INTERACTION FORCES BETWEEN RED CELLS AGGLUTINATED BY ANTIBODY

III. Micromanipulation

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ABSTRACT In the flow studies described in two previous papers (Tha, S. P., and H. L. Goldsmith, 1986, *Biophys. J.* 50:1109–1116; Tha, S. P., J. Shuster, and H. L. Goldsmith, 1986, *Biophys. J.* 50:1117–1126), hydrodynamic forces of the order of 10^{-11} N (μdyn) were applied to measure the force of separation of doublets of hardened, sphered human red blood cells cross-linked by anti–B antibody. The same cell preparation and hyperimmune antiserum has here been used to carry out experiments with micropipet aspiration techniques. One cell of a doublet was aspirated onto a holding pipet, and a second aspiration pipet was brought into proximity of the other cell so that the two pipets and the doublet were colinear. Suction was then raised until the two cells separated. Some doublets were assembled by aspiration of a singlet, bringing a second singlet into apposition with the first, and releasing it from the pipet which was then withdrawn. Cells could be repeatedly assembled and separated. At 3.56% vol/vol antiserum, the mean normal force of separation was 0.45 ± 0.11 nN in phosphate-buffered saline suspensions containing 2.5×10^4 cells/μl; at 1.22% vol/vol antiserum, the value was 0.22 ± 0.11 nN. The above values of the force were ~ 2.5× greater than those from the flow studies. The data could be fitted to a Poisson distribution with 0.05 nN as the force needed to break a single cross-bridge (c.f. 0.024 nN from the previous hydrodynamic data). The forces of separation of randomly assembled doublets were lower than those of preexisting doublets. Repeated assembly and separation of doublets showed that the cell surfaces are nonuniform in adhesion strength both over the local scale <0.25 μm^2 and the cell population.

INTRODUCTION

Cell adhesion is the signal event in many biological processes. Central to our understanding of adhesion mechanisms is knowledge of the forces and energies involved. Yet the ability to measure such forces and to interpret their significance has not proven facile. To this end we have developed a simple system whereby hydrodynamic forces of microdyne range are applied to a preparation of hardened, sphered human red blood cells cross-linked by anti–B antibody, as described in parts I and II of this work (Tha and Goldsmith, 1986; Tha et al., 1986). The advantages of this arrangement follow from the lack of cell deformability and from the focal contact area bound by cross-bridges of known specificity. The work presented in this paper seeks to confirm and extend the observations by using the same cell preparation but micromechanical techniques: specifically, the application of forces to red cell doublets by micropipet aspiration.

Pressurization of single red blood cells (rbc) by micropipet to understand their material characteristics has proved to be a powerful experimental tool (Evans and Skalak, 1980; Evans, 1981). After some theoretical refinements (Evans, 1980; Evans and Buxbaum, 1981), the technique has become applicable to certain problems in cell adhesion. In particular, the affinity between rbc bound by dextran and plasma (Buxbaum et al., 1982), and *Helix pomatia* lectin (L. Sung et al., 1985*a* and *b*) has been determined by this method. The adhesion energy and force required to separate two red cells bound by wheat germ agglutinin has been measured by micropipet suction (Evans and Leung, 1984), as have the negative pressures needed to distract a cytolytic T cell from its target cell (K.-L. Sung et al., 1986).

A major consideration in the decision to use this technique is that it provides data on individual cell–cell interaction forces. It can therefore stand as a test of the hydrodynamic theory given in part I of this work (Tha and Goldsmith, 1986) and corroboration of results obtained therefrom in part II (Tha et al., 1986) using our traveling microtube apparatus. While the latter results are generated within a well-founded theoretical framework, in a strict sense the theory is externally unverified. Also, it is necessary to confirm that some assumptions made in the theoretical treatment, such as cell rigidity, are valid, and that departures from ideality, such as imperfect sphericity,

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surface roughness, and aberrant torques, do not significantly diminish the theory's applicability. In addition, micropipet aspiration has unique exploitable advantages over the traveling microtube device. It is capable of producing a broad range of forces in one experiment without resorting to viscous and unphysiologic glycerol-based suspending media. Especially valuable is the availability of comparatively large pressures ($>10^3$ Pa) and corresponding forces (>10 nN), so that truer frequency distributions can be obtained. By micromanipulation it is also possible to examine any dynamic, history-dependent effects on adhesion because doublets may be separated and constituted at will. This allows the study of how singlets, the major component of most preparations, differ from the agglutinated cells.

EXPERIMENTAL METHODS

1. Apparatus

The micropipet aspiration and micromanipulation system used has been previously described (Evans, 1980) and is illustrated in Fig. 1. An inverted microscope (Leitz Diavert, Wetzlar, GDR) was mounted on a 10 × 50 × 60 cm granite slab resting on 15 tennis balls for vibration isolation. The microscope was fitted with a beam-splitter and videocamera (Dage-MTI, model 65, Michigan City, IN), the output of which was directed to a high-resolution black and white monitor (RCA, TC 1217, Lancaster, PA). The microscope magnification was 1,250× and the final screen magnification was 9,700×. The depth of field was ~0.5 μm. The video output was recorded on a videorecorder (Sony VO-2800 VCR, Willowdale, Ontario) and analysed off-line on a Sony VO-5800 VCR.

A stainless steel frame mounted on the specimen stage held a pair of glass cover slips, which were sealed by vacuum grease and created a chamber of dimensions ~2 × 1 × 0.1 cm. The chamber was open at the sides so that micropipets could be inserted and a cell suspension could be injected and held by capillary action. Two custommade micromanipulators driven by air pistons controlled the positions of two micropipets to a resolution of <1 μm. The pressure within a pipet could be controlled by mouth suction. Alternatively a pipet could be coupled by a continuous water system to a micrometer-driven water manometer. Negative pressures were measured through the continuous water system by a digital pressure transducer (Validyne Engineering Corp., CD 23, Northridge, CA) with a resolution of 0.1 Pa. By means of video multiplexing, the pressure data and time could be displayed and recorded on the video monitor as shown in Fig. 2. All experiments were conducted at room temperature.

