Epithelial Layer Formation in Differentiating Aggregates of F9 Embryonal Carcinoma Cells

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ABSTRACT F9 embryonal carcinoma (EC) cells, cultured in suspension in medium containing 5 × 10^{-8} M retinoic acid, aggregate and differentiate into embryoid bodies with an outer layer of visceral endoderm cells that synthesize and secrete alphafetoprotein (AFP) (Hogan, B. L. M., A. Taylor, and E. Adamson, 1981, Nature (Lond.). 291:235-237). Here we analyze the formation of the outer layer of cells as a model for epithelial differentiation. Three morphological phases are described, but analyses of cell numbers and the synthetic rates of some proteins, as well as the appearance of markers of visceral endoderm and basement membrane, show that the formation of the outer layer occurs as an orderly progression of multiple events. The markers used to follow the ontogeny of epithelial layer formation include SSEA-1, I, and i blood group antigens, laminin, fibronectin, type IV collagen, cytoskeletal intermediate filament proteins (vimentin, Endo A, and B), and AFP. The onset of epithelium formation occurs between the third and fourth day of culture, but its function is maximally expressed only when it is well organized. We found the rate of AFP secretion to be a measure of the proper alignment and maturity of the epithelium which occurs at the seventh or eighth day. This model of epithelium formation may help to explain how similar processes occur during embryogenesis.

F9 cells are a clonal cell line of mouse teratocarcinoma-derived embryonal carcinoma (EC) cells. Normally, F9 cells show very little spontaneous differentiation in vivo or in vitro. However, given certain conditions, F9 cells have been shown to differentiate into two distinct populations of extraembryonic cell types, parietal and visceral endoderm. F9 monolayers treated with retinoic acid (1) and dibutyryl cAMP (2) differentiate into an early embryonic cell type, parietal endoderm. Recently, it has been shown that F9 EC cells treated with 5 × 10^{-8} M retinoic acid for 6 to 8 d in suspension culture differentiate into aggregates called embryoid bodies. Embryoid bodies are so-called because they morphologically resemble early mouse embryos at the two-layered stage. The outer layer of embryoid bodies synthesizes and secretes alphafetoprotein (AFP) (3) which is characteristic of visceral endoderm (4).

The formation of embryoid bodies may be useful as a three-dimensional model of some of the processes occurring in early embryogenesis, such as endoderm differentiation, cavitation, and epithelial layer formation. In common with most epithelial layers, the visceral endoderm layer in this system consists of a sheet of cells underlaid by a thin basement membrane. We used this model to investigate the formation and maturation of an epithelial layer.

We made a detailed analysis of the growth and differentiation of the aggregates using metabolic radiolabeling and immunofluorescent staining. The distribution of marker proteins and antigens in the process of formation of the epithelial layer during the differentiation of the F9 embryoid bodies is described. Following the aggregation of EC cells, overlapping but clearly defined stages (cell proliferation, differentiation, basement membrane formation, and outer cell alignment) occur which lead to the formation of an epithelial layer of functioning visceral endoderm cells.

MATERIALS AND METHODS

Cells: F9 embryonal carcinoma (EC) cells (5) were cloned, and we selected a subclone (B1) based on its increased ability to differentiate in suspension cultures (measured by AFP production, see below). Clone B1 was used in all experiments (referred to as F9). The cells were maintained and passaged as described previously (6).

Antisera: For immunofluorescence, the antisera used were affinity-purified rabbit antibodies to mouse AFP (7) and mouse type IV collagen (8), rabbit antiserum to rat laminin (9), and rabbit antiserum to mouse fibronectin, absorbed with human fibronectin (10). In some experiments, the antifibronectin serum was also absorbed with bovine fibronectin before use. Both antilaminin and antifibronectin were provided by Dr. Eva Engvall (La Jolla Cancer Research Foundation). Rabbit antiserum to purified mouse Endo A and Endo B (11) reacts with endodermal cytoskeletal proteins. Human autoantibodies to human blood group antigens I (anti-I-Ma) and i (anti-i-Denb) were obtained from Dr. E. R. Gibble.
(Puget Sound Central Blood Bank) and from Dr. M. C. Crookston (Toronto General Hospital), respectively, and were provided to us by Dr. Michiko Fukuda (La Jolla Cancer Research Foundation). Monoclonal mouse antibodies (obtained from the culture fluid) to stage-specific embryonic antigen (SSEA-1) (12) were provided by Dr. D. Solter (Wistar Institute).

