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Quantitative Evaluation of Tissue-Specific Cell Adhesion at the Level of a Single Cell Pair

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Tissue specificity of cell adhesion was directly characterized in a unit cell interaction using a novel laser trapping cell manipulator in combination with a fixed micropipet. We quantified the adhesive specificity of endodermal and ectodermal epithelial cells from *Hydra*, which are known to sort out within hours after being dissociated and then randomly reaggregate. It was shown that homotypic pairs of cells from the same tissue source could adhere to each other within a certain period, while heterotypic pairs could not form an adhesion. It was also found that the adhesion probability was higher in endodermal epithelial cell pairs than in ectodermal epithelial cell pairs. The former pairs could adhere with a contact period of less than 30 sec, while 60% of the latter remained nonadherent even after a 6-min forced contact. The adhesive strength of the latter was estimated to be as large as 30 pN, while that of the former was much larger than 50 pN. The tissue-specific adhesivity quantitatively measured provides a new insight into the mechanism of cell sorting.

INTRODUCTION

When dissociated cells from several tissue sources are mixed to form an aggregate, they do not remain disorganized but sort out according to their tissue origins (Townes and Holtfreter, 1955; see Armstrong, 1989, for review). Processes of cell recognition and the ensuing selective adhesion, which become evident in cell sorting, are not merely artifacts *in vitro* but are recognized as fundamental steps observed in development and differentiation *in vivo*. Epithelial morphogenesis in animal embryos (see Takeichi, 1991; Gumbiner, 1992, for reviews), projection to the target cell in nervous systems (Harrelson and Goodman, 1988), and interactions of T cells with antigen-presenting B cells in immune systems (Kupfer *et al.*, 1987) are well-known examples controlled by the specific adhesion of cells.

Recent efforts to elucidate the molecular nature of adhesion have led to the discovery of a large family of adhesion molecules (e.g., Edelmann, 1986; Takeichi, 1987). Analysis of adhesion on the cellular level, however, has been restricted to the stochastic approach using *in vitro* cell attachments. The adhesivity was investigated through the attachment of radiolabeled cells to cell aggregates (McGuire, 1976) or to confluent cell layers (Buultjens and Edwards, 1977) and through the contact between two cell aggregates (Moyer and Steinberg, 1976) or the contact between aggregates and cell layers (Cassiman and Bernfield, 1976). Observation of temporal evolution in the cell sorting process also provided useful but indirect information on the cell-cell contact affinity (Thomas and Yancey, 1988; see Armstrong, 1989, for review). Although the above methods indicate the qualitative difference, direct quantification of adhesion specificity in unit cell interaction, i.e., the interaction between two cells, was inaccessible.

We report here a novel experimental method in which direct observation and quantitative analysis of the interaction between two dissociated cells forced to contact are made possible. A specially designed two-beam laser cell manipulator using levitation trapping force (Tashiro *et al.*, 1993) enabled us to control cell-cell contact spatially and temporally and to measure the adherent property or strength of interacting cells. Optical manipulation with the focused spot of a laser beam, invented and applied to biology by Ashkin (1980; Ashkin *et al.*, 1987), can be a powerful method for characterizing cell-cell interactions.

In these binary cell contact tests, we chose dissociated hydra epithelial cells, aiming to characterize the adhesion specificity of cells organized in tissue. In hydra, two layers of epithelium make a tube-like body column, where ectodermal epithelial cells form the outside layer and endodermal epithelial cells form the inside layer. Cell aggregates of hydra, made by dissociating tissues into a suspension of cells and then reaggregating them by centrifugation, can regenerate a complete adult form by 1 week (Gierer *et al.*, 1972). In the first step of the

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regeneration, cell aggregates establish the inside and outside cell layers by sorting and arranging endodermal and ectodermal epithelial cells (Gierer *et al.*, 1972). The existence of different adhesive properties is suggested by the fact that endodermal epithelial cells formed bigger cell clumps than ectodermal cells when they were incubated in a stirred suspension culture (Technau and Holstein, 1992).

In the present study, we have directly demonstrated the tissue specificity of cell adhesion in a unit cell interaction and quantified the adhesion specificity in terms of differences in adhesion probability and adhesion strength.

