DESMOSOME DEVELOPMENT IN AN IN VITRO MODEL

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ABSTRACT

A model has been devised to study the in vitro formation of desmosomes. The model is based on the differential labeling of two subpopulations of a desmosome-forming human cancer line (C4I). The labeled subpopulations are dispersed, preincubated separately on a shaking water bath for 24 h to allow the internalization of desmosome fragments and the repair of the cell surface, and then mixed, and allowed to aggregate. Aliquots of the mixed suspension are fixed at various intervals. The time between mixing and fixation represents the maximum age of any junction between dissimilarly labeled cells.

The beginnings of desmosome formation were observed within a few minutes after the beginning of aggregation. Close apposition of cell membranes was seen immediately after mixing, followed within 15 min by the appearance of a submembrane density in one or both of the interacting cells. Intracytoplasmic filament formation takes place at between 15 and 30 min. Desmosome formation is complete by 90 min. The process is accompanied by a progressive widening of the extracellular space and the densification and organization of the extracellular material and the submembrane plaques.

The primary function of desmosomes is considered to be that of intercellular adhesion (42, 50, 67). These structures are therefore vital for the maintenance of tissue integrity. Desmosomes also appear to be foci for the organization of cytoskeletal elements within the cell and thus are important in the determination of cell shape and internal organization (51). Therefore, a study of desmosome development may be expected to shed some light on the dynamics of tissue organization and development (43). Furthermore, the morphology of the desmosome, consisting, as it does, not only of apposing plasma membranes, but also involving extensive, symmetrical associations of cytoskeletal components (28, 31, 32), suggests that an elucidation of the formation of this complex structure may lend insight into the coordinated interactions of cells in tissue formation.

When considering the characteristics necessary for a suitable model for the study of desmosome formation, two requirements appear essential. First, the model must permit the unequivocal determination of the precise sequence and timing of the morphologically recognizable events that occur during the process of formation. Second, it must allow the investigator to intervene experimentally in the process in order to study the mechanism(s) of desmosome formation and permit the straightforward interpretation of the results.

Regenerating tissue or the developing animal satisfy the first requirement. These systems, however, suffer from at least two fundamental drawbacks. The interpretation of the effect of any agent that may modify desmosome formation will always be complicated by the possibility of indirect effects via other tissues. Furthermore, in these

models, desmosome development per se cannot be separated from the maturational processes of the interacting cells (1, 16, 44, 65).

An alternative lies in the use of dispersed, reaggregating cultured cells that form desmosomes in vitro. In such systems the cells may be subjected to a variety of experimental procedures, the effects of which act directly on the interacting cells themselves, thus satisfying the second requirement mentioned above.

There are, however, three significant difficulties associated with the use of dispersed cultured cells in studying desmosome formation. The first is the unknown, perhaps lingering, effect of the dispersing procedure on the cell surface and on desmosome formation (10, 14, 21, 40, 72, 73, 77). The second is the persistence of desmosomal remnants at the surface of the dispersed cells (48), making it difficult to judge whether a desmosome seen during or after reaggregation is the result of true, *de novo* formation or simply the reapposition of two remnants. Thirdly, even the best dispersing procedure yields a suspension containing at least a few small cell aggregates. The latter may include not only mature desmosomes but also some in various stages of development (47, 56, 69).

The present report describes a model of desmosome formation which is based on the use of dispersed, cultured cells and which overcomes the three difficulties described above. A preliminary report of this work has been presented elsewhere (15).

MATERIALS AND METHODS

The overall strategy of the model (Fig. 1) is to differentially label two subpopulations of a desmosome-forming continuous cell line with two distinct electron microscopically identifiable tracers. Each labeled subpopulation is dispersed and preincubated separately for 24 h to allow internalization of desmosomal remnants at the cell surface and recovery from the effects of the dispersing procedure. The differently labeled subpopulations, consisting of single cells and small aggregates of two or three cells, are mixed and allowed to aggregate for various time intervals before fixation. Interfaces between differently labeled cells are examined for junctional specializations which necessarily formed after the mixing of the two subpopulations. Junctions between similarly labeled cells are ignored because their time of formation is uncertain.

