Ultrastructural Modifications of the Cell Surface and Intercellular Contacts of Some Transformed Cell Strains

A. Martinez-Palomo, C. Braislovsky, and W. Bernhard

SUMMARY

Normal contact-inhibited and transformed cell cultures have been fixed and embedded in situ for an electron microscopic study of the cell surface and intercellular connections. In a number of experiments, ruthenium red was added to the fixatives following Luft's technic for visualizing the mucopolysaccharide layer on the cell surface. Cultures of normal rat, Chinese and Syrian hamster embryo cells were compared with 5 different strains of transformed cell lines: Chinese hamster cells transformed in vitro by adenovirus 12, Syrian hamster cells transformed in vivo by adenovirus 12, Syrian hamster cells transformed in vivo by SV40 virus, spontaneously transformed BHK21 cell line, and finally a spontaneously transformed rat fibroblast strain. Two main observations are reported: (a) The character of intercellular contacts was changed in the transformed cells. Whereas close junctions and desmosome-like structures are as frequent as in controls, the so-called tight junctions practically disappeared in the transformed cultures. (b) The ruthenium red layer was considerably increased in all the five transformed strains compared to the normal controls, suggesting an increase of the mucopolysaccharide deposit at the cell surface.

INTRODUCTION

Since the pioneering work by Coman (11), modifications of the surface properties of cancer cells have been considered by a number of authors as an important part of malignant transformation [see reviews by Abercrombie and Ambrose (1), Ambrose (2, 3), Weiss (49)]. The surface alteration may be reflected by a diminished adhesiveness of tumor tissue, which in turn may lead to invasion and metastases. The loss of contact inhibition exhibited by many cells transformed in vitro by oncogenic viruses resulting in multilayered foci with disorganized growth patterns, in contrast to the organized monolayer cultures of contact-inhibited normal cells, may be related to the changes in the behavior of malignant cells in vivo.

The presence of contact inhibition of normal cell cultures and its absence in cells transformed by oncogenic viruses (34, 42, 48) or by chemical carcinogens (8) have been repeatedly studied in different laboratories. The observation of foci with "criss-cross" growth in cell cultures transformed with an oncogenic virus has become an important criterion for the detection of malignant transformation in vitro, although such disorganized growth is not necessarily followed by the development of tumors when cells are transplanted to a homologous host. Attempts to understand the complex changes occurring at the surface of malignant cells have included a variety of methods. Among them, light microscopic cytochemical methods have revealed an increased amount of mucopolysaccharides on chicken fibroblasts infected with Rous virus (17) and on hamster fibroblasts transformed with polyoma virus (13). Furthermore, biochemical and ultrastructural differences between plasma membranes of a normal liver and experimental hepatoma cells have been reported recently (6, 16).

It seemed to us rewarding to search for similar changes in various types of cultured transformed cells using, in addition to classical electron microscopic technics, recent methods of ultrastructural cytochemistry which allow the detection of minute amounts of mucopolysaccharides on the cell surface by means of the ruthenium red stain proposed by Luft (30). Preliminary observations on this subject have already been reported (33). The present work is based on the use of various additional cell systems and takes more particularly into account the fine structure and frequency of distribution of the different types of intercellular connections.

MATERIALS AND METHODS

Cell Cultures

Cells Derived from Rat, Syrian Hamster, and Chinese Hamster Embryos. After trypsinization of embryos, cells were cultured in 250-ml prescription bottles (20 ml of cell suspension containing 300,000 cells/ml). At the 4th day, the medium was changed and at the 7th day cells were trypsinized and cultured subsequently in 60-ml flasks.

Chinese Hamster Embryo Cells Transformed in Vitro by Adenovirus 12. This cell line has been transformed in our laboratory as described in a previous paper (9). The cells used in this study were between the 28th and the 43rd passages.

Cell Line Derived from a Primary Tumor Induced in a Syrian Hamster by Adenovirus 12. The cell line T Ad12-XIV established in vitro from a tumor induced in a Syrian hamster by
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adenovirus 12 has been kindly supplied by Dr. G. Lorans (28). Cells between the 109th and 175th passages were used.

