EXPERIMENTAL MANIPULATION
OF DESMOSOME FORMATION

JANE OVERTON
From the Whitman Laboratory, University of Chicago, Chicago, Illinois 60637

ABSTRACT
In the corneal epithelium of the embryonic chick there is a 3- to 4-fold increase in desmosomes between the 15th and 16th days of incubation which has not been noted in earlier studies of this tissue. This finding has made it feasible to study the effects of the local cell environment on desmosome formation. Cells of 15-day corneas which were forming desmosomes rapidly, were dispersed and combined in culture with cells from 10-day corneas which were forming few desmosomes. Surfaces of the same 15-day cell which were confronted with either another 15-day cell or a 10-day cell were compared. Desmosomes formed preferentially on the surface adjacent to a like cell. When 15-day cells were confronted with pigment cells, desmosomes formed almost exclusively on the surface adjacent to a like cell. Evidence for such localized differences on the same cell surface emphasize the importance of the immediate cell environment in desmosome formation. The observation that single desmosome plaques form occasionally on lateral cell surfaces has been noted previously. This finding was confirmed.

INTRODUCTION
Desmosomes may form on the cell surface with a particular pattern (Mercer, 1965) or orientation (Kelly, 1966), or in particular restricted regions. The question posed in this report is whether local differences external to the plasma membrane influence the site of desmosome formation. In order to study this question most readily a tissue is required in which desmosome formation is rapid and of known extent. The corneal epithelium, in which desmosomes are prominent (Jakus, 1961), has been studied in the chick embryo through several days of incubation. A rapid increase in desmosome formation which has not been previously reported was observed between the 15th and 16th days, and therefore this material seemed suitable for further study. Cells in which desmosomes formed very rapidly in vivo were confronted in culture by cells in which desmosomes formed slowly. Cells of the former type were also confronted with foreign cells. Examination of different parts of the surface of a given cell then showed that in these instances the local cellular environment did indeed determine whether or not a desmosome would form.

A systematic study of normal desmosome formation in the corneal epithelium was made as a necessary background for the experimental work. In this tissue, as has been reported previously (Hay and Revel, 1969), desmosomes in the sense of maculae adherentes (Farquhar and Palade, 1963) begin to increase in number about the tenth day of incubation. Hay and Revel noted an unusual feature of the process. In early stages of desmosome formation, they described a high proportion of single plaques on lateral cell surfaces. Since such single plaques might also be expected to appear on cell surfaces in cultures, this aspect of normal development was included in the descriptive account. The role of these single plaques remains unknown.
MATERIALS AND METHODS

Experiments were carried out with White Leghorn eggs incubated in a Sears Roebuck & Co., Inc. incubator (Chicago, Ill.) at 38°C. Corneas from staged (Hamilton, 1952), intact chicks were fixed by Karnovsky's method (Karnovsky, 1965) as described by Hay and Revel (1969) and stained with uranyl acetate en bloc. Tissue was embedded in Araldite in flat embedding molds to facilitate orientation. Corneas were cut into pie-shaped pieces, and sections were made from the inner corner of the piece, according to the method of Hay and Revel, to insure that sections all came from comparable parts of the cornea. Sections were cut with a glass or diamond knife, and stained with lead citrate (Venable and Coggeshall, 1965). They were viewed with a Hitachi HU 11A electron microscope, and micrographs were taken for desmosome counts at magnifications of between 5,000 and 6,000.

Sections from three to four blocks of each cornea were examined and the entire epithelial layer was included in the micrographs. The final sample counted included between 50 and 100 nuclear cross sections. Results were expressed as desmosomes/nuclear cross section. The diameter of corneal cells was checked by measuring freshly isolated cells with an ocular micrometer. 25 cells in each sample were measured on days 12, 14, 15, and 16. Diameters averaged 4.0, 4.4, 4.4, and 4.5 μm, respectively. Desmosome counts were made from negatives viewed with a dissecting microscope at a magnification of 6. The number of nuclear cross sections for each micrograph was estimated to the nearest half nucleus. Desmosomes were classified as either "double" or "single". Double plaques were either symmetrical or asymmetrical images. An asymmetrical image of a double plaque could be due to the angle of section as well as actual asymmetry in the plaques. Plaques counted as single had little or no density associated with the opposite cell surface. Very frequently, desmosomes were obviously present, but could not be counted because the section cut through them so tangentially that no definite density immediately next to the membrane was distinguishable. Examples of desmosomes classified as single are given in Figs. 2 and 3. Although in this work a count of six desmosomes/nuclear cross section in the cornea was the highest average obtained, the actual number along a given cell boundary was often much higher, since in thin sections many plaques were not able to be classified. Hemi-desmosomes on the basal surface were not counted.