Routine Cell Culture Procedures

The cell line used was C4I, a continuous line derived from a human cervical squamous cell carcinoma provided to us by Dr. N. Auersperg (University of British Columbia), its originator (7), after 81 passages in that laboratory. Its karyotype has been shown to be close to normal (6, 26) with 41–43 chromosomes and, unlike most other epithelial cells (78), it has retained the ability to form true desmosomes in culture.

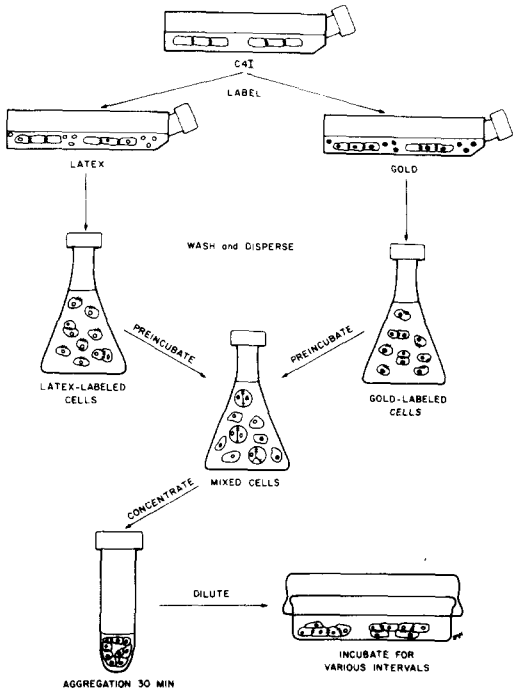

FIGURE 1 Diagrammatic representation of the overall strategy of the model. For details see the text.

Cells were grown as monolayers, in plastic T-75 flasks (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.). They were fed on alternate days with Eagle's basal medium (cat. No. 320-1015, Grand Island Biological Co. [GIBCO], Grand Island, N. Y.) supplemented with 11% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (complete basal medium, Eagle [BME]). The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C and were subcultured weekly with trypsin:EDTA as described below. The cells used in the present study had been in continuous culture in this laboratory for ~3 yr.

Cell Labeling

Cells were labeled with either latex particles or colloidal gold by incorporating the label into the culture medium. Dialyzed, 63°C-sterilized, latex particles (polystyrene spheres, 0.085 μm in diameter, Dow Corning Corp., Midland, Mich.) were added to complete BME to a final concentration of 0.5% solids. Lange's colloidal gold (Fisher Scientific Co., Pittsburgh, Pa.) was diluted to a final concentration of 0.002% colloidal gold (wt/vol) in complete BME and sterilized by filtration through a 0.22 μm filter (Falcon Labware). Cells were fed three times with their respective media on alternate days over a 7-d period.

Dispersion of Labeled Cells

Labeled cell monolayers were thoroughly washed three times with 10 ml of Earle's calcium- and magnesium-free balanced salt solution (GIBCO) to remove residual extracellular label.

The monolayers were dispersed by treating twice with 5–7 ml of 0.05% trypsin:0.02% EDTA in calcium- and magnesium-free Hanks' balanced salt solution (GIBCO) for 3–5 min followed by

vigorous pipetting. The dispersed cells were washed free of trypsin:EDTA by two rinses in complete BME and then resuspended to a final concentration of ~5 × 10^4 cells/ml.

Preincubation of Labeled Cell Suspensions

After dispersion, and before mixing, both the latex- and the gold-labeled cell suspensions were preincubated in tightly closed Erlenmeyer flasks for 24 h. Preincubation was carried out in a shaking water bath at 37°C at 85 oscillations per min (OPM) with an excursion of 1½ inches.

