Centrifugation Assay of IgE-Mediated Cell Adhesion to Antigen-Coated Gels

Lily Chu, Linda A. Tempelman, Cynthia Miller, and Daniel A. Hammer
School of Chemical Engineering, Cornell University, Ithaca, NY 14853

The adhesion of biological cells to substrates is often mediated by binding between cellular receptors and substrate-bound ligand. In this work, we used a centrifugation assay to measure the adhesion of rat basophilic leukemia (RBL) cells coated with immunoglobulin E (IgE) to substrates coated with the ligand dinitrophenol (DNP). Increasing force, decreasing DNP substrate density, and decreasing cell surface IgE density all led to decreasing adhesion. Experiments performed at low IgE cell surface densities, in which few tethers from between cell and substrate suggest individual tethers have a binding strength of 2 to 4 microdyne, in agreement with previous measurements of the force to uproot receptors from the plasma membrane. We use this system to show how subpopulations expressing different numbers of cell surface receptors may be separated by exploiting their differential adhesiveness to substrates.

Introduction

Biological cells adhere to substrates through highly specific binding between cell surface receptors and substrate ligand. Receptor-mediated cell-substrate interactions regulate many cellular physiological responses such as cell growth and differentiation (Dike and Farmer, 1988; Watt et al., 1988), cell motility (Goodman et al., 1989; DiMilla et al., 1991), and cellular immunological response (Springer, 1990). For example, there appears to be a direct relationship between the area of contact between a cell and a substrate and the extent of cellular DNA synthesis (Ingber et al., 1987). Also, a cell's motility will be inhibited if a cell is too tightly or too weakly adherent (Goodman et al., 1989). Currently, there is a great deal of research on the proper chemistry for biomaterials for artificial organs and vascular grafts which optimize adhesion and adhesion-dependent cellular physiological responses (Roy et al., 1993; Mikos et al., 1993; Hubbell et al., 1991; Lin et al., 1992; Pettit et al., 1992).

Cell-substrate interactions may also be used to separate cells. Mixed populations of cells can be separated into component subpopulations using differences in subpopulations' adhesiveness to surfaces (Wysocki and Sato, 1978; Hammer et al., 1987). Subpopulations of cells often possess unique identifying surface molecules which can be targeted with substrate-bound antibodies or other ligands to extract that subpopulation from a mixture. The design and optimization of such cell separation devices requires knowledge of the factors controlling cell adhesion.

Receptor-mediated cell adhesion can be analyzed using a force balance involving both chemical (receptor-ligand and nonspecific) and applied forces (Hammer and Lauffenburger, 1987; Hammer and Apte, 1992; Ward and Hammer, 1993). Chemical forces depend largely on the number of receptor-ligand tethers which form between the cell and substrate, and their micromechanics, such as their reactive and mechanical compliances. The number of tethers which form between a cell and substrate during encounter depends on the kinetics and affinity of binding between receptor and ligand, and the surface density of both receptors and ligands. Applied forces constitute all externally applied stresses. For example, in cell affinity chromatography, these forces would be the hydrodynamic fluid forces exerted on cells by fluid passing through the column.

Adhesion is measured with standardized assays in which

Correspondence concerning this article should be addressed to D. A. Hammer.
Current address of L. Chu: Dept. of Chemical Engineering, Univ. of Illinois at Urbana-Champaign, Urbana, IL 61801.
Current address of L. A. Tempelman: Naval Research Lab., Center for Bio/Molecular Science and Engineering, Code 6900, Washington, DC 20375.
Current address of C. Miller: Air Products and Chemicals Inc., P.O. Box 7119, Cumberland, RI 02864.

known forces are applied to cells. Such assays include the centrifugation assay (McClay et al., 1981; Pless et al., 1983; Hertl et al., 1984; Lotz et al., 1989; Schnaar et al., 1989; Foxall et al., 1992), flow chamber assays (Cozens-Roberts et al., 1990a,c; Wattenbarger et al., 1990; Truskey and Proulx, 1990; Tempelman and Hammer, 1990; Lawrence and Springer, 1991; Lawrence et al., 1990) and micropipette aspiration (Evans, 1985a,b; Tozeren et al., 1989; Sung et al., 1986; Tozeren et al., 1992; Berk and Evans, 1991; Evans et al., 1991a,b). While there are several examples in which these assays have been used to measure how substrate chemistry affects cell adhesion strength (for example, Lawrence and Springer, 1991; Cozens-Roberts et al., 1990a,c), careful analysis of these experiments show that in relatively few cases involving real biological cells have both cell and substrate chemistries been altered systematically. Furthermore, the kinetics of binding and dissociation of receptor-ligand pairs involved in adhesion is rarely, if ever, known in real biological adhesion systems.

To develop a system for the study of adhesion in which receptor and ligand densities, as well as the kinetics and affinity of their mutual recognition, can be manipulated, several avenues are possible. One is reconstituted systems in which hard spheres or substrates, or both, can be chemically derivatized with biological molecules. This was the approach of Cozens-Roberts and coworkers, for example, who measured the fluid shear force necessary to detach hard spheres coated with antibody which were recognized by counterantibodies on the substrate (Cozens-Roberts et al., 1990a,c). Another is to use biological cells which express a receptor whose binding for a ligand has been well characterized. One method for studying the effects of receptor density with such a system is to transfect cells and select subclones which express the receptor at different densities. If the transfectants are not stable, then this transfection must be performed repeatedly, which is very time consuming. The third avenue is to use an intermediary which can cross-link receptor and ligand. Provided the interaction of the intermediary with both the receptor and ligand is well-characterized, and if many different intermediaries are available, this provides a means for systematically altering the number of surface sites, the density of ligand, and the kinetics or affinity of binding between intermediary and ligand, as well as studying the resultant effects on adhesion.

In this article, we describe adhesion experiments performed using an intermediary. We use rat basophilic leukemia (RBL) cells, an immortalized cell line with intracellular, surface, and secretory biochemistry similar to that of basophils (Metzger et al., 1986). Basophils are involved in the immediate allergic response, and release histamine and serotonin after cross-linking of cell surface Fc_ϵ receptors by a foreign multivalent antigen (Metzger et al., 1986; Alberts et al., 1991). We chose RBL cells for a variety of reasons. First, the Fc_ϵ receptor on RBL cells has been well characterized (Metzger et al., 1986); the binding affinity between the *Fc* portion of IgE and the Fc_ϵ receptor, and the number of Fc_ϵ receptors on the surface of RBL cells are known. Second, a number of hapten/IgE pairs have been well-studied in connection to their ability to modulate the secretory response of RBL cells, which occurs when surface-bound IgE is cross-linked by multivalent antigens. The kinetics and affinity of binding between many available hapten antigens and IgE are known (Erickson et al., 1986, 1987, 1991; Posner et al., 1992). Third, although the experiments in this article focus on adhesion, the development of adhesive systems using substrates made of immobilized antigen might also ultimately be useful studying the effect of IgE binding and cross-linking on cellular physiological response, such as secretion of histamine. And finally, since the RBL cell line is a cultured cell line, a large number of morphologically homogeneous cells can be obtained easily and relatively cheaply, without resorting to sorting.