The number of cell layers in the corneal epithelium between the 10th and 16th days was estimated by counting the number of cells which lay under a line lying normal to the free epithelial surface. Five such counts were averaged for each day.

Epithelium was cultured intact or after dissociation and pelleting. The epithelium of 15-day chick cornea was removed from the mesenchyme by incubating whole corneas for 1 h in 2.5 mg/ml of collagenase (Worthington CLS, Worthington Biochemical Corp., Freehold, N. J.) made up in calcium-magnesium-free Tyrode's solution (CMF) at room temperature. In some cases the 10-day cornea was isolated by treatment for 50 min with 0.04% EDTA (Matheson Co., Inc., East Rutherford, N. J.) in CMF (Dodson and Hay, 1971). The epithelium was gently lifted off the underlying tissue, rinsed, and placed for culture on a Millipore filter (Millipore Corporation, Bedford, Mass.) which was either floating on the surface of the culture medium on a raft of siliconized lens paper in a 35-mm Falcon Petri dish (Falcon Plastics, Div. of B-D Laboratories, Inc., Los Angeles, Calif.), or supported by a plastic frame at the air-liquid interface in a 60-mm Falcon organ culture dish. Tissues were cultured in 2 ml of L15 tissue culture medium (Difco Laboratories, Detroit, Mich.) with 10% fetal calf serum and 50 U/ml each of penicillin and streptomycin.

In the first method for dissociation, both 10- and 15-day epithelia were treated with trypsin (Armour Tryptase, 10,000 U/ml in CMF) Armour Industrial Chemical Co., Chicago, Ill. for 30 min at 38°C. After trypsinization, cells were rinsed twice in culture medium, then dissociated in culture medium by pipetting. Dissociated cells were then filtered through four layers of lens paper in a Millipore Swinny adapter to remove clumps. Cell suspensions were counted, and viability was checked by the eosin exclusion test (Cahn et al., 1967). 95% or more of the cells excluded eosin. 5-10% of the cells were in clumps of five cells or fewer. Cell suspensions were pelleted by gentle centrifugation, and after 1 h of incubation pellets were transferred to culture dishes. Examination of 15-day corneal pellets after 24 h of culture showed some cell death, but, as seen by Table I, gave no indication that desmosome formation was inhibited.

In a second method for dissociation, epithelia were incubated in 0.5% trypsin (Difco 1:250) and 0.25% EDTA in CMF for 25 min at 38°C, rinsed, and dissociated by pipetting in culture medium. By this method, dissociation was almost complete and filtering was not deemed necessary. The dissociated cells were pelleted and cultured 24 h in 1 cm³ of medium as pellets in centrifuge tubes on a slowly rotating drum. Using this method, cultures generally appeared healthy.

For isolation of pigment cells the pigmented retina of the 10-day chick was used as a source. Eyes were dissected out and incubated 15 min in 3% trypsin (Difco 1:250) in CMF at 38°C, after which the entire outer choroid layer was removed (Trinkaus and Lentz, 1964). The pure retinal pigment layer could
then be dissected free. Pigment cells were treated by incubation in 0.5% EDTA 10 min at room temperature followed by 3% trypsin for 15 min at 38°C. After this procedure, cells could be easily isolated by pipetting. Foreign cells were mixed 50:50 with 15-day corneal epithelial cells before pelleting. Cultures were prepared for electron microscopy in the same manner as intact tissue.

In cultures with mixed cell types, 15-day corneal cells sorted out from either 10-day cells or from pigment cells, but in many regions where clusters were in contact, or where an occasional foreign cell became trapped in a group of 15-day cells, micrographs could be obtained of fields including two cell types. These regions were thus selected parts of the cultures, but within a given field there was no selection of 15-day cells which would be used for a sample. Every 15-day cell in a field which made contact with a foreign cell on one part of its surface and with another 15-day cell at some other region of the surface was included. Micrographs were printed at magnifications of 16,000-25,000 and the relevant sides of a given 15-day cell were measured with a Dietzgen map measurer (Eugene Dietzgen Company, Chicago, Ill.). Desmosomes were counted on these surfaces and the results were expressed as microns of surface cross section/desmosome (double plaques).