MATERIALS AND METHODS

Preparation of Isolated Cells

Hydra vulgaris, strain K9, were obtained from Dr. T. Sugiyama (National Institute of Genetics, Mishima, Japan) and cultured in a hydra culture medium, "M" solution (Takano and Sugiyama, 1983). They were fed five times a week with *Artemia nauplii*, which have a natural orange color. The animals were starved for 1 day before use. Isolated cells were collected from about 20 hydra bodies with the same genetic background, after removing their head and foot.

Cells were dissociated as described previously (Sato *et al.*, 1990). First, tissue pieces from the gastric region were minced into small fragments and then dissociated mechanically by repeated pipetting into a cell suspension in a hyperosmotic dissociation medium (DM) described by Flick and Bode (1983): 5 m*M* $CaCl_2$, 1 m*M* $MgSO_4 \cdot 7H_2O$, 2.8 m*M* KCl, 11 m*M* TES, 0.67 m*M* Na_2HPO_4, 0.44 m*M* KH_2PO_4, 5 m*M* sodium pyruvate, and 5 m*M* sodium citrate. The osmolarity of the DM in which cell aggregates can start regeneration is 70 mOsmol (Gierer *et al.*, 1972; Trenkner *et al.*, 1973). After filtration with a mesh of 45–53 μm, cells were collected by low-speed centrifugation (80–150*g*) and then resuspended into 1 ml of fresh DM and filtered with the mesh again. The cell suspension was added to the DM-filled chamber of the micromanipulator. From a mixture of various kinds of hydra cells lying on the bottom of the chamber, isolated epithelial cells larger than 15 μm in diameter were selected for further use. We did not choose broken cells, occasionally found in the cell suspension.

Some of the cell samples, hereafter referred to as collagenase-treated cells, were treated additionally with 5 mg/ml of crude collagenase (Sigma type designation IA) in DM. After incubation with collagenase for 30 min, the cells were washed with fresh DM, resuspended in 1 ml DM, and then transferred to the chamber for examination.

The viability of the dissociated cells was checked using the trypan blue exclusion method. In DM kept at 18°C, most of epithelial cells (92.7 ± 1.3%) were viable 2 hr after dissociation. In general, the survival rate for epithelial cells seemed to be higher than that for other types of cells.

Laser Micromanipulator

The design concept and details of this manipulation system were already described in a separate paper (Tashiro *et al.*, 1993). Briefly, the three-dimensional laser micromanipulator system consisted of a cw-Nd:YAG laser (Line Lite 607C, 1 W, 1.064 μm), a microscope (Nikon Optiphoto XF), and a sample chamber fixed to the three-dimensional translational stage of the microscope (Fig. 1). The sample chamber was an open cell whose bottom was made of an optically flat glass. A 5-μm-opening-diameter micropipet was introduced horizontally through the Teflon wall. The pipet was connected to a suction pump (Narishige, IM-200) and arranged to rotatable around its axis. Captured in the counterpropagating focused beams by levitation trapping, a cell could be manipulated relative to the three-dimensionally translating stage. The observation was recorded with a Hi-band 8-mm formatted video recorder (SONY, EVO-9500A) through a CCD TV camera which accepted visible and infrared light (Hitachi, KV-26). A video printer (SONY, CVP-G500) was used for printout. All the manipulation procedures were performed at 18°C. From our experience, it is necessary to soak the bottom glass of the chamber in DM for at least 1 hr before introducing cells, otherwise many cells tend to attach firmly to the glass. Possible deformation by the solid surface of the glass as well as adhesion to it during contact tests explains the advantage of three-dimensional cell manipulation by optical levitation trapping.

Viability Assay of Cells Optically Manipulated in Contact Tests

To check the viability of cells manipulated by the laser beam, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) was used. Active mitochondria in viable cells will turn dark blue as a result of MTT reduction and the accumulation of the blue formazan product (Mosmann, 1983; Barres *et al.*, 1992). Using a spectrophotometer, we confirmed that DM containing MTT (MTT-DM) did not absorb the infrared laser light. Accordingly, laser manipulation in MTT-DM was considered to be carried out under a condition similar to that of DM only. Having been manipulated by laser beams for up to 10 min and then kept in MTT-DM (final 0.045% MTT) for 2 hr, the cells were microscopically examined for active mitochondria stained dark