In this article, using a single hapten/IgE pair (2,4-dinitrophenol (DNP) as hapten, and IgE against DNP (anti-DNP IgE) as antibody), we report the adhesion of RBL cells coated with various densities of IgE to substrates coated with different densities of DNP. Adhesion is measured using a centrifugation assay, in which the detachment force is applied by the angular velocity of a normal desktop centrifuge. The substrates are polyacrylamide gels to which DNP has been covalently attached using a bifunctional linker. The substrate chemistry in our system is similar to that developed by Schnaar and coworkers (Pless et al., 1983; Guarnaccia and Schnaar, 1982). We clearly show how DNP substrate density and cell surface IgE density influence adhesion strength. From these experiments, we can estimate the force to break single molecular bonds, and suggest how centrifugation can be used to separate cell types with different levels of surface receptor expression. To our knowledge, this is the first time receptor number, ligand density, and applied force have been simultaneously varied in adhesion experiments involving real biological cells.

Materials and Methods

RBL cells

RBL cells, subline 2H3 (Barsumian et al., 1981), were grown in 75 cm^2 Falcon T-flasks (Becton Dickinson, Lincoln Park, NJ) at 37°C in supplemented Eagles minimal essential medium with Earles salts (Gibco, Grand Island, NY), as described by Taurog et al. (1979). Media was supplemented (MEM-S) with 10% fetal calf serum (heat inactivated), 10% newborn calf serum, 1 v/v % penicillin-streptomycin, 1 v/v % anti-PPLO, and 2 v/v % L-glutamine (all from Gibco). Cells were passed into new T-flasks every seven days in 20 mL of fresh MEM-S seeded at 3×10^6 cells/flask. Cells to be used for an experiment were harvested by washing RBL cells adherent in a flask with 3 mL 1× Trypsin-EDTA (Gibco), pouring off the Trypsin-EDTA, adding an additional 3 mL 1× Trypsin-EDTA, incubating for 10 min at 37°C, rapping the flask to dislodge the cells, and adding 20 mL of MEM-S to quench the further action of Trypsin-EDTA. The solution was removed and centrifuged (1,000 RPM, 10 min). Trypsin-EDTA is standardly used for RBL cell harvesting and does not affect Fc_ϵR1 surface expression (Taurog et al., 1979; Metzger et al., 1986). Cell density and viability determined in a hemocytometer by Trypan blue exclusion, and cells resuspended in a modified Tyrodes buffer (125 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5.6 mM glucose in deionized water) at a concentration of 2.4×10^6 cells/mL. Viability was in excess of 98%.

Antibody-labeling of cell surfaces

The number of cell surface binding sites directed against the hapten dinitrophenol (DNP) was manipulated by the simul-

taneous addition of two IgE antibodies, anti-DNP, and anti-dansyl at different concentrations. Purified mouse monoclonal anti-DNP IgE from hybridoma H1 26.82 (Lui et al., 1980) was used. Mouse monoclonal antidansyl was from clone 2774 (PharMingen, San Diego, CA). Each antibody's *Fc* portion is identical and binds RBL cell Fc_ϵR1 receptor, but only anti-DNP will bind the DNP hapten.

To label the cells with antibody, microcentrifuge tubes were prefilled with a 10 μL antibody mixture of anti-DNP/anti-dansyl. A cell suspension of 2.4×10^6 cells/mL in modified Tyrodes buffer was distributed in 0.5 mL aliquots to the antibody-containing microcentrifuge tubes. The final total antibody concentration after cellular addition was 20 nM. Because binding between IgE and Fc_ϵR1 on RBL cells is high affinity, with $K_D=10^{-10}$ M, 99% of the Fc_ϵR1 receptors will be filled with IgE at this concentration (Metzger et al., 1986). Cells and antibody were incubated for 30 min at 37°C with gentle shaking, and microcentrifuged to remove unbound supernatant antibody. IgE-treated cells were resuspended in fresh modified Tyrodes buffer at a concentration of 1.5×10^5 cells/mL in preparation for centrifugation experiments.

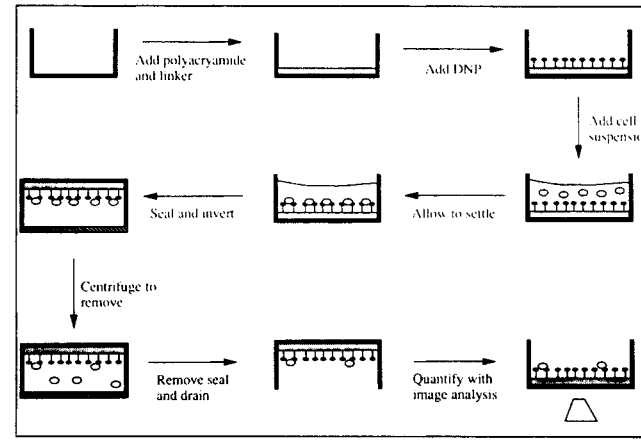

Figure 1. Steps followed in the centrifugation assay.

Acrylamide monomer solution with a bifunctional linker is added to wells of 96 well plates and polymerized. The hapten dinitrophenol (DNP) is then covalently linked to the gel. Cells coated with various amounts of immunoglobulin E (IgE) directed against DNP are added to the well. Cells are allowed to settle and bind the DNP substrate, after which the gels are sealed and inverted and centrifuged under various applied fields. Gels are then drained, and the level of adhesion is quantified with image analysis.

Preparation of DNP derivatized polyacrylamide gels

Polystyrene 96-well plates were obtained from Falcon. A polyacrylamide gel containing a bifunctional linker was created by polymerization of an acrylamide/bisacrylamide monomer solution in the well. The linker, N-succinimidyl acrylamidohexanoic acid (N-6) was synthesized in our laboratory following the method of Pless et al. (1983). Small quantities of the linker necessary to facilitate crystallization of the final product from solution were obtained from Dr. B. Brandley (Glycomed, Alameda, CA). Deaerated distilled, deionized water was used for all stock solutions; water was deaerated by exposure for $\geq$1 h to aspiration vacuum.

8 mL bis/acrylamide stock (25% acrylamide, 1.25% bisacrylamide) (BioRad, Richmond, VA), 2 mL 0.25 M HEPES, and 15 μL tetramethylethylenediamine (TMED) (BioRad) were mixed and adjusted to pH 6. The final concentration of acrylamide is 20%. N-6 linker was added to this monomer solution to bring the solution to the appropriate linker density (for example, for 20 μmol/mL, 0.0565 g N-6) and dissolved by vortexing. The monomer solution was then deaerated under aspiration vacuum for several minutes.

Our method of gel polymerization under water is from Guarnaccia and Schnaar (1982). Wells were prefilled with 275 μL deaerated water. 500 μL aliquots of monomer solution were transferred to microcentrifuge tubes. An initializer, ammonium persulfate, was dissolved in water (0.0175 g/mL). 15 μL ammonium persulfate was added to 500 μL of monomer solution in a microcentrifuge tube. Polymerization began within 2–3 min. Within this three minute period, 50 μL aliquots of the monomer solution were pipetted to the bottom of each well; polymerization was usually complete within 30 min. Gels formed at the bottom of the wells, and were in most cases the diameter of the well itself. Gels that failed to fill the entire well often fell out during centrifugation, and data from those wells was discarded.

To covalently bind DNP to the gel substrate, a DNP solution was prepared by adding 0.1395 g 2,4-DNP-ϵ-lysine-HCl (Sigma, St. Louis, MO) to a solution of 10% ethanol in HEPES (7.5 ml ethanol, 0.804 g HEPES, and 67.5 mL water at pH 8.0). 200 μL aliquots of DNP solution were added to wells containing linker-containing gels, and incubated for 12–24 h at room temperature. Finally, the DNP solution was removed and replaced with 100 μL aliquots of a capping solution containing 75 μL ethanolamine in 75 mL of 10% ethanol in HEPES for 2–4 h, to cap any N-6 linker molecules which had not been derivatized with DNP. In control experiments in which DNP was not added to the wells, reactive sites were passivated with ethanolamine. Polyacrylamide gels were stored under water at 4°C and wrapped in aluminum foil to prevent light-mediated degradation of DNP.