Cell Line Derived from a Tumor Induced in a Syrian Hamster by SV40 Virus. The cell line C12TSV5 derived from a tumor induced in a newborn Syrian hamster by SV40 virus has already been described elsewhere (44). Cells between the 52nd and the 237th passages were examined.

BHK Cell Line. We have studied BHK 21 Clone 13 hamster cells obtained by Macpherson and Stoker (31) and maintained in our laboratory from a culture that reached the 40th generation; this cell line, which is clearly transformed and highly oncogenic, has been examined between the 120th and 180th passages.

Spontaneously Transformed Rat Embryo Cell Line. Rat embryo fibroblasts, cultured with usual techniques, began to grow more rapidly than usual after the 17th passage. The morphology of the cells was modified, and confluent cultures showed a lack of contact inhibition as judged by their crisscross pattern appearance and the formation of multilayers. Cultures were examined between the 30th and the 50th passages in vitro. The malignancy of the transformed cells has not yet been ascertained.

The Culture Medium

The cells were cultured in prescription bottles with Eagle's Minimum Essential Medium supplemented eight times more with vitamins, 10% decomplemented calf serum, 10% Tryptose Phosphate Broth (Difco), and finally 100 IU of penicillin and 100 μg of streptomycin per ml. This medium was used in two different forms: the first one contained a weak concentration of sodium bicarbonate (0.35 gm/liter) for the cell passage. The second one, with a higher concentration of bicarbonate (2.20 gm/liter), was only used for changing the supernatant.

Cell Passage

The different cell strains were cultivated in prescription bottles of 60 ml, each bottle containing 5 ml of medium with initial cell concentration of 100,000/ml. Twenty-four to 48 hours after inoculation, the cell layers were generally confluent. The cells were usually fixed at 48 hours, but in some experiments, fixation was practiced at various times from 24 to 120 hours.

Electron Microscopy

Cells were fixed within the prescription bottles with a 2.5% glutaraldehyde-cacodylate buffer solution at pH 7.3 for 15 min at 4°C. Postfixation was carried out with a 2% osmium tetroxide in cacodylate buffer solution for 1 hour. When ruthenium red was used to detect the surface layer of cells, the dye was added to both fixatives at a concentration of 50 mg/100 ml; glutaraldehyde fixation was then prolonged to 1 hour and osmium tetroxide postfixation to 3 hours. After dehydration in increasing concentrations of acetone, cells were embedded in situ directly in the prescription bottles with a 2–3-mm thick layer of Epon. Following polymerization at 60°C, the bottles were broken and the layer of Epon containing the culture was then detached from the glass after a brief immersion in hot water. Blocks approximately 1 x 3 mm were cut and oriented in order to obtain vertical or horizontal sections. Thin sections were examined without additional stain or post-stained with lead nitrate. Examination was carried out by means of an electron microscope Siemens Elmiskop I.

RESULTS

The in situ embedding technic used in the present work was a necessary condition for the study of the intercellular relationships present in normal and in transformed cultured cells. In vertical thin sections of normal, contact-inhibited cultures, cells appeared flattened with few contacts with neighboring cells. Cell overlapping was minimal, and when present, involved only thin cytoplasmic processes. In contrast, similarly oriented sections of transformed cell foci showed two to six layers of cells piled on top of each other. A large proportion of the surface of adjacent cells formed parallel contacts (Figs. 1, 2). The surface activity of normal and transformed cells, as judged by the formation of pinocytic vesicles and microvilli, varied considerably from cell to cell and showed no particular relation to the transformation process.

Contact-inhibited Cells

Intercellular relationships of normal cells were best studied in horizontal sections since they generally formed a monolayered culture. The surface of adjacent cells came in contact at irregular intervals at sites where cell membranes parallel each other, leaving in between a more or less regular intercellular space of 120 to 200 Å (Fig. 3). These zones will be referred to as close junctions (45). They represent by far the most commonly observed type of intercellular connection. At these sites, condensation of cytoplasmic fibrils is scanty, and the intercellular space is occupied by moderate amounts of a fluffy material (Fig. 3). A second type of cell attachment was rarely observed as dense short plates at each side of contiguous cell membranes; the plates are formed by condensation of cytoplasmic fibrils. This type of cell contact is considered as an incomplete desmosome and has been described in other tissues as intermediate junction (Fig. 4) (18, 38). The third type of cell attachments frequently encountered in normal cells are tight junctions (14). They are formed by the fusion of the external leaflets of the cell membranes with the resulting obliteration of the intercellular space; at these sites there is no condensation of cytoplasmic fibrils (Fig. 5). The fusion line varies in length and may extend over considerable distances (Fig. 6).