The pipets were drawn on a vertical pipet puller from 1-mm i.d. borosilicate glass tubing (Kimble, Owens-Illinois, Toledo, OH). The pipet was trimmed flat to the required diameter by using an electrically heated loop of nichrome wire mounted on a three-dimensional micromanipulator and viewed with a stereomicroscope. The internal diameters of the pipet tips were measured by a videoposition analyzer (Vista Scientific Corp., model 305, La Mesa, CA) calibrated with a micrometer scale; they ranged from 1 to 5 μm. The pipets were filled by boiling under reduced pressure in phosphate-buffered saline at pH 7.4 (PBS).

2. Cell Suspension

The rbc were swollen, sphered, and fixed as previously described in detail in part II (Tha et al., 1986). Briefly, citrated type B blood from a single healthy donor was extensively washed in isotonic phosphate buffer (PB) at pH 7.4, after which the rbc were immersed for 12–20 s in a glycerol buffer containing 8×10^{-5} M sodium dodecyl sulfate, then fixed for 12 min with glutaraldehyde (Ladd Research Industries, Burlington, VT) at a final concentration of 0.085% vol/vol. After washing six times in PB, the cells were stored for up to 2 mo at 4°C in PBS with sodium azide 0.1%. For some experiments, an additional step of lysine treatment was introduced. Here, after fixation in glutaraldehyde, 1 ml of packed rbc were washed four times in PB, then stirred for 1 h at room temperature in 250 ml of 0.2 M lysine monohydrochloride, with sufficient dibasic PB to adjust the pH to 7.0. These cells were then washed four times in PB. Subsequent storage and use was as for other fixed cells.

A dilute cell suspension was used for ease of visualization in most experiments. This comprised a final fixed rbc concentration of 1.70×10^3 cells/μl in PBS and human hyperimmune anti-B antiserum (Ortho Diagnostic Systems, Don Mills, Ontario) at a final concentration of 0.25 to 50% vol/vol. Optionally, 0.5% wt/vol human serum albumin (Sigma Chemical Co., St. Louis, MO) was added to prevent cells from sticking to glass surfaces. In those experiments intended to replicate the flow studies,

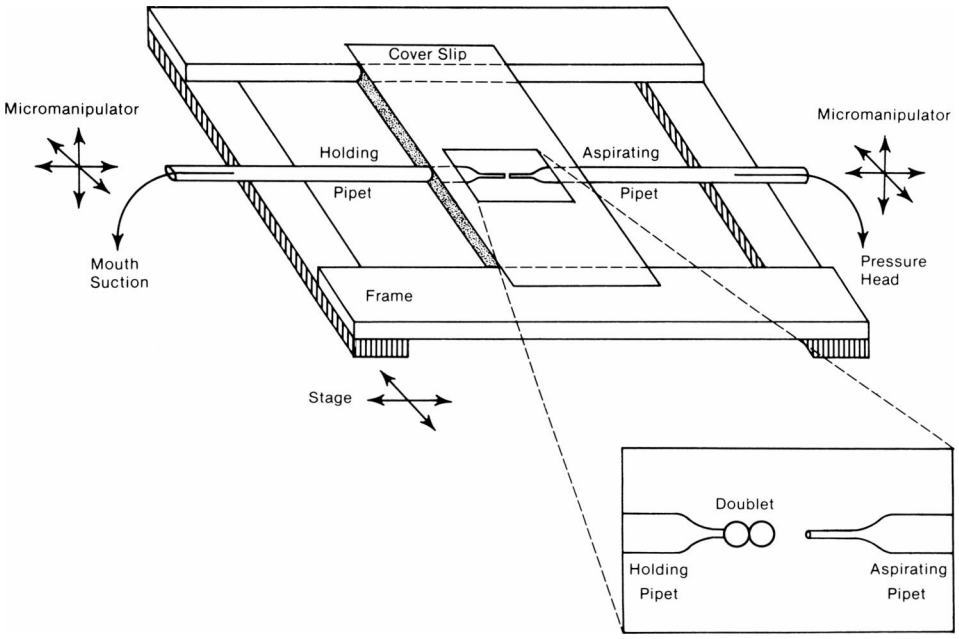

FIGURE 1 Schematic diagram of a microscope stage–mounted stainless steel frame on which are mounted coverslips to form a chamber. A cell suspension has been injected into the chamber and the pipets are inserted into its open sides. The pipets are moved by micromanipulators, and their pressures are controlled by mouth suction in the case of the holding pipet and by a micrometer-driven hydrostatic pressure head in the case of the aspirating pipet.