For immunoprecipitation, we used IgG fraction of rabbit anti-mouse laminin (13), rabbit antinmouse Endo A and Endo B (11), and rabbit antiserum to baby hamster kidney (BHK) vimentin (a gift from Dr. S. J. Singer, University of California, San Diego).

For ELISA, affinity-purified goat anti-AFP IgG (14) was provided by Dr. Ruoslahti (La Jolla Cancer Research Foundation).

**Differentiation:** To stimulate the differentiation into embryoid bodies, exponentially growing F9 EC cells were dissociated with a trypsin-versene-protein solution (TVP) (15) into single cells and small clumps of up to 5-10 cells. The dissociated cells were seeded at indicated densities in 96-well untreated polystyrene microtiter plates (Linbro/Titertek, cat. no. 76-202-05; Flow Laboratories, Inc., Hamden, CT) in 200–300 µl of a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DME) and Ham’s F-12 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.01 M HEPES buffer, and 5 x 10^-6 M retinoic acid added from a stock of 1 mg/ml (3 x 10^-5 M) in dimethyl sulfoxide. The medium was replaced on days 2, 4, 6, and 7 and the culture was continued for 8 d.

**Measurement of Growth Rates:** At appropriate times after the initiation of suspension cultures in 96-well plates the aggregates were washed in phosphate-buffered saline (PBS), and 200 µl of 1 M glycine, 2 mM EDTA, pH 7.4, was added, and the mixture was incubated at 37°C (16). After ~3 h of incubation, vigorous pipetting disrupted the aggregates into single cells. The cell suspensions were allowed to settle, and the cells were counted with Artek TV camera and cell counter (Artek Systems Corp., Farmingdale, NY).

**Quantitation of AFP:** At appropriate times up to 8 d, the culture medium was collected and frozen at −20°C until assayed; AFP was determined by a ELISA assay as previously described (17). The sensitivity of the assay was such that 10 ng/ml AFP was readily detectable, and the upper limit of the linear portion of the standard curve was at 400 ng/ml AFP.

**Immunofluorescence:** Aggregates of F9 cells were washed with PBS and fixed for 10 min in freshly prepared, cold acidified ethanol (99 ml of 95% ethanol to 1 ml of glacial acetic acid) (18). The aggregates were then washed with cold 70% and 50% ethanol and finally several times with PBS at room temperature. The fixed aggregates were embedded in 1% agar (wt/vol) and immediately frozen in liquid nitrogen. The agar blocks were sectioned on a cryostat and 6-µm sections were air dried onto gelatin-coated slides.

Washed sections were treated for 30 min at room temperature with anti-AFP (1:20), anti-type IV collagen (1:20), anti-rat laminin (1:40), antifibronectin (1:40), anti-Endo A (1:10), anti-Endo B (1:10), anti-i (1:50), anti-I (1:50), or anti-SSEA-1 (1:50). The controls were normal rabbit serum, normal human serum, or normal mouse serum. The sections were washed with PBS and treated with rhodamine-conjugated goat anti-rabbit or fluorescein-conjugated rabbit anti-mouse second antibody (1:40, Cappel Laboratories, Cochranville, PA).

**Metabolic Radiolabeling and Immunoprecipitation:** The F9 aggregates were rinsed in methionine-free medium, then incubated for 4 h in methionine-free DME medium supplemented with 10% FBS and 50 µCi/ml of [35S]methionine (>1,000 Ci/mmol; New England Nuclear, Boston, MA). The aggregates were washed three times in cold PBS containing 10 µg/ml of soybean trypsin inhibitor. The aggregates were lysed by mixing in 2-4 ml of 0.1% SDS, 10 mM Tris-HCl, pH 7.5, 3 mM MgCl2, 0.1 mM CaCl2, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM N-ethyl maleimide, 0.19 trypsin inhibitor units of Aprotinin (Sigma Chemical Co., St. Louis, MO), and 10 µg/ml soybean trypsin inhibitor. After ~30 s of mixing, 0.04 vol of 10% SDS and 0.1 vol of 0.1 M EDTA, pH 7.5, were added. The lysates were heated at 100°C for 2 min, cooled on ice, and a 0.1 vol of 10% Nonidet P-40 was added. The lysates were stored at –85°C.

We performed immunoprecipitation analyses as previously described (19). The immunoprecipitates were solubilized in SDS sample buffer and analyzed by 15% PAGE in the presence of SDS (20, 21). Gels were fluorographed (22) with Kodak XAR-5 x-ray film at −85°C.