Mixing and Aggregation

After preincubation, pairs of separate flasks containing latex- and gold-labeled cells were mixed together by rapidly pouring one into the other and agitating briefly. The suspension resulting from one pair of flasks was fixed immediately with glutaraldehyde (see below), and this sample constituted the 0-h specimen.

The remaining mixtures were centrifuged at 200 *g* for 3 min and resuspended in complete BME to a final concentration of ~1 × 10^6 cells/ml. 1-ml aliquots of the suspension were transferred to 15-ml sterile, plastic centrifuge tubes (Falcon Labware) and placed at 37°C in a CO_2 incubator. Samples were fixed at 15 and 30 min after mixing. Those samples to be incubated for longer periods of time were transferred after 30 min into 35 ml of complete BME in siliconized (to minimize cell attachment), sterile, 150-mm glass petri dishes to facilitate gas exchange between the cells and the atmosphere. They were then incubated for various time intervals up to 24 h before fixation.

Preparation for Electron Microscopy

Cell suspensions were fixed by mixing with an equal volume of 5.0% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The cells were stored in fixative for 30 min at 4°C, rinsed in cold phosphate buffer, and postfixed in cold Dalton's (12) chrome-osmium for 45 min. The fixed cells were dehydrated in alcohol, rinsed in two changes of propylene oxide, and embedded in Luft's (37) Epon. Thin sections were prepared, stained in uranyl and lead salts, and examined in the electron microscope.

Gold and Latex Measurement

To avoid the possible uptake of latex by gold-labeled cells or vice versa after mixing, it was important to remove the extracellular label from the cultures before dispersion. The effectiveness of the washing procedure was tested by measuring the concentration of label in the culture medium and washings of the cell monolayers being prepared for dispersion.

The gold content of the culture medium and washing fluid was measured by the use of an argon plasma spectrometer (Princeton Testing Laboratory, Inc., Princeton, N. J.). The minimum sensitivity of the method was 0.1 mg of gold/liter. The polystyrene latex concentration was measured spectrophotometrically by the dioxane extraction method (59, 74).

The concentration of gold dropped from 6.8 mg/liter to below the lower limit of detection (0.1 mg/liter) by the second wash. Measurement of the latex content of the washings also showed a drop to below the limits of detectability by the second or third washing.

RESULTS

The Model

C4I cells fixed while still in monolayers and scraped off the growth surface with a rubber policeman appear flat in section with moderate to large numbers of microvilli at their free surface. As reported by others (6), the cells form moderate numbers of desmosomes and are rich in 10-nm tonofilaments. Structures reminiscent of gap and intermediate junctions were present but rare.

When grown in the presence of the label, the cells accumulated easily recognizable latex (Fig. 2) or gold (Fig. 3) particles within membrane-bounded inclusions. Except for the presence of these characteristic particles, the fine structure of the cells appeared identical to that of C4I grown in label-free medium. The frequency with which label was detected ranged from about one-fourth of the cell profiles in a single section to one-third or one-half. It should be pointed out that the latex and gold were never found together in a single cell, thus ruling out any significant transfer of label between cells.

The trypsin:EDTA method of dispersion resulted in a high proportion of rounded, single cells as judged by light microscope inspection. Trypan blue exclusion tests (58) indicated that >90% of the cells were viable. Fragments of desmosomes were found at the cell surface immediately after dispersion, some of which were in the process of internalization. Preincubation of the cells for 24 h at 37°C and 85 OPM resulted in the internalization of desmosomal fragments which became extremely rare at the cell surface.

After mixing, the preincubated, labeled cells were allowed to aggregate in a small volume (1 ml) of medium at the bottom of a conical centrifuge tube. By 15 min, some small aggregates had formed, as judged by light microscope inspection, but most cells were still single. After 30 min, however, the bulk of the cells was in large, loosely clustered aggregates.

