Kinetics and Locus of Failure of Receptor-Ligand-Mediated Adhesion Between Latex Spheres. II. Protein-Protein Bond

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ABSTRACT In an extension of the previous paper, we describe the force dependence of break-up of doublets of latex spheres cross-linked by protein G–IgG bonds via the Fc region of the antibody. The receptor, the monoclonal Bear-1 antibody, was either covalently linked to 4.75-μm aldehyde/sulfate (A/S) latex spheres in a one-step reaction, or physically adsorbed to the 4.63-μm carboxyl-modified latex spheres used in Part I of this paper. The spheres were suspended in 19% buffered Dextran 40 containing the ligand, the bivalent recombinant protein G (Gamma-Bind G), and observed in the counter-rotating cone and plate Rheoscope. Break-up of doublets, tracked individually under the microscope, as well as in populations of 50–150 particles, was studied over a range of normal force from 20 to 260 pN. In individual particle studies, the fraction of doublets of spheres with covalently linked IgG breaking up in the first 10 rotations, increased from 16% in the low-force to 63% in the high-force range. In population studies, the fraction broken up increased with duration and magnitude of the applied force, and decreased with increasing ligand concentration. Moreover, doublets of physically adsorbed IgG spheres required significantly lower force than doublets of covalently linked IgG spheres for the same degree of break-up, possibly because of surface detachment of IgG molecules rather than rupture of receptor-ligand bonds. Computer simulation, using the Bell stochastic model of break-up and a Poisson distribution for the number of bonds, described in Part I, showed that the parameters of the protein-protein bond differed significantly from those of the carbohydrate-protein bond studied in Part I of this paper, the former being much more responsive to force than the latter.

INTRODUCTION

In Part I, we described the rupture of protein-carbohydrate bonds in the break-up of doublets of latex spheres with covalently coupled synthetic blood group B antigen, cross-linked by a monoclonal IgM. Here, we report an extension of this work using a protein-protein receptor-ligand system, in which the receptor was coupled onto the latex microspheres either covalently or by physical adsorption. The receptor is a monoclonal IgG, and the ligand cross-linking the latex spheres is divalent Gamma Bind G (a recombinant form of protein G that binds to the Fc region of the IgG). Break-up of individual doublets of microspheres was examined over a range of hydrodynamic forces from 20 to 260 pN, using the counter-rotating cone and plate rheoscope. The forces were computed using Eqs. I-3 and I-4 (meaning Eqs. 3 and 4 of Part I) with the coefficients $\alpha_{12}(h)$ and $\alpha_3(h)$ computed for the minimum distance of approach of spheres surfaces, $h = 10$ nm.

Together with a study of the time and force dependence of the break-up of individual doublets, we also report on the break-up of populations of doublets in the rheoscope. The fraction of doublets broken up was determined as a function of the duration and magnitude of the shear stress, and the concentration of cross-linking agent. As in the previous paper, the measured force dependence of the lifetime of doublets was related to the force dependence of the lifetime of the bond, t_b, using the theory of Bell (1978) in a simulation of doublet break-up.

MATERIALS AND METHODS

Receptor and ligand

Bear 1 (AMAC, Inc., Westbrook, MN), a monoclonal mouse IgG_1, in lyophilized form with 1% albumin, was reconstituted in water at a final concentration of 0.2 mg/ml. The recombinant protein G (Gamma Bind G, Type 2, Pharmacia Biotech, Inc., Baie d'Urfé, QC, Canada), containing two Fc binding domains, in lyophilized form, was reconstituted in phosphate-buffered saline (PBS) at a concentration of 1 mg/ml. Both Bear 1 and Gamma Bind G were used without additional purification, and aliquots were stored at −20°C.

Latex spheres

It was important to be sure that doublet break-up involved the rupture of Bear-1-Gamma Bind G bonds, rather than the detachment of nonspecifically bound Bear-1 molecules from the surface of the latex spheres. As in Part I, we initially chose carboxyl modified latex (CML) spheres to compare the time and force dependence of doublet break-up for Bear-1 covalently linked to the latex with that for Bear-1 physically adsorbed onto the latex. Unfortunately, CML spheres with covalently bound Bear-1, prepared using the carbodiimide method (Illum and Jones, 1985), were found to aggregate nonspecifically. However, we found that aldehyde/sulfate (A/S) spheres (with very similar surface charge density and diameter), to which Bear-1 was covalently coupled in a single-step reaction without the addition of an activator, did not aggregate, but these could not be used to physically adsorb the IgG. We therefore compared the break-up of doublets of the covalently coupled A/S spheres with that of break-up of doublets of CML spheres to which the IgG was physically adsorbed.

The surfactant-free 4.52-μm-diameter hydrophobic aldehyde/sulfate latex spheres (Interfacial Dynamics Corporation, Inc., Portland, OR) contain aldehyde groups grafted onto the surface of the hydrophobic sulfate

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charge-stabilized microspheres. The particles exhibit high sphericity and have approximately 10^{10} aldehyde groups per sphere. The size distribution of a population of suspended particles showed the average diameter to be 4.93 ± 0.46 μm, larger than that specified, with a bimodal size distribution, 87% of the particles having a diameter equal to 4.75 ± 0.26 μm, and 13% a diameter of 6.10 ± 0.23 μm. The surfactant-free hydrophobic CML spheres used to physically adsorb the antibody were those described in Part I of this paper.

Both native A/S and CML spheres contained a small number (<0.5%) of doublets. These nonspecifically bound aggregates could not be broken up, either by sonication or by application of high shear stress.