**RESULTS**

**Morphological Observations**

Starting almost immediately after seeding in microtiter plates, F9 cells aggregated and formed small tight colonies in
suspension culture. In the absence of retinoic acid, the aggregates were smooth spheres that grew up to 0.5 mm in diameter (Fig. 1a). After 4-5 d in suspension culture, the aggregates were dark and appeared to be necrotic in the center.

In the presence of $5 \times 10^{-8}$ M retinoic acid, F9 EC cells were stimulated to differentiate into embryoid bodies. The process was divided into three stages. In the first stage, the aggregates formed and increased in size. In the second stage, the cells in the aggregates were discernible. The first phase was from days 0 to 3 and the aggregates formed and increased in size. In the second stage, their appearance as observed by phase-contrast microscopy started to change after 3-5 d. A distinct single-celled outer layer became visible (Fig. 1b). We found that the number and size of the aggregates and the time of appearance of the outer layer of cells depended upon the cell numbers of F9 EC cells seeded per well at the start of the culture. The aggregates formed from a higher number of F9 EC cells seeded per well were greater in number and small in size and the outer layer of cells appeared after only 3 d in culture, whereas the aggregates formed from a lower number of EC cells seeded per well were comparatively fewer in number, larger in size and the outer layer of cells appeared after 5-6 d in culture. The third stage occurred after 5-6 d, when most of the embryoid bodies were cystic (Fig. 1c). Only at this stage were the cells of the outer layer stained for AFP in immunofluorescence tests (Fig. 1d).

**Cell Proliferation**

The cells in the aggregates cultured in the presence of $5 \times 10^{-8}$ M retinoic acid proliferated more slowly than in its absence (at about one-half of the rate on average). The number of cells seeded in one well of a 96-well plate (in 0.3 ml of medium) also affected the growth rate of aggregates. Fig. 2 is a plot of cell numbers during 8 d of culture (for simplicity, only the data of seeding densities 150, 1,200, and 4,800 cells are plotted and the data of seeding densities 75, 300, 600, 2,400, and 9,600 cells are not included in Fig. 2). Two phases of cell growth were discernible. The first phase was from days 0 to 3 when rapid proliferation occurred at all densities. The cells in the aggregates formed from lower numbers of starting EC cells (75, 150, or 300) grew more slowly than those formed from intermediate (600, 1,200, or 2,400) or higher seeding density (4,800 or 9,600). From day 3 to 6, however, the cell number of aggregates formed from the higher seeding densities declined, and this was not prevented by daily feeding. The second phase was after day 6 with a new rise in cell number of all seeding densities. It was thus clear that densely seeded cultures proliferated more rapidly and, as shown below, had a striking effect on the time of appearance and the level of AFP.

**Quantitation of Secreted AFP**

It has previously been shown that F9 EC cells cultured in suspension in the absence of retinoic acid show a few spontaneously differentiated cells. These cells could be detected by immunoperoxidase reaction for AFP on sections (3), but AFP was not detectable in the medium by ELISA. When retinoic acid was present, the cultures seeded at high densities produced detectable levels of AFP at 6 d. There was a sharp rise on day 7 and it leveled off by day 8 (Fig. 3). The detectable level of AFP appeared 2 d later in cultures of lower seeding densities. One explanation for the earlier differentiation of aggregates of high cell densities is that the medium is conditioned in these cultures. Components secreted into the medium that appear to affect the course of differentiation are currently being analyzed.

**Appearance of Endodermal Markers and Laminin Synthesis**

We used immunoprecipitation analysis and PAGE to determine the time at which increased synthesis of endodermal marker proteins could be detected. We treated high density cultures of F9 cell aggregates with retinoic acid for various times up to 7 d, washed, incubated for 4 h with [35S]methionine, and solubilized (19). The cell lysates were immunoprecipitated with antisera to vimentin, endodermal cytoskeletal proteins Endo A and B, AFP; and laminin. Fig. 4 compares the products immunoprecipitated by vimentin and Endo A and Endo B antisera. Control cultures not treated with retinoic acid synthesized detectable levels of vimentin (Fig. 4a, lanes 1 and 2) but very low levels of Endo A and B (not detectable at the exposure times shown) (Fig. 4b and c, lanes 1 and 2). Vimentin synthesis increased by the second day of culture (Fig. 4a, lane 4).
then decreased or remained relatively constant through the rest of the experiment (Fig. 4a). The transient increase in the amount of [35S]methionine-labeled vimentin immunoprecipitated from a constant amount of trichloroacetic acid-insoluble lysate was reproducible in three replicate experiments (data not shown).

