further work is required to obtain greater statistical accuracy in the probabilities. The standard deviation and the number of liposomes observed for each case shown in Fig. 8 appears in Table 2.

We have been able to find semiquantitative agreement between these data and a previous theoretical analysis of dynamical cell adhesion (Hammer and Lauffenburger, 1987). Assuming that adhesion is "rate-controlled" (that is, the likelihood of adhesion depends on the rate of bond formation during the contact time), this analysis predicts

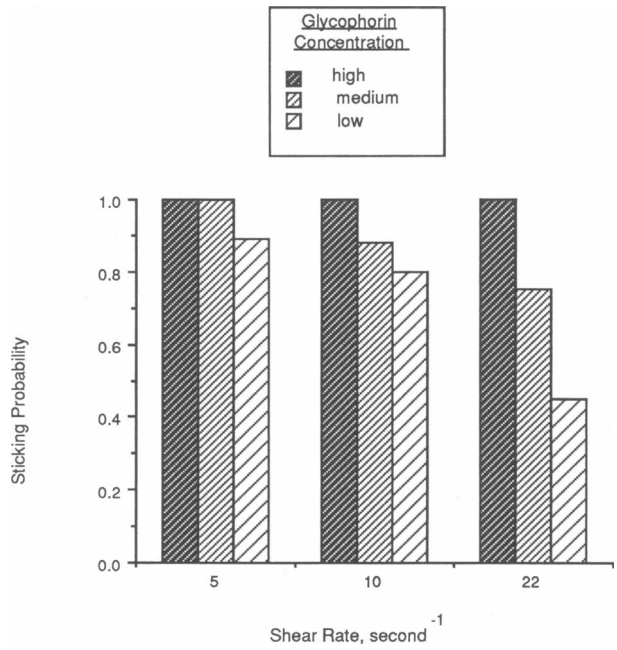

FIGURE 8 Sticking probability at different shear rates for liposomes with 4–5 μm average diameter. High, medium, and low glycophorin concentrations represent glycophorin/lipid ratios of 0.013:1, 0.0004:1, and 0.0001:1, respectively. The standard deviation and number of liposomes observed for each case appears in Table 2.

an asymptotic approximation for the minimum number of cell receptors, N_T^*, necessary for adhesion in the face of shear rate S (see Eq. 21 of Hammer and Lauffenburger, 1987):

$$N_T^* = \left[\left(\frac{A_c}{4\pi R^2}\right)\left(1 - \exp\left\{-k_f \rho_L \left(\frac{A_c}{\pi}\right)^{1/2} \Big/ sR\right\}\right)\right]^{-1}, \quad (10)$$

where R is the cell radius, k_f is the bond formation rate constant, A_c is the contact area, and ρ_L is the surface ligand density. If, as generally expected for membrane reactions, k_f is diffusion-limited, then $k_f = 2\pi D/[\ln(b/s) - 3/4]$, with D the receptor diffusivity in the cell

TABLE 2 Sticking probability data with standard deviation and number of liposomes observed.

Glycophorin/lipid weight ratio	Shear rate $5\,s^{-1}$ P	Shear rate $5\,s^{-1}$ Number of liposomes	Shear rate $10\,s^{-1}$ P	Shear rate $10\,s^{-1}$ Number of liposomes	Shear rate $22\,s^{-1}$ P	Shear rate $22\,s^{-1}$ Number of liposomes
0.013:1	1.0*	4	1.0*	5	1.0*	18
0.004:1	1.0*	4	0.88 ± 0.23	8	0.75 ± 0.27	6
0.001:1	0.89 ± 0.22	9	0.80 ± 0.31	9	0.45† ± 0.44	16

*All liposomes adhered on first contact. †Six liposomes stayed near the surface and some stuck repeatedly but they did not adhere. The data was included in the probability, accounting for these liposomes with a sticking probability of 0.0.

membrane, b the mean distance between free receptors, and s the receptor/ligand encounter complex radius (Lauffenburger and DeLisi, 1983). For the present rough comparisons, we will simply use $k_f = 2\pi D$ as an approximation. The data in Fig. 8 give the sticking probability, p, as a function of S and N_T. We can compare this probability with $N_T/N_T^*(S)$ as predicted from Eq. 10. Given some reasonable estimates for the relevant parameters, $D = 10^{-10}$ cm^2/s, $A_c = 10^{-1}$ μm^2, $R = 2$ μm, and $\rho_L = 10^{11}$ cm^{-2}, we can obtain the results shown in Table 3. Notice that when $N_T/N_T^* > 1$, $p_{exp} = 1.0$, whereas when $N_T/N_T^* < 1$, $p_{exp} < 1.0$. Certainly, this is only a semiquantitative comparison because of the unknown parameter values that required rough estimation. However, the capability for interpretation of this sort of fundamental data by this theoretical model is promising.