Centrifugation assay

A schematic for the centrifugation experiments is shown in Figure 1. Gels were rinsed under clean, deionized water several times, until the supernatant was clear of DNP (DNP is yellow), to remove unbound DNP molecules. 330 μL of IgE-treated cell solution at 1.5×10^5 cells/mL was delivered into each well. The wells were closed with a standard adhesive sealing tape for 96 well plates, taking care that air bubbles were not entrapped in the wells. Any wells containing air bubbles were discounted since cell death and lysis are common when cells are exposed to air.

Cells were allowed to settle to the gel surface at room temperature under an acceleration of 1 g for 20 min. One plate of gels was inverted (subject to a removal field of 1 g) for 10 min. This plate served as a control where maximal levels of adhesion were expected (see text). Other plates were inverted and placed in a temperature-controlled Jouan (Winchester, VA) CR412 desktop centrifuge equipped with 96 well-plate buckets and exposed to acceleration fields of 50, 100, 200, or 300 g at 25°C (corresponding to 500, 750, 1,060, and 1,300 RPM, respectively). After applying a centrifugation force for 10 min, the sealing tape was gently removed with the plate still inverted, and the supernatant drained by absorbing the liquid

onto paper towels. A small layer of liquid remained on the gel surface, keeping the cells hydrated during measurement of the cell density. Image analysis was used to quantify cell adhesion. Four distinct areas in each well were video-recorded using a Dage-MTI (Michigan City, IN) 70S Newvicon videocamera connected to a Nikon (Garden City, NY) Diaphot-TMD inverted stage microscope, and stored on VHS videotape on a JVC (Univisions, Syracuse, NY) BR-S611U recorder. Videoframes were digitized from videotape with TCL-Image software (Perceptics, Knoxville, TN) and a Data Translation (Marlboro, MA) frame grabber running on an Apple (Cupertino, CA) Macintosh IIx computer. Since four duplicate wells for each condition were used, this gave a total of 16 observations under each condition in a single experiment. Furthermore, experiments were performed as many as five times on separate days. The data is reported as a fraction of cells adherent, normalized to systems at which 1 g applied field, 100/0::anti-DNP/antidansyl, and 10 μmol/mL linker was used, as justified in the text. Negative controls included trials in which no DNP was added to the gel (and the gel was capped with ethanolamine), no IgE was added to the cells, or no anti-DNP was added to the cells.

Quantifying DNP derivatization density

The density of DNP on the gel surface was estimated by measuring the total amount of DNP covalently attached to the gel, and using geometrical considerations to estimate the amount exposed on the gel surface. To relate DNP concentration to its absorbance, a calibration curve taken at an absorbance at 365 nm, and the absorbance peak for DNP, was generated using a Beckman ACTA MVI spectrophotometer. Absorbance at 365 nm was linear with DNP-lysine-HCl density in solution up to 0.035 mg/mL. Absorbance as a function of DNP density (ρ_{DNP}) was fit to the equation Abs (units) $=0.019515$ (units) $+29.471$ (units mL/mg) $\times \rho_{DNP}$, with a correlation of $r \geq 0.999$. To determine the amount of DNP in a gel, two derivatized gels which were generated in parallel with those used in the actual experiments were treated with a strong base (5N NaOH) for 16.5 h to hydrolyze DNP from the gel surface. The absorbance at 365 nm of the solubilized DNP was then measured spectrophotometrically and converted to concentration using the calibration equation given above.

To estimate the surface concentration of DNP, we measured the height and the diameter of each gel by Vernier caliper to be 1.3 and 7 mm, respectively. The size of DNP-lysine was assumed to be 20 Å, based on lengths of carbon-carbon bonds in a chain (1.5 Å), carbon-carbon bonds in a benzene ring (1.4 Å), and carbon-hydrogen bonds in a chain (1.1 Å). We considered 10 Å thick slices of the gel, and assumed that half of the DNP molecules in the upper 10 Å slice would be accessible to cell bound IgE, since DNP molecules will be in a random orientation; we assumed the other half would be pointed downward and inaccessible. Furthermore, we have evidence that these dense 20% gels may not derivatize uniformly. In these experiments, 25% of the available linker is derivatized (see Table 1). In other experiments in our laboratory in which DNP has access to both sides of a gel of 0.25 mm thickness, 50% of the linker derivatizes. This leads us to believe the gels derivatize nonuniformly, with the upper half of the gel containing twice the density suggested by the average DNP concentration. Therefore, our estimates for DNP surface density represent twice the level expected in the upper 10 Å of a uniformly derivatized gel.

Table 1. Estimated Relationship Between Linker Concentration and DNP Surface Density

Linker Conc. (μmol/mL)	Meas. DNP Conc. (μmol/mL)	Est. DNP Surface Dens. (molec./cm$^2 \times 10^{-10}$)
20	4.79 ± 0.11 ($n=12$)	57.5 ± 1.29
10	2.63 ± 0.16 ($n=6$)	31.6 ± 1.95
5	1.32 ± 0.048 ($n=8$)	15.9 ± 0.58
1.25	0.30 ± 0.0025 ($n=4$)	3.65 ± 0.04
0.625	0.17 ± 0.00 ($n=2$)	2.1 ± 0.0
0.3125	0.080 ± 0.00 ($n=2$)	1.0 ± 0.0
0.08	0.025 ± 0.005 ($n=2$)	0.32 ± 0.016

The correlation between the linker concentration used in our experiments, the measured DNP concentration per volume of gel, and the calculated (estimated) DNP surface density (molecules/cm^2) is shown in Table 1. In our work, we strictly report adhesion as a function of linker concentration, due to obvious uncertainties in our estimate of surface ligand density.

Results

To vary the number of ligand (antigen) binding sites on the cell surface, we change the fraction of Fc_ϵ receptors which are filled with IgE antibody directed against DNP. We do this by filling the remaining Fc_ϵ receptors with IgE directed against an antigen which is not present on the substrate, dansyl. Antibody against DNP (anti-DNP) and antibody against dansyl (anti-dansyl) are incubated with the cells in various ratios (x/y::anti-DNP/anti-dansyl) where x is the percentage of Fc_ϵ receptors filled with IgE against DNP (Weetal, 1992). Since both antibodies have identical Fc regions, they bind the Fc_ϵ receptor with the same affinity and partition on the cell surface according to their ratio in bulk concentration.

Our system can distinguish between adhesion mediated with specific receptor-ligand interactions and adhesion mediated by nonspecific forces (Figure 2). Three types of cells were prepared: cells sensitized with 100% anti-DNP and 0% antidansyl (100/0); cells sensitized with 0% anti-DNP and 100% antidansyl (0/100); and untreated cells with no IgE (0/0). Gels labeled +DNP had DNP covalently bound using a linker concentration of 10 μmol/ml; in gels labeled −DNP, no DNP was present. Figure 2 shows significant adhesion only occurs when cells are coated with anti-DNP (100/0) and the gel contains DNP (+DNP); all other combinations result in very low levels of adhesion. The highest level of background adhesion is seen with the 0/100 system on +DNP gels; however, this adhesion was never greater than 10% of the adhesion seen with the 100/0, +DNP combination, even at the lowest force tested (1 g). At all higher forces, control binding (0/100 on +DNP surfaces) was negligible compared to that seen with 100/0 and +DNP combination, indicating that IgE does not mediate binding nonspecifically. As expected, increasing the centrifugal field decreases adhesion in all cases. With 100/0 cells on +DNP substrates, a field of 300 g reduces adhesion to 75% of that seen at 1 g. For calibration, the adherent cell surface density at 1 g with 100/0 cells on 10 μmol/mL+DNP gels was 504 cells/mm^2. This amounts to a substrate area coverage of 13%.

