Cell Adhesion to Fibronectin and Tenascin: Quantitative Measurements of Initial Binding and Subsequent Strengthening Response

Margaret M. Lotz,* Carol A. Burdsal,* Harold P. Erickson,‡ and David R. McClay*

*Department of Zoology, Duke University, Durham, North Carolina 27706; and ‡Department of Cell Biology, Duke University School of Medicine, Durham, North Carolina 27710

Abstract. Cell–substratum adhesion strengths have been quantified using fibroblasts and glioma cells binding to two extracellular matrix proteins, fibronectin and tenascin. A centrifugal force–based adhesion assay was used for the adhesive strength measurements, and the corresponding morphology of the adhesions was visualized by interference reflection microscopy. The initial adhesions as measured at 4°C were on the order of 10^{-5} dynes/cell and did not involve the cytoskeleton. Adhesions to fibronectin after 15 min at 37°C were more than an order of magnitude stronger; the strengthening response required cytoskeletal involvement. By contrast to the marked strengthening of adhesion to FN, adhesion to TN was unchanged or weakened after 15 min at 37°C. The absolute strength of adhesion achieved varied according to protein and cell type. When a mixed substratum of fibronectin and tenascin was tested, the presence of tenascin was found to reduce the level of the strengthening of cell adhesion normally observed at 37°C on a substratum of fibronectin alone. Parallel analysis of corresponding interference reflection micrographs showed that differences in the area of cell surface within 10–15 nm of the substratum correlated closely with each of the changes in adhesion observed: after incubation for 15 min on fibronectin at 37°C, glioma cells increased their surface area within close contact to the substrate by ~125-fold. Cells on tenascin did not increase their surface area of contact. The increased surface area of contact and the inhibitory activity of cytochalasin b suggest that the adhesive "strengthening" in the 15 min after initial binding brings additional adhesion molecules into the adhesive site and couples the actin cytoskeleton to the adhesion complex.

CELL–SUBSTRATUM adhesion to fibronectin (FN)[1] or to glass often has been associated with several morphological and cytological changes including cell spreading and the formation of focal contacts, close contacts, and stress fibers (1, 9, 12, 19, 32–34, 40, 42, 45, 49, 51). While a spread morphology and focal contacts provide physical evidence that an adhesion has occurred, the actual adhesion and its relationship to the cell's behavioral changes that follow must be defined sequentially and on functional terms. As in cell–cell adhesion (39, 48), cell–substrate adhesion has been suggested to involve at least two measurable steps: the first involves initial contact between cell and substrate and the second step strengthens the adhesion through processes requiring metabolic energy (10, 27, 35, 44, 48).

A number of extracellular matrix proteins including FN and laminin have been found to serve as adhesive substrates for cells. A more recently discovered extracellular matrix protein has been given a variety of names (24) including myotendinous antigen, hexabrachion, J1, GMEM, cytotactin, and tenascin (4, 14, 15, 22, 28, 36). In this paper it will be called tenascin (TN). The discoveries of TN, its biochemistry, tissue distribution, and biological function have been reviewed recently by Erickson and Lightner (23).

The function of TN as a substrate molecule remains somewhat controversial. TN has been reported to be both adhesive (3, 16, 25, 28, 30, 36) and relatively nonadhesive (15, 24, 47). Its varied distribution in embryonic mammals and birds has not yet provided a definitive insight as to its role in development. For example, TN is very prominent in smooth muscle of gizzard and dense connective tissue of chick embryos (14, 37); in mesenchyme surrounding fetal hair follicles, mammary glands, and the condensing mesenchyme of cartilage anlagen (24, 37); and in embryonic chick retina, optic tectum, and cerebellum (18). TN is in restricted locations in many adult tissues and is prominently expressed by many tumors (15). Whether TN is acting as an adhesion molecule in these different sites might best be answered by applying both morphological and functional adhesion criteria to its interactions with cells.

1. *Abbreviations used in this paper*: FN, fibronectin; IRM, interference reflection microscopy; RCF, relative centrifugal force; TN, tenascin.

In this paper we compare the adhesion of various cells to FN with adhesion to TN using both a centrifugal force–based assay, which can quantify the two major components of adhesion (39), and by interference reflection microscopy (IRM), which we used to detect and quantify the two morphological markers, focal and close contacts (34). In doing so, not only is cell adhesion to TN quantified in comparison with FN but a functional description of adhesion to these proteins is related temporally to the morphological appearance of cells at the site of cell–substrate contact. Adhesive strengths were found to vary over two orders of magnitude according to the cell type and the protein being tested. Furthermore, cells increased their adhesive strength to FN rapidly upon incubation at 37°C, while adhesion to TN, when measurable at 4°C, did not increase significantly or decreased at 37°C. As a part of the study, we obtained IRM images of the cells on different substrates and at different time points. Thus, we could examine adhesive strength and also measure the area of the cell in close contact with the substrate at any time after initial contact.

Materials and Methods

Adhesion Assay

This assay was modified from that of McClay et al. (39) so that all cells in the microtiter well, bound and free, could be counted with a low experimental error. The design of the assay avoids any shear forces except those introduced by centrifugal force. This permits one to quantify the force necessary to separate cells from a substrate. The assay is diagrammed in Fig. 1. One set of microtiter plate wells (Microtest III; Falcon Labware, Oxnard, CA) was coated with either FN or TN followed by a background blanking of nonspecific adhesive surfaces with BSA. Cells labeled metabolically with [³H]leucine (10 μCi/ml) were added to these wells. Another set of wells, filled with media, were inverted on the first. Pairs of communicating wells formed assay chambers. The wells were held together without leaking by a layer of double-sided carpet tape (3M Corp., Minneapolis, MN) lining the top surface of the microtiter plates.

The assembly was centrifuged at 17 g for 8 min at 4°C to bring all cells gently into contact with the substrate. For time points representing "0 min" in contact (actual time of contact was up to 8 min), the assembly was immediately inverted and centrifuged again at 4°C (for 8 min) at the relative centrifugal force given in the figure legends. A table-top, refrigerated centrifuge (Jouan, Inc., Winchester, VA) was used along with microtiter plate carriers on a rotor with a radius of 16 cm. Cells that did not adhere at a given force were centrifuged into the opposite well. The plates were then frozen in a slurry of dry ice-ethanol, and the bottom 3 mm of both the substrate-coated (bound cells) and opposing wells (free cells) were clipped and then counted separately by liquid scintillation methods. For details on other time points see figure legends.

Percent cells bound was calculated as dpm bound cells/(dpm bound cells + dpm free cells). After subtracting backgrounds (binding to BSA alone, which was ≤5% at forces >5 g), results are reported as the mean percent bound of three to eight experimental replicates ± SEM. A control in each assay also measured the maximum number of radioactive counts bound to the substrate since 10–15% of the counts in a given cell suspension were due to released counts or cellular debris. These counts were used to normalize the maximum number of cells bound to 100%. Dyne force per cell was calculated by F = (specific density of the cell − specific density of the medium) × volume of the cell × relative centrifugal force (RCF), where specific density of the cell = 1.07 gm/cm^3 and specific density of the media = 1.00 gm/cm^3 (39). Cell volumes were calculated assuming spheres with the diameter of the cells in question.

Proteins and Substrate Preparation

Human serum FN was purchased from New York Blood Bank (New York). The FN was purified using a gelatin-agarose (Sigma Chemical Co., St. Louis, MO) column according to Ruoslahti et al. (43). TN was prepared

from the human glioma cell line U251-MG by immunoaffinity chromatography of cell supernatant using the monoclonal antibody 81C6 (24). Wells were incubated for 2 h at room temperature with 50 μl of a 10 μg/ml protein solution. Wells were then washed twice with DME containing 50 mM Hepes (DME-H). Experimental wells were then blanked with 100 μl of 10% BSA in DME-H at room temperature for 2 h; control wells were treated with the BSA solution alone. After washing the BSA from the wells twice with DME-H, 100 μl of DME-H was added to each well. The plates were placed on ice for a few minutes until cells were added.