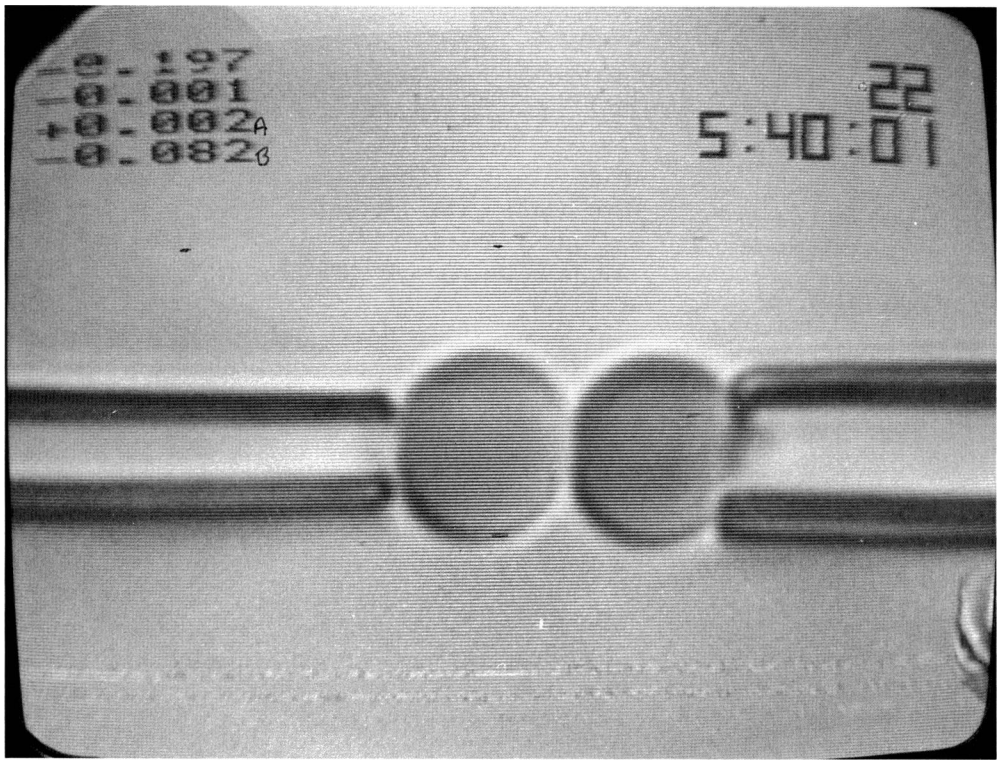

FIGURE 2 A video image of a doublet of sphered red cells in PBS-albumin containing 50% vol/vol antiserum. One sphere of the doublet has been firmly aspirated using mouth suction by the holding pipet of 2.65 μm i.d. to the right of the screen. The aspirating pipet of 1.70 μm i.d. to the left has been aligned and brought into close proximity of the other sphere, and the suction in it is being increased via a water manometer. The negative pressure is displayed at the top of a column of figures in the left upper quadrant of the screen in units of Pa $\times$ 10^{-3}. The three other figures are unused channels. The figures in the screen right upper quadrant are, from left to right, the hour, minute, second, and hundredth of second above. The horizontal lines near the bottom of the screen are caused by freezing the frame.

we used a concentrated cell suspension which was essentially identical to that of previous experiments (Tha et al., 1986). Here, fixed rbc were at a final concentration of 2.5×10^4 cells/μl in a solution of glycerol 76% wt/wt in 0.15 M NaCl. Antiserum was present at either 1.22% vol/vol or 3.56% vol/vol as before. At times the glycerol and NaCl were omitted and replaced by PBS with human serum albumin 0.5% wt/vol. Once prepared, dilute and concentrated cell suspensions were processed in the same way. Polypropylene microcentrifuge tubes (Elkay Products, Shrewsbury, MA) were used throughout all experiments. The suspensions were mixed for 12 h at room temperature on a rotary mixer at 3 rpm. In some experiments, the suspension was then centrifuged in 1-ml aliquots at 2,000 *g* for 2 min; in others it was vortexed at high speed for 3 min. To all suspensions, before their transfer to the microscope chamber, was added 10 μl/ml of a 0.5% rbc suspension in PBS of thrice-washed, fresh type A cells, which were nonreactive to the antiserum. This gave a fresh cell concentration in the suspension of ~500 cells/μl. As the fresh cells were discoid in shape, they could easily be distinguished from fixed cells during an experiment. Their purpose was to serve in zeroing the manometer before measuring pressures as, being deformable, they moved readily in the pipets. The cell suspension was then gently transferred to the microscope chamber by Pasteur pipet.

3. Procedure

To measure separation forces, doublets chosen at random from the suspension were aspirated by mouth suction with the holding pipet of i.d. 2.50–3.50 μm so that they were held in a coaxial orientation. The aspirating pipet of i.d. 1.70–5.15 μm was then held as close as possible to the doublet, and aligned such that the two pipets and the doublet were all colinear and in focus, thus ensuring that their axes were perpendicular to the optical axis as shown in the photograph of Fig. 2. The suction was then raised at a rate of $\leq$15 Pa s^{-1} until the two cells of the doublet separated. In some experiments, doublets were assembled by aspiration by the holding pipet of a singlet; a second singlet was maneuvered close to the other by the aspirating pipet and they were gently apposed. The second singlet was released by the aspirating pipet, which was withdrawn. If the strength of the contact between the two rbc was greater than the gravitational force (~10^{-5}–10^{-4} nN), they remained in contact, and could be separated as above. In one experiment, the cells of the same doublet were repeatedly separated and reassembled.

4. Image and Force Analysis

A frame-by-frame video replay established the negative pressure at the time the doublet separated. As the pressure rose fairly linearly and was updated every 0.14 s, they were averaged over the preceeding and subsequent updates to diminish any noise. The entire sequence from positioning of the doublet to break-up was reviewed in slow-motion to exclude all doublets that were knocked or compressed by the pipets, those where the doublet and pipets were not colinear, or where any large gap existed between the aspirating pipet and the doublet. Also excluded was any doublet showing independent motion of the two cells before their separation, i.e., rolling arising from torques about their contact point.

Micropipet aspiration had not been used previously to study hardened cells. The cells' rigidity posed an obvious problem, for a seal could not be made between the pipet tip and a cell. The separation forces reported herein have been calculated from the pressure as recorded by the transducer multiplied by the cross-sectional area of the pipet tip: $F_{sep} = \Delta P \pi R_p^2$ where R_p = ½ pipet i.d. This provides an exact result only in the case where there is a perfect seal between the cell surface and the pipet orifice. In our experiments, the doublet is positioned so that a perceptible gap of $\geq$1 μm is maintained between the aspirating pipet and the outermost cell. The presence of this gap affords the visual evidence of doublet separation (translocation of the outermost cell to the aspirating pipet orifice), and also ensures that there has been no mechanical disruption or compression of the doublet. However, the gap with its attendant fluid flow into the pipet complicates the analysis of separation forces. Lubrication theory predicts that when the separation distance between a body and the pipet entrance is less than the contact length over which the body makes close approach to the surface, there is an added force in excess of the suction force. In the present case, the force is likely to be negligible because of the geometry, i.e., a planar pipet surface with very sharp edges (radii of curvature <0.1 μm) and a rigid spherical cell, and because of the relatively large gap dimensions. Rather, at these gap dimensions, the fluid leakage means that not all the suction pressure exerted (as recorded downstream by the pipet) would be transmitted to

the cell–cell contact. This effect is examined in Results and Discussion 1(*a*) below.

5. Error

Measurement uncertainties were determined from the precision of the relevant instruments. The measurement precision of pipet diameters was estimated to be <0.1 μm. The more sensitive transducer for pressures ≤100 Pa had a resolution of 0.1 Pa and was essentially noise-free. The less sensitive transducer had a resolution of 1 Pa and sometimes was used at its lower limit, <100 Pa. In this range noise could account for error of ±3.5%, which we attempted to reduce by averaging the values around the time of break-up. Also in this range the less sensitive transducer could give falsely low readings by as much as 5%; most readings were above this region where these errors were smaller. All pressure readings were updated every 0.14 s, during which pressures rose on an average by ≤2.1 Pa. At low pressures this error could be up to ±2.5% for those break-ups occurring midway between updates.