Increased synthesis of Endo A and B were detectable in cultures treated for 3 d with 5 × 10^-8 M retinoic acid (Fig. 4b and c, lane 5). This corresponds to the earliest time at which a distinct endodermal cell layer is observed and is still within the period of rapid initial cell growth (Fig. 2). High levels of Endo A and B synthesis continued through the remaining 7 d of the test period (Fig. 4b and c, lanes 6 and 7).

Low levels of intracellular AFP could be detected by immunoprecipitation on the third day of culture (Fig. 5a, lane 5). It took at least two additional days of culture to detect AFP in the culture medium (Fig. 3). Immunoprecipitation with laminin antisera detected a basal level of synthesis by control cultures and cultures exposed to retinoic acid for 1 d (Fig. 5b, lanes 1, 2, and 3). However, a large increase in the amount of immunoprecipitable laminin was detected on day 2 (Fig. 5b, lane 4). It is of interest that increased synthesis of laminin on day 2 is also accompanied by increased vimentin synthesis (Fig. 4a, lane 4). This increased synthesis of laminin clearly preceded the increased levels of Endo A, Endo B, and AFP detected on day 3 (compare lanes 4 of Fig. 4b and c and Fig. 5a) and may be a controlling factor in the formation of an epithelial layer.

**Organization of the Basement Membrane and Formation of the Epithelial Layer during Differentiation**

We examined sections of aggregates and embryoid bodies cultured at high density by immunofluorescence using antibodies against laminin, fibronectin, and type IV collagen to study the organization of the basement membrane. Day-8 aggregates cultured in the absence of retinoic acid were stained uniformly for laminin, fibronectin, and type IV collagen (Fig. 6, column A). In contrast, sections of 8-d bodies cultured in the presence of retinoic acid showed laminin, fibronectin, and type IV collagen located mainly as a distinct deposit underneath the outer layer of cells (Fig. 6, column D). Only the outer layer of cells was stained for AFP (Fig. 1d), Endo B (Fig. 7, column C), and Endo A (data not shown). It is thus clear that, after 8 d of culture in retinoic acid, F9 aggregates have a distinct basement membrane underneath an outer epithelial layer of visceral endoderm cells.

We studied the formation of the epithelial layer and the organization of the basement membrane by staining sections of embryoid bodies at various days in culture. Up to 3 d of culture, the sections of aggregates were uniformly stained for laminin, fibronectin, and type IV collagen (Fig. 6, column B, data for day-1 and day-2 aggregates not shown). The intensity of stain for laminin was stronger in sections of day-3 aggregates when compared to sections of day-1 aggregates. The observation agrees with the increased synthesis of laminin observed on day 3 (Fig. 5b). In sections of day-4 embryoid bodies, in addition to staining the core of the body, most of the staining for laminin, fibronectin, and type IV collagen was located underneath and around the outer layer of cells (Fig. 6, column C), indicating the start of the formation of the basement membrane. A complete layer was seen by day 6: thus, the organization of the basement membrane begins after 3 d in culture and is complete by day 6.

We studied the onset of the differentiated phenotype of the epithelial layer using antibodies against cytoskeletal markers (Endo A and Endo B), blood group antigens I and i whose expression changes during differentiation of the early mouse embryo (23), and a stage-specific cell surface antigen of the embryo (SSEA-1) (13). F9 EC cells express SSEA-1 and I...
antigen but not i antigen on their cell surface. Embryoid body cultures up to 3 d show uniform distribution of SSEA-1 and I antigen but are negative for i antigen and Endo B as shown by immunofluorescent staining (Fig. 7, column A). For I antigen, day-1 sections are shown. Day-3 sections show a similar distribution. At 4 d, Endo A (data not shown), Endo B and i antigen appear, while SSEA-1 is less well stained and has disappeared altogether from the outermost layer (Fig. 7, column B). The distribution of I antigen is also different; it is now found mainly in the outer layer (Fig. 7, column B). By 8 d, epithelial markers (Endo A and B) and blood group antigens (I and I) are restricted to the outer layer while SSEA-1 (EC cell marker) has almost completely disappeared from all cells (Fig. 7, column C).