When ruthenium red was added to the fixatives, a dense surface layer 120–130 Å thick appeared at the cell surface and at the intercellular space of close junctions and intermediate junctions (desmosomes). Tight junctions, however, are not permeated by ruthenium red (Figs. 6, 7), as revealed by the study of unstained sections in which the density of the intermediate line is not increased by the dye (Fig. 8). The quintuple-layered appearance of tight junctions is barely visible in unstained sections due to the slight contrast given to the membranes by postfixation with osmium tetroxide.
Transformed Cells

As in normal cells, the most common type of cell attachment in multilayers of transformed cells is represented by tight junctions (Fig. 9). The intercellular space present at such junctions is partially or completely filled by ruthenium red material (Figs. 10–12), which appears over the external leaflet of the trilaminar cell membrane (Fig. 12). Intermediate junctions were rarely seen in hamster embryo cells transformed in vitro or in hamster cells derived from an in vivo tumor induced by adenovirus 12; they were absent in spontaneously transformed cell cultures.

Of particular interest is the fact that tight junctions were extremely scarce in transformed cultures. Only exceptionally were such junctions observed in spontaneously transformed rat cells and in vitro transformed hamster embryo cells (Table 1). No tight junctions were found in the other two types of transformed cultures.

Thickness of the Ruthenium Red Layers

The overall thickness of the ruthenium red layer, including the outer leaflet of the cell membrane and the deposited ruthenium red material, averages 120 Å in normal hamster embryo cells and 130 Å in rat embryo cells. In contrast, the surface layer positive to ruthenium red was found to be increased in thickness and more irregular in all cultures of transformed cells examined (Table 1). The maximal increase of ruthenium red layer in transformed cells represents in general more than two-fold the amount of ruthenium red found in normal cells (Figs. 13–18). It is important that all these figures have been obtained from cells cultured under the same conditions and fixed after 48 hours of growth. The possible influence of the age of cultures on the ruthenium red layer was studied with the strains C12 and TSV5 and with Chinese hamster cells transformed with adenovirus 12. The cells were fixed at various intervals between 24 and 120 hours with ruthenium red added to the fixative and compared to normal control cells of the same age. Neither the thickness of the ruthenium red layer nor the density of the stain were changed with the increasing age of the cultures. However, in preliminary experiments, primary confluent cultures of hamster embryos infected with high multiplicities of adenovirus 12 showed a strong increase of the ruthenium red layer after the 12th hour of infection, coinciding with the appearance of virus-induced nuclear antigens (A. Martinez-Palomo, C. Brailovsky, and R. Wicker, 1968, unpublished data).

DISCUSSION

Our results indicate that, in the examined material, there are two types of ultrastructural changes which can be visualized in cultured transformed cells: those visible as cell contacts with classical electron microscopic technics, and those which can be shown on the whole cell surface only by means of a cytochemical stain. Both phenomena may be intimately linked to and be responsible for the loss of contact inhibition.

Intercellular Contacts of Normal and Transformed Cells

Despite extensive tissue culture work on normal and transformed cells and the recognition of the importance of surface phenomena in cell growth regulation, only a few reports have dealt with the ultrastructural specializations present at the surface of cells in tissue culture. The fine structural features of the attachment regions between adjacent cells have been mainly observed in epithelial tissues where a tripartite junctional complex is usually found (18, 19). Recent work suggests the existence of a spectrum of attachment structures rather than the existence of distinct types (26, 27). Studies on connective tissue cells have demonstrated less distinct intercellular junctions. In cultured fibroblasts, sites of close apposition of adjacent membranes have been described (14), whereas in fibroblasts of developing tissues, a different type of contact has been described as “attachment sites” (38). In the present study the use of an in situ embedding technic offered great advantage over the usual method employed for the ultrastructural study of cell cultures. Normal intercellular relationships are preserved, the whole culture can be studied under a light microscope, and adequate orientation of the blocks prior to sectioning can be obtained.