RESULTS AND DISCUSSION

1. Experimental Design

(*a*) *Trials.* Overestimation of separation forces caused by fluid flow into the pipet was of concern. To examine the extent to which fluid leakage influenced the results, we compared the pressures and forces recorded from the same cell sample using pipets of i.d. 3.42 and 1.70 μm, as shown in Table I. The ratio of their cross-sectional area was ~4:1. An inverse relation between pipet size and pressures is seen, but the calculated forces are still significantly greater in the larger pipet by a ratio of ~1.5:1. This indicates that leakage existed which increased with pipet size and produced falsely inflated values. As will be seen in Results 3(*b*) (Table IX), in relation to estimates from the flow studies (Tha et al., 1986) the forces are inflated by a factor of ~2.5. Very small pipets of i.d. <1.5 μm are hard to align and plug easily, so pipets of ~1.7 μm i.d. represent a compromise and were used for all experiments.

(*b*) *Direction of Separation.* Spherical geometry specifies that the two cells of the doublet have an approximately circular, small contact area (≪0.25 μm^2) lying perpendicular to the doublet axis (Tha and Goldsmith, 1986). The experimental design just described is intended to exert only forces normal to the contact acting along the line joining the sphere centers. Such forces should result in simultaneous breakage of all cross-bridges between cell surfaces, and the magnitude should vary directly with the number of cross-bridges. Nonnormal forces tend to impart rolling motion to the outer sphere creating torques about the contact area. Such torques have a significant mechanical advantage in the rupturing of bonds. Bond breakage could then occur sequentially across the contact region, and the resultant forces would not be expected to depend much on bond numbers. The possibility of "stray" torques resulting from minor deviations from the ideal flow regime was of concern in the previously described experiments (Tha et al., 1986).

In the present experiments, nonnormal forces certainly could be exerted, and visible rotation of one sphere relative to the other was sometimes seen. In Table II, the separation forces measured for these doublets was significantly less than for those where no apparent relative rotation existed. This finding confirms our expectations. That forces of separation are strongly dependent on antiserum concentration (and thus on bond density) is seen in Table III, indicating that bond breakage is not sequential. It seems reasonable to assume then that the separation force acts normal to the contact area in the absence of observable rolling motion. Hence doublets that exhibited rolling motion were excluded from analysis.

2. Characterization of Red Blood Cells

(*a*) *Deformability.* The analysis and interpretation of separation forces produced by both aspiration and hydrodynamic means rests on the assumption that cells were not deformed by the applied stresses. Aspiration of single fixed rbc by micropipets of 1.70–5.15 μm showed no extensional deformation of the cells at negative pressures of >3,000 Pa. This pressure is vastly in excess of that required to deform normal rbc, and greater than any pressures or forces that cells are subjected to in any experiment described in this series.

(*b*) *Glutaraldehyde Effects.* The use of glutaraldehyde was required to harden the red cells and to maintain their spherical shape. Glutaraldehyde is a good fixative for our purposes as it is very rapid acting and does not interact with sugar residues (Hopwood, 1972), which are the antigenic determinants of the ABO blood group (Hakomori, 1981). Its interactions with proteins leave more intact their higher-order structure (Lenard and Singer, 1968) and their immunogenicity (Rockoff et al., 1979; Van

TABLE I
EFFECT OF PIPET DIAMETER ON SEPARATION FORCES (DILUTE CELL SUSPENSION, 1% VOL/VOL ANTISERUM)

Pipet diameter (i.d.)	Pipet cross-sectional area	Ratio of areas	*n*	Negative pressure	Force
μm	μm^2			*Pa ± SD*	*nN ± SD*
3.42	9.19	4:1	11	303 ± 104	2.79 ± 0.95*
1.70	2.27		8	824 ± 202	1.87 ± 0.46*

*Values significantly different, $P < 0.05$.

TABLE II
EFFECT OF NORMALITY ON SEPARATION FORCES (DILUTE CELL SUSPENSION, 1% VOL/VOL ANTISERUM)

Description	*n*	Force
		nN ± SD
Normal forces: no visible relative rotation of spheres of doublet	15	1.43 ± 0.61*
Nonnormal forces: visible relative rotation of spheres of doublet	11	1.00 ± 0.37*

*Values significantly different, $P < 0.05$.

TABLE III
CONCENTRATION DEPENDENCE OF SEPARATION FORCES (DILUTE CELL SUSPENSION)

[Antiserum]	*n*	Force
% vol/vol		*nN ± SD*
1.00	15	1.43 ± 0.61*
0.25	15	0.71 ± 0.22*

*Values significantly different, $P < 0.001$.

Ewijk et al., 1980) than do most other fixatives. Nonetheless, the membrane structure is evidently changed during fixation. Of particular concern in our experiments is glutaraldehyde's capacity for polymerization via an aldol condensation with itself (Peters and Richards, 1977), and its bifunctionality. Conceivably, this could result in polymers of various lengths forming covalent cross-links directly between two different red cell surfaces, or to glutaraldehyde's fixing antibody molecules to the cell surfaces and as a consequence altering antibody uptake and binding characteristics. This ability to attach proteins to membranes is well known and is exploited by the clinical laboratory in passive hemagglutination tests (Herbert, 1978).

With regard to polymer cross-links, we used only electron microscope–grade glutaraldehyde confirmed spectrophotometrically to be polymer-poor according to the method of Anderson (1967). There was no absorption peak at 235 nm. In addition, the results given below show that the magnitude of the force to break a single cross-bridge (<0.1 nN) is too low by at least an order of magnitude to be accounted for by covalent bonds (Levinthal and Davison, 1961; Bell, 1978). However, a few doublets were encountered, both in newly fixed rbc and after exposure to antiserum, that could not be distracted by forces up to 25 nN; this may represent covalent linkage of red cells taking place during the fixation process. Otherwise, covalent linkage by glutaraldehyde between rbc seem unlikely to figure in the present results.

There remains the question of residual reactive glutaraldehyde moieties on the rbc surface. As glutaraldehyde is highly reactive with primary amino groups (Rembaum et al., 1978), the rbc were postfixed with lysine before their incorporation into cell suspensions. In Table IV are shown the separation forces for lysine-treated cells. They do not differ from untreated cells.[1]

These results are consistent with the negative experimental controls, i.e., the absence of agglutination by anti-A antiserum of the experimental-type B cells; and by the experimental anti-B antiserum of fixed type A cells. In immunoradioassays intended to measure antibody uptake by rbc, the results were not consistent (data not shown). Some experiments showed the fixed cells having increased uptake, others that the fixed and fresh cells had the same uptake. Such variable and small differences between fresh- and fixed-cell antibody uptake have also been reported in the literature (Rockoff et al., 1979; Van Ewijk et al., 1980; Capo et al., 1982). Matsukura (1972) found no differences between glutaraldehyde-fixed and fresh rbc uptake of ferritin-labeled anti-A antibody. We conclude that reactive moieties, if present, do not markedly affect antibody activity, and that if antibody attachment to the rbc surface occurs via glutaraldehyde, it is immaterial to adhesion.