Thus, by immunofluorescence tests, differentiated expression of the outer cells occurs after 4 d (Endo A, Endo B, i appearance, SSEA-1 disappearance and change in distribution of I antigen); basement membrane formation and epithelial layer alignment also occur after 4 d; AFP secretion (which characterizes visceral endoderm) is a product of the mature epithelial layer after 6 d of culture.

DISCUSSION

We have investigated the process of the formation of an epithelial layer during embryoid body formation in F9 cells.

![Figure 5](image) Immunoprecipitation with AFP and laminin antisera. Cell lysates labeled with [35S]methionine were immunoprecipitated with antiserum to AFP (a) or laminin (b) and analyzed by SDS-PAGE followed by fluorography. Lanes 1 and 2 of each panel represent control F9 lysates grown either as monolayers (lane 7) or as aggregates (lane 2) for 2 d in the absence of retinoic acid. F9 cells exposed to retinoic acid for either 1 d (lane 3), 2 d (lane 4), 3 d (lane 5), 5 d (lanes 6 and 9) or 7 d (lanes 7 and 10). Lane 8 (PC13 EC cells). Exposure time for a was 9 d and for b, 1 d. Position of migration of [35S]leucine marker proteins actin (Ac), Endo B (B), Endo A (A), and vimentin (V) and nonradioactive AFP marker are indicated on the left margin. Arrows on the right margin indicate the two subunits of laminin.

These studies show that an orderly progression of events accompanies the development of an epithelial layer before the establishment of functional maturity (see below). In comparing the time courses of morphological changes, correlations with changes in the rate of synthesis and localizations of several biochemical markers were apparent. The following model is suggested. A phase of rapid cell proliferation for 3 to 4 d produces a colony of similar cells, an aggregate, with similar and uniform distributions of laminin, fibronectin, type IV collagen, and SSEA-1. Laminin synthesis is greatly increased on day 2 (Fig. 4), and sections of day-3 aggregates show a homogeneous distribution slightly more intense than earlier. We conclude that the increased production of laminin is an important event because it precedes the appearance of cells expressing the differentiated phenotype (Endo A, Endo B, and AFP) which are first detected on d 3 (Figs. 4 and 5). It is possible that laminin stimulates in some way either the growth or the differentiation of the endoderm cell, and evidence for this will be presented elsewhere (Grover, A., G. Andrews, and E. D. Adamson, manuscript submitted for publication). By day 4, it is apparent that the outer cells are synthesizing large amounts of laminin but now this matrix component is being accumulated under the outer layer (Fig. 6). In addition, morphological observation shows an outer ring of cells. The fourth day is also the crucial stage when differentiated endodermal expression is observable by immunofluorescence (appearance of Endo A, Endo B, and i antigens; Fig. 7). At this time, the outer layer of cells lose the ability to stain for SSEA-1 but still show high intensity of I antigen.

By the fifth day, the basement membrane is well formed and cystic spaces are observable in the interior. In this model, we see falling cell numbers (Fig. 2) as a part of the orderly progression of embryoid body formation, just as it is a part of normal embryogenesis (24). Only after the formation of internal cysts does the cell number start to rise again, and this correlates with the appearance of AFP in the medium and possibly with an increase in the number of visceral endoderm cells. Although it is impracticable to count the proportions of cells in the two main compartments, it is likely that an expansion of all cell layers occurs as the body enlarges and becomes cystic.

Expressed on a per cell basis, the production of AFP is greatly accelerated at 7 d and, since only the outer cells appear to make AFP (Fig. 1d), it suggests that these cells rapidly become much more efficient in expressing their visceral endoderm phenotype. By the eighth day, the cytoskeleton of the epithelial layer is maximally organized (Fig. 7), and this correlates with almost maximal rates of AFP synthesis. We suggest that AFP secretion may require the formation of a mature polarized epithelium and the formation of a complete contiguous basement membrane, together with an organized cytoskeleton (Endo A and B), in order to attain a maximal rate. In this system, we assumed that a criterion of the functional maturity of the epithelial layer is the rate of AFP secretion (Fig. 3). This occurs only when the epithelial layer is maximally expressing its characteristic markers and when it is well aligned on a thin basement membrane. The importance of basement membrane on epithelial integrity has also been reported by Sugrue and Hay (25). As a final measure of the maturity of the outer visceral endoderm layer, we followed the progress of AFP production in cultures as old as 23 d. The rates of AFP synthesis remain at a high plateau over this time period. In addition, SSEA-1 cell surface antigen reappears on the outer surface of the endoderm cells (not shown). This was not
FIGURE 6 Immunofluorescent staining of sections of F9 aggregates cultured in the absence and presence of retinoic acid. Column A shows sections of aggregates cultured for 8 d in the absence of retinoic acid. Columns B, C, and D show sections of aggregates cultured in the presence of retinoic acid for 3, 4, and 8 d, respectively. (L) Stained with anti-laminin; (F) stained with anti-fibronectin; (C) stained with anti-type IV collagen. Bar, 50 μm. × 250.