Close Junctions. The term “close junction” has been used in the present study to identify the most common type of cell attachment found in normal and transformed cells. Close junc-

### Table 1

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Degree of contact inhibition</th>
<th>Intermediate junctions [desmosomes]</th>
<th>Tight junctions</th>
<th>Close junctions</th>
<th>Average thickness of ruthenium red layer (Å)</th>
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<tr>
<td>Normal hamster embryo</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>130</td>
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<tr>
<td>(Chinese Syrian)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>+++</td>
<td>-</td>
<td>+</td>
<td>±</td>
<td>120</td>
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<tr>
<td>Hamster embryo cells</td>
<td>-</td>
<td>+</td>
<td>±</td>
<td>+++</td>
<td>330</td>
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<tr>
<td>transformed in vitro</td>
<td></td>
<td></td>
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<td></td>
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<td>by adenovirus 12</td>
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<tr>
<td>Hamster cells transformed</td>
<td>-</td>
<td>11</td>
<td>±</td>
<td>+++</td>
<td>330</td>
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<td>in vivo by adenovirus 12</td>
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<td>Hamster cells</td>
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<td>in vivo by SV40 virus</td>
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<td>+++</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>±</td>
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<td>transformed</td>
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tions were described in embryonic chick cells as regions where adjacent cell membranes approximate, leaving in between an intercellular space less than 100 Å wide (45). In the different types of cell cultures we have examined, close junctions have an intercellular space less than 200 Å wide, filled with a material stainable with ruthenium red. This focal accumulation of surface material, probably mucopolysaccharide in nature, at sites of membrane apposition may have a functional significance in the maintenance of cellular adhesion. It seems doubtful that close junctions in cultured cells may be related to contact inhibition, since these junctions were found to be similar in structure and distribution both in monolayered, contact-inhibited cells, as well as in multilayered transformed cultures. The possible function of close junctions as regions of intercellular communication remains hypothetical.

Intermediate Junctions ("Desmosomes"). Such junctions as shown on Fig. 4 resemble poorly developed desmosomes. They are seldom seen in normal and transformed hamster cells and may be likened to the "attachment sites" found in developing connective tissue cells (38) and to the intermediate junctions (zonula adherens) of epithelial cells (18). Such connections are characterized both by the parallel alignment of adjacent cell membranes with an intercellular space less than 200 Å and by condensation of fibrillar material of the subjacent cytoplasmic matrix. They appear to function mainly as devices for cell-to-cell attachment; no basic fine structural differences were seen in the present work between intermediate junctions of normal and transformed cells.

Tight Junctions. The presence of tight junctions in cultured fibroblasts has been reported by Devis and James (14). Our study demonstrates that normal rat embryo fibroblasts are frequently joined by regions of fusion of external membranes similar to those originally described as tight junctions in various epithelial tissues (18). Moreover, our findings have shown that ruthenium red does not permeate the interior of tight junctions, indicating the existence of cell membrane fusion and the absence of a well-defined intercellular space at the level of these junctions. Considerable attention has been recently given to the elucidation of the role of tight junctions in organoid tissues. A function of this cell attachment is suggested by the resistance offered by tight junctions to mechanical tension (18, 19). These junctions may also restrict exchanges along the intercellular space since they appear to be impermeable to macromolecules, small water-soluble molecules, ions, and even water (19). On the contrary, tight junctions may be sites of intercellular communication across the membranes, perhaps representing areas of low electrical resistance through which ionic exchange is facilitated. This assumption is supported by the demonstration of electronic coupling between cells which are connected by tight junctions (39, 46). The possibility that tight junctions may play a role in the phenomenon of contact inhibition has been suggested by an ultrastructural examination of cell junctions in migrating chick embryo tissues (45). Probably related to this problem is the finding that growth regulation of cells in culture may be mediated by the transmission of an inhibitor substance through close intercellular contacts (40). Considering our results on the relative absence of tight junctions in transformed cultures which have clearly lost contact inhibition, and taking into account present-day knowledge on the function of these contacts, the lack of tight junctions may represent a morphologic basis for the alteration of the adhesive properties of transformed cells and for the modification of intercellular communication and exchange of growth-regulating substances. A fine structural basis for the reduced adhesiveness of tumor cells studied by Coman (11) was initially found by Easty and Mercer (15), who observed that "smooth edge-to-edge contacts" in cultures of kidney tumor cells were less numerous than in normal kidney cells. Bruni et al. (10) also emphasized the reduced number of desmosomes in HeLa cells originally derived from a human cervical carcinoma. Our finding of a relative absence of tight junctions in transformed cells is supplemented by the observation of a decreased incidence of such junctions in isolated rat hepatoma cell membranes, as shown by Benedetti and Emmelot (6, 16). Interestingly enough, hepatoma cells do not show electrical coupling as normal hepatic cells do (29).