RESULTS

Normal Development of the Corneal Epithelium

Fig. 1 summarizes the findings on desmosome development in the normal intact corneal epithelium in vivo. Desmosomes seen as double plaques (solid circles) occur infrequently on day 10 in this sample, with only 0.3 plaques/nuclear cross section. In the course of the succeeding 5 days there is a gradual and consistent increase to between one and two desmosomes/nuclear cross section on day 15. During the next 24 h period a rapid 3-4-fold increase in desmosome frequency occurs. Each symbol represents the results from one cornea, and each cornea was taken from a different embryo. The 29 embryos used to obtain the data were all fixed during a 2-3-wk period so that there was little chance for seasonal variation of the eggs. The results appear to be consistent, except for a wide spread of values on day 16. However, such a spread might be expected since when a rapid change occurs the onset would be somewhat variable in individual embryos.

Counts of single plaques (open circles) also increased to day 15 and showed the greatest increase between day 15 and day 16, at the time when desmosomes (double plaques) were being formed most rapidly. Because a high frequency of single plaques in early stages has been noted by others (Hay and Revel, 1969), the ratio of single to double plaques was examined. This ratio is illustrated in Fig. 1 (triangles) and shows that the highest proportion of single plaques occurs early in the process. On day 12 the proportion of single plaques is 0.33 and, as the total number of desmosomes increases over the next 4 days, the proportion of single plaques drops.

Figs. 2 and 3 illustrate single plaques in the 12-day cornea. Single plaques may consist of material of considerable electron opacity next to the plasmalemma, or distinct plaques which may have filaments associated with them (Fig. 3). The distance between cells at the sites where these single structures appear may be variable. The plasma membrane of the cell in which the plaque lies is generally flattened in the plaque region, but the surface of the cell immediately opposite may not be. In 16-day corneas it is more common to find both opposed cell surfaces flattened, one with and one without a plaque.

As the desmosome number in the epithelium increases, the number of epithelial cell layers increases concurrently. Estimates of the number of cell layers in the epithelium are plotted in Fig. 1 (squares). Between days 10 and 16 the number of layers increases from three to six. This increase coincides with the time during which desmosomes are increasing in number, but the kinetics differ. The rate of increase in thickness of the epithelium...
Desmosome formation in corneal epithelium. Solid circles, solid line: double plaques. Open circles, solid line: single plaques. Open triangles, dashed line: average ratio of single to double plaques. Solid triangles on days 12 and 16: individual ratios of single to double plaques; there is no overlap. Open squares, dashed lines: average number of cell layers in the epithelium.

is a consistently gradual one, while that of desmosome formation is not. A comparison of Figs. 4 and 5 illustrates the change in epithelial thickness and the increase in desmosome frequency. It can be seen from Fig. 4 that desmosomes are more numerous in the upper layers of cells. This was not noted in earlier studies of the cornea, but it is a common feature of epithelial development (Mercer, 1965). This difference may be related to the differentiation of these outer two to three layers as flattened and especially electron opaque. No study was made of the frequency of single plaques at different levels of the epithelium but they were not confined to any particular level.

Single or asymmetrical plaques occur not only in association with developing desmosomes as seen here, but also when desmosomes degenerate during tissue turnover (Odland and Reed, 1967; Listgarten, 1964; Horstmann and Knoop, 1958). In the 16-day cornea, cells of the outermost epithelial layer occasionally stain very lightly, which could be due to incipient desquamation. In such cells a number of instances were found suggestive of desmosome degeneration such as the distinctly asymmetrical desmosomes in Fig. 6. The evidence taken as a whole suggests that if degeneration is responsible for producing some single plaques, it is probably confined to the terminal part of the period studied. Although the single plaques recorded in Fig. 1 may include some degenerating desmosomes or even some asymmetrically cut desmosomes, this could not account for the bulk of single plaques counted.