Covalent coupling of protein to aldehyde/sulfate latex

Bear-1 was covalently coupled to the spheres in a one-step reaction (Illum and Jones, 1985; Rembaum et al., 1978). The aldehyde groups of the latex form stable bonds with primary amino groups of the protein (e.g., lysine). A suspension of 7.5×10^5 spheres · μl^{-1} in 0.1 M HEPES buffer, pH 6.5, was washed three times with the buffer. Bear-1 was added to the suspension at a final concentration of 10 μg · ml^{-1} and mixed at room temperature for 4 h. The spheres were washed and suspended in PBS, pH 7.4, containing 0.1% bovine serum albumin, and mixed overnight to block any nonspecific binding sites that remained on the sphere surface. The spheres were stored in 0.1 M phosphate buffer with 5% glycerol, 0.1% bovine serum albumin, 0.1% NaN_3, pH 7.4, at 4°C at final concentrations of $\sim 2 \times 10^5$ spheres · μl^{-1}. After covalent linking, examination of the spheres in a hemocytometer revealed <0.5% doublets in the suspension. The spheres were used within 1 month of coating with IgG.

Physical adsorption of protein to CML

Antibodies are known to adsorb strongly onto the hydrophobic domains of latex spheres by van der Waals–London forces, rendering the spheres more hydrophilic (Illum and Jones, 1985). Briefly, CML particles were washed with 0.1 M HEPES buffer, pH 6.5, and incubated with Bear-1 at a final concentration of 10 μg/ml overnight at room temperature. The spheres were washed and incubated with PBS, pH 7.4, containing 0.1% albumin and subsequently stored in the 0.1 M phosphate storage buffer, pH 7.4.

Characterization of protein-coated spheres

Before coupling, the electrophoretic mobility of Bear 1-coated A/S and CML spheres corresponded to zeta potentials of −73.5 ± 1.7 and −69.8 ± 4.2 mV, respectively. After coating with Bear-1, the potential decreased to −34.7 ± 4.1 and −35.7 ± 2.6 mV (n = 15), respectively, values very similar to those of A/S and CML spheres coated with albumin, −34.4 ± 3.2 and −38.0 ± 1.7 mV, respectively.

The surface receptor density was quantified using a standard IgG enzyme-linked immunosorbent assay as described in Part I. Here, the Bear 1-coated spheres were incubated for 20 min with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Southern Biotechnology Associates, Birmingham, AL), and the color was developed with Sigma 104. The standard curve was made by incubating increasing concentrations of Bear-1 and polyclonal mouse IgG (Bio/Can Scientific, Mississauga, ON, Canada) in Dulbecco's minimum essential medium containing 15% fetal calf serum in each of the wells of microtiter plates precoated with goat anti-mouse IgG + IgM (Southern Biotechnology Associates) for 1 h at 37°C. The receptor density (Bear 1 molecules per sphere) on A/S and CML spheres was found to be 16,600 ± 5000 and 11,400 ± 3000, respectively (SD, n = 3); however, this difference was not statistically significant (p > 0.1).

Controls (A/S and CML spheres coated with albumin) showed only background levels of conjugation with the alkaline phosphatase-conjugated goat anti-mouse antibody.

Maximum number of bonds

Both A/S and CML spheres coated with Bear-1 exhibited significant shear-induced aggregation in the presence of Gamma Bind G. Thus, when suspended at a concentration of 4000 spheres · μl^{-1} in 19% Dextran 40 (w/w) with 0.9 nM Gamma Bind G, 20% of the spheres had formed doublets after shearing at $G = 8\ s^{-1}$ for 30 min. In contrast, after shearing for 30 min in the absence of Gamma Bind G, <0.5% of the spheres were present as doublets, not significantly different from the number observed before shearing or that in the native suspension. Albumin-coated spheres also did not exhibit any shear-induced aggregation with Gamma Bind G.

The number of bonds available for cross-bridging depends on the surface area for contact, which is restricted by the spherical geometry of the spheres and its receptor density. The closest distance of separation between the two spheres of a doublet depends on the size of the molecule on the sphere surface and is taken to be ~10 nm (diameter of one IgG molecule, MW 160,000; van Oss, 1979), yielding α_{12} = 19.33 and α_3 = 7.02 in Eqs. I.4 and I.3. Allowing for the diameter of the cross-linking molecule Gamma Bind G (MW 22,000), we take the maximum separation permitted to be 24 nm. The maximum area over which bonding can occur, given by the surface area of the spherical cap of thickness = (24 nm − 10 nm)/2 = 7 nm, and radius b of the A/S sphere = 2.38 μm, is 0.10 μm^2. For ~17,000 Bear-1 antibody sites on the surface of A/S spheres, each of 71 μm^2 surface area, assuming uniform distribution, there would be 24 Bear-1 molecules in the 0.10-μm^2 area available for binding.

At the experimentally used concentration of 4000 spheres · μl^{-1}, shear-induced aggregation of Bear-1 spheres at low G ($8\ s^{-1}$) was observed at a Gamma Bind G concentration of ≥0.45 nM, corresponding to a ~4 times excess of soluble ligand to surface receptor. However, only a fraction of the sites on the spheres are likely to be occupied at equilibrium, because not all of the Bear 1 molecules would be in the correct orientation for binding. The actual number of cross-bridges may therefore be less, as low as one or two in some cases. With increasing concentration of soluble Gamma Bind G, we observed an increase in the fraction of doublets formed, as well as the formation of triplets, and higher order multiplets.

Procedures

All of the shear application experiments were carried out using the transparent, counter-rotating cone-and-plate rheoscope previously described (Tees et al., 1993; Part I of this paper).

Antibody-coated spheres were suspended in PBS, pH 7.4, containing 19% Dextran 40, 0.1% albumin (19% Dextran-PBS; $\eta_{23°C}$ = 18 mPa · s). Gamma Bind G, diluted to 2 μg/ml in PBS, pH 7.4, containing 0.1% albumin, was added to the sphere suspension at final concentrations from 0.9 to 3.6 nM. The spheres were mixed for 1 h at room temperature before use. All experiments were carried out at room temperature (22–24°C).

Data acquisition: break-up of individual doublets

As described in Part I, break-up of doublets was assumed to occur when F_n was a maximum, i.e., when the angle factor $\sin^2\theta_1 \sin 2\phi_1$ in Eq. I.3 was a maximum. As before, the value of $F_{n,\ max}$ was obtained from the experimentally recorded rotational orbit of the doublet at high magnification and low shear rate (Tees et al., 1993).