Cell Maintenance

NIL cell fibroblasts (hamster) were a gift of Dr. R. Hynes (Massachusetts Institute of Technology, Cambridge, MA). The glioma cell line U251-MG, clone 13, was a gift of Dr. D. Bigner (Duke University Medical Center, Durham, NC). The astroglioma cell line 1321 NI was obtained from Lineberger Cancer Research Center (Chapel Hill, NC). All three were grown in DME supplemented with 10% FCS, 1% antimycotic/antibiotic (Gibco Laboratories, Grand Island, NY), 2 mM glutamine (Gibco Laboratories), and 20 mM glucose. NIL and glioma cells were routinely passaged when ~70% confluent in 100-mm tissue culture dishes (Falcon Labware). The glioma cells were removed from the tissue culture dishes for passaging with 0.02% EDTA in calcium- and magnesium-free PBS (CMF-PBS) consisting of 140 mM NaCl, 1.5 mM KH$_2$PO$_4$, 3 mM KCl, and 8 mM Na$_2$HPO$_4$. NIL cells were removed for passages using 0.01% trypsin (Gibco Laboratories), 0.04% EDTA in CMF-PBS.

Cell Preparation for Assay

Cultures of NIL and glioma cells were split and replated at half confluence the day before use in the assay. Cells were labeled in leucine-free modified Eagle's medium (Selectamine kit; Gibco Laboratories) containing 10 μCi/ml of [³H]leucine (ICN Radiochemicals, Irvine, CA), 1% antimycotic/antibiotic, 2 mM glutamine, and 20 mM glucose. 50 μCi were used per 100-mm plate of cells. After 4 h of labeling, the plates were washed with CMF-PBS and the cells were removed from the plates and dissociated using 0.02% EDTA in CMF-PBS. The cells were washed, resuspended in cold DME-H to 1.2 × 10^5 cells/ml, and kept on ice for a few minutes until addition to assay wells. 100 μl of cell suspension was added per well. For cytochalasin b treatment, cytochalasin b (Sigma Chemical Co.) was added to NIL cells at a final concentration 10 μg/ml during the last hour of labeling. After washing the plates and cell dissociation, cytochalasin b was maintained at 10 μg/ml throughout the remainder of the assay.

Chick brain cell suspensions were prepared from 10- or 13-d embryonic chick brains using the light trypsin and EDTA dissociation as published (5). Brain cells were labeled at 37°C on a rotary shaker at 120 rpm with 300 μCi [³H]leucine per brain. After 2.5 h, the cells were pelleted at 210 g for 5 min, resuspended in 0.02% EDTA in CMF-PBS, and triturated to dissociate residual clumps. The cells were centrifuged again, resuspended in cold DME-H to 4.5 × 10^5 cell/ml, and kept on ice.

Preparation for IRM Analysis

Chambers were constructed on No. 1 thickness glass coverslips (Corning Glass Works, Corning, NY) so that the optical requirements of the interference reflection microscope could be satisfied. Cylinders were made from the wide ends of plastic yellow pipette tips (Pipetman; Rainin Instrument Co., Woburn, MA). These cylinders had an inner diameter of 5 mm and were 6 mm long. Melted paraplast (Fisher Scientific Co., Pittsburgh, PA) was applied around the outer circumference to seal the cylinder onto the glass. The area of the glass bottom surface equaled the area of an adhesion assay well bottom.

The bottoms of these chambers were coated with protein solutions as in adhesion assays. 3 × 10^4 glioma cells, dissociated as for adhesion assays, were added to the chambers on ice. The cells were gently centrifuged onto the chamber bottom at 17 g for 4 min at 4°C. For 0-min time points, chilled formalin was added to the wells immediately after centrifugation at a final concentration of 3%. Other chambers were kept at 4 or 37°C for 15 min and then chilled formalin was added. The cells were fixed at room temperature for 20 min, the formalin solution was gently removed by aspiration, and fresh media were gently added. To view the cells, the cylinders were pulled off and any remaining paraplast was carefully scraped away.
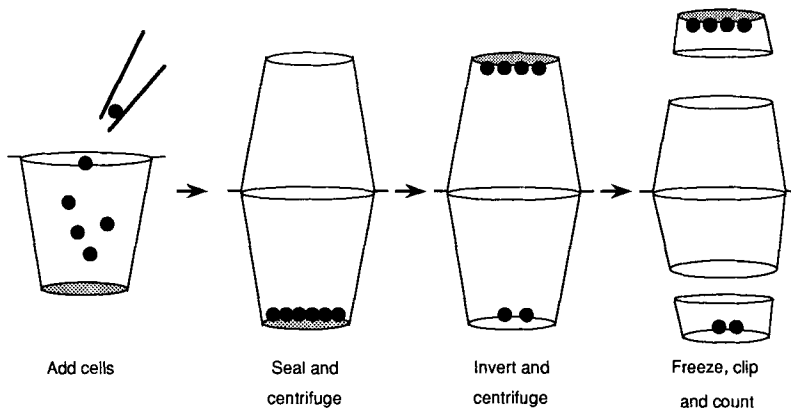
Figure 1. The centrifugal force–based adhesion assay. Labeled probe cells are added to a substrate-coated microtiter well. A second, fluid-filled microtiter well is inverted over the first and the two are sealed with a gasket of double-sided carpet tape. The cells are gently centrifuged onto the substrate at 4°C. The plate is then incubated or immediately inverted (depending on the experiment) and centrifuged at 4°C, providing a known force tending to pull cells away from the substrate. The chamber is then quick-frozen, the top and bottoms are cut, and the cell number in each is quantified by scintillation methods.

Add cells   Seal and centrifuge   Invert and centrifuge   Freeze, clip and count

Results

Cells of Different Types Have Diverse Affinities for FN and TN

To gauge the strength of adhesion, centrifugal force was used to measure resistance to detachment of cells from either FN or TN in an assay modified from McClay et al. (39) (Fig. 1). A graph of percent cells bound vs. force applied demonstrates the adhesive strengths of the different interactions. At 4°C, fibroblasts bound to FN with a much higher affinity than glioma cells bound to FN (Fig. 2 a). To dislodge 50% of the fibroblasts, an RCF of >700 g or 3.6 × 10⁻⁴ dyne/cell was required (the absolute force was not determined since the microtiter plate carrier could not be centrifuged faster). For the glioma cells on FN at 4°C, only ∼40% of the cells bound even at rather low detachment forces. Thus, the NIL fibroblasts withstood a detachment force >20 times higher than the gliomas on FN.

On TN, the NIL fibroblasts did not bind at all, as defined by cells remaining attached at 12 g. About 40% of the glioma cells bound to TN at a low detachment force (12 g = 3.6 × 10⁻⁶ dynes/cell). About half of these cells were detached from TN at a force of 45 g compared with the 90 g that was required to detach half the bound glioma cells from FN. Thus, glioma cells attached to both FN and TN at 4°C, and attachment was measurably, but not remarkably, stronger to FN at this 0-min time point (immediately after centrifugation).

Incubation at 37°C Has Different Effects on Adhesion Depending on the Substrate

Studies performed at 37°C reveal other differences in the responses of cells to FN and TN. In 2 h, fibroblasts and glioma cells strengthen their adhesion to FN but not to TN when incubated on these substrata at 37°C (Table I). Experiments then examined the speed at which strengthening occurs. The strength of fibroblast adhesion to FN increased dramatically during the first 15 min of contact with this substrate at 37°C (Fig. 3). In <15 min at 37°C, essentially all fibroblasts continued to adhere when challenged with the maximum centrifugal force available. Glioma cells required a longer incubation at 37°C to exceed the maximum strength measurable, though the rate of strengthening may not differ from the rate increase of fibroblasts (Fig. 3). Thus, incubation at 37°C led to an increase in the adhesion of both cell types to FN, and

the rate of increase is on the order of minutes to achieve more than an order of magnitude increase in adhesive strength.