Preliminary experiments had shown that if the cells were left in the bottom of the centrifuge tube for 1 h or longer, they began to show anoxic changes and lysis. This was prevented by transferring the aggregating cells to shallow pools of medium in siliconized petri dishes after 30 min in the centrifuge tube. Under these conditions the cells remained well preserved for up to 24 h of incubation, the longest interval studied. Frequent mitotic figures were observed at all time intervals.

Desmosome Formation

In each experiment some cells were fixed immediately after mixing. Centrifugation of these

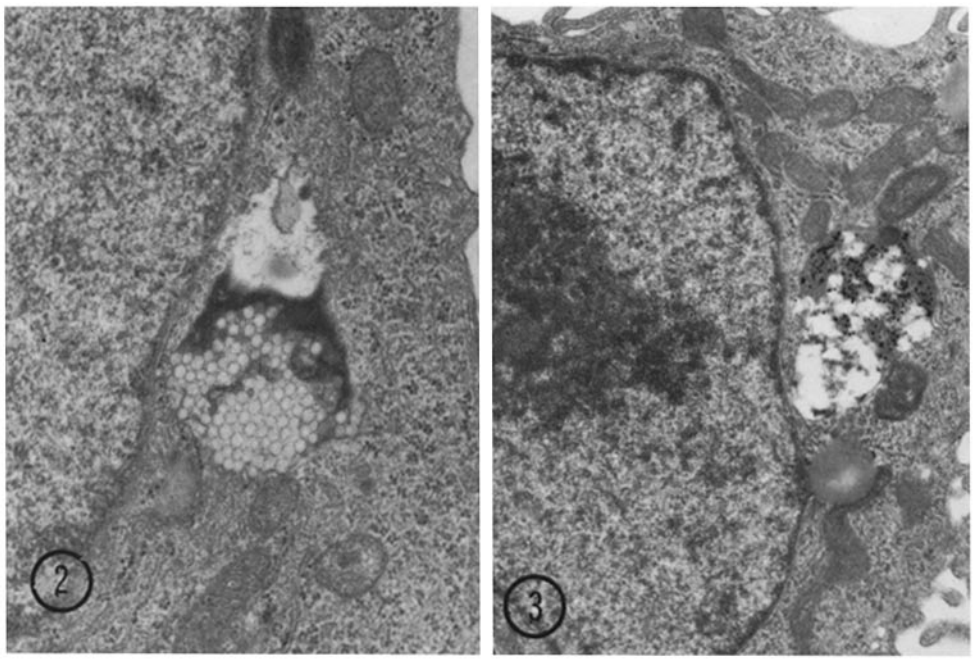

FIGURE 2 Latex spheres in a juxtanuclear inclusion in C4I. × 25,000.

FIGURE 3 Gold particles in a juxtanuclear inclusion in C4I. × 14,000.

samples was performed only after fixation as a means of concentrating the cells before processing for electron microscopy. The maximum time interval between actual mixing of the cells and the addition of fixative in these "0 h" samples was <15 s. Even within these extremely short intervals, apposed plasma membranes could be detected between dissimilarly labeled cells (Fig. 4). These contacts were always punctate and occurred either between apposing microvilli or between the microvillus of one cell and small protuberances of the cell body of another. No modification of the cytoplasm subjacent to such regions could be detected. These areas contained fine, filamentous material and, except for pinocytotic vesicles, were devoid of organelles including ribosomes or glycogen granules.

15 min after mixing, areas of increased cytoplasmic density were seen at one (Fig. 5) or both sides of the region of membrane apposition. The distances between the apposing plasma membranes were not >12 nm (Fig. 6). Dense material was present in the intercellular space but was not organized in any recognizable pattern and remained finely filamentous or granular. At this stage the cytoplasmic densities were often somewhat out of register, and the width of the intercellular space between the apposing membranes was variable. The cytoplasmic densities seen at 15 min after mixing were, for the most part, usually confined to the narrow organelle-free zone underlying the plasma membrane. On some occasions, however, dense filamentous extensions, penetrating deeper portions of the cytoplasm, were sometimes observed. In these instances the cytoplasmic densities on adjacent cells were always paired and had a more organized, plaquelike configuration.