TABLE IV
EFFECTS OF POSTFIXATION OF RBC WITH LYSINE ON SEPARATION FORCES (CONCENTRATED CELL SUSPENSION, 1.22% VOL/VOL ANTISERUM)

Cell Treatment	*n*	Force
		nN ± SD
No postfixation	11	0.257 ± 0.135*
Postfixation in lysine, 0.2 M	16	0.230 ± 0.129*

*Values not significantly different.

(*c*) *Albumin Effects.* Red blood cells, fixed or fresh, can adhere to a wide variety of surfaces. Pertinent to micromanipulation work is their strong adhesion to glass in simple buffers (Trommler et al., 1985), which is greatly reduced by the addition of plasma proteins to the medium (George et al., 1971). Human serum albumin was added at a concentration of 0.5% wt/vol which had proved adequate in previous micromanipulation work. The previous flow studies in part II had used albumin-free media. We therefore compared separation forces of a cell preparation in the presence and absence of albumin. As shown in Table V, at this concentration there is no significant difference.

(*d*) *Specificity of Adhesion.* In most preparations of fixed rbc, a few doublets and higher order aggregates could be found, irrespective of the presence of albumin or antiserum. These aggregates composed <1% of the total cell population. Our query was whether these "nonspecific" doublets differed from those produced by antiserum.

24 "nonspecific" doublets suspended in PBS were examined; in some cases albumin was present. On separation, 19 showed independent motion of the spheres, and usually, as

TABLE V
EFFECTS OF ALBUMIN ON SEPARATION FORCES (DILUTE CELL SUSPENSION, 1% ANTISERUM)

Suspension	*n*	Force
		nN ± SD
No added albumin	23	1.43 ± 0.58*
Human serum albumin 0.5% wt/vol added	13	1.36 ± 0.56*

*Values not significantly different.

[1]The forces at comparable antiserum concentration are lower in Table IV than in previous tables. This is due to the effective antigen concentration (rbc) being higher by a factor ~15 in the concentrated cell suspension, and follows from the Law of Mass Action.

pressures increased, the gradual appearance of a visible separation between cells that still maintained material contact. These tethers' breadth was too small to be resolved visually, but their length was up to ~3 μm. They were broken by forces of ~0.2–2.5 nN. The remaining five doublets were rigidly linked with no tethers, and did not break up even at high pressures (<25 nN). Both of these behaviors were seen infrequently in suspensions containing antiserum. Presumably "nonspecific" doublets remain interspersed with antibody-mediated aggregates, where they could be identified and discarded.

This nonspecific adhesion was investigated by attempting to construct such doublets by randomly apposing singlets in antiserum-free medium of PBS-albumin 0.5% wt/vol. Of 36 trials, seven resulted in doublets which were seen to be tethered on distraction. All but one of the seven came from the same single rbc preparation. The singlets generally stuck to each other only after repeated apposing and rubbing of their surfaces.

We suspect that the tethers were composed of cell debris, perhaps polymerized hemoglobin. We had noted previously that those cell samples that had major hemolysis during their preparation showed more spontaneous aggregation than those without hemolysis. Similarly, those singlets that were made to adhere had the most traumatized surfaces. As to those doublets resistant to break-up, this stability is consistent with some sort of covalent linkage, perhaps provided by glutaraldehyde, as discussed in Results 2(*b*). It could also be produced by multiple noncovalent linkages.

More important than the actual identity of these linkages is the capacity to distinguish this nonspecific adhesion from aggregates formed by antibody. That one can do so reinforces the evidence of the negative control discussed in Results 2(*b*). In the present experiments, specific antibody-mediated adhesion is being examined. Practically, it allows one to analyze data free of such artifacts.[2]

3. Antibody-mediated Adhesion

(*a*) *Variability of Binding Strength.* We noted that when two randomly chosen singlets were apposed by manipulation to form a doublet in the presence of antiserum, their forces of separation were lower than those of "preexisting" doublets in the same population, i.e., those preformed in the suspension; in other words, "assembled" doublets were weaker than "preexisting" doublets, as shown in rows 1 and 2 in Table VI. We wished to know whether these results signified that agglutination occurs preferentially in a more adherent subpopulation, or whether they reflect agglutination occurring randomly, but with increasing strength over time. Such time-dependent adhesion has been seen with rbc attached to glass (Mohandas et al., 1974; Trommler et al., 1985).

TABLE VI
EFFECTS OF VORTEXING AND CENTRIFUGATION ON SEPARATION FORCES (DILUTE CELL SUSPENSION, 1% VOL/VOL ANTISERUM)

Description	*n*	Force	*P*
		nN ± *SD*	
Standard cell prepartion: "preexisting" doublets	15	1.43 ± 0.61*	<0.02
Same cell preparation; singlets apposed then separated: "assembled" doublets	14	0.87 ± 0.49‡	<0.02
Standard cell preparation: vortexed	13	0.90 ± 0.51‡	<0.01
Standard cell preparation: vortexed and centrifuged	15	1.45 ± 0.47*	<0.01
Standard cell preparation: centrifuged	12	1.38 ± 0.34*	

*Values not significantly different.
‡Values not significantly different.

To test these alternatives, a standard dilute cell suspension with antiserum 1% vol/vol was subjected to vigorous vortexing for 3 min before viewing. Only a few aggregates remained, and these had lower separation forces which were comparable with those of "assembled" doublets, as shown in row 3 of Table VI. The smaller adhesion forces may arise from doublets surviving the vortexing but with weakened binding therefrom, or the beginning of reaggregation. In either case, the cell suspension was nearly monodisperse.