Because of the demonstrated relationship between FN and the actin cytoskeleton (7, 8, 11, 13, 31, 33, 45), the role of actin
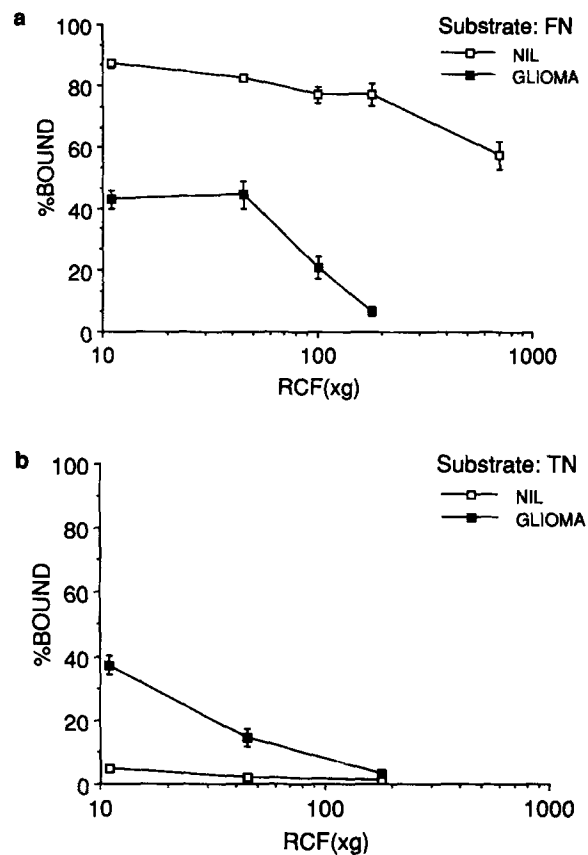

Figure 2. Cell–substratum binding and initial adhesion. Fibroblasts and glioma cells on FN (a) or TN (b). At 4°C, cells were added to coated wells and the plates were assembled. Immediately after centrifugation of the cells onto the substrates at 4°C, the plates were inverted and centrifuged again at 4°C. Replicate plates were centrifuged at different RCFs. Background binding at all RCFs >10 g was ≤10% and is subtracted from these and all subsequent results.

Table I. Differential Changes in Adhesion to FN and TN upon Warming

Cell type	FN		TN	
	4°C	37°C	4°C	37°C
NIL	99 ± 3*	116 ± 1	11 ± 4	19 ± 8
Glioma	50 ± 1	110 ± 1	26 ± 7	8 ± 1
Astroglioma	25 ± 3	61 ± 3	36 ± 4	10 ± 4
Chick brain	30 ± 2	96 ± 6	18 ± 2	6 ± 5

The effect of 37°C incubation on the strength of cell–substratum binding. For 4°C measurements, the assay was performed as in Fig. 2. For 37°C measurements, cells were spun onto the protein at 4°C and plates were incubated for 2 h at 37°C in a CO_2 incubator. After incubation at 37°C, plates were assembled and then inverted and centrifuged again at 4°C. The various cell types were detached from the protein using an RCF that would yield 1.31×10^{-5} dynes/cell.
* Percent cells bound at 1.3×10^{-5} dynes/cell.

polymerization in the strengthening response was examined. Cytochalasin b is an inhibitor of microfilament polymerization (50) and therefore is useful in determining the importance of actin polymerization for cell behavior. That cytochalasin b has dramatic effects on fibroblasts can be seen in Fig. 4, which shows the altered morphology due to disturbed actin polymerization. Fibroblasts treated with cytochalasin b were incubated on FN for 15 min at 4 and 37°C (Fig. 5). At 4°C, treated cells and untreated cells adhered to FN equally well. After incubation at 4°C for 15 min, treated and untreated cells continued to adhere to FN equally well with a modest increase in binding relative to 0-min time levels. After incubation at 37°C, a considerably lower percentage of cytochalasin b–treated cells adhered to FN relative to control cells. In fact, the binding of cytochalasin-treated cells remained at levels observed with the 4°C incubation. A simple interpretation suggests that the affinity at 4°C is unaffected by cytochalasin b and also is present at 37°C. The strengthening seen at 37°C is blocked by cytochalasin b and thus is likely to involve actin polymerization.

When glioma cells were incubated at 37°C on TN, a strengthening response was not observed (Fig. 6), nor was it observed for any of the other cell types tested on TN (Table I). In fact, adhesion of glioma and embryonic brain cells actually decreased with time when these cells were incubated on TN at 37°C. In the case of glioma cells, the decrease occurred after 15 min of incubation on TN (Fig. 6). This decrease was not due to possible artifacts introduced by temperature shifts because, when the cells were maintained at higher temperatures for the entire experiment, still <10% of the glioma cells bound to TN (data not shown). Thus, of the cell types tested, none exhibited a strengthening response on TN as they did after incubation on FN, revealing another manner in which adhesion to substrate components can differ.

Since there were very different cell responses to FN and TN upon incubation at 37°C, we next asked how cells would respond to a mixed substrate containing both FN and TN. Glioma cells were used since these cells adhered to both substrates and showed the greatest range of responsiveness to incubation at 37°C. The initial binding at 4°C and the adhesion after 15 min at 37°C was measured by comparing substrates of FN, TN, and FN plus TN (Fig. 7). For the mixed substrate, FN was applied at 5 μg/ml followed by TN at 20 μg/ml

(wells coated with a single protein were incubated with FN or TN solutions at the same concentrations). At 4°C, adhesion to TN alone was higher than to FN alone, due at least in part to the fourfold higher concentration of TN (compare with Fig. 2 where adhesion to TN was weaker than to FN when both were applied at 10 μg/ml). Adhesion to the mixed substrate was essentially the same as to TN alone (at 50 g) or slightly reduced at higher forces. The experiment at 37°C was more dramatic. Virtually 100% of the cells bound to 5 μg/ml FN, but adhesion to 20 μg/ml TN was essentially at background at the three forces tested. This loss of adhesion to TN at 37°C is similar to that observed on 10 μg/ml TN (Table I). Adhesion to the mixed substrate was clearly weakened relative to FN alone, demonstrating that TN blocked cell binding to FN.

Morphological Correlation of Cell–Substrate Adhesion as Seen by IRM

How do the cell–substrate interactions detected by the adhesion assay appear morphologically? IRM was chosen because it provides a means to measure the actual area of cell-to-substrate contacts. In IRM, black areas of the image correspond to the approach of a portion of a cell to within 10–15 nm of the substrate, grey areas correspond to a separation of 15–100 nm, and white areas correspond to a separation of >100 to ~200 nm where the image fades (34). Past adhesion studies by IRM have focused on the morphology of adherent fibroblasts after lengthy incubation at 37°C (see Discussion). These studies revealed that adherent fibroblasts exhibit distinctive focal contacts after incubations on the time scale of hours. Focal contacts appear as black regions 2–10 μm long and 0.25–0.5 μm wide (29, 34, 46).

Using IRM, one can ask whether the initial adhesion involved substantial areas of close contact and whether the strengthening observed in the assay could be correlated with any changes in the IRM image. Glioma cells had the greatest range of adhesive strengths to FN (at 0 and 15 min) and ad-
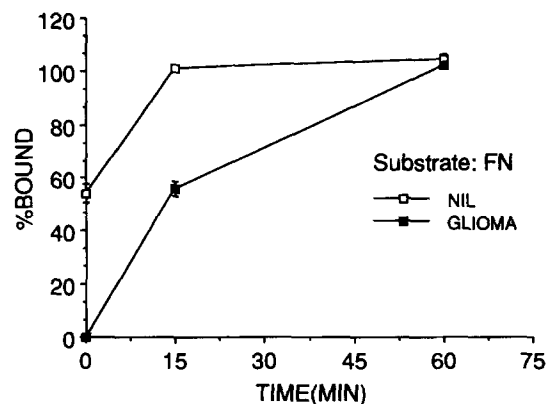