Filamentous extensions of cytoplasmic densities were more common in cells allowed to aggregate for 30 min. At this time the apposing membranes were still separated by only ~12 nm. The cytoplasmic densities, by now in strict register, showed regions of enhanced density and organization and assumed somewhat more distinct plaquelike configurations. The associated cytoplasmic filaments arranged at approximately right angles to the plane of the developing plaque were poorly defined and not susceptible to precise measurement. The plaque formation was accompanied by a densification of the extracellular material in the corresponding region of cell apposition. Yet, the extracellular material still showed little organization (Fig. 7).

60 min after mixing, the plaques of the forming

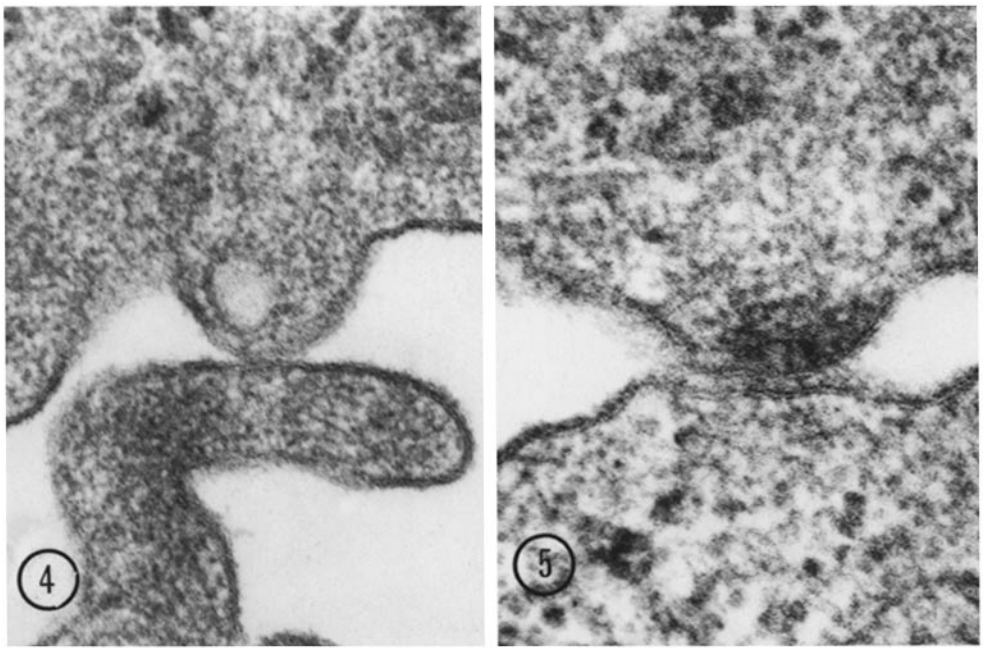

FIGURE 4 A close contact between dissimilarly labeled cells fixed immediately (within 15 s) after mixing. × 114,000.

FIGURE 5 A junction between dissimilarly labeled cells fixed 15 min after mixing. A submembrane density is visible in one cell. × 160,000.

desmosomes had a more organized, less diffuse appearance. The filaments were more discrete and began to assume a more oblique orientation towards the plaques. The width of the intercellular space increased to 20 nm. The dense material had taken on a distinctly granular appearance. Fully mature desmosomes, with organized extracellular dense material in a 30-nm intercellular space and well-defined plaques, were present by 1½ h after mixing (Fig. 8). They were associated with 10-nm tonofilaments oriented obliquely to the plane of the plaques.

