Proc. Natl. Acad. Sci. USA
Vol. 78, No. 8, pp. 4975–4979, August 1981
Cell Biology

Intercellular recognition: Quantitation of initial binding events

(neural retina/adhesive specificity)

DAVID R. MCCLAY*, GARY M. WESSEL*, AND RICHARD B. MARCHASE†

*Department of Zoology, Duke University and †Department of Anatomy, Duke University Medical Center, Durham, North Carolina 27706

Communicated by Saul Roseman, April 22, 1981

ABSTRACT **The hypothesis that intercellular adhesion can be subdivided into two separable phenomena—an initial recognition event and a subsequent stabilization—is supported by the use of a cell binding assay that provides a quantitative measure of intercellular binding strengths. Radioactive single cells are brought into contact with cell monolayers at 4°C in sealed compartments. The compartments are inverted and a centrifugal force is then applied to dislodge the probe cells from the monolayers. By varying the speed of centrifugation, the force maintaining associations between embryonic chicken neural retina cells was determined to be on the order of 10^{-5} dyne. Topographic specificities of single neural retina cells for retinal monolayers from various regions of the retina were detected with this assay and corresponded to those observed in more traditional assays at 37°C. Also observed were two time- and temperature-dependent stabilization processes in which the force required for dislodgment increased. One of the stabilization processes was sensitive to dinitrophenol and was inactive at 4°C; the second was still active in metabolically blocked cells. The metabolic-dependent process resulted in interactions at least 13 times as strong as the initial binding. The metabolic-independent process resulted in about a 2-fold increase in binding strength and had a temperature dependence similar to that of membrane diffusional phenomena.**

Precise cellular interactions play an essential role in the assembly of tissues during embryogenesis and are attributed, in part, to adhesive specificities (1). Studies by Umbreit and Roseman (2) on embryonic chicken tissues *in vitro* suggested that cell adhesion could be subdivided into an initial reversible phase and a subsequent stabilizing process. Reversible adhesions were characterized by their dissociation by small fluid shear forces. The stabilizing process was strongly dependent on time and temperature and led to more permanent bonds between cells.

Data obtained by using a newly developed cell binding assay support these results and suggest that interactions determining recognition specificity take place during the reversible phase of cell interactions. In addition, the assay provides a quantitative measure of intercellular binding strengths. Radioactive probe cells are brought into contact with monolayers in sealed compartments by a low centrifugal force. The compartments are then inverted in the centrifuge and the force required to dislodge the probe cells from the monolayers is determined. Probe cells were found to bind at 4°C with a strength that resisted about 10^{-5} dyne of dislodgment force per cell and exhibited recognition specificity and sensitivity to trypsin and calcium. When the bound cells were incubated for 8 min at 37°C, the resistance to dislodgment was at least 13 times stronger than that of the associations formed at 4°C. This stabilization was dependent on the cells' being metabolically active.

With metabolism inhibited, a second type of strengthening of cell binding was detected. This increase was also temperature dependent and approximately doubled the force required for dislodgment. Rate-versus-temperature studies suggest that membrane diffusional properties may be limiting for this second type of increase in binding strength.

MATERIALS AND METHODS

Neural retina tissue was dissected from 10-day chicken embryos (Pittsboro Hatchery, Pittsboro, NC), placed into calcium- and magnesium-free saline for 10 min, and then incubated in 0.2% trypsin (1:250; GIBCO) in the saline containing 0.1 mM EDTA. After 20 min at 37°C the tissues were washed three times with culture medium [Eagle's minimal essential medium containing: 10 mM Hepes (pH 7.2), penicillin at 50 units/ml, streptomycin at 50 μg/ml, DNase (Sigma) at 15 μg/ml, and 2% fetal calf serum (GIBCO)]. The tissues were gently dissociated by pipetting, and the cell suspensions were washed twice in culture medium. The cells in suspension excluded trypan blue (>95%), incorporated radiolabeled leucine as well as or better than cells prepared by other published procedures for dissociation that were compared, and aggregated well by rotary methods (3).

Sheep erythrocytes were washed and stored in phosphate-buffered saline and used within 2 weeks.