Figure 3. Binding to FN over time at 37°C. For the 0-min time point, the assay was performed as in Fig. 2. For the 15-min time point, the plates were assembled at 4°C and incubated at 37°C. They were then inverted and centrifuged at 4°C. For the 1-h time point, cells were centrifuged onto the protein at 4°C; the plates were incubated at 37°C for 1 h and then assembled and centrifuged at 4°C. A detachment force of 700 g or 3.6×10^{-4} dynes/cell was used.
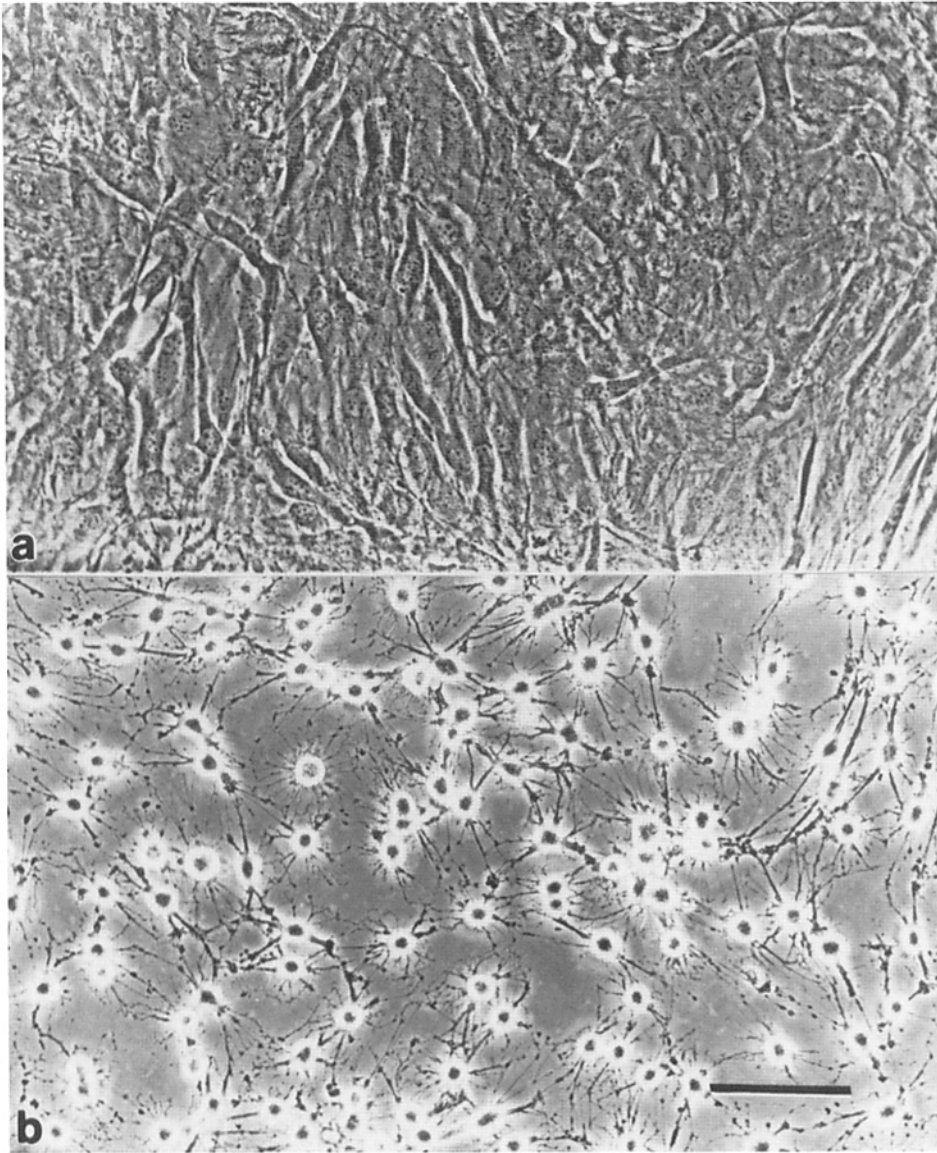
Figure 4. Effect of cytochalasin b on fibroblast morphology. Fibroblasts were exposed to cytochalasin b at 10 μg/ml for 1 h. The photograph was taken under phase–contrast optics. The effect is reversible if cytochalasin b is removed. Bar, 100 μm.

hered to TN as well. Therefore, experiments with these cells are shown. Correlations similar to those reported below for glioma cells between adhesive strength and IRM image have been found for a number of cell types tested. Three aspects of glioma cell images were measured to quantify the cell-to-substratum contacts: the cell area as seen in the phase image, the entire area of the cell detectable by IRM (this includes black, grey, and white zones), and the area within 10–15 nm of the substrate (the black areas in IRM images). These measurements are presented in Table II.

Can cell–substrate contacts formed during initial binding be correlated with the strength of initial binding? Initial binding of glioma cells to either FN or TN survived a detachment force of 2.8 and 1.4 × 10^{-5} dynes/cell, respectively (the force that left 20% of the cells in contact). Thus, the data indicate that initial binding of glioma cells to both proteins is similar (differs by a factor of less than two). Contact areas were compared for glioma cells centrifuged onto either FN or TN and fixed immediately for viewing (Fig. 8, *a* and *b*, and Fig. 9, *a* and *b*). The contact areas formed during initial bind-

ing were similar in all three aspects according to *t* tests, *p* < 0.05 (Table II). Cells on both substrates appeared round in the phase–contrast images, and the IRM images (the grey halos) are speckled similarly with black and white points. Thus, the similar strength of initial binding correlates within a factor of two with a similarity of the IRM image.

After 15 min at 4°C on FN, the strength of adhesion had increased modestly but significantly. IRM showed a twofold increase in the area of 15–100-nm contact and a tenfold increase in the area of <15-nm contact, correlating with the increased adhesion measured in the assay. The strength of adhesion of glioma cells to TN decreased after 15 min at either 4 or 37°C. IRM showed a slight decrease in the 15–100-nm contact and no change in the already minimal <15-nm contact. Phase–contrast images (Figs. 8 and 9) showed no significant change in total cell area on either FN or TN, implying that no cell spreading is initiated at 4°C on either substrate.

IRM images after incubation at 37°C on FN revealed large increases in the <15-nm contacts (Fig. 8 and Table II). There
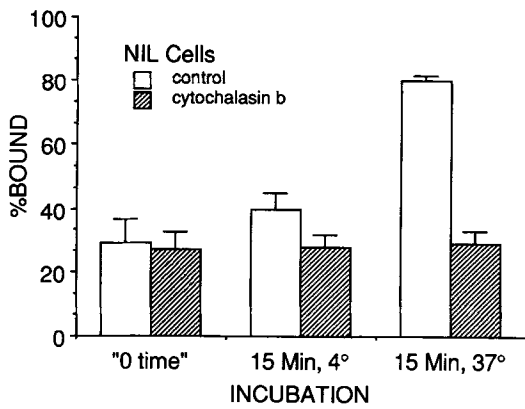
Figure 5. Effect of cytochalasin b on the strengthening of adhesion of fibroblasts to FN. FN was applied to wells at 5 $\mu g/ml$. Fibroblasts were either treated with cytochalasin b, including a 1-h pretreatment and continuous exposure during the assay, or they were left untreated. The assembled assay plates were immediately inverted and centrifuged (0-min time point) or were incubated for 15 min at either 4 or 37°C and then inverted and centrifuged. The detachment force was 500 g or 2.6 × 10^{-4} dynes/cell. A t test indicates that the average percent bound of treated and untreated fibroblasts at 15 min at 4°C is not significantly different, $p < 0.05\%$, whereas the difference at 37°C is highly significant.

was no such increase in the IRM image of cells that had been incubated on TN (Fig. 9 and Table II). Cell spreading occurred on FN (Fig. 8 e) and this correlated well with the area measured in the phase image, which increased to twice that seen at 0-min time points (Table II). The largest change was in the area of close contact with the substrate, which was ~125 times as large as the area seen at 0 min. The adhesive strengthening response on FN also was manyfold larger than the initial unincubated measurements (Fig. 10), correlating well with the IRM images.

Glioma cell adhesion to TN at 37°C (Table II) also can be correlated with surface area in contact with the substrate. By IRM, contact measured after incubation at 37°C on TN is essentially the same as that measured for initial contact (Table II). From the adhesion assay data one might have expected