We next vortexed a standard cell preparation and then immediately centrifuged it at 2,000 *g* for 2 min before viewing. As a control, a standard cell preparation was centrifuged without any prior vortexing. These results are shown in rows 4 and 5 of Table VI. The separation forces for both samples are essentially the same as for "preexisting" doublets. In this instance, centrifugation produces a high rbc collision frequency and terminates in a no-shear state, these being conditions that promote aggregation in any suspension. Although not quantitated, centrifugation did not seem to change the extent of aggregation of the control sample, but did induce aggregation to about the same level in the vortexed sample. Irrespective of its effects on the extent of aggregation in the sample, centrifugation per se apparently had no effect on the strength of the adhesive bond. This is as would be expected of undeformable cells, which are unable to increase their contact area, but it is reassuring that there are no secondary, nonspecific adhesive effects. In addition, there was no time dependence of adhesion strength at least in the range of 2 min to 12 h, and it can be dismissed as the cause of the force difference between "assembled" and "preexisting" doublets. This must result from the aggregates being intrinsically more adherent than the unaggregated segment of the population. A question raised by this conclusion is: on what scale

[2]Although this is not the case with the flow studies as discussed in Tha et al., 1986, *Results.* (N.B. There, "nonspecific" doublets are referred to as "preexisting.") However, "nonspecific" doublets are a small minority of the total.

TABLE VII
EFFECTS OF REPEATED APPOSITION AND SEPARATION ON SEPARATION FORCES (DILUTE CELL SUSPENSION, 1% VOL/VOL ANTISERUM)

Part I

Description	*n*	Force	*P*
		nN ± *SD*	
Standard cell preparation: initial separation	17	1.61 ± 0.66	<0.01
Reapposed, second separation	17	1.05 ± 0.34*	
Reapposed, third separation	17	0.91 ± 0.40*	
Reapposed, fourth separation	10	0.86 ± 0.28*	
"Assembled" doublets (from Table VI)	14	0.87 ± 0.49*	

Part II

Description	*n*	Force	*P*
		nN ± *SD*	
Top-ranked values: initial separation	5	2.44 ± 0.62	
Top-ranked values: all subsequent separations (pooled)	12	1.12 ± 0.32	<0.001
Bottom-ranked values: initial separation	5	0.88 ± 0.27*	<0.01
Bottom-ranked values: all subsequent separations (pooled)	14	0.78 ± 0.28*	

*Values not significantly different.

is this variation in binding strength, i.e., are individual red cell surfaces nonuniform in this regard?

The experiment performed to test this was to separate a doublet, then repeatedly reappose and distract it. The likelihood of the cells' sticking at the same spot is remote, as the area of contact is $<0.25\ \mu m^2$, and therefore each distraction is measuring separation forces at different regions of the same cell surfaces. The same cell preparation was used as above. The results in Table VII, Part I show a significant difference in separation forces between the initial distraction and all those subsequent. There is no significant difference between the second, third, and fourth distractions, which also are statistically indistinguishable from "assembled" doublets. This suggests that some regions of the cell surface support stronger adhesion than others, and that adhesion would appear to occur preferentially at these sites. Yet analysis of these data also reveals that there are differences between individual doublets. As shown in Table VII, Part II, the 17 values of the initial distraction were ranked, and the top- and bottom-ranked five values were grouped and compared with regard to initial and subsequent distractions. The latter values were pooled, for no differences between them were found. There is a threefold difference between the initial distraction values of the top- and bottom-ranked doublets. Although the "restick" values of the top-ranked group are diminished by 50% compared with the initial distraction, still the difference between top- and bottom-ranked groups persists.

These experiments, considered jointly, lead one to conclude that in the present preparation the cell surface is nonuniform in its adhesiveness over a small scale of $<0.25\ \mu m^2$, and also over a large scale of individual cells. Some cells are "stickier" than others over extensive portions of their surface. It is noteworthy that these findings concerning separation forces parallel the electron microscopic evidence that A sites are nonrandomly distributed on rbc. Lee and Feldman (1964) first reported clustering of A sites on electron micrographs of individual red cells as detected by ferritin-labeled anti–A antibody. Voak and Williams (1971) later confirmed this statistically using antibody and also *Dolichos biflorus* lectin (which is A-specific), both of which were ferritin-labeled (Williams and Voak, 1972). The clustering of A sites was demonstrated statistically for areas of $\sim 10^{-2}\ \mu m^2$; this figure is within the possible range of contact areas for our antibody-linked doublets (Tha and Goldsmith, 1986). In addition, all these investigators noted highly variable labeling of cells from the same sample, with a few cells being very heavily labeled and others virtually unlabeled. The same observation was also reported by Matsukura (1972) using anti–A ferritin–labeled antibody and by Fischer and Stege (1967) using fluorescent anti-A and -B antibody on neonatal erythrocytes. Factors other than nonuniformity of A site distribution must figure in the variance of the above results. Electron microscopy of our sphered, fixed rbc show that surface roughness can result from the fixation process; this will cause variation in contact area. Furthermore, cell trauma may result from repeated manipulation. We cannot be certain to what extent these factors have influenced our results. Nevertheless, it seems likely that adhesion is favored at regions with properties unrepresentative of the surface as a whole. This situation has been predicted to be the case (Pethica, 1961; Pethica, 1980) and found for red cells adherent to glass (Trommler et al., 1985).

(*b*) *Comparison with Flow Studies.* The conditions of the flow studies (Tha et al., 1986) were duplicated as far as was possible, excepting, of course, the substitution of negative pressure for hydrodynamic force as the disrupting agent. The influence of additional albumin in the micromanipulation work was shown to be nil, and the influence of the nonreactive cell suspension added before viewing, which formed <1% of the total preparation volume, must be nugatory. Two concentrations of antiserum were employed: 1.22% and 3.56% vol/vol, respectively the second lowest and the highest of the four concentrations used in the flow experiments. The cell concentrations were more concentrated than most of those described elsewhere in this paper, but were the same as in the flow experiments. To generate sufficient viscosity (and thus forces) in Poiseuille flow, glycerol had been used at high concentrations in the medium. Here, the doublets were initially separated at 3.56% vol/vol antiserum in glycerol 76% wt/wt (a representative concentration), the results of which are shown in Table VIII. Because manipulation of cells in such a highly

TABLE VIII
EFFECT OF GLYCEROL AND CENTRIFUGATION ON SEPARATION FORCES (CONCENTRATED CELL SUSPENSION, 3.56% VOL/VOL ANTISERUM)

Modification	n	Force	Coefficient of variation
		$nN \pm SD$	%
Standard cell preparation	16	0.449 ± 0.108*	24
Glycerol 76% wt/wt in medium	14	0.474 ± 0.205*	43
Standard cell preparation: centrifuged	11	0.496 ± 0.190*	38

*Values not significantly different.

viscous medium proved awkward, the question of whether substitution of PBS for glycerol had any effects on the results was tested. Comparing row 1 and 2 of Table VIII, glycerol apparently does not influence separation forces, despite its lower dielectric constant and protein stabilizing effects (Gekko and Timasheff, 1981). All further experiments were therefore performed substituting PBS for the glycerol-saline solution.