Fifty microliters of a suspension of antibody-coated Bear-1 spheres containing Gamma Bind G was pipetted into the rheoscope and sheared at the lowest G (~$8\ s^{-1}$, corresponding to $F_{n,\ max}$ = 15.7 pN for an orbit having $C = \infty$ [Eq. I.7] and η = 18 mPa · s) for 30 min to allow doublets to form through two-body collisions between spheres. Doublets close to the midplane, the rotational orbits of which were analyzed at low shear, were observed and videotaped at a preset shear rate until break-up or until they disappeared from view. The value of $F_{n,\ max}$ was computed from Eq. I.3 using the measured shear rate and doublet orientation corresponding to the maximum value of the angle factor.

Data acquisition: population studies

The effect of the magnitude and duration of the applied force on break-up at various concentrations of soluble cross-linking ligand was studied in populations of 50–120 doublets in the rheoscope. The fraction of doublets breaking up at a given shear stress and time was determined by counting the number of doublets before and after application of shear.

For each experiment, 50 μl of the sphere suspension containing from 0.9 to 3.6 nM Gamma Bind G was pipetted into the rheoscope and sheared at the lowest G ($\sim$ 8 s^{-1}) for 30 min to allow doublets to form. Because the density of the spheres (1.055 g · cm^{-3}) was slightly less than that of the suspending medium (1.081 g · cm^{-3}), the particles rose with a velocity of $\sim$1 μm min^{-1}. After 30 min, most of the spheres were close to the upper (cone) wall and traveled with velocities close to those of the cone wall. Thus, these particles reappeared in the field of view after 70 s, the time for one cone rotation through 360°. This enabled the total number of doublets in the observed volume to be accurately counted, a procedure that was carried out over two rotations of the cone, and the average of the two measurements was recorded. It was found that the number of doublets counted in successive rotations of the cone did not change significantly after 30 min. The desired shear stress, $\tau = G\eta$, from 0.8 to 1.7 Pa, corresponding to $F_{n,\,max}$ from 85 to 185 pN, was then applied for periods from 5 to 60 s. After the flow was arrested and all particles had risen to the surface of the cone, the number of doublets remaining was counted at the low shear rate as described above. Each experiment was repeated eight times with freshly pipetted sphere suspensions.

Error analysis

Errors in the measurement of viscosity, particle diameter, angle factors, and shear rate were as described in Part I. Because of excursions of up to 10% in the shear rate over the course of 1 min, errors of up to 10% in G computed from the measured dimensionless period of rotation, TG (Eq. I.6), could occur in the population studies. Furthermore, for the population studies, there is a remaining error in the counting of doublets. From repeat counts over one cone rotation, this was estimated to be ±10%.

Computer simulation

A Monte Carlo simulation of doublet break-up, similar to that described in Part I, was used to relate the results of individual doublet break-ups and of the population studies to the force and time dependence of the rupture of the receptor-ligand bond. In both simulations, each doublet rotation was divided into N_s equal time steps of duration $\Delta t = T/N_s$; here N_s was chosen to be 1000. The viscosity of the suspending medium was taken to be 18 mPa · s. Bond formation was allowed in the simulation by incorporating a bond formation time t_f. At each time step a test for bond formation was made.

The bond parameters supplied to the simulation were t_0, the bond lifetime at zero force; t_f; c, the sensitivity of the bond to force; and the average value of the Poisson distribution of the bonds linking the cells, $\langle N_b \rangle$. The simulated experimental variables supplied for the individual break-up experiments were the maximum normal force, $F_{n,\,max}$, and the number of simulated doublets, n. For the population studies, the simulated experimental variables were the applied shear stress, duration of shear, number of doublets, and the number of repeated experiments. As before, the bond parameters were systematically varied under simulated experimental conditions resembling those in the rheoscope, in an attempt to achieve a match to the experimental data.

RESULTS

Temporal distribution of break-up of individual doublets

In this study, the shear rates were varied from 20 to 120 s^{-1}, over a range of F_n from 20 to 260 pN. Of the 154 doublets analyzed, 58 (38%) broke up before being lost from the field of view, <1 to 10 s after the flow commenced. As in Part I, the results of the temporal distribution of break-ups were standardized by plotting time as the dimensionless number of rotations, and break-up statistics were compiled from those doublets that broke up within 10 rotations from the onset of flow.

The time and the number of rotations until break-up or disappearance from the field of view were determined for the 154 doublets of spheres with covalently linked IgG in suspensions containing 0.9 nM Gamma Bind G. The data were grouped into three force ranges: $20 \leq F_n < 100$ pN (low force), $100 \leq F_n < 180$ pN (mid-force), and $180 \leq F_n < 260$ pN (high force). Table 1 shows the number and fraction of doublets that broke up in each force range within 10 rotations. There is a marked increase in fraction of break-ups with increasing force from 16% in the low force, to 63% in the high force range. Fig. 1 shows the fraction of doublets that broke up in a given rotation, i.e., the fraction of the total number of doublets observed in that rotation that broke up plotted against the elapsed number of rotations. With increasing force, there is a marked increase in the fraction broken up within the first two rotations: from 16% in the low force range to 26 and 55% in the mid- and high force ranges, respectively. Moreover, 95% of all break-ups occurred within the first four rotations.