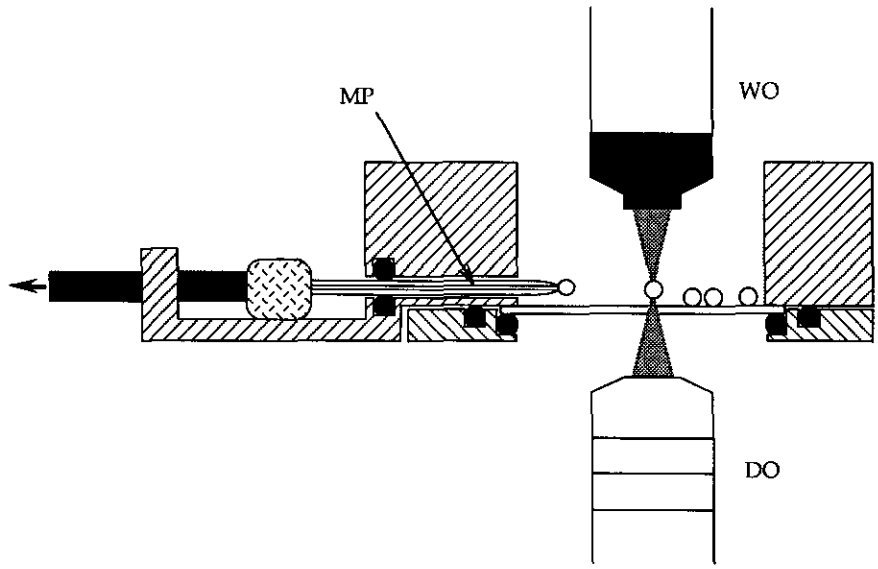

FIG. 1. Cross-sectional view of the sample chamber for cell contact tests. A cell trapped with a counterpropagating beam through two objective lenses is moved to the micropipet by translating the three-dimensional microscopic stage. DO, dry object lens; WO, water-immersed object lens; MP, micropipet.

blue. When numerous intracellular vacuoles in the endodermal cells prevented observation, cell viability was confirmed by the trypan blue exclusion method.

RESULTS

Classification of Dissociated Cells

We used cells prepared from the gastric region of hydra because epithelial cells in this region are not differentiated in form and function as are those in the head and foot regions (Dubel, 1989), and they do not display position-dependent adhesion in cell aggregates (Sato *et al.*, 1992; Technau and Holstein, 1992). After dissociation, most of the isolated cells looked round in shape. However, by careful observation of cell size and intracellular vacuoles, we could easily distinguish epithelial cells from other cell types of smaller size, such as nerve cells, nematocytes, gland cells, and interstitial stem cells (Campbell and Bode, 1983).

Endodermal epithelial cells of hydra can be vitally stained by injection of Evans blue or India ink into the gastric cavity after feeding (e.g., Teragawa and Bode, 1991). Having used this method at first, we then realized that we could identify these epithelial cell types even without staining. Differences in the color and number of intracellular vacuoles helped us to classify epithelial cells as either endodermal or ectodermal: endodermal epithelial cells have a natural orange color in their vacuoles due to *A. nauplii* (see Materials and Methods). We confirmed this by observing dissociated endodermal or ectodermal epithelial cells prepared after the separation of two epithelia using the procedure of Bode *et al.* (1987).

Freshly prepared cell suspension in the present work could sort out and reconstruct epithelial cell layers if they had been forcibly gathered into a cell aggregate by centrifugation, and the sorting pattern was the same as the ones already observed by other workers (Graf and Gierer, 1980; Sarras *et al.*, 1993). Meanwhile, small cell clumps (2–5 cells) due to incomplete tissue dissociation were found to exist in the cell suspension together with isolated cells. They tended to dissociate within 120 min. Most cells were still alive according to the viability tests (see Materials and Methods), and the cells still maintained their ability to aggregate and sort out if they had been forcibly gathered using a centrifuge (data not shown). However, since this dissociation suggests a decrease in the adhesive capability of cells in an isolated state or in small cell clumps, all the adhesion experiments were carried out within 90 min. In the case of collagenase-treated cells, they were used within 60 min because the treatment required an extra 30 min after the dissociation. Using MTT, we also confirmed that ectodermal and endodermal epithelial cells were alive after laser manipulation of up to 10 min (Fig. 2).