Fig. 1 shows the binding assay. Cell monolayers were constructed on flat-bottom polyvinyl microtiter plates (Dynatech, Alexandra, VA; tissue-culture treated). The monolayers were established by first treating the microtiter plates with M_r 60,000 poly(L-lysine) in distilled water (50 μg/ml; Sigma) for 1 hr at 24°C. The plates were washed with distilled water, and 0.1 or 0.2 ml of cell suspension in culture medium was added. The cells were centrifuged onto the polylysine-coated bottoms (50 × g; 3 min). The plates were incubated for 0.5–1 hr at room temperature or 37°C. The monolayers were washed first with culture medium containing M_r 30,000 poly(L-glutamic acid) (10 μg/ml; Sigma), which was found to neutralize the binding of probe cells to cell-free polylysine-treated wells, and then with culture medium. When cells were centrifuged onto polylysine-treated plates containing no monolayers (10 × g; 10 min; 4°C), >95% of the population resisted a dislodgment force of >3000 × g. However, when the polylysine layer was treated with polyglutamate before addition of probe cells, <3% of the probe cell population remained bound to the wells after exposure to 50 × g. The polyglutamate treatment did not affect probe cell binding to monolayer nor did polyglutamate remove bound monolayer cells. Therefore, polyglutamate was used to minimize background binding of probe cells to any polylysine-exposed areas within the monolayer (exposed areas never exceeded 10% of the total area).

Probe cells were labeled with [^{3}H]leucine (10 μCi/ml in leucine-free Eagle's medium; 16 hr; New England Nuclear; 1 Ci

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Abbreviations: RCF, relative centrifugal force; Con A, concanavalin A; DNP, dinitrophenol.

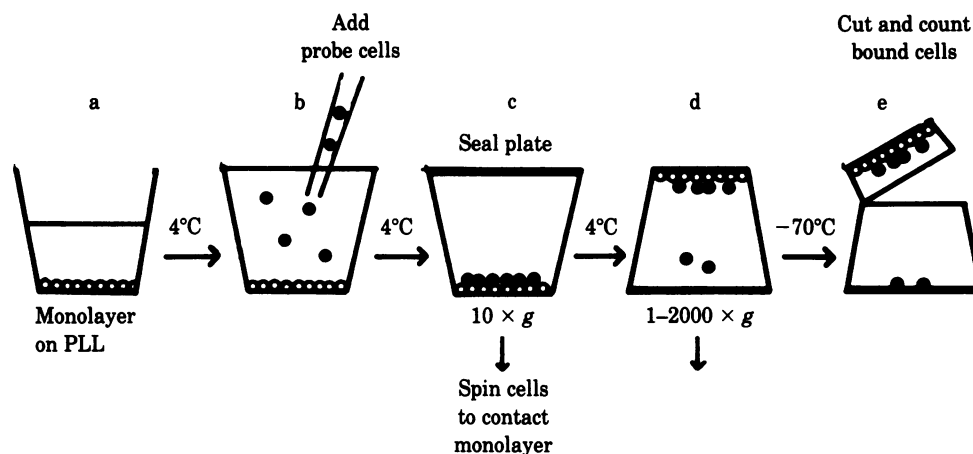

FIG. 1. Cell binding assay. (*a*) Monolayer cells are spread onto polylysine (PLL)-treated microtiter wells. (*b*) Radioactive probe cells are added and each well is filled to the brim. (*c*) Wells are sealed with plate sealing tape and the probe cells are centrifuged to contact the monolayers. (*d*) Plates are inverted (immediately or after an incubation period) and centrifuged to provide a dislodgment force tending to remove probe cells from the monolayers. (*e*) After centrifugation, the plates are quick frozen in the inverted position in ethanol/dry ice; then the bottom 3 mm of each well is removed for assay by scintillation methods.

= 3.7 × 10^{10} becquerels), with ^{32}P (carrier-free $H_3{}^{32}PO_4$; 2 hr; 20 μCi/ml in culture medium; New England Nuclear), or with ^{51}Cr (100 μCi/ml in culture medium; 2 hr; 37°C; New England Nuclear). Probe cell suspensions in culture medium were added to the microtiter wells at 4°C to give a final concentration of 1 × 10^5 cells per well. The final volume in each well was brought to 0.3 ml. This volume resulted in a slightly positive meniscus, a necessary step in the assay to prevent air bubbles when the wells were sealed. When a 37°C step was included in an experiment, the wells were not sealed until after the return to 4°C. The wells were sealed by rolling on an adhesive microtiter plate sealer (Dynatech). Every other row on a microtiter plate was used. The empty rows received approximately 10 μl of spillover as the wells were sealed (this was found to be a necessary contribution to the 5% experimental error of the assay). Probe cells were then centrifuged into contact with the monolayers (10 × g; 10 min; 4°C). This force was sufficient to bring >90% of the neural retina cell population into contact with the monolayers in control experiments when no dislodgment force was applied. The sheep erythrocytes were centrifuged at 50 × g for 20 min to achieve 95% contact.