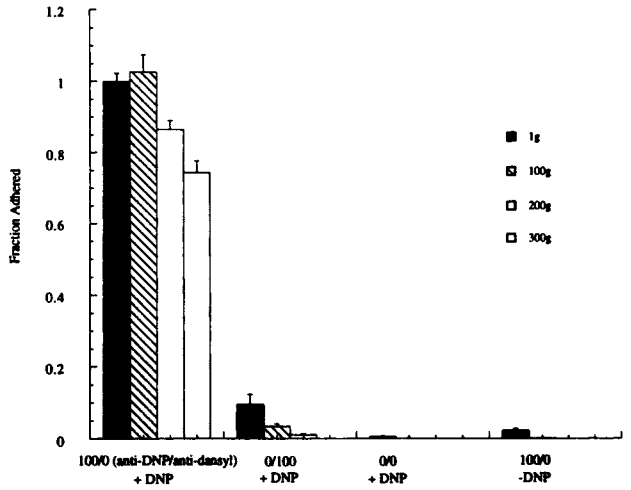

Figure 2. Example results showing the ability of this system to distinguish specific (receptor-mediated) cell adhesion from nonspecific background levels of adhesion.

Binding is quite strong if cells are labeled with IgE against DNP, and the gels contain DNP. However, binding is quite low in all controls: (1) for cells without IgE anti-DNP on gels containing DNP; (2) for cells without any IgE on DNP gels; and (3) for cells with IgE anti-DNP but on gels lacking DNP.

The force to break a receptor-ligand bond is not known unambiguously. The force to uproot glycophorin molecules from a red blood cell membrane has been measured to be 1–3 μdyne (Evans et al., 1991a). Experiments at 1 g expose cells to a force of 0.04 μdynes (see Discussion), almost two orders of magnitude below that likely to uproot the receptor. Figure 2 shows that in the absence of specific antibody-antigen binding, significant adhesion is not observed under this small force, suggesting 1 g experiments are a test for the existence of any antibody-antigen binding. Figure 3 represents experiments performed at 1 g in which both the linker density and the number of surface IgE directed against DNP are varied. Data in this experiment are expressed as the fraction adherent, where the number of cells bound is normalized to the number of 100/0 cells bound on 10 μmol/mL, +DNP gels under 1 g applied field. We varied the density of sites against DNP on the cell surface by varying the ratio of anti-DNP/antidansyl from 0/0 to 100/0. Figure 3 shows the expected trend for increasing recognition as either the density of DNP or the number of anti-DNP molecules on the cell surface increases. Greater than 90% adhesion occurs for a significant range of linker densities (>1 μmol/mL) when greater than 50% of the Fc_ϵ receptors are filled with anti-DNP IgE. When 10 to 30% of the receptors are filled with anti-DNP, adhesion is maximal at 1 g when the linker density is ≥ 10 μmol/mL. As in Figure 2, when DNP, anti-DNP, or both are absent, very little adhesion is seen.

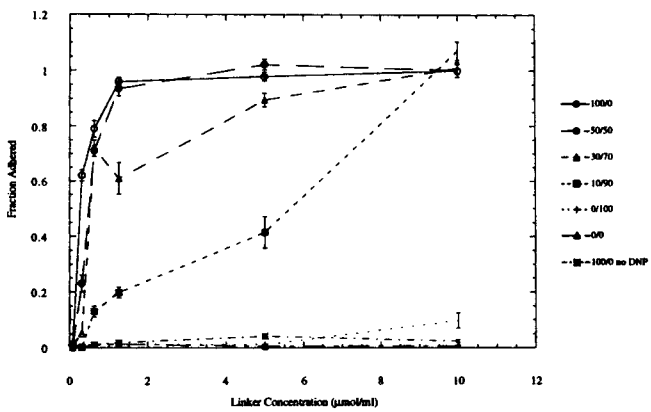

Figure 3. Fraction of adhesion plotted against linker concentration for various anti-DNP IgE cell surface densities at 1 g.

For cells with plenty of IgE binding sites (100/0 and 50/50 cells), adhesion is modulated by linker (ligand) density. For 30/70 and 10/90 cells, both cell surface site density and ligand density both control adhesion. Since 1 g is a weak field, this is an assay for any receptor-ligand binding. Control levels of binding were quite low, as shown.

At high anti-DNP IgE cell surface densities (50 or 100% coverage), DNP surface density may be limiting adhesion, especially at low DNP substrate densities; if so, adhesion is likely controlled by DNP surface availability (Figure 3). When anti-DNP surface coverage decreases ($\leq 30\%$), both anti-DNP cell surface and DNP substrate density limit adhesion. Since the applied force in these experiments is quite weak, combinations of anti-DNP and DNP densities which the fractional adhesion is significantly below 1 are likely in a limit where cells that remain bound have formed very few tethers (see Discussion). Figure 3 also shows that maximal adhesion readily occurs at 1 g with cells completely covered with anti-DNP on surfaces of 10 μmol/mL linker density. Therefore, we will continue to use these conditions as a positive control; all levels of adhesion will be given as a fraction of the adhesion seen under these conditions.

Using phase contrast microscopy, we measured the projected area of RBL cells fully decorated with anti-DNP incubated on two types of gels, one without DNP and one with DNP at 10 μmol/mL, after a 20 min surface incubation. Area measurements on the two surfaces indicate the diameter of cells on gels without DNP to be 12.1 ± 1.4 μm ($N=15$), as compared to 11.5 ± 1.6 μm ($N=15$) on +DNP gels. The mean cell diameters on the two surfaces are not significantly different, and this lack of difference was confirmed by a statistical t-test ($p \leq 0.278$ that cell sizes are significantly different). These measurements indicate that although there is significant specific recognition of anti-DNP coated cells on 10 μmol/mL linker gels (Figure 3), no significant spreading of these cells on DNP-coated surfaces occurs compared to that of controls during the time scale of our experiments.

In Figure 4, the fraction of adhesion is measured as centrifugation force, linker density, and IgE surface coverage are varied. Each panel (a through f) shows a different linker, and therefore, ligand density, with 4a being the highest linker density tested. As the ligand density or receptor density decreases, or as the applied force increases, adhesion decreases. Figure 4a shows that at the highest ligand density, it is impossible to distinguish between cells that are completely coated with anti-DNP antibody (100/0), and those with a tenfold reduction in anti-DNP (10/90). For all receptor coverages tested in Figure 4a, adhesion decreases with increasing applied force, from a fraction adherent of 1 at 1 g to 0.8 at 300 g.