Binary Contact Tests of Cell Adhesion

Binary contact between two kinds of epithelial cells and the judgment of adhesion were carried out according to procedures shown in Fig. 3. Among cells lying on the bottom of the chamber, we first chose an appro-

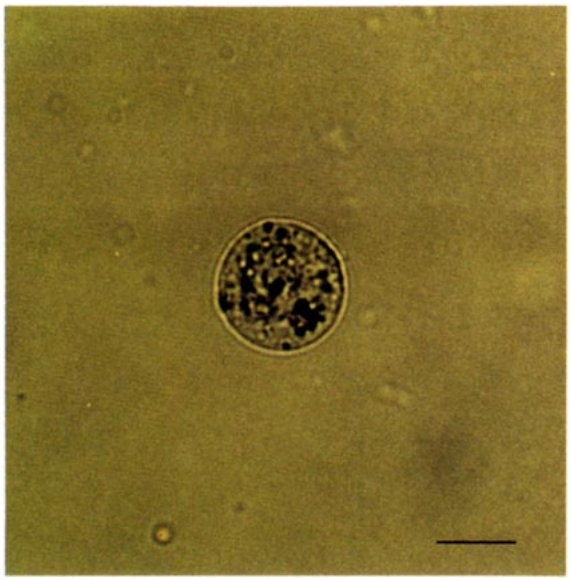

FIG. 2. An ectodermal epithelial cell manipulated with laser beams, in which mitochondria metabolized MTT into an insoluble dark blue reaction product. Bar, 10 μm.

priate cell as the pipet-side cell, trapped it by focused beams, and then lifted it up to the top of a micropipet to which the cell was to be clamped by suction. Then a second cell was selected and, being trapped in the laser beams, it was brought above the pipet side and pushed down to the first cell. After a 3-min contact, the laser beams were cut off. The pair of cells was regarded as adherent if they remained attached to each other firmly enough to resist gravity even after axial rotation of the micropipet. If the second cell detached and fell down from the first cell, another forced contact was tried between the same cells; this procedure was repeated at most three times.

Table 1 shows the results of the binary cell contact tests in four combinations depending on cell type and position. The following two characteristics in the binary cell adhesion are clearly demonstrated here. (1) Only pairs of cells from the same tissue source can adhere to each other. (2) The probability of adhesion formation differs between two kinds of homotypic pairs. For pairs of endodermal epithelial cells, stable adhesion reaches nearly 100%, while only 40% of ectodermal pairs can make stable adhesion.

Hydra has a natural extracellular matrix, the mesoglea, between two epithelial cell layers (Day and Lenhoff, 1981: Sarras *et al.*, 1991). Sarras *et al.* (1993) have shown that epithelium formation proceeded even if cell aggregates of hydra were treated with monoclonal and polyclonal antibodies generated against isolated mesoglea, although further morphogenesis was blocked by them. They also showed that no immunofluorescent signal for the mesoglea appeared until about 17 hr after pellet formation. To ensure that no mesoglea fragments remained to mediate cell adhesion, in the present work the same contact test was repeated with collagenase-treated cells. Before this experiment, we checked whether collagenase-treated cells could sort out to form epithelial bilayers in the regeneration of aggregates. It was confirmed that the collagenase treatment used here did not seriously affect sorting ability. The results with collagenase-treated cells included in Table 1 show the characteristics already mentioned. To sum up, hydra epithelial cells have the specificity to adhere to each other only in homotypic pairs, and endodermal cell pairs are more adherent than ectodermal pairs.

In some cases, endodermal epithelial cells in contact were found to move, actively changing their shape (Fig. 4). Ectodermal epithelial cell pairs, however, scarcely showed such motility or plasticity of cell shape and remained almost spherical even after adhesion formation was achieved (Fig. 5).