Surface Layer in Normal and Transformed Cells

Most types of cells are covered with a polysaccharide-rich coat named "glycocalix" by Bennett (7). This surface coat, very complex biochemically, is visualized with the aid of biochemical methods specific for the detection of mucopolysaccharides or glycoproteins. It has been demonstrated that light microscopically on ascites tumor cells by means of Hale's iron reaction (21). This layer was also shown in the electron microscope in different types of animal cells with iron stain (50) or with phosphotungstic acid (32, 35, 36) in a large variety of tissues with the silver-methenamine technic (37) and, recently, with thiocarboxyhydradize silver (43). A closer approach has been reported in a combined biochemical and ultrastructural study on the considerable increase of sialic acid-containing substances in isolated hepatoma plasma membranes (6). The ruthenium red technic of Luft (30) used in the present work has also been applied to detect surface coats in a number of tissues (26, 27, 30). The specific reaction of the dye with mucopolysaccharide substances is indicated by the binding of ruthenium red with heparin mast cell granules and by the selective blockage of the staining reaction with cetylpyridinium chloride (22, 23). Furthermore, digestion of cultured Rous sarcoma cells prior to fixation and ruthenium red staining by means of hyaluronidase reduced considerably the thickness of the ruthenium red layer. However, ruthenium red-positive residues could not be removed with this enzyme (H. R. Morgan, 1968, unpublished data). There are perhaps glycoproteins or glycolipids which are not digestable. The mechanism of the interaction between ruthenium red, osmium tetroxide, and the cell substrate remains unknown. From our findings it is evident that ruthenium red can be used for the detection of surface coats in cultured cells only when cells are fixed and embedded in situ; otherwise, the poor penetration of the dye and the loss of ruthenium red material during centrifugation of cells produces unreliable results. Another important factor is the concentration of ruthenium red in the fixative. Too much stain gives heavy, uncontrollable precipitates, whereas too little leads to an uneven contrast of the surface layer. Correct measures of the ruthenium red layer are sometimes difficult to

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make because of these irregularities. The data shown in Table 1 represent an average thickness based on a series of measurements.

Surface mucopolysaccharides may have a significant function in the maintenance of repulsive forces between adjacent cells. Electrostatic repulsion between like surface charges constitutes an important factor in cell repulsion (1, 2), and sialic acid contributes significantly to the negative surface charge of the cell (3, 4). Early studies on the electrophoretic mobility of cells revealed an increase in the negative charge of certain tumor cells (4). However, it soon became evident that such an increase may also be observed in regenerating and embryonic tissues (5). On the other hand, the surface charge of different types of tumor cells was not increased (12, 47). Furthermore, the contribution of sialic acid residues to the increased electrophoretic mobility found in certain tissues varies widely among different cells. Nevertheless, it appears that at least in some tumor tissues, an increase in sialic acid enhances the negative surface charge, which in turn may result in a reduced adhesiveness of such cells (16). Our finding of an increased thickness of the ruthenium red stainable layer in spontaneously and virus-transformed cells extends the earlier report by Defendi and Gasic (13) of an augmentation of surface mucopolysaccharides in polyoma virus-transformed hamster cells which have been shown to possess an increased electrophoretic mobility susceptible to neuraminidase treatment (20). An increased production of acid mucopolysaccharides, as well as a rise in the concentration of an enzyme involved in the production of hyaluronic acid, have also been described in chick embryo fibroblasts transformed by Rous sarcoma virus (17, 25, 41), whereas SV40 polyoma virus-transformed mouse (3T3) or human fibroblasts were reported to have a reduced production of hyaluronic acid (24). This latter rather unexpected result is still difficult to understand.