Figure 7. Glioma cell binding to a mixed substrate of FN and TN. To test cell attachment to a single substrate, wells were incubated with a 5 $\mu g/ml$ solution of FN or a 20 $\mu g/ml$ solution of TN and then blanked. To assay cell attachment to a mixed substrate, wells were incubated sequentially with 5 $\mu g/ml$ FN and 20 $\mu g/ml$ TN and then blanked. Replicate plates were centrifuged at increasing RCFs at 4°C in a and after warming for 15 min at 37°C in b.
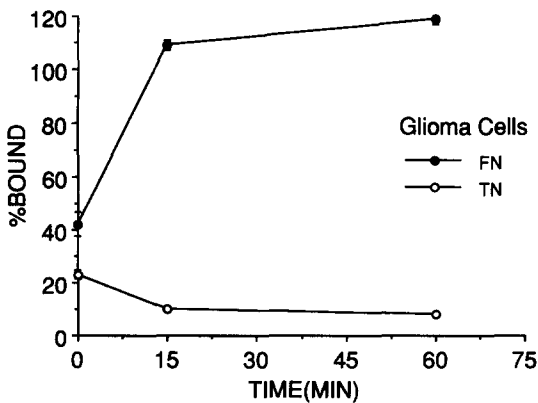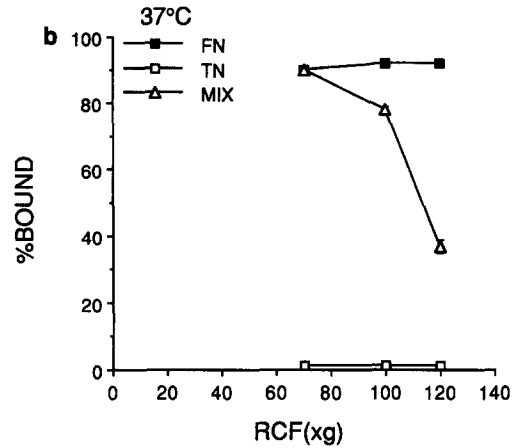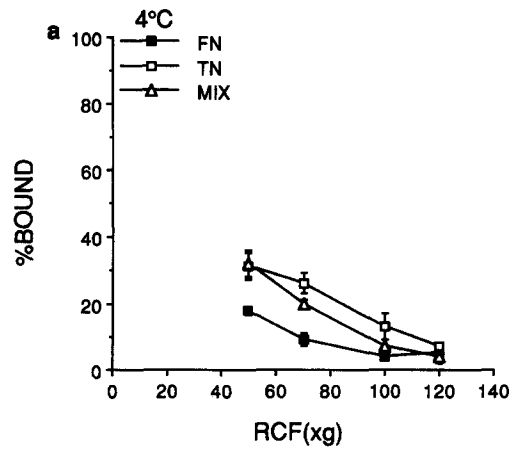

Figure 6. Glioma cell binding to FN and TN over time at 37°C. For the 0-min time point, the assay was performed as in Fig. 2. The 15-min and 1-h time points were performed as in Fig. 3. A detachment force of 1.3 × 10^{-5} dynes/cell was used.

Table II. Area of Glioma Cell–Substrate Contact Compared to Cell–Substrate Adhesion

Incubation	Phase	15–100 nm	<15 nm
FN			
0 min	236 ± 31*	113 ± 23	1.1 ± 0.2
15 min, 4°C	376 ± 49	270 ± 40	13.4 ± 4
15 min, 37°C	599 ± 175	396 ± 41	138 ± 34
TN			
0 min	271 ± 23	123 ± 14	1.3 ± 0.4
15 min, 4°C	306 ± 23	111 ± 20	1.0 ± 0.4
15 min, 37°C	258 ± 25	86 ± 17	2.0 ± 0.1

Area measurements of glioma cell–substratum contact. Three types of cell–substrate contact area measurements as discussed in the text were quantified as in Materials and Methods and are presented here. Phase areas were quantified from phase images as in Fig. 8, a, c, and e, and Fig. 9, a, c, and e. Grey areas (representing cell areas within 15–100 nm of the substratum) and black areas (representing regions of close contact where <15 nm separate cells and substratum) were quantified from corresponding IRM images.
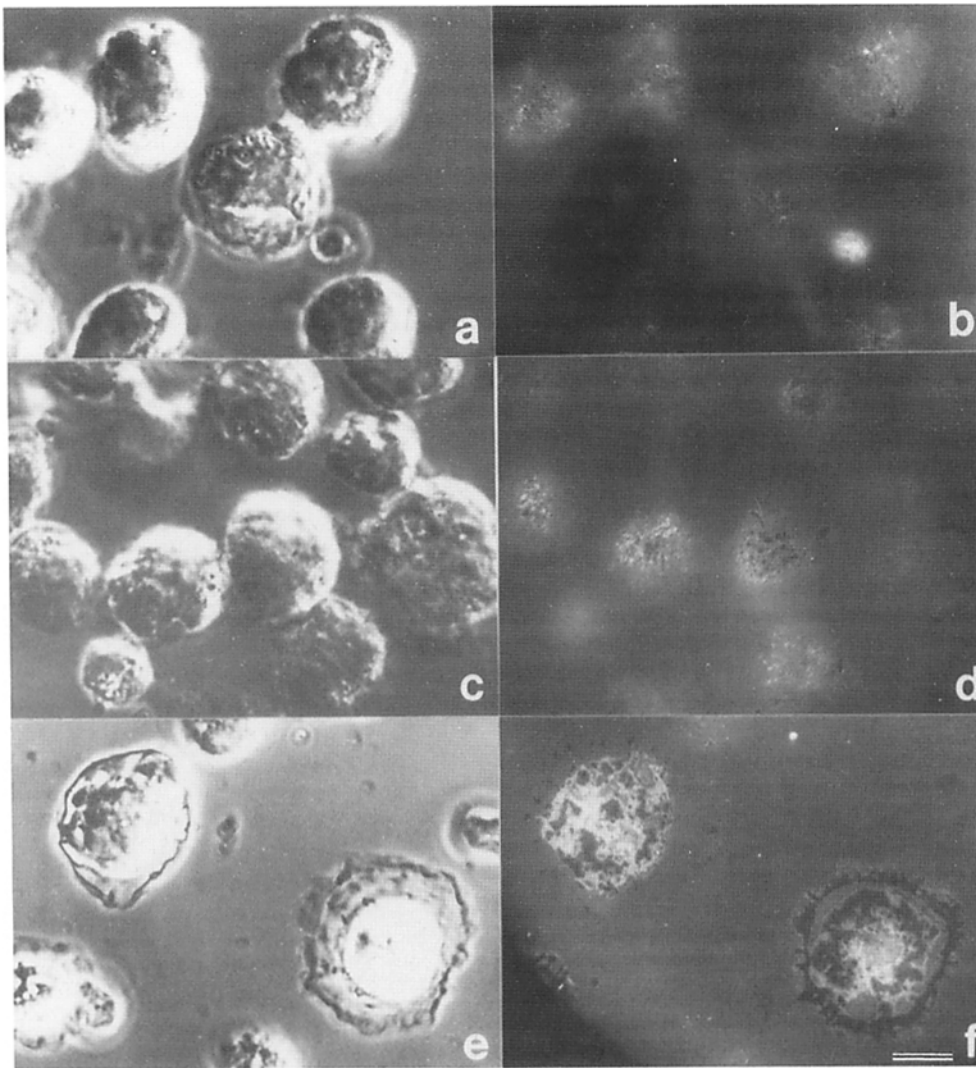* Average area in square micrometers ± SEM.

Figure 8. The effect of various incubations on the contact between glioma cells and FN. Glioma cells were centrifuged onto glass coverslips coated with 10 μg/ml of FN and then incubated for various times before being fixed for observation by phase–contrast microscopy and IRM. (*a*) Phase-contrast image of glioma cells during initial contact with FN at 4°C. Glioma cells were centrifuged onto FN at 4°C and immediately fixed. (*b*) IRM image of field in *a*. (*c*) Phase-contrast image of glioma cells after a 15-min incubation on FN at 4°C. (*d*) IRM image of field in *c*. (*e*) Phase–contrast image of glioma cells after a 15-min incubation on FN at 37°C. (*f*) IRM image of field in *e*. Bar, 10 μm.

a reduction in the area of contact with TN at 37°C since the percentage of cells in contact dropped. However, since cells in the IRM analysis were subjected to shear forces during the addition and removal of fixative, a shear not experienced during adhesion assays, it is likely that only the most adherent cells remained bound to the substratum after the cells were fixed. This selection process would prejudice the IRM data against detection of a decrease in the amount of surface area within extremely close range of the substrate.

Discussion

The role of TN in cell adhesion has been a subject of considerable study and some controversy. Erickson and Taylor (24), using a simple microtiter assay that demonstrated robust adhesion to FN, found no detectable cell adhesion activity for TN. Other laboratories, however, using a very similar microtiter assay, have observed adhesion of a variety of cells to TN (3, 16, 25). Chiquet-Ehrismann et al. (15) observed adhesion of human fibroblasts, and Bourdon and Rouslahti (3) observed adhesion of human fibroblasts, glioma, epithelial, and mesenchymal cells to TN. Friedlander et al. (25) reported adhesion of chicken fibroblasts, but no adhesion of

neurons or glial cells to TN in this assay. Using a more sensitive centrifugal assay, Friedlander et al. (25) could demonstrate adhesion of neurons and glial cells to TN. Their centrifugal assay is rather different from the one we have used. It apparently is sensitive to very weak adhesion, but has not been developed to permit a measure of the force of adhesion.