The plates were inverted and again centrifuged so that the centrifugal force now tended to pull the probe cells away from the monolayers. In control experiments, monolayers were not dislodged by forces 3-fold greater than the maximum used in most experiments. Unless otherwise indicated, the centrifugation time was 10 min. At the completion of a centrifugation, the plate was placed, still sealed and in the inverted position, into ethanol/dry ice. The wells were frozen and the bottoms of the wells containing the monolayers were clipped off (with a pet toenail clipper modified to cut exactly at 3 mm). The well bottoms, containing the monolayers and any attached probe cells plus ≈40 μl of frozen culture medium, were transferred while still frozen to scintillation vials and assayed either in Aquasol (New England Nuclear) or in a Triton X-100/toluene-based cocktail. ^{51}Cr-Labeled cells were assayed in a gamma counter.

In the binding assay, measurements were made in at least quadruplicate; SEMs generally were <5%. Results of identical experiments performed on separate days were generally within 5% of one another after probe cell populations were normalized for cpm per cell.

At a contact force of 1 × g it took 90–120 min to bring 90% of the neural retina probe cells into contact with the monolayers; 10 min was more than sufficient when a force of 10 × g was applied. The dislodgment force did not differ significantly when 1 × g and 10 × g contact forces were compared. Higher contact forces (50 × g) did lead to a significant increase in the dislodgment force. Therefore, the 10 × g contact force was used for all neural retina experiments reported.

A small amount of soluble label was present in the 40 μl of medium that was counted with the monolayers, creating a background of <3%. At low centrifugal forces, several minutes were required for removal of unbound probe cells from the monolayer area; at higher forces, probe cells quickly were removed (<1 min at 100 × g) and therefore were not contained in the 3-mm portion of the well that was assayed.

The forces of centrifugation did not appear to damage cells in the present experiments: polylysine-bound monolayer cells were not released even after 1 hr at 2000 × g; probe cells were not released from monolayers at 2000 × g if they first had been in contact with the monolayer for a period of time at 37°C.

Cell specific densities were calculated by weighing a volume of cells and correcting for the volume between cells by using [^3H]polyethylene glycol (4).

RESULTS

Dislodgment Force. The relative centrifugal force (RCF) required to dislodge populations of single, radioactive, neural retina cells from neural retina monolayers was measured after incubation at 4°C. At RCF <50 × g nearly all the cells resisted dislodgment (Fig. 2). With increasing RCF, progressively more cells were removed during the 10-min centrifugation; 85% of the probe cells were removed at RCF between 50 and 400 × g.

The force per cell required for dislodgment was calculated by

$$F_D = (\rho_{cell} - \rho_{medium}) \cdot V_{cell} \cdot RCF$$

in which F_D is dislodgment force per cell; ρ_{cell} is specific density of the cell (1.07 g/cm^3); ρ_{medium} is specific density of the medium (1.00 g/cm^3); V_{cell} is cell volume (a diameter of 10 μm was used for retina cells). With the RCF range 50–400 × g the retina probe cell population was removed from monolayers by forces