As the ligand density decreases (proceeding from Figure 4a

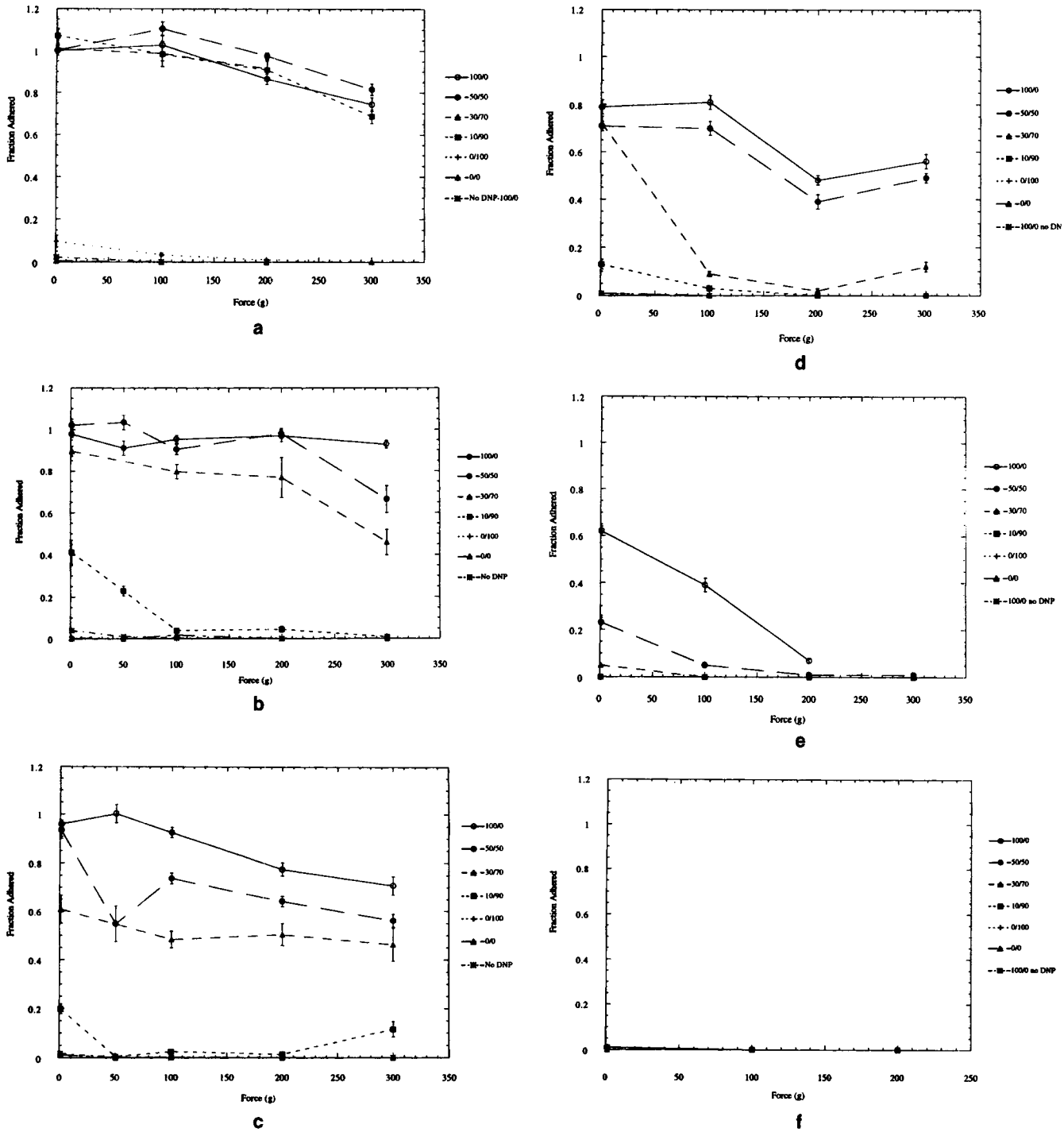

Figure 4. Fraction of adhesion as a function of applied centrifugation field at different anti-DNP/anti-dansyl surface ratios.

Each panel corresponds to a different linker density. (a) 10 μmol/mL; (b) 5 μmol/mL; (c) 1.25 μmol/mL; (d) 0.625 μmol/mL; (e) 0.3125 μmol/mL; (f) 0.08 μmol/mL.

through 4f), a number of dramatic changes occur in the fraction of cells adherent. For example, as we proceed from 4a to 4b, in which the linker density changes from 10 to 5 μmol/mL, we see a change in the behavior of cells in which only 10% of receptors are coated with anti-DNP (10/90). The fraction of adhesion of these cells on 5 μmol/mL gels is only 0.41 at 1 g applied force, and at forces 100 g or greater, adhesion is indistinguishable from control (Figure 4b). Furthermore, at a linker density of 5 μmol/mL, it becomes possible to distinguish between 100/0, 50/50, and 30/70 cells over a range of applied forces. In general, as the ligand density decreases in the sequence of Figures 4a through 4f, it becomes possible to distinguish between the adhesion behavior of cells with different surface densities of anti-DNP. In Figure 4d, in which the linker density

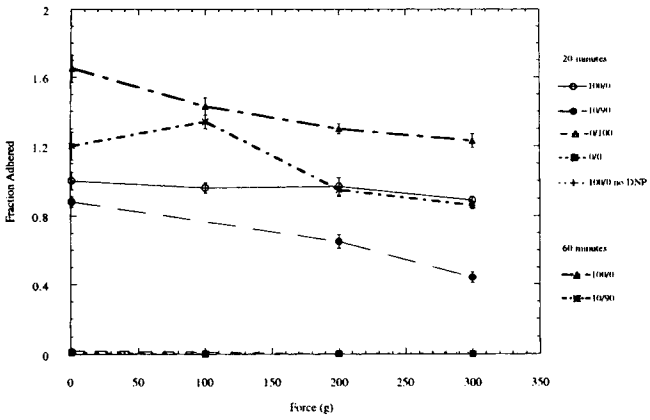

Figure 5. Comparison of binding of 100/0 and 10/90 cells on 10 μmol/mL gels for 20 and 60 min.

Curves indicate a greater level of adhesion for both populations, likely due to increased deposition, but little strengthening response.

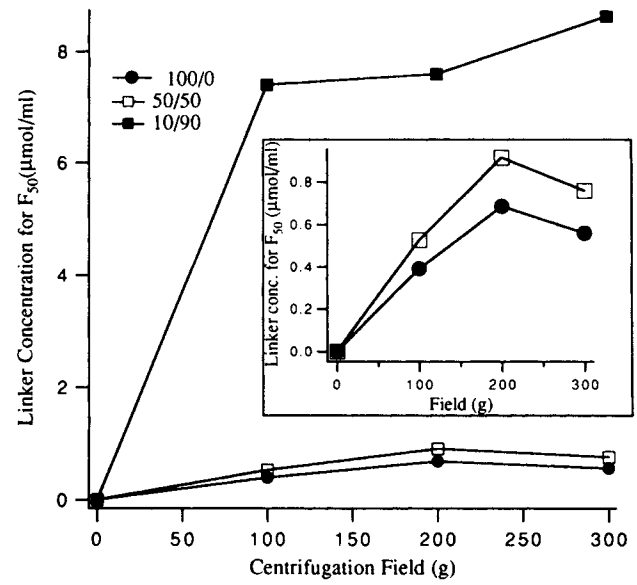

Figure 6. F_{50} curve generated showing the ligand density necessary to maintain 50% adhesion as a function of applied field, an often used measure of the average strength of adhesion.

Inset of figure is an expanded view of F_{50} for 100/0 and 50/50 cells.

is 0.625 μmol/mL, the adhesion of 30/70 cells is indistinguishable from control at forces ≥ 100 g; likewise, 50/50 cell adhesion is indistinguishable from control at forces ≥ 100 g when the linker density is 0.3125 μmol/mL (Figure 4e); finally, 100/0 cells show negligible adhesion at a linker density of 0.08 μmol/mL (Figure 4f).