Comparing Parts I and II of Table IX, it is apparent that the separation forces given by micromanipulation are on average ~2½ times greater than those given by the flow technique, i.e., the normal force (F_3) data (Tha et al., 1986). We favor the latter as having greater accuracy because of the inflation of pressures attendant upon pipet leakage. Also figuring in the higher values may be the considerable numbers of doublets breaking apart during manipulation before any measurements were made (Table IX, Part I). These doublets probably had relatively low strength adhesion. This assertion is supported by their proportion being larger at 1.22% vol/vol antiserum concentration than at 3.56% vol/vol. This unmeasured break-up may account for the ratio of forces for micromanipulation: flow technique being greater at 1.22% than at 3.56% vol/vol, as more low values at the former concentration were preempted.

Various statistical parameters were examined in an effort to see whether they revealed information about the adhesion process, for the formation of cross-bridges at low ligand concentration is thought to be a Poisson process (Capo et al., 1982). A scatter diagram of the force data is displayed in Fig. 3. For comparison, the normal force data derived from the flow studies is included. The distribution in all cases is positively skewed. It is possible that the skewness is an artifact of low-end data truncation caused by the aforementioned unmeasured break-up, rather than being indicative of a Poisson distribution. Micromanipulation probably provides undistorted data for the higher end of the distribution because there is no ceiling on pressures, whereas the hydrodynamic technique probably causes some truncation of both tails of the distribution, as previously discussed (Tha et al., 1986). In Table IX, Part I, the coefficients of variation derived from micromanipulation

TABLE IX
CONCENTRATION DEPENDENCE OF SEPARATION FORCES: COMPARISON WITH FLOW STUDIES (CONCENTRATED CELL SUSPENSIONS)

Part I. Micromanipulation

	Antiserum concentration		P
	1.22% vol/vol	3.56% vol/vol	
n	14	16	
Force (nN ± SD)	0.218 ± 0.106	0.449 ± 0.108	<0.001
Coefficient of variation (%)	49	24	
Percent of total breaking up during manipulation (raw data)	44 (39/89)	22 (13/60)	

Part II. Flow Studies

	Antiserum concentration		P
	1.22% vol/vol	3.56% vol/vol	
n	17	28	
Normal force (nN ± SD)	0.083 ± 0.032	0.197 ± 0.083	<0.001
Coefficient of variation (%)	39	42	
Ratio micromanipulation: flow forces	2.63	2.28	
P value vs. same concentration, micromanipulation technique	<0.001	<0.001	

data are greater than those from the flow technique (Part II) at the lower antiserum concentration, but less than those from the flow technique at higher concentration. Although this pattern of an inverse relation between coefficient and antiserum concentration is consistent with a Poisson distribution for forces, the value of 24% for the 3.56% vol/vol concentration is unusually low, cf. the coefficients for the same antiserum concentration in Table VIII, which are greater by a factor of ~1.6–1.8 even though the means are statistically indistinguishable.

The chi-square goodness-of-fit test and the Poisson heterogeneity test are two statistical methods that explicitly assess conformance of data to a Poisson frequency distribution (Armitage, 1971). By this means, the raw micromanipulation data were successfully fitted above the 5% significance level to Poisson distributions with 0.052 nN (5.2 μdyn) as the force needed to break a single cross-bridge. According to this calculation, the average number of cross-bridges per doublet at the higher concentration is 8.6 and at the lower concentration is 4.2. These results are to be compared with the 0.024 nN (2.4 μdyn) force per cross-bridge calculated by the same means from the previous hydrodynamic data (Tha et al., 1986). These figures stand in approximately the same relation, viz., a little more than double (here, 2.17), as do the means of the

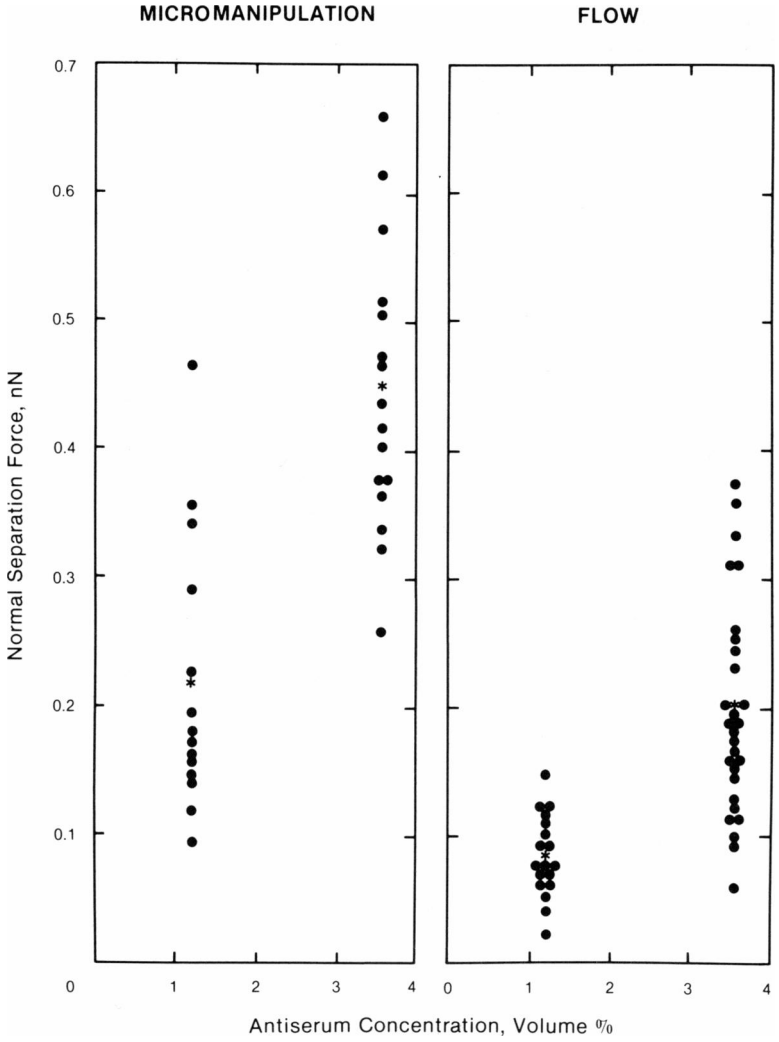

FIGURE 3 Scatter diagrams of separation forces at 1.22% and 3.56% vol/vol antiserum concentration. (*Left panel*) Data from micromanipulation experiments. (*Right panel*) Normal force data from traveling microtube technique (Tha et al., 1986). Stars represent mean values of Table IX of this paper and Table II of Tha et al., 1986, respectively.