Cultured Corneal Epithelium

It was felt that the pronounced increase in the number of desmosomes between days 15 and 16 might be used as an assay to study desmosome formation if the effect were obtained in culture.
Figure 2  Corneal epithelium, day 12. Arrows indicate single plaques. Bar, 0.5 μm. X 32,000.

Figure 3  Corneal epithelium, day 12. Arrows indicate single plaques. Bar, 0.5 μm. X 50,000.

Figure 4  Corneal epithelium, day 16. Arrow indicates cluster of desmosomes. Bar, 1 μm. X 6,000.
Inset, arrows indicate desmosomes which cannot be included in a count. Bar, 1 μm. X 75,000.

Figure 5  Corneal epithelium, day 10. Arrow indicates characteristic endoplasmic reticulum. Bar, 1 μm. X 8,000.
15-day corneal epithelia were cultured for 24 h. The results appear in Table I. Cultured epithelia show a 3-4-fold increase in desmosomes as does cornea in vivo. The presence of mesenchymal components of the cornea thus does not appear to be essential for this process.

**Dissociated Corneal Cells**

If, as mentioned above, conditions immediately external to the plasma membrane can determine where a desmosome will form, then by altering conditions one might alter the pattern of desmosome formation. To achieve this, cells from 10-day corneal epithelia which were at the very beginning of the process of desmosome increase and which after 24 h in vivo could be expected to have less than one desmosome/nuclear cross section, were dissociated and combined 50:50 with dissociated 15-day cells.

The latter should be forming desmosomes very rapidly over the next 24 h. The quantitative results of this experiment are reported in Table I. It is clear that after 24 h fewer desmosomes are formed in the 10- and 15-day cell cultures than in 15-day cell cultures alone. If 15-day corneal cells are diluted by one half with 10-day cells which form a negligible number of desmosomes, one might expect half as many desmosomes to form (2.86) as in 15-day cells alone (5.72, see Table I), provided these corneal cells are unaffected by their new environment. The actual value (2.33) approaches this.

Observation of the mixed cultures showed that in certain regions the number of desmosomes was very high, while in other regions desmosomes were few or lacking (Figs. 7-11). In these cultures it was possible to identify many of the cells as being characteristic of cells from either 10- or 15-day corneas. They could thus be tentatively considered as coming from a particular source and appeared to cluster in the culture with cells of their own type. The cytoplasmic characteristics of maturing corneal cells have been described previously (Hay and Revel, 1969). The most useful diagnostic feature of the 10-day cell is the abundant endoplasmic reticulum which is of relatively regular diameter, has a slightly electron-opaque content, and tends to lie in parallel cisternae (Figs. 5, 10, and 11). In normal 10-day cornea (Fig. 5) this trait is pronounced, but by 11 days, in keeping with the changing synthetic activity of the cornea (Trelstad, 1970), a few cells have begun to lose this trait, and by 12 days it has been lost by many. The endoplasmic reticulum becomes much more irregular in outline. Although it was not possible to characterize the source of every cell in a culture, distinctions could be made in a large number of cases.

There are three distinctive surface regions in epithelial cells of the 15-day cornea: lateral, free, and basal. Since the epithelium is five to six cells thick, many cells have only lateral surfaces. In pellets the cells with free or basal surfaces generally seem to reach the exterior of the culture, but when individual cells are trapped inside they form a basal surface (Fig. 8) or a free surface (Fig. 9) around a spherical vesicle or cavity in the culture. The basal cytoplasm is characterized by hemidesmosomes and numerous microfilaments; the free surface can be distinguished by microvilli.

It is the lateral cell surfaces that most concern us here. When cells of epithelia are dispersed, desmosome plaques are often taken into the cytoplasm in vacuoles (Overton, 1962, 1968; Berry and Friend, 1969; Fischman and Moscona, 1969) and in some tissues appear to break down completely. Desmosomes which form in culture on lateral surfaces may all be newly organized. However, in rare instances such as that shown in Fig. 7 desmosomes associated with vacuoles or channels in the cytoplasm have been observed after 24 h of culture. Such cases could possibly have persisted from the original experimental disruption of the tissue or could have arisen later in the culture period. Figures of this kind have been observed only once in the intact cornea.