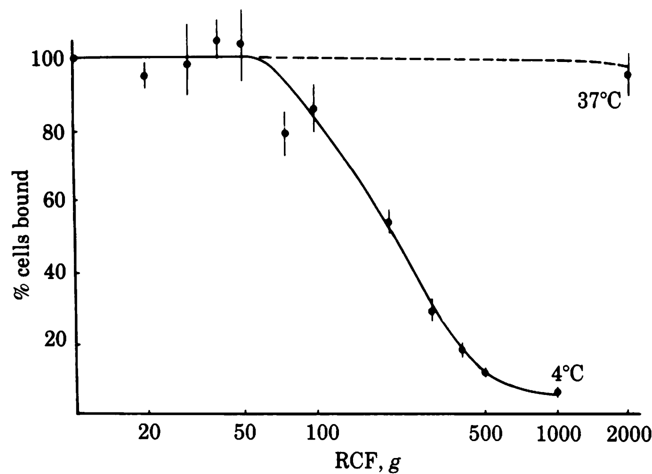

FIG. 2. Removal of neural retina probe cells from neural retina monolayers at different RCF. Monolayers were established on polylysine. Probe cells were 10-day embryonic chicken neural retina cells labeled with [^{3}H]leucine at 0.05 cpm per cell; they were suspended in culture medium and added to wells (100,000 cells per well) at 4°C. The probe cells were brought into contact with the monolayers (10 × *g*; 10 min; 4°C). The plates were then inverted and centrifuged for 10 min at 12 different RCF. Each point represents the mean ± SEM of cells remaining bound (n = 8 wells). The dashed line represents one point for which the wells were warmed to 37°C for 8 min prior to inversion and centrifugation at 2000 × *g*; after this brief warming period the entire population of cells resisted a 2000 × *g* dislodgment force.

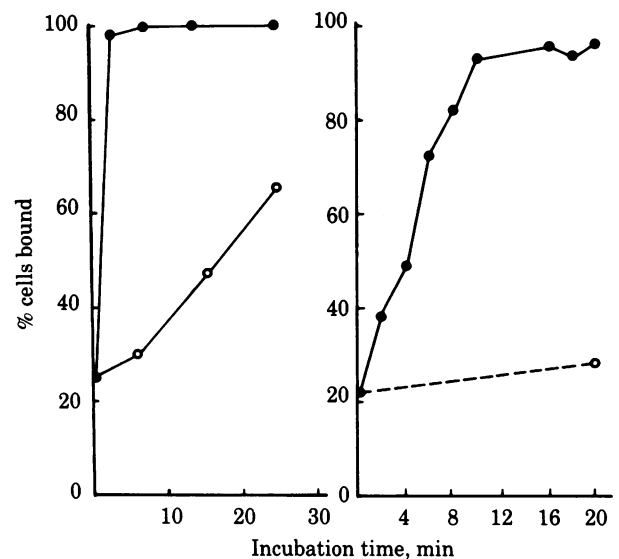

FIG. 3. Effect of time of contact at 37°C (●) or 4°C (○) before application of a removal force. Neural retina probe cells (108,000 cells per well; 0.04 cpm per cell) were added to wells containing monolayers of neural retina and cells were centrifuged into contact with the monolayer. The plates were incubated for different times at 37°C or 4°C and then placed back on ice; the plates were then inverted and centrifuged at 200 × *g* (*Left*) or 400 × *g* (*Right*).

of 1.8×10^{-6} to 1.4×10^{-5} dyne per cell; the median force for removal was 7×10^{-6} dyne per cell. Continued application of a centrifugal force beyond the 10-min period resulted in gradual removal of additional cells but at a rate that was lower than the initial removal rate.

With increased time of contact between probe cells and the monolayer at 4°C, there was an increase in the binding of retina cell populations to retina monolayers at lower dislodgment forces (Fig. 3). However, nearly all of the probe cells were dislodged by a RCF of 750–1000 × *g*. By contrast, when the plates were warmed to 37°C while the probe cells were in contact with the monolayers, a much larger increase in binding strength occurred. After only 6 min at the higher temperature, most of the probe cells resisted a dislodgment force of 400 × *g* (Fig. 3) and by 8–10 min, almost no probe cells were dislodged even at 2000 × *g* (Fig. 2). The exact magnitude of this stabilization could not be determined due to limitations on the centrifuge carriers. However, from these experiments we conclude that there are two phases to the cellular interactions: first, an initial binding at 4°C, which is reversible in moderate shear forces; and second, a stabilization that requires incubation at 37°C and results in intercellular bonds that are relatively irreversible. These data thus support the findings of Umbreit and Roseman (2) that two separable phenomena contribute to adhesive interactions.