micromanipulation and hydrodynamic data. On the whole, there is good agreement of results from these two different experimental methods. It is remarkable that the fit of data to statistical expectation exists despite many potential factors that modify the distribution. However, there is marked departure from statistical expectation in other micromanipulation data, presented below.

(*c*) *Adhesion at High Antiserum Concentration.* Micropipet aspiration readily provides negative pressures well in excess of those needed to disrupt doublets cross-linked by antibody. It is therefore well-suited to measure the relatively large forces prevailing at high concentrations of antiserum. To this end, a dilute cell suspension at 50% vol/vol antiserum concentration was prepared, and a mean force of 2.33 ± 1.23 nN ($n = 16$) found for "preexisting" doublets and 1.24 ± 0.53 nN ($n = 8$) for "assembled" doublets. Although these experimental conditions differ from those of the previous section, where a concentrated cell suspension was used, the results admit of comparison: saturation is a defined state, however it is arrived at. The mean force of 2.33 nN at 50% vol/vol antiserum concentration must represent a maximum of cross-linkages, for it is less than double that for a 1% vol/vol antiserum concentration (1.43 nN); it is an order of magnitude greater than the results for the concentrated cell suspensions of 1.22 and 3.56% vol/vol antiserum, being 10.7 times greater than for the lower concentration (0.218 nN). The variation at 50% vol/vol antiserum is high, the coefficient of variation being 52.8%, and is in excess of expectation ($P < 0.001$) if the force exerted by a single cross-bridge were 0.052 nN (5.2 μdyn). The lowest value for a single cross-bridge that can be fitted is an implausible 0.29 nN (29 μdyn). What statements can now be made about cross-bridge strength?

Increased variation will have the effect of increasing the estimated value of a single cross-bridge. As aforementioned, the causes of increased variation are numerous. They include nonuniformity of contact area and receptor distribution, heterogeneity of antibody affinity, and experimental error; all but the last factor would become more prominent as antiserum concentration (and cross-bridge numbers) increase. Also, the posited deletion of low values (and therefore decreased variance) would figure less in the distribution at higher concentrations. A further complica-

tion is introduced by immunoglobulin M's ability to bind by two sites to each surface when cross-linking, (as opposed to immunoglobulin G's single site), further increasing the heterogeneity of cross-bridge strength. Lastly, it is doubtful whether a Poisson model for cross-bridge formation is appropriate at high antibody concentrations. Obviously any statement on the subject must be tentative. The value of 5.2 μdyn is somewhat less than the lowest recorded value (0.094 nN = 9.4 μdyn) for a doublet separation using micropipet suction. It may be viewed as an upper bound of cross-bridge strength for these data, which in any event are over double the results from the flow technique. The lower bound for a single cross-bridge is as yet unknown and under investigation. Using the flow technique at the lowest antiserum concentrations (<0.5%) that still agglutinate rbc, we have so far not succeeded in recording any separation forces of less than 1 μdyn. This range encompasses the value of 4 μdyn proposed by Bell (1978) on theoretical grounds as the critical force to rupture an "average" antigen–antibody bond.

How many bonds then are estimated to exist at saturating antiserum concentration? If one takes the ratio of 0.052 nN for bond strength and 2.33 nN for the mean force of saturation, the bond number is 45; as both figures are inflated, their ratio is unaffected. Another approach is to use the figure of 0.01 nN from the flow studies as the cross-bridge strength, and the mean force at saturation 2.33 nN, but reduced by a factor of 2.45 (which is the average of flow: micromanipulation forces from Table IX, Part II). The bond number using these figures is 95. The number of receptors in the maximal contact area (0.25 μm^2) is ~2,000. The number of cross-bridges is expected to be much lower given the steric restrictions on packing of bulky IgM molecules and the absence of receptor mobility of fixed cells (Bell, 1978). The above results would suggest that <5% of receptors in the contact region are bound by cross-linking antibody. This figure is comparable to Bell's analysis (1978) of the experiments of Edelman and colleagues (1971) and Rutishauser and Sachs (1975), from which he deduces that $\leq$10% of concanavalin A lectin molecules bound to nylon fibers within the contact region with a cell participate in cross-bridging. Also comparable is Chien and colleagues' recent estimate that ~3% of the *Helix pomatia* lectin in the contact region between two red cells was cross-bridging (L. Sung et al., 1985*a* and *b*).

We think it likely, from the above, that the total number of cross-bridges stabilizing a doublet is <100 at high antibody concentration, and a very few at low concentrations. This range seems plausible in view of the typical free energies of antigen–antibody bonds (e.g., 8.5 kcal $mol^{-1} \simeq$ 35.6 kJ $mol^{-1} \simeq$ 15 kT per molecule) which should stabilize cells by a few bonds. Capo et al. (1982) have calculated that one concanavalin A bond between cells is sufficient to initiate aggregation of thymocytes, and radiolabeled antibody uptake studies show that microscopic hemagglutination is visible at average antibody densities of 25 molecules per red cell (Greenbury et al., 1963).

CONCLUDING REMARKS

The results reported in this paper are overall in good agreement with those of the previous papers (Tha and Goldsmith, 1986; Tha et al., 1986) inasmuch as the separation forces are well within the same order of magnitude and the force of a single antigen–antibody cross-bridge is again estimated to be on the order of $\leq 10^{-2}$ nN (10^{-6} dyn). These results do not resolve whether shear or tensile forces (F_{shear} or F_3) are responsible in the flow technique for separating the cells of the doublets. They do however provide corroborative evidence that the method of the traveling microtube technique as applied to this cell system is sound.

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