Population studies

These studies were carried out to compare the break-up of doublets of spheres covalently coated with IgG (A/S), with those in which IgG was physically adsorbed (CML), as a function of the magnitude and duration of shear stress, and of ligand concentration. Fig. 2 shows a plot of the time course of the percentage of break-up of doublets at 0.9 nM Gamma Bind G after the application of shear stress at 0.8 and 1.7 Pa corresponding to $F_{n,\,max}$ = 85 pN and 185 pN, respectively. Table 2 gives values of the percentage break-up as a function of time. The extent of break-up for the CML spheres was markedly greater than that of the A/S spheres, and a two-way analysis of variance verified that the difference was highly significant ($p < 0.001$) at both 0.8 and 1.7 Pa over the whole 60-s time course (Kwong, 1995). Both A/S (Fig. 2, *solid lines*) and CML spheres (Fig. 2,

TABLE 1 Break-up of individual doublets of aldehyde/sulfate spheres: comparison of observed and simulated fraction broken up within 10 rotations in three force ranges; [Gamma Bind G] = 0.9 nM

Applied force (pN)	Fraction of doublet break-ups (%)	
	Experimental	Simulation (±SD)*
$20 \leq F_n < 100$	16.4 (n = 55)	12.4 ± 5.0 (n = 50)
$100 \leq F_n < 180$	36.0 (n = 50)	36.0 ± 7.3 (n = 50)
$180 \leq F_n < 260$	63.3 (n = 49)	68.4 ± 5.9 (n = 50)

n, no. of doublets.
*Computed using the best-fit bond parameters with $\langle N_b \rangle$ = 3.

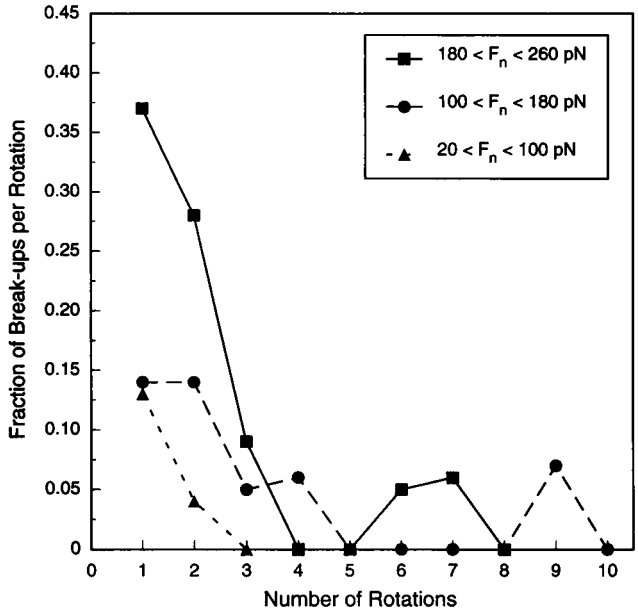

FIGURE 1 Individual doublet break-ups. Plot of fraction of doublets of A/S spheres cross-linked by 0.9 nM Gamma Bind G, breaking up per rotation (the fraction of the total number of doublets observed in that rotation that broke up) during the first 10 rotations after the onset of shear, for high ($180 \leq F_n < 260$ pN), intermediate ($100 \leq F_n < 180$ pN), and low ($20 \leq F_n < 100$ pN) force ranges.

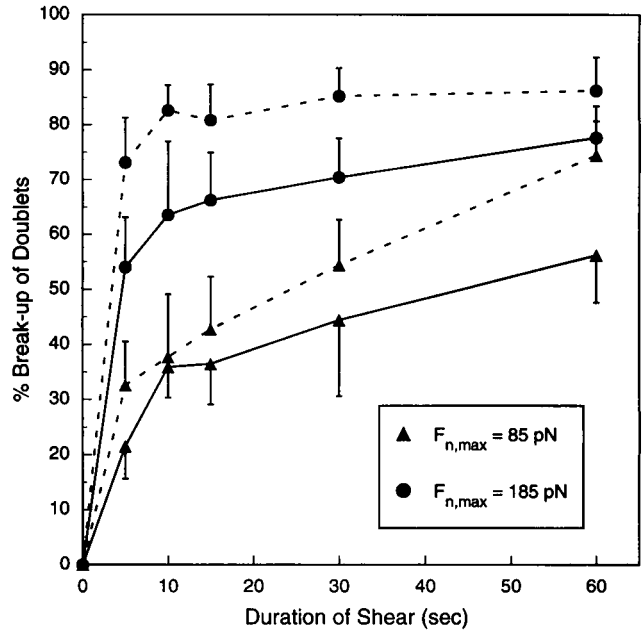

FIGURE 2 Population studies. Plot of percentage of break-up of doublets of A/S spheres (——) and CML spheres (– – –) cross-linked by 0.9 nM Gamma Bind G as a function of the duration of shear stress at 0.8 Pa (▲) and 1.7 Pa (●) corresponding to a maximum hydrodynamic normal force of 85 and 185 pN, respectively. Bars represent one SD of the mean ($n = 8$).

dashed lines) exhibited a rapid rise in percentage break-up during the first 10 s, followed by a slower, almost linear increase from 10 to 60 s, with the exception of CML spheres at $\tau = 1.7$ Pa, where the fraction of break-ups reached a plateau at ~80% after 10 s.

Fig. 3 shows a plot of percentage doublet break-up as a function of Gamma Bind G concentration at shear stresses of 0.8 and 1.7 Pa applied for 60 s. As found at [Gamma Bind G] = 0.9 nM, the degree of break-up of the CML doublets (Fig. 3, *dashed lines*) was markedly greater than that of the A/S doublets (Fig. 3, *solid lines*) over the whole range of [Gamma Bind G], from 0.9 to 3.6 nM, and at both shear stresses. For both A/S and CML spheres, the degree of break-up decreased with increasing [Gamma Bind G]. However, the decrease was much greater for the A/S than for the CML spheres: 51% compared to 28% at $\tau = 0.8$ Pa and 36% compared to 4% at $\tau = 1.7$ Pa. A two-way analysis of variance (Kwong, 1995) indicates that this difference is significant ($p < 0.001$) over the whole range of [Gamma Bind G].

DISCUSSION

Covalent linking versus physical adsorption

The objective of this study was to extend the work described in Part I on the time and force dependence of rupture of antigen-antibody bonds (blood group B–IgM, a carbohydrate-protein interaction) to that of antibody-Fc–protein G bonds (a protein-protein interaction).