Properties of the Initial Binding at 4°C. *Specificity.* Tissue specificity in adhesive interactions has been observed in the sorting out of embryonic cells within aggregates (5–7) and in the selective collection of single cells prepared from different tissues by aggregates (8) and monolayers (9). Adhesive specificity based on topographic location within a tissue type has been shown to exist between cells of the retina and optic tectum (10) and among cells within the retina (11). This topographic specificity also was detectable with the current assay in the initial binding step (Table 1). More dorsal neural retina cells bound to ventral retina monolayers at 4° than to dorsal monolayers, and ventral neural retina probe cells preferred dorsal monolayers.

When sheep erythrocytes were tested in this assay they fell away from sheep erythrocyte monolayers or from neural retina monolayers at 1 × *g* (Table 2). The maximal adhesive interaction for sheep erythrocytes was thus calculated to be less than 1×10^{-9} dyne. Furthermore, because erythrocytes did not bind to neural retina cells even after only a 1 × *g* dislodgment force, it can be argued that the initial binding observed between adhesive cells is a property that requires reciprocal recognition between the two cell surfaces.

Concanavalin A (Con A). To examine how a known bridging molecule affected initial binding parameters, cells were treated with Con A. As shown in Table 2, Con A-mediated binding of the previously nonadhesive erythrocytes to erythrocyte monolayers resisted removal forces of about 10^{-6} dyne per cell. The threshold for a Con A effect was about 0.5 μg of Con A per ml. Neural retina cells displayed an augmented resistance to dislodgment from monolayers in the presence of Con A (Table 2). For neural retina cells, the threshold for augmentation occurred at Con A concentrations of 0.5–1 μg/ml. The latter data suggest that there is a quantitative component to the initial binding that is sensitive to the number of binding sites between cells.

Trypsin sensitivity. Binding of retinal probe cells to the monolayer also was sensitive to trypsin (Table 2). The median force for removal of retinal cells that had been allowed to recover

Table 1. Specificity of binding of dorsal and ventral neural retina cells

Time of incubation at 4°C, hr	Probe cells	Binding, %*: To dorsal monolayers	Binding, %*: To ventral monolayers	Specificity ratio†
0	Dorsal	13	25	1.92
	Ventral	33	19	1.71
1	Dorsal	23	41	1.78
	Ventral	57	40	1.43

* Percentage of cells bound after 300 × *g* dislodgment centrifugation.
† From (D – V)/(D – D) or (V – D)/(V – V). For all ratios, for difference from 1, $P < 0.05$.

Table 2. Effects of trypsin and concanavalin A (Con A) on binding

Cell type	Treatment	% bound after 200 × *g* removal force	Force to remove 50% of input cells,* dyne per cell
Sheep erythrocyte	None	0†	$<1 \times 10^{-9}$
	Con A (10 μg/ml)	74	1.17×10^{-6}
Neural retina	Con A (5 μg/ml)	76	1.62×10^{-5}
	Trypsin‡	26	3.6×10^{-6}
	Recovered	48	7.1×10^{-6}

* Calculated from experiments with eight different removal forces.
† To sheep erythrocyte monolayers and to retina monolayers.
‡ Both the probe cells and the monolayers were incubated in 0.2% trypsin for 10 min at 37°C.

for several hours after dissociation (12) was twice that required for removal of freshly trypsinized cells from monolayers. The dorsal/ventral specificity among retinal cells was also lost when monolayers and probe cell populations were freshly trypsinized.

Calcium sensitivity. Cell binding at 4°C was sensitive to Ca^{2+} concentration. A reduction in binding of ≈75% was observed in low Ca^{2+} (0.1 mM), and binding was further reduced in the presence of 2 mM ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (to about 10% of control levels).

Properties of the Stabilization Process. The stabilization phenomenon observed at 37°C was divisible into two distinct processes based on sensitivity to dinitrophenol (DNP). In the absence of DNP, cells rapidly adhered to monolayers and resisted dislodgment forces in excess of 10^{-3} dyne per cell (the highest dislodgment force tested). In the presence of DNP, cell-to-monolayer binding also was increased in strength but only to a maximum of about 5×10^{-5} dyne per cell. This moderate increase in binding strength appeared to be the same process as that which occurred with time at 4°C although at 37°C it occurred faster. Again, even after 20 min of contact at 37°C, cells in DNP were completely dislodged by a RCF of 750–1000 × *g*. By contrast, at 37°C in the absence of DNP, binding of the entire cell population resisted these higher dislodgment forces.