Effect of surface lectin density on attachment

The number of WGA molecules on the surface which are available for binding to glycophorin is expected to limit the number of bonds formed with a liposome and therefore the number of adhering liposomes at low lectin surface coverage. However, it is difficult to measure the number of available binding groups for a particular receptor present due to the small surface area involved, the monolayer coverage, and the difficulty in labeling an active site without altering the receptor's binding behavior. The total amount of protein may be measured, but the number of active binding groups may not always be directly related to the amount of protein present because of protein denaturation, improper orientation of the binding site, etc. The number of active groups may vary considerably when proteins are covalently linked to a surface because the binding sites may be hidden, distorted, or destroyed in the coupling process. We are presently developing enzyme linked immunosorbent assay techniques for future measurements, but for these initial studies, we varied the concentration of lectin in the solution applied to the activated surface to permit qualitative observations on the effect of receptor concentration.

The amount of lectin placed on the slide was 96, 43, or 5 μg. For an area of ~6.5 cm^2, 43 μg should correspond to roughly monolayer coverage by lectin, assuming that all of the lectin binds to the surface and that each lectin molecule occupies 0.7 nm^2. The higher concentration was selected to see if the WGA had enough time to completely cover the surface and form a monolayer, and the lower concentration was selected to see if an order of magnitude decrease in the amount of lectin would affect the attachment process.

TABLE 3 Correlation between sticking probability and N_T/N_T^*.

	$N_T = 3 \times 10^4$		$N_T = 1 \times 10^3$		$N_T = 2.4 \times 10^2$	
S (s^{-1})	p_{exp}	N_T/N_T^*	p_{exp}	N_T/N_T^*	p_{exp}	N_T/N_T^*
5	1.00	40	1.00	1.3	0.89	0.32
10	1.00	25	0.88	0.83	0.80	0.20
22	1.00	14	0.75	0.45	0.45	0.11

For each flow rate and glycophorin concentration, liposome attachment experiments were done at the three different lectin surface concentrations. In no case did we see a significant variation in the type of sticking behavior with lectin density. It is very likely, however, that even the lowest concentrations of lectin used produced a lectin surface with enough binding groups to bind all of the available glycophorin molecules in the binding region. From the estimate of 3×10^4 glycophorin molecules on a 4-μm diam liposome mentioned previously, we can calculate the surface concentration to be 6×10^{10} glycophorin molecules/cm^2. Assuming that the cross-section of a glycophorin molecule embedded in a membrane is ~1 nm^2 (Goodwin et al., 1982), we can also calculate that a monolayer coverage would be ~10^{14} molecules/cm^2, which is much greater than the highest glycophorin concentration used in these experiments. Thus, because the glycophorin density on the liposome surface was much less than a monolayer, it may be necessary to also have much less than a monolayer coverage of lectin on the surface to see the effect of lectin density on adhesion. In other words, there may have been more than enough WGA to bind all available glycophorin molecules even at the lowest protein level studied. We can conclude that under the conditions investigated, the lectin coverage was a much less significant parameter than the flow rate, liposome diameter, and glycophorin concentration. However, further work is necessary to evaluate the importance of lectin density at lower surface concentrations. It was not possible to measure the lectin surface concentration and glycophorin membrane concentration with our current facilities, but future experiments are planned to clarify the effect of surface lectin density on adhesion by measuring the surface lectin concentration and the glycophorin membrane concentration.

In contrast to these results, Rutishauser and Sachs (1975) did see decreased cell adhesion to lectin-coated fibers for some cell types under similar reduced surface coverages. They measured the binding of three types of cells to fibers with 100–1.5% monolayer lectin coverage. At a lectin coverage of 50%, only 20% of the number of cells bound at the monolayer lectin coverage were bound the fiber, whereas the two other cell types bound ~90–95% of the number at a monolayer lectin coverage. At 1.5% lectin coverage, 10% of the number of cells at full

coverage were bound for all three cell types. The difference in binding with the three types of cells studied was attributed by the authors to receptor mobility. The cells with the least mobile receptors were seen to lose their ability to bind more rapidly than the cells with mobile receptors as the lectin concentration decreased. Thus, whereas their data suggest that the glycophorin liposomes may have been expected to show some decrease in binding at the lowest surface WGA concentration, Rutishauser and Sachs did see significant variation in binding among different cell types. The insensitivity to WGA level seen in our experiments may be due to the high mobility of glycophorin in the liposomes and the low receptor concentration. Cells commonly have on the order of 10^5 receptors, which is an order of magnitude higher than the highest glycophorin concentration used in our experiments. The liposome receptor would be expected to have a higher mobility than the cell receptor because there are no intercellular components (cytoskeleton) interacting with glycophorin and no membrane components other than lipid to hinder the mobility.

SUMMARY

We have developed and tested a model experimental system consisting of glycophorin liposomes and a WGA surface for use in a parallel plate flow chamber to study some fundamental aspects of receptor-mediated cell adhesion to a surface in fluid shear flow. Our results have shown that the liposomes approach the bottom surface of the chamber as predicted by fluid mechanical theory, although the factors which influence initial cell-surface contact within ~1 μm of the surface were not directly measurable. The sticking probability parameter correlated well the dependence on glycophorin membrane receptor concentration and fluid force. The experiments indicated that as the membrane receptor concentration decreases and the fluid force increases, the liposome has a lower probability of permanent attachment per contact.

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