A monoclonal IgG antibody was linked to latex spheres, either covalently or by physical adsorption, and the spheres were cross-linked by divalent protein G. Other investigators have used latex spheres to model the biophysical aspects of cell-to-cell or cell-to-surface adhesion (Cozens-Roberts et al., 1990a,b; Kuo and Lauffenburger, 1993). Well-documented techniques for immobilizing biomolecules to latex spheres involve the use of an activator such as carbodiimide, which covalently couples proteins to latex spheres bearing carboxyl groups, or glutaraldehyde, which covalently cross-links the amino groups between a protein and a latex particle, or polyglutaraldehyde-bearing latexes, which react readily with the primary amino groups of pro-

TABLE 2 Population studies: comparison of time course of fraction of break-ups of doublets of latex spheres with covalently coupled and physically adsorbed IgG; [Gamma Bind G] = 0.9 nM

Duration of shear (s)	Fraction of doublet break-ups (% ± SD)			
	$F_{n,max}$ = 85 pN		$F_{n,max}$ = 185 pN	
	Covalent*	Adsorbed*	Covalent*	Adsorbed*
5	21.5 ± 5.9	32.6 ± 7.9	54.0 ± 9.1	73.1 ± 8.2
10	35.9 ± 5.6	37.7 ± 11.4	63.5 ± 13.4	82.6 ± 4.6
15	36.5 ± 7.4	42.7 ± 9.6	66.2 ± 8.7	80.8 ± 6.5
30	44.4 ± 13.8	54.4 ± 8.3	70.4 ± 7.1	85.2 ± 5.1
60	56.2 ± 8.6	74.4 ± 6.2	77.6 ± 5.8	86.2 ± 6.1

*IgG was covalently linked to A/S latex spheres, physically adsorbed onto CML spheres.

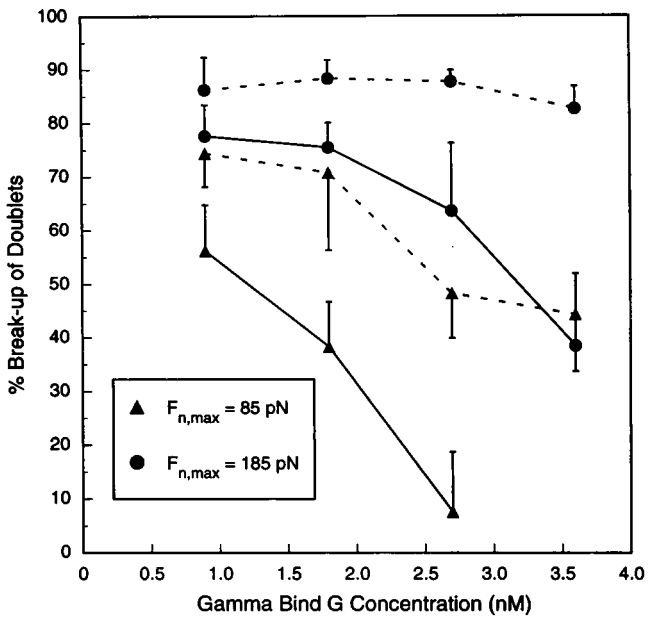

FIGURE 3 Population studies. Plot of percentage of break-up of doublets of A/S (——) and CML (– – –) spheres as a function of Gamma Bind G concentration after 60 s at shear stresses equal to 0.8 Pa (▲) and 1.7 Pa (●), corresponding to a maximum hydrodynamic shear force of 85 and 185 pN, respectively. Bars represent one SD of the mean (n = 8).

teins to form covalent bonds (Illum and Jones, 1985). It should be noted that initial adsorption of protein is a requirement for such covalent coupling reactions to occur. Proteins also readily adsorb onto the hydrophobic domains of the hydrophilic CML spheres. If a receptor is covalently bound to the surface of latex spheres, break-up of doublets of spheres cross-linked by a multivalent ligand is expected to take place at the specific receptor-ligand linkage, because the strength of a covalent bond is known to be 2–3 orders of magnitude greater (Levinthal and Davison, 1961). In the flow-induced detachment studies of red blood cells adhering to a glass surface (Xia et al., 1994), it was shown that the mean hydrodynamic force needed to detach sphered, swollen, and fixed red cells (SSRC) adhering to antibody covalently bound to glass was appreciably greater than that to detach SSRC adhering to antibody physically adsorbed to the glass. Although in both cases it was possible that bond rupture occurred by antigen extraction from the cell membrane (Evans et al., 1991), the difference in the magnitude of the detachment force could have been due to the physically adsorbed antibody being pulled off the glass surface. However, results were inconclusive, because the antibody surface density in the two cases was not compared. In our case, it was shown that the surface density of the physically adsorbed IgG was somewhat lower than that of the covalently bound IgG, although the difference was not statistically significant. We demonstrated that, at each shear stress, the degree of break-up of doublets with covalently bound IgG cross-linked by Gamma Bind G was significantly lower than that of doublets with physically adsorbed IgG (Figs. 2 and 3, and Table 2). It can be argued that the differences in the degree of break-up are due to the lower number of cross-bridges in the case of the doublets of CML spheres, and not to a difference in the character of the bond. However, this cannot explain the fact that, with increasing [Gamma Bind G], the degree of break-up at high shear stress did not significantly decrease in the case of the CML spheres (Fig. 3). These differences may therefore be due to breakage of a nonspecific attachment between antibody and the sphere rather than breakage of a specific bond between antibody and Gamma Bind G, because the former process is thought to require less energy than the rupture of specific receptor-ligand bonds (Lauffenburger and Linderman, 1993). However, because of the stochastic nature of bond rupture, break-up of some Gamma Bind G-IgG bonds between doublets bearing adsorbed IgG cannot be ruled out.

It should again be pointed out that the spheres to which the IgG was covalently coupled had surface functional groups different from those to which the antibody was physically adsorbed. The fact that the measured zeta potential was the same for both IgG-coated A/S and CML spheres, however, lends some credence to the hypothesis that the observed differences in the population studies were due to the greater forces required to rupture protein-protein bonds than to detach IgG molecules from the surface of the CML spheres, rather than to the difference between the two types of spheres.