Adhesion Probability as a Function of Cell Contact Time

The lower adhesion probability of ectodermal cell pairs shown in the above experiments raises the ques-

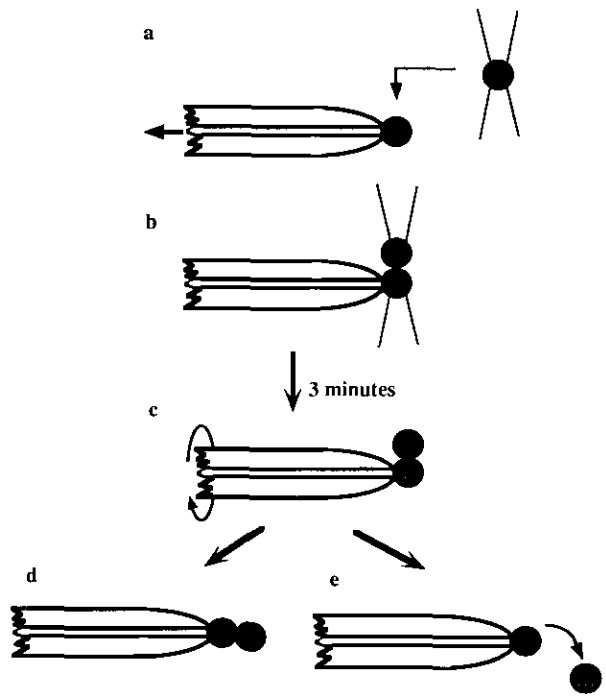

FIG. 3. The procedure of the contact tests between two isolated cells is shown. (a) An epithelial cell is lifted and transported by laser beams to the micropipet where the cell is clamped by suction, and then another epithelial cell is trapped with laser beams and brought near the cell at the micropipet. (b) The second cell is transported above the micropipet and pushed down to get in contact with the cell at the micropipet. (c) After a 3-min forced contact, the laser beams are cut off and the pair is judged as to whether the cells remain adherent (d) or separate (e).

TABLE 1
NUMBER OF PAIRS WHICH MADE ADHESION

Cell combinations[a]	Ectodermal / Ectodermal	Ectodermal / Endodermal	Endodermal / Ectodermal	Endodermal / Endodermal
Cell preparation				
Mechanical dissociation	6 (15)[b]	0 (8)	0 (6)	14 (15)
Collagenase treatment after the mechanical dissociation	5 (9)	0 (3)	0 (4)	6 (7)

[a] Top, forthcoming cell manipulated by laser beams. Bottom, cell held on the pipet.
[b] The number of pairs in which the adhesion formed is presented. In parentheses, the number of sample pairs examined is shown.

tion whether the 3-min contact is long enough for the formation of stable adhesion. We then measured the adhesion probability as a function of the contact time. In the measurement, a forced contact was made for a given period, from 30 sec to 6 min, and then the formation of adhesion was observed in the same manner as described above. Thirty seconds were technically the minimum in order to make reproducible contacts by the laser manipulator method.

The time dependency of the adhesion probability is shown in Fig. 6. For endodermal epithelial cell pairs, even a 30-sec contact leads to complete adhesion on every occasion. On the other hand for ectodermal epithelial cell pairs, a slight increase of adhesion probability is found when the contact time is prolonged from 0.5 to 6 min. However, it appears that the probability reaches a saturation and even a further increase of the contact period will not lead to full adhesion of all the ectodermal pairs.

Estimation of Adhesive Force

The criteria of adhesion formation used in the contact test show that the force keeping the cells in contact is greater than gravity, although, precisely speaking, the force that pulls a cell down is made of the difference between gravitative and buoyant forces. Such a force of downward pulling was evaluated by measuring the falling velocity of a cell when it reached a constant value. It was found that it took 36 sec for a typical endodermal epithelial cell to land on the bottom when it was released from laser trapping at a hight of 100 μm. Applying the constant falling velocity, the diameter of the cell (17 μm), and the medium viscosity (1.32×10^{-3} pascal sec) to the Stokes' drag equation, $f = 6\pi r\eta v_e$, where f is the force, r the radius of a trapped target, η the viscosity of the medium, and v_e the velocity, we could obtain $f =$ 0.5 pN. Accordingly, the attaching force of adherent cell pairs is found to be larger than 0.5 pN.

In principle, the adhesive force existing between cells can be more precisely evaluated by calibration of the trapping force required to detach a cell from an already formed pair. In a previous paper (Tashiro *et al.*, 1993), we measured the transverse trapping force generated by this optical levitation trapping system, although estimated values of the forces for cells scattered and were dependent on the size of cells. The trapping forces estimated were 25 ± 17 pN for endodermal cells of 17.6–23.6 μm in diameter and 27 ± 9 pN for ectodermal cells of 14.7–22.4 μm in diameter, when a moderate Nd:YAG laser power (110 mW), that is, half of the maximum available power, was used. So, the trapping force can be doubled because it is approximately proportional to the laser power.