FIGURE 7 Immunofluorescent staining of sections of F9 aggregates cultured in the presence of retinoic acid for 3 d (column A, for I antigen a day 1 section is shown), 4 d (column B) and 8 d (column C). Column D shows sections treated with normal serum (controls). NRS, normal rabbit serum. NHS, normal human serum. NMS, normal mouse serum. (E) Stained with anti-Endo B. Bar, 20 μm. (I) Stained with anti-I. Bar, 50 μm. (S) Stained with anti-SSEA-1. Bar, 50 μm. (I) Stained with anti-I. Bar, 50 μm. (E) × 420. (I, S, and I) × 250.
unexpected since the visceral endoderm of the egg cylinder stages of mouse embryo development expresses this antigen (26).

It is possible that part of the explanation for the late production of high levels of AFP is related to the stage of the cell cycle of the outer visceral endoderm cells. If this is the case, then AFP production in this system is highest when cell numbers have become steady (8 to 10 d), a situation which is the reverse of that described for rat fetal hepatocytes (27). It seems then that the mechanism of control of gene expression in primary cultures is quite different from that of the model system described here in which the epithelial layer is still undergoing development and differentiation.

There is no reason to think that cells can relocate in the embryoid body during the process of differentiation, and therefore we favor the hypothesis that an outside cell position is important in the cell's ability to differentiate into a functioning visceral endoderm cell. This is also supported by the findings of Rosenstraus et al. (28), who showed that if PSA1 or F9 EC cells differentiated in suspension cultures in the presence of a distinguishable cell line that grew on the aggregates as an outer layer, then the EC cells in the aggregate no longer formed endoderm. We believe that the internal layers of less-well-differentiated cells are important in some unknown way to the maintenance of the endoderm layer since all attempts to isolate an independent line of visceral endoderm cells which continue to secrete AFP have failed. The inner cell layer, however, never achieves an ordered pseudostratified epithelium which occurs when some multipotent cell lines differentiate into embryoid bodies (29).

During the process of differentiation of F9 aggregates, SSEA-1 (the cell surface marker expressed by EC cells) is lost, first by the outer layer of cells and later from the inner cells (Fig. 7). This is not true for the embryoid bodies formed from OTT6050 cells (26), in which inner cells continue to express SSEA-1. Similarly, antigen disappears from the interior cells of F9 aggregates (Fig. 7). The expression of these antigens may depend on the cell line and hence on the degree of differentiation achieved. It is also possible that retinoic acid affects the expression of SSEA-1 and I antigen either directly, or indirectly, by allowing a state of differentiation distinct from that of spontaneously differentiating cell lines such as OTT6050.

Our experiments have shown that the laminin component of the new basement membrane was likely to have been derived by de novo synthesis which increases on the second day (Fig. 5 b). It is likely that type IV collagen was also synthesized by the aggregates (6) and was not derived from the fetal bovine serum in the medium. We also used a species-specific antifibronectin antiserum to show that most if not all of the fibronectin which organizes into the basement membrane is synthesized by the differentiating F9 cells. Since we have shown that higher cell densities differentiate earlier and produce higher rates of AFP synthesis, we may conclude that either the cells are stimulated by interaction or that they produce materials which condition the medium and that this then stimulates differentiation. It is therefore possible that basement membrane components are stimulatory (Grover, A. G. Andrews, and E. D. Adamson, manuscript submitted for publication) since they are synthesized at early stages.

On the basis of these data, the process of differentiation of F9 embryonal carcinoma cells into an epithelium consists of the following stages: (a) aggregation, (b) proliferation, (c) differentiation, (d) organization, (e) maturation, and (f) secretion. We are currently investigating in further detail these events of epithelial layer formation and maintenance. Experiments are in progress to study basement membrane breakdown as an approach to the analysis of epithelial layer degradation. The process of embryoid body formation appears to provide a useful model for both normal and pathological processes.

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