Under the culture conditions used here, despite the possibility that some desmosomes may have persisted from the original 15-day tissue, we know that most of them are newly formed, since the desmosome frequency rises so rapidly between the 15th and 16th day. Therefore, this material should provide an answer to the question of whether or not desmosomes form more readily on a surface with a particular microenvironment. In Table II the amount of surface seen in section/desmosome is compared on two different sides of the same 15-day cells. In combinations of 10- and 15-day cells, 42% of the measured cell surface lay between cells from different sources. Of the 50 cells in the sample, only two showed a smaller amount of surface/desmosome on the side opposed to the 10-day cell. In these two cases the number of desmosomes was low. In six cases there were no desmosomes on either surface.
FIGURE 6 Corneal epithelium, day 16. The cell in the upper part of the figure at the free surface of the epithelium takes the stain more lightly than the cell immediately below it. This corresponds with a lighter stain at desmosome plaques. Bar, 0.5 μm. X 48,000.

FIGURE 7 Pellet of corneal epithelium from 10- and 15-day embryos. Arrows indicate desmosomes which appear to be in channels or vacuoles. Bar, 0.5 μm. X 37,000.

FIGURE 8 Pellet of corneal epithelium from 10- and 15-day embryos. A vesicle in the pellet (V) is surrounded by the basal surface of a cell. Arrows indicate densities which are spaced like hemidesmosomes. Bar, 1 μm. X 29,000.

FIGURE 9 Pellet of corneal epithelium from 10- and 15-day embryos. A vesicle (V) is surrounded by the free surface of a cell which possesses microvilli. Bar 1 μm. X 29,000.
TABLE II
Desmosome Frequency on Different Sides of the Same 16-Day Corneal Epithelial Cell

| Cell types combined | Number of cells | Micra/desmosome (double plaques) | Number of single plaques observed |
|---------------------|----------------|----------------------------------|---------------------------------|
|                     | side A | side B | (same cell type) | side A | side B |
| 10- and 15-day corneal epithelium | 50     | 2.04  | 25.1            | 1     | 6     |
| 15-day corneal epithelium and pigmented retina | 50     | 1.75  | 35.0            | 3     | 1     |

In the remaining 42 cases the side of the cell adjacent to a cell of unlike type showed a higher number of micra of surface cross section/desmosome than the side adjacent to a cell of the same type. Thus, desmosomes form preferentially on the latter side. It appears that a desmosome is more than ten times as likely to form next to another 15-day cell as next to a 10-day cell. The number of single plaques observed in this sample is recorded in Table II. There are so few single plaques that no firm conclusions can be drawn, but it is interesting to observe that more single plaques were found on the side adjacent to the 10-day cell than on the side adjacent to a cell of the same type. 10-day cells were not forming plaques as rapidly as 15-day cells.

The results of mixing corneal epithelium cells of different ages were compared with the results of mixing 15-day corneal cells with cells from a different tissue. Pigmented cells were chosen since they provided an unambiguous cytoplasmic marker (Fig. 12). Nuclear markers were not considered, since the nucleus of a cell frequently does not appear in a section. It can be seen from Table II that essentially the same results were obtained. 41% of the measured cell surface lay next to pigment cells. 46 cases had less surface/desmosome on the side adjacent to a like cell. There was a single exception, in which the number of micra/desmosome was less on the side of the cell adjacent to a pigment cell. A total of six cells had desmosomes on the surface facing the pigment cell. This is consistent with the finding of Armstrong (1970) that pigment cells can form desmosomes with unlike cells. However, in the present case, misclassification of cell types could also contribute to such results. By ten days of incubation the retinal pigment cells have numerous pigment granules throughout the cytoplasm but it still could be possible to obtain a section of such a cell that did not include pigment. Despite these uncertainties of cell identification, the major result seems clear.

In comparing results of the two types of cultures recorded in Table II, it can be seen that the number of desmosomes formed between 15-day cells under different culture conditions was roughly the same. There are approximately 2 μm of surface cross section/desmosome in both instances. One may calculate the micra of surface cross section/desmosome, using the measurements of cell diameter (Materials and Methods section) and the number of desmosomes per nucleus in 16-day cells (Table I). In the normal intact 15-day cornea this value is also approximately 2 μm. Thus, a localized difference in external environment causes a corresponding localized deficiency in desmosome number with little or no interference in desmosome formation elsewhere in the cell.