Although initially viewed as contradictions, there are two points in which these studies all agree and which can perhaps be presented as a consensus. The studies show, first, that adhesion to TN is much weaker than to FN and, second, that cells attached to TN do not flatten and spread, but maintain a rounded configuration. The results of our present study confirm both of these points, give a quantitative measure of the weakness of adhesion to TN relative to FN, and provide a possible explanation for the major adhesive differences between the two molecules.

The weak, transient adhesion we observe for glioma cells to TN indicates that they could have receptors with an affinity for TN, and Bourdon and Rouslahti (3) recently appear to have isolated an integrin receptor for TN from these cells. Nevertheless, one must question whether cell adhesion is a primary physiological function of this receptor. Adhesion to TN at 37°C was virtually eliminated by the weakest forces
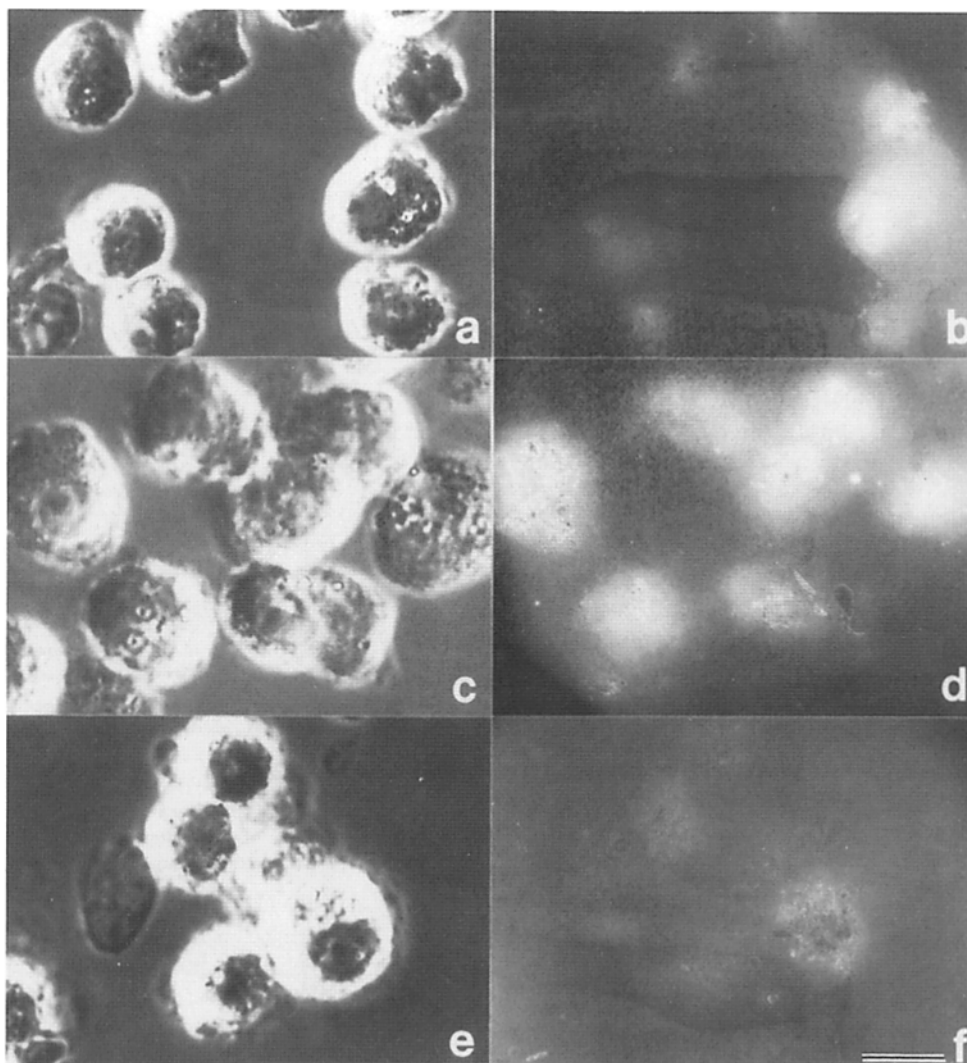
Figure 9. The effect of incuba-
tion on glioma cell contact
with TN. Glioma cells were
centrifuged onto glass cover-
slips coated with 10 μg/ml of
TN and then incubated for
various times before being
fixed for observation by phase–
contrast microscopy and IRM.
(*a*) Phase image of glioma
cells during initial contact
with FN at 4°C. Glioma cells
were centrifuged onto TN at
4°C and immediately fixed.
(*b*) IRM image of field in *a*.
(*c*) Phase–contrast image of
glioma cells after a 15-min in-
cubation on TN at 4°C. (*d*)
IRM image of field in *c*. (*e*)
Phase–contrast image of gli-
oma cells after a 15-min incu-
bation on TN at 37°C. (*f*)
IRM image of field in *e*. Bar,
10 μm.

we could apply. It is orders of magnitude weaker than adhe-
sion to FN, which is considered a typical physiological adhe-
sion. Indeed, the loss of adhesion 15 min after the centrifu-
gation that forms the initial contact suggests that adhesion to
TN is too weak to resist the cell's own movements or shape
changes after centrifugation.

Recently there have been several reports suggesting that a
role of TN may be the opposite of adhesion, specifically to
inhibit or modulate adhesion to substrates like FN. Tan et al.
(47) studied migration of neural crest cells on substrates
coated with alternate strips of TN or FN. The cells migrated
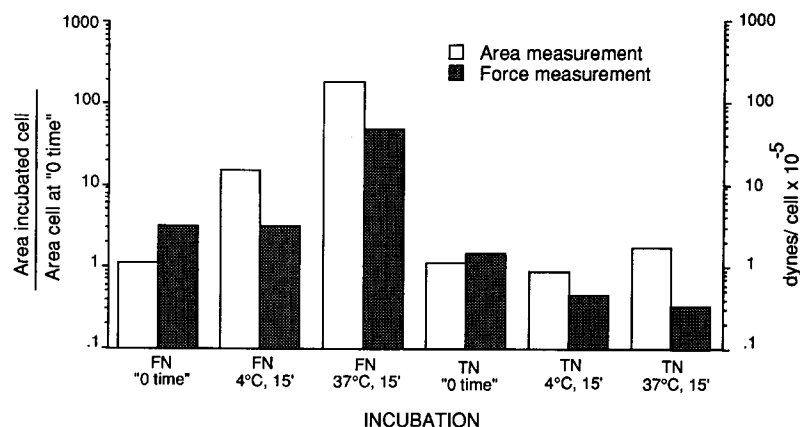almost exclusively on the FN substrate. The few cells that

Figure 10. Area measurements of glioma cell–sub-
stratum contact compared to strength of cell–sub-
stratum adhesion. Area measurements presented
here are the areas of close contact listed in Table
II. Strength measurements represent the force re-
quired to remove 50% of the cell population that
initially bound to each substrate after the indicated
incubation. The strength of glioma cell initial
binding (0 min at 4°C) to FN and TN was taken
from Fig. 2 and the strength measurement to FN
after 15 min at 37°C was taken from Fig. 3. Other
adhesion assays were performed to determine the
remaining strength measurements. On TN sub-
strates after a 15-min incubation at both 4 and
37°C, >50% of the initial cell population was re-
moved at the lowest RCF assayed. Therefore the
strength measurements for these two incubations
are an approximation.

1802

wandered onto the TN strips assumed a rounded shape and did not migrate further. Mackie et al. (38) made very similar observations in frog and rat embryos, and these authors, as well as Bronner-Fraser (6), have suggested that TN might somehow modulate cell migration on FN or other substrates.

Tan et al. (47) observed that TN can inhibit attachment of cells to FN when the two molecules were applied to plastic as a mixed substrate. Mackie et al. (38) reported that TN added in solution with cells inhibited adhesion to an FN substrate, and this was studied further by Chiquet-Ehrismann et al. (15). Our present results confirm the inhibition observed for TN coated on the substrate after the FN. Although the initial adhesion of glial cells was similar on FN, TN, and FN plus TN, the strengthening response on FN plus TN was significantly inhibited relative to FN alone. An attractive explanation for how TN inhibits cell adhesion to FN is steric blocking. The large TN molecule, the hexabrachion, can straddle the FN molecules and literally cover up a significant fraction of the FN adhesion domains (Lightner, V. A., and H. P. Erickson, manuscript in preparation).