The qualitative interpretation of these experimental results is straightforward. When the ligand density is high, levels of binding of cells with different levels of receptor expression are the same (Figure 4a), since an excess of ligand can compensate for a dearth of receptors. At low ligand densities (Figure 4c-4e), receptor site densities need to be high to guarantee recognition. Cells below the requisite IgE surface density at a given DNP concentration detach. Therefore, low ligand densities exert a "selection pressure" which allows one to distinguish between cells of different IgE density. Also, the effect of IgE and DNP surface densities depends on the applied force, since the number of bonds which form depend on both of these densities, and greater numbers of bonds are more likely to withstand a given applied stress.

In Figure 5, we show results from incubation time experiments, in which we assess the effects of long time deposition and binding incubation (60 min) on adhesion. The RBL cell is a transformed leukocyte, and although it is stimulated by the presence of antigen (Metzger et al., 1986; Oliver et al., 1988), its spreading and response are entirely different from cells of the vascular system (endothelial cells) and connective tissue (fibroblasts). Extensive spreading is not observed over the 20-60 min time scale of these experiments. Measurements of the projected area of RBL cells fully coated with IgE on surfaces with and without ligand show no statistical difference between 20 and 60 min incubation times (results not shown). For cells completely coated with anti-DNP (100/0), the increase in adhesion was 60% at 1 g, but only 30% at 300 g. (Fraction of adhesion is greater than 1 in these experiments because the fraction of adhesion uses the level of binding at 20 min, 10 μmol/mL, 1 g as a reference, and there is greater binding at 60 than 20 min). More cells are able to settle to the bottom of the well after 60 min, which leads to an increase in adhesion at 1 g. In the Discussion, we show how the increased binding at 60 min can be used to estimate the cell's Stokes settling velocity. Although RBL cells incubated for 60 min have a longer time on the substrate in which they can form a greater number of antigen-antibody bonds to withstand the applied stress, there does not appear to be an increase in adhesion at higher forces. This reinforces that these cells are unlike connective tissue cells, and that there may be complex biochemical changes which occur upon contact in this system which do not necessarily increase adhesion strength.

A common practice in adhesion experiments, and centrifugation experiments in particular, is to determine the parameter values (force or ligand density) at which half of the cells are bound (fraction adherent = 0.5). There is some inaccuracy in such a determination, which results from interpolating curves. Linear interpolation of the data in Figure 4 is highly inaccurate; very few of the curves in these figures cross the fraction adhered = 0.5 ordinate. However, we were able to linearly interpolate data at fixed applied force from curves that were replotted from the data in Figure 4. These plots were in the form fraction adherent vs. linker density as a function of receptor coverage; each plot was at a separate centrifugation field of 100, 200, and 300 g. These plots are similar in format to Figure 3, which was generated for a 1 g applied force. Such a determination gives the linker density which supports 50% adhesion at each force, and represents a description of the average adhesive behavior of the population. The F_{50} linker concentration (fraction adherent at 50%) plots are shown in Figure 6.

Figure 6 shows there is little change in the linker concentration necessary to maintain 50% adhesion as the applied field changes from 100 to 300 g. This is likely because these are mild (factor of three) changes in applied force. There appears little difference in the linker concentration necessary for

50% adhesion as IgE surface density changes by a factor of two (from 50/50 to 100/0). Only when IgE surface density is altered by a factor of ten (from 100/0 to 10/90) do the requirements on ligand density dramatically increase. Clearly, the increase in ligand is compensating for the relative absence of receptors to achieve the proper level of binding to maintain adhesion.

Discussion

In this study, we have used the RBL cell as a model system to study the effects of ligand density and IgE cell surface density on adhesion. There are several reasons why such a study is of value. First, it demonstrates proof of the concept that such model studies can be performed with this system. With the availability of many different antigen-IgE pairs of different kinetics and affinity, we should now be able to assess the role of kinetics and affinity in cell adhesion. Second, these experiments are the first in which the strength of adhesion has been measured as a function of receptor site number and substrate ligand density with real biological cells. Qualitatively, adhesion changed as expected when these parameters were varied, which confirms that our gross picture of adhesion is correct. Third, by performing these quantitative experiments, we hope to move from a gross qualitative understanding of adhesion to a detailed quantitative one. This will likely come when we can develop appropriate mathematical models which recreate the trends seen in this data and which can also be independently verified.

We can use the data from Figures 3 and 5 to estimate the density difference between cell and medium. In a 20 min deposition (Figure 3), a typical cell surface coverage for highly adhesive substrates (that is, 100/0 cells on 20 μmol/mL linker substrates at 1 g) is 504 cells/mm^2. The liquid volume in the wells is 330 μL (area = 0.38 cm^2; height = 0.87 cm). This gives a maximum binding of 19,200 cells/well. Since cells are added at a concentration of 1.5×10^5 cells/ml, or 49,500 cell total, to the well, 39% of the cells deposit in the first 20 min. The settling velocity, V_s, is 2.82 μm/s, and given $V_s = 2g\Delta\rho R_c^2/9\mu$, with $\mu = 0.01$ g/cm·s and $R_c = 6$ μm, we calculate $\Delta\rho = 0.036$ g/cm^3. Based on this velocity, all cells should settle in $t_s = 3.1\times10^3$ s. We can also use data at 1 g from 60 min settling experiments (Figure 5) to determine $\Delta\rho$ and t_s. Since the fraction adherent increases to 1.65 at 1 g after 60 min (compared to 20 min), and if we assume all cells which settle bind, this suggests the settling time for all cells is $t_s = 1.98\times10^3$ s, which gives $\Delta\rho = 0.056$ g/cm^3. Clearly, these two methods represent lower and upper bounds on $\Delta\rho$ (0.036 g/cm^3 $< \Delta\rho <$ 0.056 g/cm^3) and t_s (1.98×10^3 s $< t_s < 2.9\times10^3$ s). There are many reasons why either bound might be in error, but given the reported values of $\Delta\rho = 0.04$–0.05 g/cm^3 for leukocytes (Bongrand and Bell, 1984), we feel confident in assuming $\Delta\rho = 0.045$ g/cm^3 and $t_s = 2.5\times10^3$ s. The long settling time suggests that during the 20 min experiments, there is a wide range of residence times for cells on the surface (between 0 and 1.2×10^3 s).

Our measurements of adhesion strength can be compared to measurements made by other laboratories. Even at high IgE surface coverage and high DNP substrate density, adhesion could be partially reversed with forces of 12 μdyne (see Figures 4a and b). This force is based on a density difference of 0.045 g/cm^3, as stated above. This force is higher than for the adhesion of glioma cells on fibronectin at 4°C (1–5 μdyne) but weaker than the strength of glioma cell adhesion to fibronectin at 37°C (60 μdyne) (Lotz et al., 1989). Glioma cells do exhibit some spreading after 15 min on fibronectin at 37°C; RBL cells do not exhibit spreading after 20 min at 25°C. In contrast, fibroblasts show much greater levels of adhesion, especially at 37°C in which they are completely resistant to 360 μdyne after only a 15 min incubation (Lotz et al., 1989). Fibroblasts are cells with active cytoskeletal reorganization, and probably form cytoskeletally driven patches, which increase adhesion strength (Ward and Hammer, 1993). The strength of chicken hepatocyte strength on carbohydrate surfaces is 9.7 μdyne at 4°C and 146 μdyne at 37°C (Guarnaccia and Schnaar, 1982), once again spanning the range of observed RBL cell behavior. In general, RBL cell adhesion is much closer to the glioma cell and chicken hepatocyte cases, with RBL cell adhesion falling between that for glioma cells and hepatocytes at 4°C and 37°C. This may simply be an effect of temperature at which these experiments were performed. However, RBL cell adhesion to DNP appears much weaker than fibroblast adhesion to fibronectin.