DISCUSSION

The work presented here demonstrates that the location in which a desmosome forms on the cell surface is determined at least in part by the local environment of the plasma membrane. 15-day corneal epithelial cells in vivo and in culture have the capacity to form desmosomes rapidly on lateral surfaces, yet when cells of different types are confronted desmosomes show a selective distribution. They form preferentially between 15-day cells. It is not the intent to suggest that an environmental influence can result in formation of a desmosome anywhere on the cell surface. In the normal corneal epithelium, cells in the central layers form desmosomes (matched plaques) on all surfaces, but those at the free and basal epithelial surfaces do not. In the present experiments cells excluded their free and basal surfaces from cell contact so that desmosomes formed only on lateral surfaces where their appearance was normal. Thus, the experiments do not negate the idea that only certain parts of the surface are responsive to environmental influence.

Although the experimental demonstration of environmental influence on desmosome formation is new, the idea itself is an old one suggested by observations of normal tissue (Campbell and Campbell, 1971). One of the most striking exam-
Figure 10 Pellet of corneal epithelium from 10- and 15-day embryos. Arrows indicate desmosomes. 10-day cell (10) possesses characteristic endoplasmic reticulum. Bar, 0.5 μm. × 37,000.

Figure 11 Pellet of corneal epithelium from 10- and 15-day embryos. Arrows indicate two desmosome plaques in the 15-day cell which were classified as single plaques. Bar, 1 μm. × 25,000.

Figure 12 Pellet of corneal epithelium from 15-day and pigmented retina cells (P) from 10-day embryos. Arrows indicate desmosomes. Bar, 1 μm. × 12,000.
examples is shown by fine structure studies on the epidermis (Breathnach and Wyllie, 1967). Here Langerhans cells which are characterized by the lack of desmosomes lie in the stratum spinosum where desmosomes are abundant. Stratum spinosum cells have an abrupt change in surface organization in those regions lying adjacent to Langerhans cells where no desmosomes occur, although desmosomes are numerous over the rest of the cell surface. In this case and in the experiments with mixed cells presented in this report, there appears to be a strictly local response to environmental differences.

Experiments with mixed cells do not only show that desmosome formation is a localized response, but also provide an example of cell interaction resulting in inhibition. There are numerous cases in the literature of inhibiting effects between experimentally mixed cells: for example, liver cells cultured with cartilage cells prevent chondroitin sulfate synthesis (Abbott and Holtzer, 1964) and, likewise, coaggregates of 16-day and 10-day embryonic neural retina cells reduce glutamine synthetase inducibility of the 10-day cells (Morris and Moscona, 1971). Also, contact between like cells may cause reduced synthesis. Inhibition of growth in tissue culture occurs at saturation density at which time synthesis of DNA, RNA, and protein is much reduced (Stoker, 1967). Although it is established that surface or close-range contacts between cells can inhibit synthetic activity, examples of localized inhibition are rare. So little is known of the process of desmosome formation that one cannot even determine whether the effect is a local inhibition of synthesis of desmosome components or a fault of assembly.

The interesting finding of Hay and Revel (1969) that single plaques on lateral cell surfaces are associated with early stages in desmosome formation has been confirmed here. Although it seems possible that they represent an early stage in desmosome formation, their role remains enigmatic. The presence of single plaques has not been observed in studies of desmosome formation in other embryonic tissues (Overton, 1962; Hay, 1968; Lentz and Trinkaus, 1971) but has been reported in two forms of slow growing Morris hepatomas (Hruban et al., 1972). These single plaques may be presumed to be distinct from hemidesmosomes which form on the basal surface, since hemidesmosomes have a somewhat different morphology (Kelly, 1966; Hay and Revel, 1969). There is also some indication that different factors control their development because diseased conditions are known in which desmosomes are absent yet hemidesmosomes remain unimpaired (Wilgram, 1964).

Experiments described here might be considered a study of one aspect of cell adhesion in developing tissues, since it is generally acknowledged that desmosomes have an adhesive function. However, the results are probably not applicable to many situations in which embryonic cells have been used for studies of cell sorting, adhesion, and reaggregation because it is known that desmosomes in the sense of macula adherens form late in development (Hay, 1968; Lentz and Trinkaus, 1971) when major cell movements are completed and cytodifferentiation is underway.

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