Both the microscopy and the adhesion assay reveal cell adhesion to consist of an initial binding or affinity followed by a substantial strengthening of that adhesion. The initial affinity represents the actual adhesion between the cell and the substrate component under conditions where actin and other cytoskeletal elements are prevented from participation. The strengthened adhesion measured after an incubation at 37°C represents the initial adhesion plus what appears to be the consequences of coupling the cytoskeleton to the adhesion receptors. Often the strength of the final adhesion was so large that it exceeded, technically, our ability to measure it (the force required to separate the cells exceeded the centrifuge maximum and/or the integrity of the microtiter assay chambers).

Earlier studies hinted indirectly that an initial contact event with the substratum probably was the first of several steps needed for adhesion measured at 37°C (10, 17, 26, 27, 35, 48). But, since none of those previous studies could directly detect initial contact, very little was learned about it. Other studies saw initial contact at 4°C as detected by coating microscopic-sized beads with FN and allowing these to incubate with cells (26, 44). However, this assay could not monitor later adhesive interactions between the substrate and cells because the beads were phagocytosed. Thus, the centrifugal assay is the only assay that has directly quantified the affinity of cells for substrate components. What can be done with the measurements? The following paragraphs discuss the relative importance of the measurements in the context of other chemical and biophysical measurements that have been published.

To achieve the huge increase in strength of adhesion, a large increase in surface area of contact certainly can help and, from our measurements, does correlate well with the increase in adhesive strength. But, is surface area of contact the relevant measure that accounts for increase in strength of adhesion? How does one account for the apparent need to couple to the cytoskeleton to see the large increase in adhesive strength? The answer to these questions may begin with a consideration of the material properties of the cell itself (41). Biophysical treatments suggest that the cell actually peels away from the substrate. A peeling process can be imagined using the removal of scotch tape from its substrate

as an analogy. The strength of an adhesion, given a peeling model, is a function of the length of the border where the peeling is occurring at any instance in time. The strength is not related to the total area of contact, only to the adhesive molecules along the length of the border of contact.

Now, if the material property of the cell were to change, as might occur when the receptors become tied together with the cytoskeleton, then the entire area of contact could contribute to the strength of adhesion and not just the borders of the contact surface. The analogy would be if one changed the adhesive backing of scotch tape from flexible mylar to a rigid support; one would then have to remove the entire surface area at once instead of peeling away the tape a little at a time. This "coupling" hypothesis is consistent with our data and is also supported by the photobleaching measurements of Duband et al. (20) which show that integrin receptors are recruited from a diffuse, mobile pool and concentrated at focal adhesions when cells attach to FN.

A theoretical estimate for the force that can be sustained by single bonds and by clusters of bonds, with the specific goal of estimating the force of cell adhesion, was presented by Bell (2). He concluded that if a cell were held by multiple bonds, which had to be detached as a unit, there would be a "critical force per bond, f_c," at which the cell would suddenly detach. He estimated $f_c = 0.4 \times 10^{-5}$ dynes/bond. There are many assumptions in this calculation and it was only meant to be accurate within a factor of two or so. A minor correction to this model can be added based on a recent analysis of protein–protein bonds (21). Whereas Bell used the free energy of dissociation as an estimate of bond strength, the "intrinsic bond energy," which is ~7 kcal/mol higher (21), is a better value to use in his equation 15. Thus one can calculate a value of ~10^{-5} dynes/bond as the estimate of the critical force required to detach a cell.

It is important to note that this is the average force per bond, distributed over all bonds in the cluster that are under tension at one time. If the cell membrane were rigid so that all bonds had to be detached together, the total force for detachment would be f_c times the number of receptors in each cluster. Thus, if the receptors were clustered into rigid focal contacts where 50 receptors have to be detached at once, a total force of 50×10^{-5} dynes would be required to detach the cell. If the receptors were few in number and dispersed, so that they are detached one-by-one or a few at a time as the cell peels off, a much smaller force would detach the cell. The argument leading to f_c only applies to clusters with multiple receptors, but a force near 10^{-5} dynes should be near the minimum force required to detach cells held by dispersed bonds.

The theoretical model and our experimentally measured forces approximate one another at both extremes. After strengthening on FN, ~50% of a population of glial cells were detached by a force of $>36 \times 10^{-5}$ dynes. The force that would actually detach fibroblasts could not be measured, due to technical limitations. The minimum value for glial cells already implicates clusters of >40 receptors as the attaching unit. These probably correspond to the focal contacts that are formed by this time. In contrast, the initial attachment of glial cells to TN and FN was detached by forces of $1.3–3 \times 10^{-5}$ dynes, very near the force estimated for disrupting a single bond. This very weak adhesion might suggest that the cell is held by only one to three bonds, but a

more reasonable interpretation is the peeling mechanism. If there were only one to three receptors under tension at the peeling edge at any one time, even this very weak force would detach the cell.

The adhesion measurements reported here suggest another property of cell surface receptors. Our data and that of Duband et al. (20) suggest that nonadherent cells exist with their receptors largely uncoupled from the actin cytoskeleton. At initial contact there are no focal contacts in IRM images and cytochalasin b fails to inhibit those affinities as seen at 4°C. If strengthening an adhesion includes a coupling to the cytoskeleton, the cell may also have a mechanism for rapidly uncoupling surface receptors that are in contact with their substrate. This mechanism could involve molecular conformation changes or could simply be related to receptor density at the site of contact.

References

1. Badley, R. A., C. W. Lloyd, A. Woods, L. Carruthers, C. Allcock, and D. A. Rees. 1978. Mechanisms of cellular adhesion. III. Preparation and preliminary characterisation of adhesions. *Exp. Cell Res.* 117:231–244.
2. Bell, G. I. 1978. Models for the specific adhesion of cells to cells. *Science (Wash. DC)*. 200:618–627.
3. Bourdon, M. A., and E. Rouslahti. 1989. Tenascin mediates cell attachment through an RGD-dependent receptor. *J. Cell Biol.* 108:1149–1155.
4. Bourdon, M. A., T. J. Mathews, S. V. Pizzo, and D. D. Bigner. 1985. Immunochemical and biochemical characterization of a glioma-associated extracellular matrix glycoprotein. *J. Cell. Biochem.* 28:183–195.
5. Brackenbury, R., U. Rutishauser, and G. M. Edelman. 1981. Distinct calcium-independent and calcium-dependent adhesion systems of chicken embryo cells. *Proc. Natl. Acad. Sci. USA.* 78:387–391.
6. Bronner-Fraser, M. 1988. Distribution and function of tenascin during cranial neural crest development in the chick. *J. Neurosci. Res.* 21:135–147.
7. Buck, C. A., and A. F. Horwitz. 1987. Integrin, a transmembrane glycoprotein complex mediating cell-substratum adhesion. *J. Cell Sci. Suppl.* 8:231–250.
8. Burn, P., A. Kupfer, and S. J. Singer. 1988. Dynamic membrane-cytoskeletal interactions: specific association of integrin and talin arises *in vivo* after phorbol ester treatment. *Proc. Natl. Acad. Sci. USA.* 85:497–501.
9. Burridge, K., L. Moloney, and T. Kelly. 1987. Adhesion plaques: sites of transmembrane interaction between the extracellular matrix and the actin cytoskeleton. *J. Cell Sci. Suppl.* 8:211–229.
10. Carter, W. G., H. Rauvala, and S.-H. Hakomori. 1981. Studies of cell adhesion and recognition. II. The kinetics of cell adhesion and cell spreading on surfaces coated with carbohydrate-reactive proteins and fibronectin. *J. Cell Biol.* 88:138–148.
11. Chen, W.-T., and S. J. Singer. 1982. Immunoelectron microscopic studies of the sites of cell-substratum and cell-cell contacts in cultured fibroblasts. *J. Cell Biol.* 95:205–222.
12. Chen, W.-T., E. Hasegawa, T. Hasegawa, C. Weinstock, and K. M. Yamada. 1985. Development of cell surface linkage complexes in cultured fibroblasts. *J. Cell Biol.* 100:1103–1114.
13. Chen, W.-T., J. Wang, T. Hasegawa, S. S. Yamada, and K. M. Yamada. 1986. Regulation of fibronectin receptor distribution by transformation, exogenous fibronectin, and synthetic peptides. *J. Cell Biol.* 103:1649–1661.
14. Chiquet, M., and D. M. Fambrough. 1984. Chick myotendinous antigen. I. A monoclonal antibody as a marker for tendon and muscle morphogenesis. *J. Cell Biol.* 98:1926–1936.
15. Chiquet-Ehrismann, R., E. J. Mackie, C. A. Pearson, and T. Sakakura. 1986. Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. *Cell.* 47:131–139.
16. Chiquet-Ehrismann, R., P. Kalla, C. A. Pearson, K. Beck, and M. Chiquet. 1988. Tenascin interferes with fibronectin action. *Cell.* 53:383–