At all temperatures, DNP-insensitive increases in binding were initially linear with time. The percentage of additional cells bound at 5 min, relative to initial binding, as a function of temperature provided a thermodynamic assessment of this process. When plotted in the Arrhenius form (Fig. 4), the rate of binding increase had a slope of −1.63 and a calculated activation energy of 7.42 kcal/mol (1 cal = 4.18 J). The DNP-insensitive process thus had a calculated activation energy in the same range as found for many membrane diffusion-related processes.

DISCUSSION

The cell binding assay described here detected three types of cellular interactions. The first, an initial binding, occurred at 4°C. Bound neural retina cells were dislodged from monolayers by forces of 10^{-6}–10^{-5} dyne per cell. The initial binding step exhibited recognition specificity and sensitivity to trypsin, Con A, and Ca^{2+}. The second two processes resulted in stabilization of these initial interactions. One of the stabilization processes was sensitive to DNP and inactive at 4°C; the second was still active in metabolically blocked cells. In less than 10 min at 37°C, the DNP-sensitive strengthening process resulted in interactions that were more than 13 times as strong as the initial binding. The DNP-insensitive increase resulted in interactions that were about twice as strong as the initial binding.

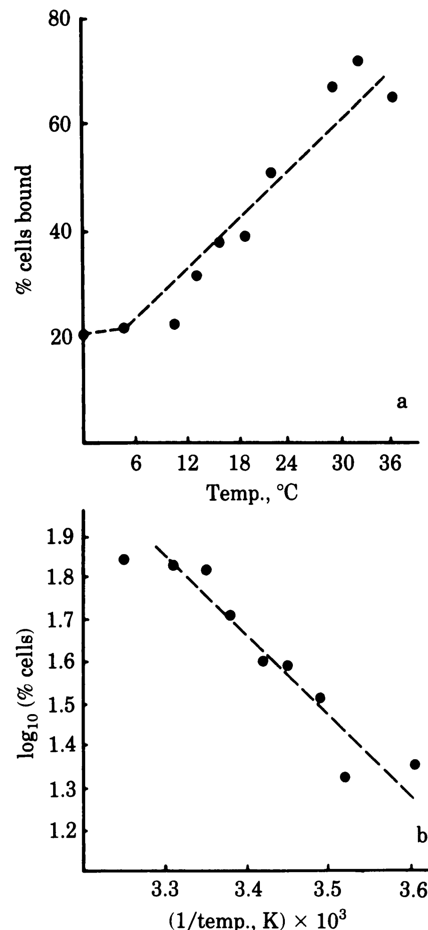

FIG. 4. DNP-insensitive binding strength increases at different temperatures. Probe cells (102,000 cells; 0.04 cpm per cell) were added to 10 replicate plates of monolayers each containing 1 mM DNP. One of the plates was inverted immediately and centrifuged (300 × *g*; 10 min; 4°C) to give the initial binding. The remaining plates were incubated at nine different temperatures for 5 min (at all temperatures tested, binding increases were occurring at a linear rate). The plates were then returned to 4°C, inverted, and centrifuged (300 × *g*; 10 min; 4°C). (*a*) Direct plot. (*b*) Plot in the Arrhenius form. The slope of the line, determined by least squares, was −1.63 which gives a calculated energy of activation for the process of 7.42 kcal/mol.

A transition from a weak initial adhesive interaction to a stronger interaction was previously observed by Umbreit and Roseman (2) using a Coulter Counter assay. Their assay distinguished between cell interactions that were resistant to gentle inversion of the counting vial and those resistant to 10 rapid inversions of the vial. Although the strengths of the adhesive interactions could not be quantitated, this assay demonstrated a transition from "reversible" to stronger "irreversible" interactions. Using a rotating drum apparatus to study *Dictyostelium* cells, Gerisch and coworkers (13) were able to detect an EDTA-sensitive and reversible bond that formed prior to permanent bonds that were resistant to EDTA. Moscona (14) has described "primary" and "secondary" phases of aggregation. These phases, however, refer to the sorting out of cells in aggregates and thus to later steps in the *in vitro* histogenetic process.