It, therefore, appears that variations among different tissues may be considerable. Contact inhibition is probably not an all-or-none phenomenon and may be based on a variety of factors still poorly understood. However, in the systems we have studied, both the absence of tight junctions and the increase in surface mucopolysaccharides of transformed cells are likely to be related to their loss of contact inhibition. Other types of cell lines and transforming agents of cultured cells and also primary solid tumors have to be studied with the same methods before any final conclusions concerning the ultrastructural substrate of contact inhibition in general can be made.

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REFERENCES


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Fig. 1. Vertical section of a pile of hamster embryo cells transformed in vitro with adenovirus 12. Due to a loss of contact inhibition, the cells grew on top of each other forming four to six layers. See cell-to-cell contacts (arrows) × 12,000.

Fig. 2. Normal hamster embryo cells. In contrast to transformed cells (Fig. 1), contact-inhibited cells form a monolayered culture. Usually a single layer of cells is seen in vertical sections; in the micrograph a slender cytoplasmic process underlies a flattened cell. Besides the stratification of transformed cells, no consistent ultrastructural differences can be observed at low magnifications between contact-inhibited and transformed cells. Numerous sites of cell-to-cell contact are seen (arrows). × 12,000.

Fig. 3—5. Normal hamster embryo cells. At higher magnification, three types of cell contacts are seen. By far the most common type is formed by the parallel disposition of adjacent cell membranes, leaving an intercellular space of variable length less than 200 Å wide (Fig. 3). These sites of cell contact are identified as close junctions (× 80,000). A second type of junction less frequently found is desmosome-like or resembles intermediate junctions (Fig. 4); they form short dense plates at the cytoplasmic side of contiguous cell membranes (× 80,000). A more commonly seen type of cell contact is represented by tight junctions (Fig. 5); these junctions are formed by the fusion of the external leaflet of two adjacent cell membranes with the resulting obliteration of the extracellular space. × 80,000.

Fig. 6. Normal rat embryo cell. Extended tight junction connects the cytoplasmic processes of two adjacent cells. Ruthenium red stains a layer of dense material both at free surfaces (top) and in the intercellular space (bottom left). × 90,000.

Fig. 7. Normal rat embryo cell. Ruthenium red-positive material covers the surface of two contiguous cells; in sections stained with lead citrate, the density of the membranes is increased and therefore it is difficult to decide whether ruthenium red contributes to the density of the midline leaflet of tight junctions. × 120,000.

Fig. 8. Normal rat embryo cell. In a section without post-stain, the ruthenium red-stainable material is seen at the surface of the cell, but not at the level of a tight junction (arrows). The slight contrast of the membranes at the junction is due to the density given by the osmium tetroxide fixative. × 120,000.

Fig. 9. Low magnification view of a vertical section of spontaneously transformed rat embryo cells. The cells are covered by a uniform thick layer of ruthenium red-positive material which fills the intercellular space at the numerous close junctions observed in this picture. × 30,000.

Figs. 10—12. Rat embryo cells spontaneously transformed. The most common type of cell junction both in normal and in transformed cultures are close junctions (Fig. 10, arrows). × 30,000. At higher magnification two close junctions are seen (arrows, Fig. 11); the material stained by ruthenium red fills the uniform space left between contiguous cells. × 60,000. The trilaminar appearance of the cell membrane is seen at a close junction (arrow, Fig. 12); ruthenium red is deposited over the external leaflet of the plasma membrane. × 120,000.

Figs. 13—18. This series of electronmicrographs illustrate the varying thickness of the ruthenium red layer in different types of cultured cells, all fixed after 48 hours of growth.

Fig. 13. Normal hamster embryo cell. × 120,000.
Fig. 14. Normal rat embryo cell. × 120,000.
Fig. 15. Hamster embryo cell transformed in vitro with adenovirus 12. × 120,000.
Fig. 16. Hamster embryo cell derived from an in vivo tumor induced by SV40 virus. × 120,000.
Fig. 17. Spontaneously transformed BHK cell. × 120,000.
Fig. 18. Spontaneously transformed rat embryo cell. × 120,000.
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Figures 13, 14, 15, 16, 17, 18
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