390.
17. Couchman, J. R., D. A. Rees, M. R. Green, and C. G. Smith. 1982. Fibronectin has a dual role in locomotion and anchorage of primary chick fibroblasts and can promote entry into the division cycle. *J. Cell Biol.* 93:402–410.
18. Crossin, K. L., S. Hoffman, M. Grumet, J.-P. Thiery, and G. M. Edelman. 1986. Site-restricted expression of cytotactin during development of the chicken embryo. *J. Cell Biol.* 102:1917–1930.
19. David-Pfeuty, T. 1985. The coordinate organization of vinculin and actin filaments during the early stages of fibroblast spreading on a substratum. *Eur. J. Cell Biol.* 36:195–200.
20. Duband, J.-L., G. H. Nuckolls, A. Ishihara, T. Hasegawa, K. M. Yamada, J.-P. Thiery, and K. Jacobson. 1988. Fibronectin receptor exhibits high lateral mobility in embryonic locomoting cells but is immobile in focal contacts and fibrillar in stationary cells. *J. Cell Biol.* 107:1385–1396.
21. Erickson, H. P. 1989. Cooperativity in protein-protein association: the structure and stability of the actin filament. *J. Mol. Biol.* 206:465–474.
22. Erickson, H. P., and J. L. Iglesias. 1984. A six-armed oligomer isolated from cell surface fibronectin preparations. *Nature (Lond.).* 311:267–269.
23. Erickson, H. P., and V. A. Lightner. 1988. Hexabrachion protein (tenascin, cytotactin, brachionectin) in connective tissues, embryonic brain, and tumors. *In* Advances in Cell Biology. Vol. 2. K. R. Miller, editor. Jai Press Inc., Greenwich, CT. 55–90.
24. Erickson, H. P., and H. C. Taylor. 1987. Hexabrachion proteins in embryonic chicken tissue and human tumors. *J. Cell Biol.* 105:1387–1394.
25. Friedlander, D. R., S. Hoffman, and G. M. Edelman. 1988. Functional mapping of cytotactin: proteolytic fragments active in cell-substrate adhesion. *J. Cell Biol.* 107:2329–2340.
26. Grinnell, F. 1974. Studies on the mechanism of cell attachment to a substratum with serum in the medium: further evidence supporting a requirement for two biochemically distinct processes. *Arch. Biochem. Biophys.* 165:524–530.
27. Grinnell, F. 1980. Fibroblast receptor for cell-substratum adhesion: studies on the interaction of baby hamster kidney cells with latex beads coated with cold insoluble globulin (plasma fibronectin). *J. Cell Biol.* 86:104–112.
28. Grumet, M., S. Hoffman, K. L. Crossin, and G. M. Edelman. 1985. Cytotactin, an extracellular matrix protein of neural and non-neural tissues that mediates glia–neuron interactions. *Proc. Natl. Acad. Sci. USA.* 82:8075–8079.
29. Heath, J. P., and G. A. Dunn. 1978. Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system: a correlated interference-reflexion and high-voltage electron microscope study. *J. Cell Sci.* 29:197–212.
30. Hoffman, S., and G. M. Edelman. 1987. A proteoglycan with HNK-1 antigenic determinants is a neuron-associated ligand for cytotactin. *Proc. Natl. Acad. Sci. USA.* 84:2523–2527.
31. Horwitz, A., K. Duggan, C. Buck, M. C. Beckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin–a transmembrane linkage. *Nature (Lond.).* 320:531–533.
32. Hughes, R. C., S. D. J. Pena, J. Clark, and R. R. Dourmashkin. 1979. Molecular requirements of the adhesion and spreading of hamster fibroblasts. *Exp. Cell Res.* 121:307–314.
33. Hynes, R. O., and A. T. Destree. 1978. Relationship between fibronectin (LETS Protein) and actin. *Cell.* 15:875–886.
34. Izzard, C. S., and L. R. Lochner. 1976. Cell-to-substrate contacts in living fibroblasts: an interference reflexion study with an evaluation of the technique. *J. Cell Sci.* 21:129–150.
35. Juliano, R. L., and E. Gagalang. 1977. The adhesion of Chinese hamster cells. I. Effects of temperature, metabolic inhibitors and proteolytic dissection of cell surface macromolecules. *J. Cell. Physiol.* 92:209–220.
36. Kruse, J., G. Keilhauer, A. Faissner, R. Timple, and M. Schachner. 1985. The J1 glycoprotein–a novel nervous system cell adhesion molecule of the L2/HNK-1 family. *Nature (Lond.).* 316:146–148.
37. Mackie, E. J., I. Thesleff, and R. Chiquet-Ehrismann. 1987. Tenascin is associated with chondrogenic and osteogenic differentiation in vivo and promotes chondrogensis in vitro. *J. Cell Biol.* 105:2569–2579.
38. Mackie, E. J., R. P. Tucker, W. Halfter, R. Chiquet-Ehrismann, and H. H. Epperlein. 1988. The distribution of tenascin coincides with pathways of neural crest cell migration. *Development (Camb.).* 102:237–250.
39. McClay, D. R., G. M. Wessel, and R. B. Marchase. 1981. Intercellular recognition: quantitation of initial binding events. *Proc. Natl. Acad. Sci. USA.* 78:4975–4979.
40. Norton, E. K., and C. S. Izzard. 1982. Fibronectin promotes formation of close cell-substrate contact in cultured cells. *Exp. Cell Res.* 139:463–467.
41. Rees, D. A., C. W. Lloyd, and D. Thom. 1977. Control of grip and stick in cell adhesion through lateral relationships of membranes glycoproteins. *Nature (Lond.).* 267:124–128.
42. Rees, D. A., J. R. Couchman, C. G. Smith, A. Woods, and A. G. Wilson. 1982. Cell-substratum interactions in the adhesion and locomotion of fibroblasts. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 299:169–176.
43. Rouslahti, E., E. G. Hayman, M. Pierschbacher, and E. Engvall. 1982. Fibronectin: purification, immunochemical properties and biological activities. *Methods Enzymol.* 82:803–831.

44. Schwarz, M. A., and R. L. Juliano. 1984. Interaction of fibronectin-coated beads with CHO cells. *Exp. Cell Res.* 152:302-312.
45. Singer, I. 1982. Association of fibronectin and vinculin with focal contacts and stress fibers in stationary hamster fibroblasts. *J. Cell Biol.* 92:398-408.
46. Singer, I. I., D. W. Kawka, S. Scott, R. A. Mumford, and M. W. Lark. 1987. The fibronectin cell attachment sequence Arg-Gly-Asp-Ser promotes focal contact formation during early fibroblast attachment and spreading. *J. Cell Biol.* 104:573-584.
47. Tan, S. S., K. L. Crossin, S. Hoffman, and G. M. Edelman. 1987. Asymmetric expression in somites of cytotactin and its proteoglycan ligand is correlated with neural crest cell distribution. *Proc. Natl. Acad. Sci. USA.* 84:7977-7981.
48. Umbreit, J., and S. Roseman. 1975. A requirement for reversible binding between aggregating embryonic cells before stable adhesion. *J. Biol. Chem.* 250:9360-9368.
49. Virtanen, I., T. Vartio, R. A. Badley, and V.-P. Lehto. 1982. Fibronectin in adhesion, spreading and cytoskeletal organization of cultured fibroblasts. *Nature (Lond.).* 298:660-664.
50. Weber, K., C. P. Rathke, M. Osborn, and W. W. Franke. 1976. Distribution of actin and tubulin in cells and in glycerinated cell models after treatment with cytochalasin B (CB). *Exp. Cell Res.* 102:285-297.
51. Woods, A., C. Smith, D. A. Rees, and G. Wilson. 1983. Stages in specialization of fibroblast adhesion and deposition of extracellular matrix. *Eur. J. Cell Biol.* 32:108-116.