For a pair of endodermal epithelial cells, no separation occurred even if we raised the laser power to the maximum. At the maximum power, the membrane of a cell which was trapped and tugged by the laser was torn before separation. On the other hand, pairs of ectodermal epithelial cells could be separated with laser powers of one-quarter to one-half maximum. From these observations, we can estimate the adhesive forces to be much greater than 50 pN for pairs of endodermal epithelial cells and to be 27 ± 9 pN for pairs of ectodermal cells.

DISCUSSION

In the present study, tissue-specific cell adhesion shown by Roth and Weston (1967) is examined directly through interactions of individual cells at the level of a single pair, and not statistically through interactions among cells in which the specificity is shown indirectly as rates of attachment or sorting pattern. The adhesion force in hydra epithelial cells is estimated to be more than 50 pN for pairs of endodermal epithelial cells and to be as great as 30 pN for pairs of ectodermal epithelial cells. On the other hand, the adherent force between endodermal and ectodermal epithelial cells will be less than 0.5 pN, if it was generated at all. It is also shown that all of the endodermal pairs become adherent immediately after the initiation of contact, nominally in less than 30 sec. Meanwhile, the adhesion probability of ec-

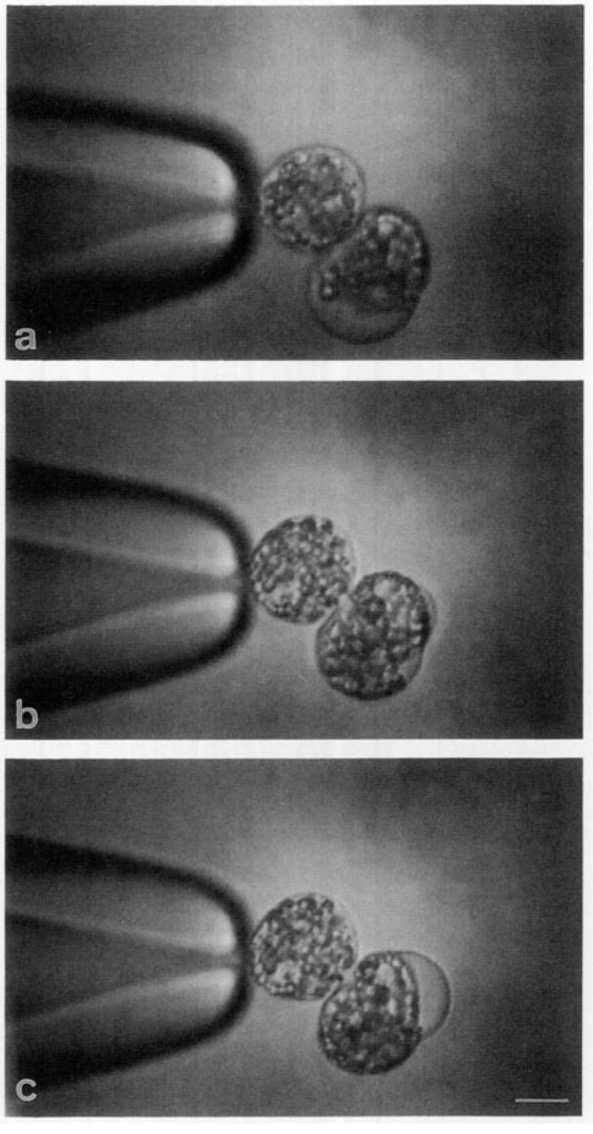

FIG. 4. Two endodermal epithelial cells which remain attached after removal of support by the laser beams. (a, b, and c) The temporal movement of the attached cell at an interval of 5 sec. Bar, 10 μm.

todermal pairs reached no more than 40% after 6 min of contact.