To further analyze the strength of adhesion, we must assess the possibility of bivalent binding between IgE and DNP. Referring to the measured densities of DNP given in Table 1, and the known distance (10 nm) between Fab binding arms of IgE antibody (Zheng, 1992), the average number of DNP molecules accessible to a single IgE molecule is 0.45 at the highest linker density (20 μmol/mL), and will fall off as linker (ligand) density decreases. This, combined with the rotational restrictions on a singly bound IgE molecule make it unlikely that there is significant bivalent binding at most, if not all, of the DNP densities of these experiments. Therefore, it is likely that any IgE-DNP binding is monovalent.

Consider the experiments performed at 1 g (Figure 3). An acceleration of 980 cm^2/s and $\Delta\rho = 0.045$ g/cm^3 gives an applied force of $4/3\pi\rho R_c^3 g = 4\times10^{-8}$ dynes, about 25 times smaller than the expected strength of a single receptor-ligand bond of 10^{-6} dynes (Evans et al., 1991a; Bell, 1978). This reinforces that 1 g experiments are an assay for the formation of any receptor-ligand tethers.

By applying forces to cells which are bound by very few tethers, we can measure the strength of single monovalent interactions. The binding of 10/90 cells at 1 g (Figure 4b), in which $F_{ad} = 0.41$, is largely due to the formation of single tethers. The remaining 59% of the cells form no tethers, since those cells do not adhere under 1 g applied field. If the distribution of bonds among all deposited cells is given by the Poisson distribution, the mean number of bonds such that 59% of the cells form no tethers would be $-\ln(0.59) = 0.53$. The fraction of cells forming one tether would be $0.53\exp(-0.53) = 0.31$, and forming two tethers would be 0.08. Therefore, if 41% of the cells are bound, 75% of these would be bound by a single tether, and 19.5% bound by two tethers; hence, 95% of the adherent cells would be bound by ≤ 2 tethers. If we then apply 2–4 microdyne stresses to these cells as we do in Figure 4b (50–100 g), we see about half the adhesion is reversed at 50 g, and the adhesion is completely reversed at 100 g, leading to individual bond strengths between 2 and 4 μdyne. While this might be an overestimate, since we cannot verify that all of the cells bound under these conditions are bound by a single tether, this range is in quite good agreement with previous measurements of the strength of antibody glycophorin bonds in red blood cells (Evans et al., 1991a), and

corresponds well with other recent estimates of antibody-red blood cell strengths (H.L. Goldsmith, personal communication) where the mechanism of detachment is uprooting molecules from the cell membrane. These forces appear weaker than the forces necessary to break individual tethers between molecules which are covalently attached to surfaces (Kuo and Lauffenburger, 1993).

Now, we consider the case when the cell is more completely covered with IgE. For 100/0 cells there are an average of 2×10^5 IgE molecules/cell (Ryan, 1989). The smallest possible area of contact between the cell and substrate is the area of a microvillus tip $A_{mv}=10^{-10}$ cm^2 (Bongrand and Bell, 1984). If the total cell area is 9×10^{-6} cm^2, the average number of receptors per microvillus tip is $\bar{n}_r=2{,}2$, assuming that the receptors are randomly distributed over the cell surface, which has been verified by immunogold antibody labeling in tandem with electron microscopy (Oliver et al., 1986). By Poisson statistics, the probability of having at least one IgE available on a tip is $1-e^{-\bar{n}_r}=0.89$. However, most cells will have multiple microvillus tips in contact with the substratum, so it is safe to assume that most of these cells have sufficient receptors available to bind ligand.

For an anti-DNP IgE molecule on a microvillus tip, the rate of forward reaction is extremely rapid. Simple kinetic theory for diffusible reactants suggests:

$$k_f=\frac{k_+k_{+1}}{k_{+1}+k_-} \tag{1}$$

where k_f is the overall rate of reaction, k_+ and k_- are transport rates of encounter and dissociation of potential reactants, and k_{+1} is the intrinsic reaction rate (Bell, 1978). From experiments of Erickson and coworkers (1986) on binding of DNP in solution to cell surface bound IgE, k_{+1} is 4×10^6 s^{-1}. We estimate k_- from the time for a receptor to disengage from a surface DNP molecule when the IgE surface diffusivity is $D=2\times10^{-10}$ cm^2/s (as measured for the Fc_ϵ receptor by Ryan (1989) and Menon and coworkers (1986)). Here, as $k_-=2D/(s^2\{1.5+\ln 0.5\})$, and $s=10^{-7}$ cm is the radius of the encounter complex (DeLisi, 1980), so $k_-=4.9\times10^4$ s^{-1}. Since $k_{+1}\gg k_-$, reaction on the microvillus tip is diffusion-limited, and $k_f=k_+$. k_+ depends on the ligand density, since the time to find the ligand depends on how the ligand are spaced. From solution of the mean capture time equation (DeLisi, 1980), one can estimate the forward transport rate constant:

$$k_+=\frac{2\pi DN_l}{\ln\left(\frac{1}{s\sqrt{\pi N_l}}\right)-\frac{3}{4}} \tag{2}$$

where N_l is the ligand density. For $N_l=5.75\times10^{11}$ cm^{-2}, $k_+=k_f=5.7\times10^2$ s^{-1}; for $N_l=3.2\times10^9$ cm^{-2}, $k_+=k_f=1$ s^{-1}. This suggests that if a receptor is present on the microvillus tip, the reaction will take place within 1 s if the ligand density is low, and much faster at higher ligand densities. Clearly, the time scale for IgE-DNP reaction is much shorter than the time scale for cellular deposition on the substrate.

Since reaction is fast and there are plenty of receptors, it is unlikely that either of these sources can explain the decrease in adhesion with increasing ligand density for 100/0 cells in 1 g experiments (Figure 3). The most likely explanation is the availability of ligand in the area of the microvillus tip. Table 2 gives the probability of finding ≥1 ligand in the area of the microvillus tip by Poisson statistics, $P(\geq1)=1-\exp(-N_lA_{mv})$, where A_{mv}, the area of the microvillus tip, is 10^{-10} cm^2. Note from Table 2 the probability of finding ≥1 ligand per microvillus corresponds well with the fraction of 100/0 cells adherent as a function of ligand density. The fact that most cells will have more than one microvillus available for binding suggests we might be overestimating the ligand density; nevertheless, the correspondence between adhesion and ligand density suggests adhesion is controlled by the availability of ligand when receptors are in excess (100/0 cells).