The upper limit of force measurement in the cell binding assay was set by the microtiter centrifuge carriers at RCF of 2000 × *g* or 5×10^{-4} dyne per cell. When a 37°C incubation period was added to the basic assay, this magnitude of force did not release probe cells from monolayers. In other studies of adhesive forces, about 1 dyne/cell was required for separation of

fibroblasts from a substrate (15) or for the separation of cancer cells from one another (16). The separation force measurements of such "irreversibly" bound cells may be underestimates, however, because, as noted by Weiss (17), at high separation forces membranes might tear before cells separate. In any event, the binding strength of the DNP-sensitive bond is beyond the limits of practical measurability by this assay. We can only conclude that the strength at 37°C is at least 13-fold greater than that of the initial binding.

The DNP-sensitive stabilization process that results in the formation of irreversible bonds may be rate limited by ATP production and availability. Aggregation assays dependent upon a DNP-sensitive step have been found to have an activation energy of 33.5 kcal/mol (unpublished data), a value that is similar (between 15°C and 40°C) to the activation energy of respiration. This does not suggest a mechanism for strengthening, however, because a number of cellular functions requiring ATP could be involved. These include cell shape changes, membrane–cytoskeleton interactions, enzymatic reactions, synthesis of, release of, or interaction with extracellular matrix components, and formation of cell junctions. The DNP-sensitive changes that occur within minutes of initial cell contact can be studied with the cell binding assay and may provide a focus for future analyses of the secondary processes that stabilize the cell interactions.

The DNP-insensitive strengthening process had a Q_{10} of 2.1, corresponding to an activation energy of 7.5 kcal/mol. These values are in the range of membrane diffusional processes as measured by photobleaching (18) and fluorescence depolarization (unpublished data) and suggest that a membrane diffusional process might be rate limiting for the DNP-insensitive increase in binding. This observation might be expected if additional intercellular bond formation were dependent on mobility of cell-surface macromolecules.

Cell recognition assays were initially designed to demonstrate histotypic specificities that were apparent during development. The demonstration of specificity has become, in a way, a test for the validity of an assay as well as an observation of presumed cell recognition properties. Specificity was used experimentally by Townes and Holtfreter (5), Moscona (6), and Steinberg (7) to describe the histotypic sorting out processes that occur within aggregates. This specificity has been proposed to fit the Weiss–Tyler model for cellular recognition (19, 20) which proposed that complementary cell-surface molecules analogous to antigen–antibody complexes are responsible for specificity. Although lock-and-key interactions such as antigen–antibody complex formation or lectin–carbohydrate binding proceed at 4°C, adhesive interactions usually have not been detected at this temperature. Sorting-out experiments require cell movement, intact metabolism, and time on the order of days. Specificity has also been observed in the collection of cells by aggregates (8) or monolayers (9) prepared from different tissues. Time on the order of minutes to hours was required for significant numbers of cells to be collected, and low temperatures or antimetabolic agents were severely inhibitory. These specificities also could be modeled after Tyler and Weiss but, again, because of the time and apparent metabolic requirements, other factors could confound the interpretation.

The cell binding assay described here differs from traditional cell aggregation assays in that the aggregation assays generally include liquid shear forces greater than 10^{-4} dyne (21). Thus, initial binding phenomena without subsequent stabilizations and adhesive events with binding strengths less than 10^{-4} dyne are not detectable with aggregation assays (8, 9). With this cell binding assay, a dislodgment force as low as 10^{-9} dyne per cell can be detected. The results with this assay show that specific interactions do occur at 4°C and that the interactions result in binding strengths on the order of 10^{-5} dyne. Following these interactions, stabilization processes (that do not need a mechanism for specificity) produce an enhancement of the strength of an interaction that allows the cells to withstand more severe disruptive forces. The specificity observed in collection assays may be due to the same mechanisms as those responsible for the specificity observed with this cell binding assay. For the mechanisms to be identical, however, the strengthening process (required for collection assays) must be similar for all tissues so as not to influence specificity differences that occur with initial binding.

The cell binding assay thus may allow a focused study of the molecular components responsible for initial recognition and specificity without complications arising from subsequent cellular processes. In addition, the assay may allow the component processes of cellular adhesion to be first separated and later synthesized to produce an integrated understanding of these phenomena.

We thank Susan Gurganus for secretarial support. This work was supported by Grants GM-22477, EY-02845, and NS-06233 from the National Institutes of Health. D.R.M. is the recipient of Research Career Development Award HD-00259.

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