Computer simulation versus experimental results

In an attempt to compare the character of the bond for our receptor-ligand system to other systems that have been described in our laboratory (Tees et al., 1993; Part I of this paper) and elsewhere (Alon et al., 1995), we used the Bell model of bond lifetime (Bell, 1978) to simulate the observed time and force dependence of the break-up of doublets of spheres bearing covalently linked IgG, both individually and in populations. We did not simulate the results of the break-up of doublets of spheres with physically adsorbed IgG, because of uncertainties in the process of detachment, some of which could still occur by rupture of the IgG-Gamma Bind G bonds.

The simulations were carried out over a range of bond parameters: t_o from 100 to 1000 s, c from 0 to 10^{12} N^{-1}, $\langle N_b \rangle$ from 1 to 15 bonds, and t_f from 1 to 1000 s. Simulations of the individual break-up studies were carried out with 50 doublets (equal to the numbers used in the experiments), and each set was repeated five times. The fraction of break-ups per rotation was averaged at F_n = 60, 118, and 219 pN, corresponding to the mean experimental values in the low, intermediate, and high force ranges, respectively. In parallel, simulations of the population studies were run over the same range of bond parameters for populations of 100 doublets, having the experimentally observed distribution of orbit constants, with each set repeated eight times, and the fraction of break-ups at τ = 0.8 and 1.7 Pa determined as a function of time.

The best fit of the experimental data to the simulation of the break-up studies was determined by computing χ^2 statistics for the first four rotations at each of the above F_n in the case of the individual break-ups, and the ratio (experimental/simulation) at 5, 10, 15, 30, and 60 s and τ = 0.8 and 1.7 Pa in the case of the population studies. The set of bond parameters that best fitted the individual break-up experiments, and which also fitted the covalently bound sphere population experiments, was found to be $c = 9.5 \times 10^{10}$ N^{-1}, t_o = 175 s, t_f = 20 s, and $\langle N_b \rangle$ = 3 bonds.

Table 1 shows the simulated mean and standard deviation (n = 5) of the fraction of individual doublet break-ups within 10 rotations, in each of the three force ranges. It is evident that these values are in good agreement with those experimentally observed. Fig. 4 shows the simulated fraction of break-ups in a given rotation plotted against the number of rotations at the three values of F_n using the above parameters, and it can be seen that the general features of the experimental values (Fig. 1) were quite well reproduced. The initial fractions of break-up were similar to the experimental values, although, in the mid- and high force ranges, the rate of break-up was faster than observed: at high and intermediate F_n, there is an initial high fraction of break-ups in the first rotation (44.4 ± 6.8% and 20.4 ± 5.9%, respectively; SD not shown in the graph) compared to the experimental values of 37% and 14% shown in Fig. 1. This is followed by a rapid decrease over the next three rotations, reaching values of 6.5 ± 4.4% and 4.1 ± 3.8% in the fourth rotation, respectively, compared to the experimental values of 0% and 5%. Thereafter, the fraction of break-ups fluctuates between 0% and 6% for the remaining rotations.

However, at the lowest F_n, the observed fraction of break-ups decreased from 14% to 0% in the first three rotations, with no more break-ups occurring thereafter (Fig. 1), whereas the simulation (Fig. 4) showed a continuous fluctuation of break-ups between 0% and 3% over the whole 10 rotations. Here, the difference between the simulated and observed break-ups may have been due to the low number of break-ups observed experimentally: 9 break-ups out of the 55 observed.

The simulation of the population studies, carried out for the covalently bound IgG on the A/S spheres, is in fairly good agreement with the experimental values, as shown in a plot of the fraction of doublet break-ups against time at τ = 0.8 and 1.7 Pa in Fig. 5. Values of the percentage break-up as a function of time are compared with experiment in Table 3. At the lower shear stress, the mean values of the observed fraction of break-ups were 14% greater than the simulated values, although these differences were not significant at any time point. At the higher shear stress, however, the observed values were significantly different from the simulated ones at times of >15 s. Here the mean experimental values were 16% lower than the simulated values, the latter tending toward complete break-up at long times of shear, reaching values of >90%.

Although one would expect the fraction of doublets broken up to approach 100% with time, the plateau observed at times of >30 s suggested the presence of a population of doublets with low orbit constants, and hence low values of

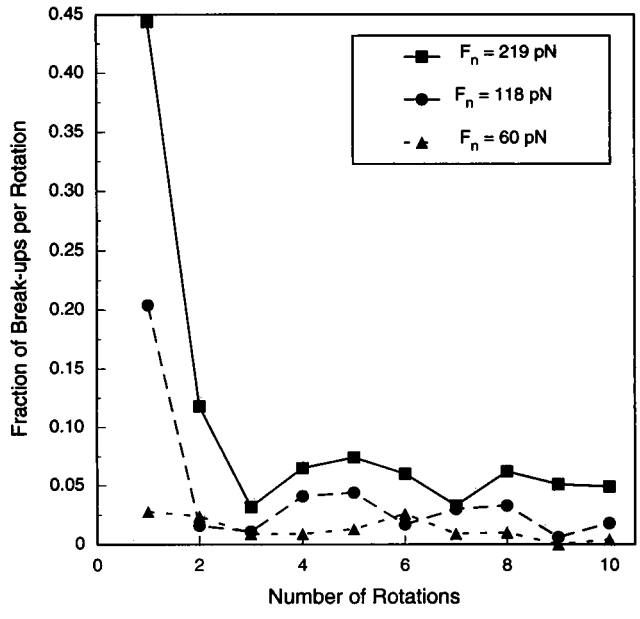

FIGURE 4 Simulation of individual break-up studies. Plot of fraction of doublets breaking up per rotation for F_n = 60, 118, and 219 pN produced by computer simulation of shear-induced rotation of doublets. The best fit bond parameters of the stochastic model were t_o = 175 s, t_f = 20 s, $c = 9.5 \times 10^{10}$ N^{-1}, with the mean value of the Poisson distribution in number of bonds, $\langle N_b \rangle$ = 3.