First, we discuss the adhesive heterogeneity observed only in the ectodermal cell population. Dissociated endodermal and ectodermal cells in the present work could sort out to each other and form epithelial cell layers when they had been forcibly gathered into a cell aggregate. Fewer than 30% of ectodermal cells, however, were found to form homotypic cell adhesion in the binary contact tests. We cannot conclude from the present results what really caused such heterogeneity in ectodermal cells, but we may suggest the following possibilities as examples. (a) Possible localized sites for cell adhesion on the ectodermal cell surface. Ectodermal epithelial cells scarcely showed the plasticity of cell shape that endodermal cells did, suggesting their low cell membrane fluidity. It seems likely that the adhesion sites of cells in the epithelium might be retained for a certain time even after dissociation. (b) A possible natural difference in adhesivity among ectodermal cells. Although epithelial cells collected from different positional origins in the body axis do not sort out to each other in cell aggregates (Sato *et al.*, 1992; Technau and Holstein, 1992), we cannot deny the possibility of a difference in adhesivity due to the positional origins of cells, which is not detectable by the cell population assay. (c) A possible artificial difference in adhesivity

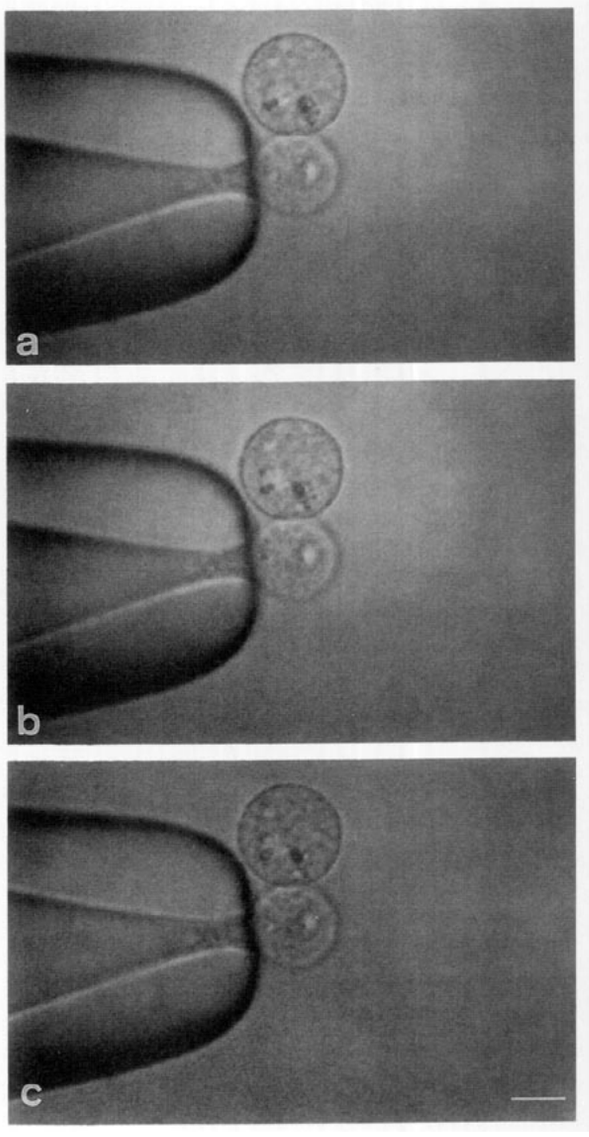

FIG. 5. Adherent pairs of ectodermal epithelial cells after the laser support was cut. In contrast to the endodermal cells, the ectodermal cells do not change the contact surface. (a), (b), and (c) are taken with a time interval of 5 sec. Bar, 10 μm.

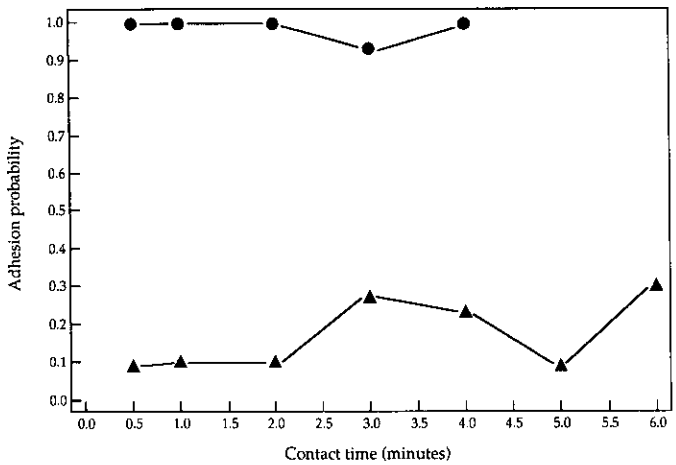

FIG. 6. Adhesion probability as a function of the contact time. Binary cell contact tests were carried out in two kinds of homotypic pairs; pairs of endodermal epithelial cells (●) and pairs of ectodermal epithelial cells (▲); 10–20 pairs were tested for each point.

among ectodermal cells, which is due to various degrees of injury to the ectodermal cell surface caused by the dissociation procedure and a difference in the duration of time to recover.