Samples fixed 3 h or more after mixing sometimes displayed occasional unpaired plaques associated with tonofilaments at the surface of a few cells. Although not studied systematically, they were apparently substantially more common than those seen immediately after preincubation.

Although larger in size, aggregates fixed 6, 12, 15, 18, and 24 h after mixing were essentially identical to those seen after 3 h. Mitotic figures were common up to 24 h after mixing, the longest interval studied.

DISCUSSION

The formation of *adherentes* junctions, including desmosomes, has been observed in a number of experimental settings. These have included normal development (1–3, 8, 13, 16, 18, 22–24, 34, 39, 44, 46, 47, 49, 61, 64, 70, 71, 76), wound healing (33), and aggregating cells (4, 14, 25, 29, 36, 38, 47, 49, 50, 52–55, 57, 63, 65).

The details of the various stages in the formation of desmosomes, however, have been systematically described in only a few reports (33, 34, 46, 47). These studies are in general agreement with one another and with the results reported here, except for some discrepancies as to whether intracellular (47) or extracellular (36, 46) specializations are the first to become visible or whether they appear simultaneously (34).

Orwin et al. (46) reported close (<5 nm) membrane apposition preceding desmosome formation, but others (33, 34, 47) did not. We have observed close membrane apposition in our 0-h specimens, but whether this represents a stage in desmosome formation is presently unclear.

The single, unpaired densities observed in our 15-min samples, although apparently precursors of plaques, should not be confused with the single plaques reported to represent stages in desmosome formation in other studies (23, 24). The latter were