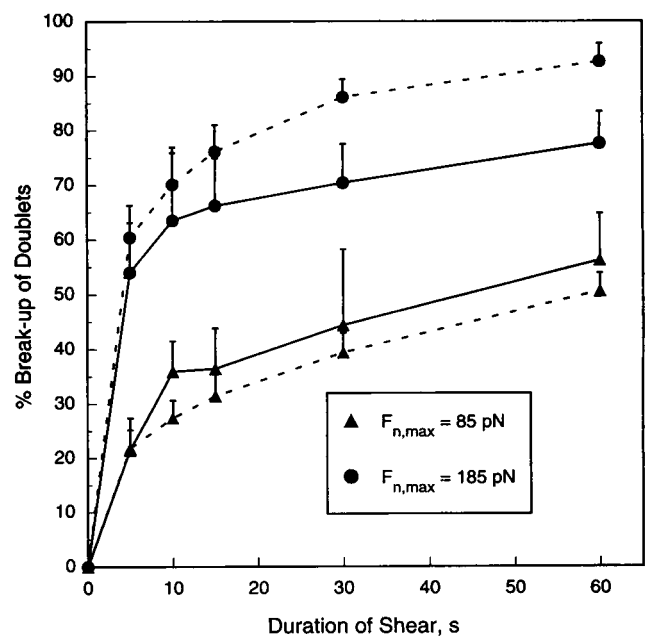

FIGURE 5 Simulation of the population studies (– – –) compared to experimental results (spheres bearing covalently bound IgG, ——). Plot of percentage of break-up of doublets as a function of duration of shear at shear stresses = 0.8 Pa (▲) and 1.7 Pa (●), corresponding to a maximum normal force of 85 and 185 pN, respectively; based on bond parameters: t_o = 175 s, t_f = 20 s, $c = 9.5 \times 10^{10}$ N^{-1}, and $\langle N_b \rangle$ = 3. Bars represent one SD of the mean (n = 8).

TABLE 3 Population studies: comparison of time course of measured and computer simulated fraction of doublet break-up; [Gamma Bind G] = 0.9 nM

Duration of shear (s)	Fraction of doublet break-ups (% ± SD, n = 50)					
	$F_{n,max}$ = 85 pN			$F_{n,max}$ = 185 pN		
	Experimental	Simulation*	Ratio (exp/sim)	Experimental	Simulation*	Ratio (exp/sim)
5	21.5 ± 5.9	21.8 ± 3.4	0.98	54.0 ± 9.1	60.4 ± 5.9	0.89
10	35.9 ± 5.6	27.4 ± 3.2	1.31	63.5 ± 13.4	70.1 ± 5.8	0.91
15	36.5 ± 7.4	31.4 ± 4.0	1.16	66.2 ± 8.7	76.1 ± 4.9	0.87
30	44.4 ± 13.8	39.4 ± 3.6	1.13	70.4 ± 7.1	86.1 ± 3.3	0.82
60	56.2 ± 8.6	50.6 ± 3.3	1.11	77.6 ± 5.8	92.6 ± 3.3	0.84
			⟨1.14⟩			⟨0.87⟩

*Computed using the best-fit bond parameters with $\langle N_b \rangle$ = 3.

the angle factor $\sin^2\theta_1 \sin 2\phi_1$ (Eq. I.3). The normal force experienced by these doublets would be significantly lower than $F_{n,\,max}$, and thus the probability of bond rupture at times of <60 s is expected to be low.

In a population of doublets having a distribution of orbit constants, C, the initial high rate of break-up is due to those doublets having the lowest number of bonds and rotating in orbits in or close to the X_2X_3 plane (Fig. I.1), with high values of C for which F_n at a given τ is close to $F_{n,\,max}$. The rate of break-up then decreases as F_n decreases in the remainder of the population having lower C, as well as greater number of bonds, with a lower probability of break-up and therefore longer times to rupture the bonds. The differences between the observed and simulated fractions of break-ups at τ = 1.7 Pa could be due to a difference in the simulated and actual distribution of orbit constants. In the simulation, 100 experimentally measured C, chosen randomly from the individual break-up studies, were used to represent the population of doublets. The distribution of this population is biased toward high values of C (>1.5), and therefore angle factors > 0.8, because of a bias in the choice of doublets in the individual break-up studies. Based on this distribution of C, as defined by a distribution of the angle $\phi_{2,max}$ (Fig. 1, Part I; tan $\phi_{2,max} = Cr_e$; Goldsmith and Mason, 1967), the arithmetic mean of the angle factor = 0.93 would yield an average force of 75 pN at 0.8 Pa and 172 pN at 1.7 Pa.

In fact, an experimentally measured steady-state distribution of $\phi_{2,max}$ for rods (r_e = 18.4; Anczurowski and Mason, 1967) yields a distribution of orbit constants C for which the mean angle factor was only 0.67. However, computer simulation of the population studies using this set of orbit constants, while resulting in a good match of the experimental points at high shear stress, leads to significantly lower values of the fraction of break-ups at the lower shear stress. It is likely, therefore, that to obtain a better match of the data, a further adjustment of the bond parameters is required.

The observed decrease in fraction of break-up of doublets with increasing [Gamma Bind G] (Fig. 3) was likely due to an increase in the number of cross-bridges. We therefore simulated the break-up of doublets of spheres of covalently linked IgG as a function of [Gamma Bind G] after 60 s of shear by changing the average number of bonds, $\langle N_b \rangle$, while keeping the other bond parameters constant. As shown in Fig. 6, the fraction of break-up of doublets decreased with increasing $\langle N_b \rangle$. A comparison with Fig. 3 suggests that $\langle N_b \rangle$ increases from ~3 to ~7 as [Gamma Bind G] increases from 0.9 to 3.6 nM.