The adhesion heterogeneity has not been demonstrated by assays with stirred suspension culture or cell aggregates, but could be detected by the binary cell contact test. This is because, if the above-discussed is the case, the heterogeneity is transiently observed only as a very early event of cell sorting and/or the statistical assays cannot detect the characteristics of individual cells.

The differential adhesion hypothesis is a well-known thermodynamic model of cell sorting, which explains why cells can rearrange themselves into the thermodynamically most stable pattern in a mixture of various cell types (Steinberg, 1970). It assumes that cells are stabilized by surface contact with each other but that the stabilization energy differs according to the contact partner. In a mixture of two types of cells, A and B, cell sorting to enclose a mass of A cells by a surrounding layer of B cells is explained by the following order of adhesivity: the contacts of A–A cells should be the most cohesive but the heterotypic contacts of A–B cells must be stronger than homotypic ones of B–B cells. Thus, this model does not emphasize that cells possess tissue-specific adhesivity (see Armstrong, 1989, for review). Also in this model, if cells display tissue-specific adhesion, that is, if heterotypic cell contacts are much weaker than homotypic ones, the complete separation of two types of cell mass will result. The experimental result that the strength of heterotypic cell adhesion was below one-fortieth of the homotypic strength does not support this hypothesis, although no separation of the ectodermal and endodermal cell masses was found in a cell aggregate of hydra.

For cell sorting, there has been another experimentally well-supported hypothesis (e.g., Moscona, 1968) which emphasizes tissue-specific cell adhesion, i.e., that homotypic adhesion is always stronger than heterotypic adhesion. This hypothesis, however, does not answer the question how cells of different origins can arrange their relative placement in order to organize tissues without heterotypic cell interactions (see Armstrong, 1989, for review).

By the binary contact tests in the present study, the tissue adhesive specificity of hydra epithelial cells was clearly shown by the fact that adhesion can be made only between the same type of cells. We must then discuss what is responsible for the mutual placement of the endodermal and ectodermal cells in a cell mixture. The following scheme can be proposed from the big differences in adhesivity between the ectodermal and the endodermal cell pairs, together with the high locomotive ability of endodermal cells. After dissociation in the course of cell sorting, small clumps of endodermal cells come in contact, flicking out ectodermal cells which are not so adherent, to make their own clump. This process results in the formation of an ectodermal cell layer outside the inner endodermal cell clump. In such a view, small adherent forces below 0.5 pN may play a role at an early stage of regeneration even though they are not measured in this experiment.

In order to make the initially sorted cell arrangement firmer between endodermal and ectodermal cells, an additional process should be required. A hint may be found in the recent work by McNeil *et al.* (1993), in which they investigated the very early steps of cadherin-mediated epithelial cell adhesion as the top of a molecular cascade that leads to the remodeling of cell structure and function. Based on the results obtained, they discussed that interactions between E-cadherin and the cytoskeleton were not required for initial cell recognition and adhesion and that the role of such interaction, which eventually occurred after the early steps, was related to further strengthening of the cell contact and the initiation of cell remodeling. Since our measurements of cell adhesion were also performed in the very early period of cell contacts, the stabilization of heterotypic epithelial cell interaction should take place in later stages beyond the time intervals checked here.

In the present study, our method revealed quantitative differences in the mechanical adhesion property such as adhesion strength between cells from different origins. E-cadherin is found on many types of epithelial cells as a cell adhesion molecule (see Takeichi, 1991, for review). On hydra epithelial cells, unfortunately, adhesion molecules have not yet been identified, but major

specific adhesivity of the membrane may be primarily determined by the nature of adhesion molecules. The adhesivity is also a function of the density and distribution of molecules on the surface and of membrane fluidity. In combination with the knowledge of the molecules involved, the method presented should provide information for the understanding of the adhesion process in multicellular organisms.

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