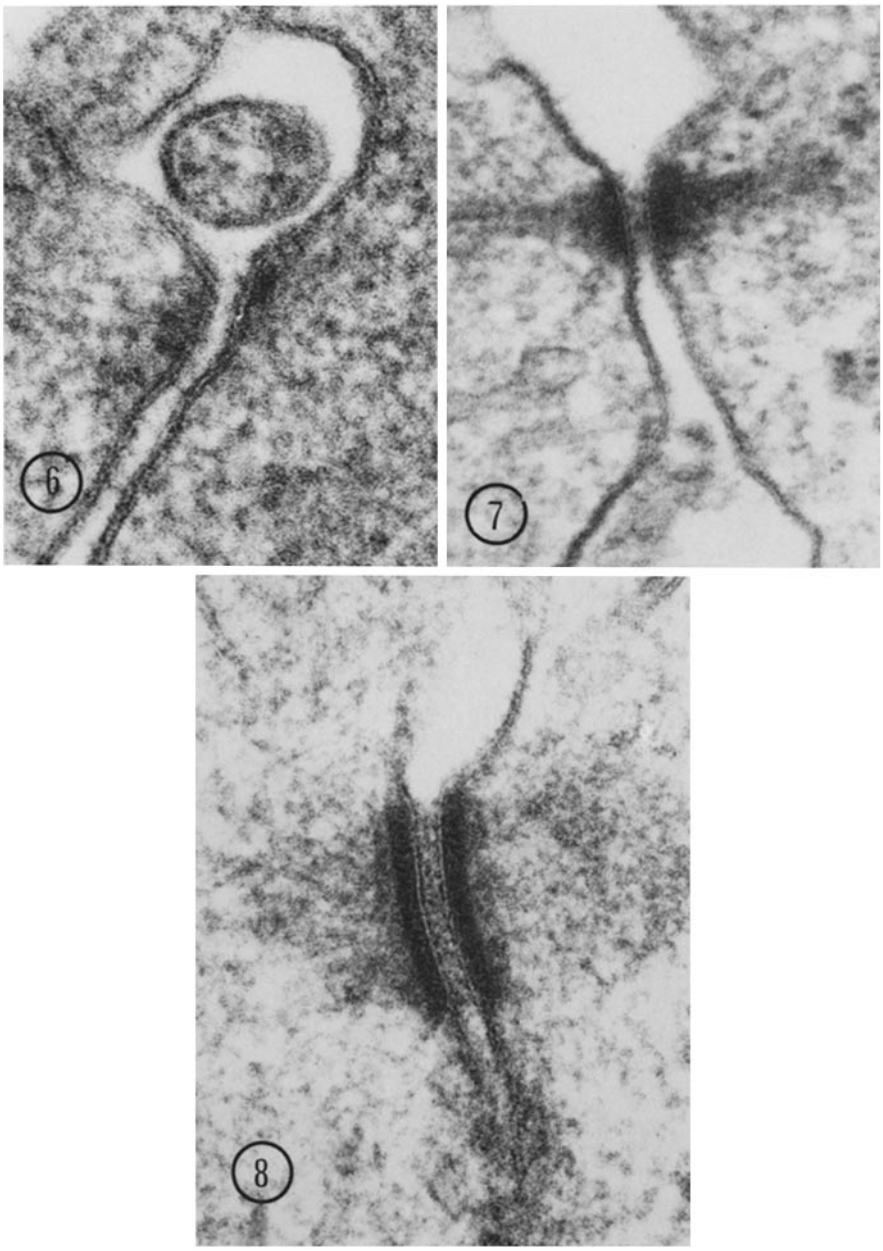

FIGURE 6 A junction between two dissimilarly labeled cells fixed 15 min after mixing. Two small, indistinct cytoplasmic densities, out of register with one another, are visible. × 190,000.

FIGURE 7 A junction between two dissimilarly labeled cells fixed 30 min after mixing. Two distinct, dense plaques, in strict register, are associated with poorly defined filamentous material oriented at right angles to the plane of the plaques. × 160,000.

FIGURE 8 A mature desmosome between two dissimilarly labeled cells fixed 90 min after mixing. The intercellular material is well organized and the tonofilaments are oriented obliquely to the plane of the plaques. × 160,000.

mature, well-organized desmosomal plaques associated with tonofilaments essentially similar to the desmosomal fragments seen immediately after dispersion, and to the occasional unpaired plaques we observed in our 3-h and older samples. It is our conclusion that these plaques represent the remains of desmosomes that have split during cell movement in the growing aggregates and are not stages in the *de novo* formation of desmosomes.

The timing of desmosome formation has been estimated from minutes (36) to hours (47, 52) to a day or more (55, 71). It is possible that these discrepancies are the result of real differences in the rate of desmosome formation in the various tissues studied. On the other hand, it seems at least equally likely that the apparent inconsistencies resulted from the imprecision inherent in estimating actual times in a faulty experimental setting. The drawbacks of using whole tissues undergoing development or cell suspensions in which desmosomes may have persisted through the dispersing procedure (56, 69) and in which no provisions were made for the internalization of desmosomal fragments or cell surface repair have been discussed in the Introduction.

It is evident from published studies using various other cell types (5, 9, 11, 17, 19, 20, 27, 30, 35, 40, 41, 45, 52, 60, 62, 66, 68, 72, 75), that the cell surface is reconstituted within 24 h after trypsinization (but see reference 10). Thus, preincubating the dispersed cells for 24 h before mixing presumably allows sufficient time for the repair of any changes to the cell surface caused by the dispersing procedure. The same preincubation period also permits the internalization of desmosomal fragments formed as the result of dispersion. Overton (48) has observed internalization of desmosomal fragments within 3 h after trypsinization of chick embryo tissues. We have observed internalization in C4I immediately after dispersion. By the end of the preincubation period, desmosomal fragments at free surfaces of the cells were extremely rare. The use of differentially labeled cells allows us to accurately determine the maximum possible age of any junction found between dissimilarly labeled cells.

The system described in the present report has allowed us to observe the sequence of events and the timing of the various stages of desmosome formation with a greater degree of accuracy than was possible with other systems. In addition, the model lends itself to the experimental interventions required for the analysis of the mechanisms involved in desmosome formation.

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