Bond parameters

In our computer simulation using Bell's model, the set of bond parameters that best fitted both types of experiments reasonably reproduced the main features of both individual break-up and population studies, and, as indicated in Tables 1 and 3, the overall statistics were well matched. Based on the bond parameters given above, values of E_o, the bond free energy minimum (0.89 eV), assuming $\tau_o = 10^{-13}$ s, from the expression $t_o = \tau_o \exp(E_o/kT_K)$, and r_o (0.39 nm)

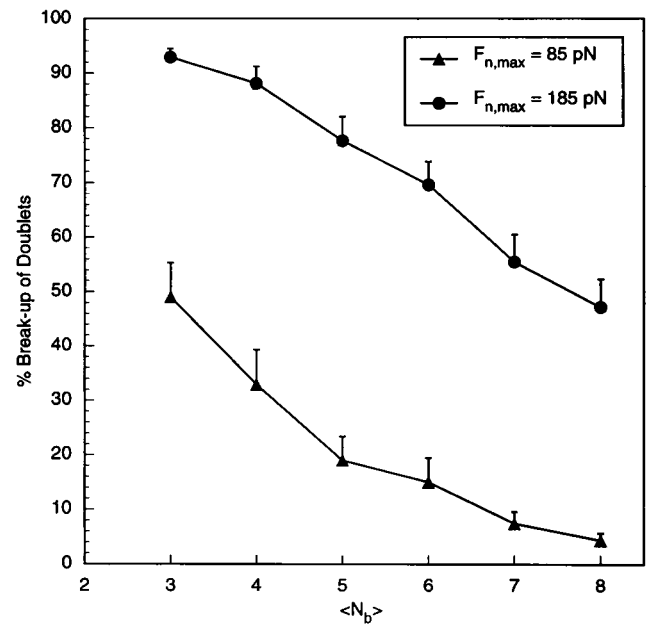

FIGURE 6 Simulation of the population studies. Plot of percentage of break-up of doublets as a function of average number of bonds, $\langle N_b \rangle$, at shear stresses = 0.8 Pa (▲) and 1.7 Pa (●), based on bond parameters: t_o = 175 s, t_f = 20 s, $c = 9.5 \times 10^{10}\ N^{-1}$. Bars represent one SD of the mean (n = 8).

were obtained. The latter is consistent with the distance, typically about the size of a water molecule, in which hydrophobic forces of the protein-protein bond operate (Erickson, 1994). The "critical" force, f_c, required to instantaneously rupture a single bond ($= E_o/r_o$) is then ~390 pN. This value seems reasonable, because 63% of the doublets broke up in the force range $180 \leq F_n < 260$ pN in less than 0.5 s after the application of force. Based on these parameters, we have plotted bond lifetimes as a function of constant applied force as shown in Fig. 7 and compared our data with those obtained in this laboratory for doublets of spheres linked by blood group B antigen–IgM bonds (Part I of this paper) and with those for P-selectin–PSGL-1 bonds as obtained from neutrophils rolling on a lipid bilayer containing P-selectin (Alon et al., 1995). The slope of the line is the sensitivity of the bond to force, c. The parameters of blood group B antigen–IgM and P-selectin–PSGL-1 bonds, both entailing carbohydrate-protein interactions, were $c = 3 \times 10^{10}$ N^{-1}, $t_o = 25$ s, $r_o = 0.12$ nm, and $c = 1.2 \times 10^{10}$ N^{-1}, $t_o = 1.05$ s (reverse rate constant at zero force, $k_r^o = 1/t_o = 0.95 \pm 0.17$ s^{-1}), $r_o = 0.05$ nm, respectively. Again, as pointed out in the previous paper, although r_o has the dimensions of length, and here has a value less than the physical dimensions of the hydrogen-bond binding cleft, it is in fact only a parameter describing the dependence of bond lifetime on force. The steep decline of the line for the Gamma Bind G–IgG bond (a protein-protein interaction) indicates that the bond lifetime is very sensitive to force and is said to have low tensile strength. In contrast, both blood group B antigen–IgM and P-selectin–PSGL-1 bonds have gentle slopes, i.e., the bond lifetimes are much less responsive to force—they are said to have high tensile strength. The high tensile strength of the P-selectin–PSGL bond, together with the measured high on and off rates, is thought to be ideal for the maintenance of the rolling adhesion between the leukocyte and the endothelium.

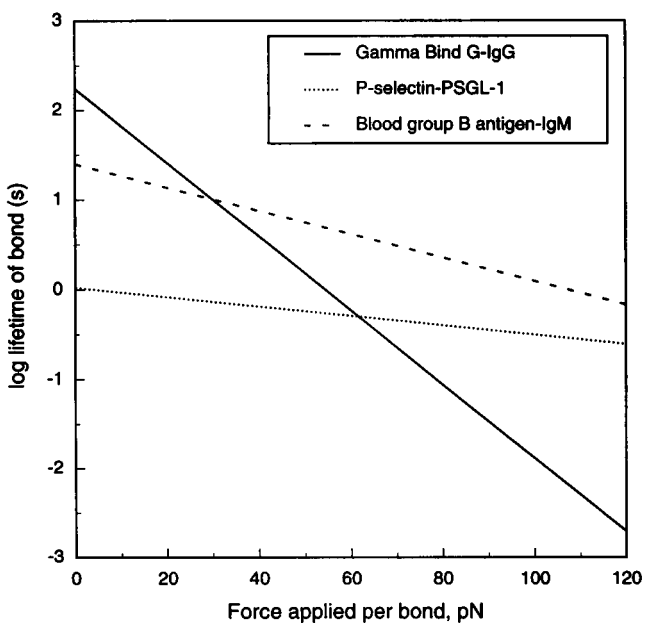

FIGURE 7 Plot of log lifetime of the protein G–IgG (——), P-selectin–PSGL-1 (· · ·; Alon et al., 1995), and blood group B antigen-IgM bonds (– – –; Part I of this paper) as a function of increasing constant applied force. The lines were calculated from Eq. I.1, using the best fit of the bond parameters to the experimental data.

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