With this explanation in hand for the binding of 100/0 cells, we examine the binding of 10/90 cells in Figure 3. At an IgE receptor density of 2×10^4 receptors/cell, the average receptor number of IgE molecules per microvillus tip is 0.22, and the probability of finding a receptor on the tip surface is $1-\exp(-0.22)=0.197$. One simple approximation for the probability of adhesion might be the product of the probability of finding a ligand and that of finding a receptor in the tip region, since both are required for adhesion. For a single microvillus, this simple approximation greatly underestimates the extent of adhesion. For example, at a linker density of 10 μmol/mL, the fraction of cells adherent is 1, whereas the probability of finding a ligand and receptor in the tip area are 1 and 0.2, respectively. This overly facile argument gives a probability of adhesion of 0.2, far below what is observed. While multiple microvilli tips are a possible explanation for the high levels of adhesion when the probability of finding a receptor on each microvillus is so low, remember multiple

Table 2. Ligand Availability as a Function of Linker Concentration

Linker Conc. (μmol/mL)	Est. DNP Surface Dens.* (molec./cm$^2\times10^{-10}$)	Fraction Adherent at 1 g for 100/0 Cells**	Probability ≥1 Ligand per Microvillus† $[P(\geq1)=1-\exp(-N_lA_{mv})]$
20	57.5	1	1
10	31.6	1	1
5	15.9	0.97	1
1.25	3.65	0.97	0.974
0.65	2.1	0.79	0.877
0.313	1.0	0.62	0.632
0.08	0.32	0	0.273

* Estimated as described in the Materials and Methods section and given in Table 1.
** F_{ad} of 100/0 cells under 1 g applied field as given in Figure 4.
† Calculated using the Poisson distribution where N_l is the DNP surface density and A_{mv} the microvillus tip area.

microvilli will force us to decrease our estimate of ligand density to maintain agreement in the excess ligand limit. It is also quite likely there is more adhesion than suggested by the average number of receptors/microvillus initially, since receptors can redistribute over the cell surface on the time scale of seconds (Menon et al., 1986). Therefore, the full analysis of this experiment would involve a calculation of the time for a receptor to find its way to the microvillus tip as a function of receptor density, and the time to react as a function of ligand density. Two additional complications are that the RBL cell population is heterogeneous in receptor density [the distribution of IgE receptors among the population is known to be log-normally distributed (Ryan, 1989)], and approximately 31% of the Fc_ϵ receptors are immobile. It is possible that different subpopulations of cells which express different receptor levels have significantly different times for encounter between receptor and ligand which then affect the population average time for encounter. Therefore, the kinetic rates of encounter and the heterogeneity of the population must be included in formulating the probabilistic analysis of binding in these 1 g experiments. In our attempts previously to perform a detailed treatment of this problem, in which we have incorporated kinetic rates of transport of receptors onto microvilli tips and their subsequent reaction, our results have consistently overestimated the binding, but we encourage other groups to consider these and other factors which might contribute to low levels of binding seen under these conditions.

Since we have not yet quantitatively explained the binding of RBL cells to the gels at different IgE and ligand densities at 1 g, we cannot adequately deal with the more difficult problem of detachment at higher accelerations. Our experiments performed at accelerations up to 300 g show in most cases significant detachment can occur at these accelerations, which are equivalent to approximately 12 μdyne of applied force. The magnitude of the force coupled with estimates of individual bond strengths given previously suggest very small numbers of bonds mediate these attachments. Being in the small bond number limit, it is likely a probabilistic binding and, with appropriate models for the kinetics of binding, a probabilistic detachment analysis will be most appropriate (Cozen-Roberts et al., 1991b; Hammer and Apte, 1992). Since we have not yet solved the probabilistic cell-substrate binding problem, investigation of the probabilistic detachment is unwarranted at the moment.

Two additional factors must be considered when analyzing these experiments in which multiple receptor-ligand binding occurs. First, one must decide how bonds are distributed in the cell substrate interface, which requires detailed knowledge of the time-dependent morphological changes of the shape of the interface during binding to ligand-coated substrata. There is currently no adequate theory for the kinetics of spreading of rough cells which might be activated. Second, when the centrifugation field is applied, one must determine how the macroscopically applied stresses are transduced to the cell substrate interface. To do this, one must know the rheological constitutive equations for this cell type, a notoriously difficult problem which occupies considerable time and effort of those in the biophysics community. Indeed, the rheology of only two cell types has been rigorously determined: red blood cells (Evans and Skalak, 1980) and neutrophils (Evans and Yeung, 1989), and there remains considerable controversy over the rheology of the latter. The rheological properties of RBL cells would have to be elucidated before the adhesion data of this article could be rigorously verified, although approximate theories may be developed.

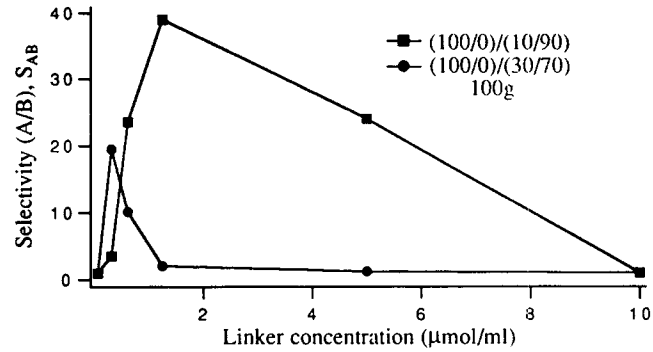

Figure 7. Selectivity for binding population *A* over population *B* (*A/B*) at 100 g as a function of linker density.

Two combinations are considered, $A = (100/0)/B = (10/90)$ and $A = (100/0)/B = (30/70)$. Selectivity is a maximum at some intermediate linker density, and selectivity can be greatest when chemical differences are greatest [as in the (100/0)/(10/90) case].

Nevertheless, the RBL cell system remains an ideal system to study the chemical basis of cell adhesion. With the availability of different antigen-IgE pairs of different chemistry, the effects of receptor-ligand chemistry on adhesion can be studied. Also, polyacrylamide gels have been shown to be ideal substrates for adhesion as they are easily biochemically modified to contain adhesion ligands, and nonspecific cell binding to these gels is very low. We plan to continue using this system for a detailed understanding of the chemical basis of adhesion.

As final demonstration of the utility of this study, we can use our results to understand how to separate cells which express different numbers of surface receptors which bind the same ligand. If we define selectivity for binding cell type A over cell type B, S_{AB} = fraction adhered A/fraction adhered B, we can plot S_{AB} as a function of linker concentration. For $A = 100/0$ cells and $B = 10/90$ cells, in which there is a factor of 10 difference in IgE density, selectivity goes through a maximum with linker concentration, with the greatest selectivity at 1.25 μmol/mL (Figure 7). This maximum in selectivity persists when comparing (100/0) to (30/70) cells, but less selectivity is possible because the chemical differences between the populations are not as great (3.3-fold difference in IgE density). Also, the linker concentration at which the selectivity is maximum is lower when the chemical differences between populations are not as great, because low ligand density provides greater selection pressure for populations which are close in chemistry. Also, the range of linker concentration over which substantial selectivity may be achieved is much more narrow when the chemical differences are smaller. This analysis once again shows that separation based on adhesion must be optimized for the proper ligand density and force to achieve maximum selectivity, and that optimization may be achieved with a detailed knowledge of adhesion.

In summary, using RBL cells coated with IgE and substrates coated with antigen, we have performed adhesion experiments which measure the effect of ligand and receptor density on the strength of adhesion. The experiments provide data to use in

the formulation of detailed quantitative models of receptor-mediated binding and detachment as outlined here. These experiments are also the first to measure the strength of adhesion as a function of ligand density and cell surface site density with real biological cells, and were performed with a desktop centrifuge. From these experiments, we were able to determine the strength of the DNP-IgE-Fc_ϵ receptor-cell linkage to be 2–4 μdyne, in agreement with previous measurements of the force necessary to uproot receptor-ligand bonds.

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