

# RUTHENIUM RED STAINING OF NORMAL AND TRANSFORMED MURINE FIBROBLASTS IN VITRO

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## INTRODUCTION

Differences between normal and transformed cells in tumorigenicity and growth control *in vitro* are thought to be correlated with differences in the structure of the cell surface (1-4). For this reason a number of cytochemical studies have been made lately in order to elucidate the ultrastructure of the cell membrane and to find consistent differences between normal and transformed cells. A number of investigators have made use of the specificity of the plant lectin concanavalin A for certain sugar groups of the glycoproteins (5-7), some others utilized ruthenium red (RR) as a specific dye for acid mucopolysaccharides present in the cell coat (8-11). Because some of the latter studies provided apparently conflicting results (9, 10), we decided to include the RR-staining technique in our electron microscope investigations of normal (3T3) and SV-40-transformed (SV-3T3) mouse fibroblasts. Although small differences were found between the RR-stained cell surface of 3T3 and SV-3T3 cells, we were unable to correlate these with differences in the structure of the cell membrane. This was because RR proved to stain not only mucopolysaccharides in the cell coat, but also serum components more or less firmly attached to the coat of cells grown *in vitro*.

## MATERIALS AND METHODS

Normal and transformed 3T3 cells were grown on carbon-coated cover slips in 5-cm plastic Petri dishes filled with 5 ml of Dulbecco's modified Eagle's me-

dium supplemented with 10% newborn calf serum and antibiotics. The dishes were seeded with  $2.5 \times 10^5$  cells and grown to confluence in a humidified CO<sub>2</sub> incubator at 37°C. The cells were fixed and stained with RR *in situ*, essentially according to the method of Luft (12) as modified by Martinez-Palomo and Brailovsky (13). Half of the cover slips with cells were given three short rinses in phosphate-buffered saline (PBS) before fixation, the others were fixed directly after removal of the culture medium. In addition, different treatments were sometimes given as described under Results. After dehydration in a graded series of ethanol solutions and in propylene oxide, the cells were embedded in a mixture of Epon and Araldite. The embedded cell layer was separated from the glass, sectioned, and studied in a Philips 300 electron microscope operating at 80 kV without additional staining.

## RESULTS

Normal 3T3 cells that were not rinsed before fixation and staining showed big, irregular patches of RR-positive material on or near the densely stained free plasma membrane (Fig. 1). The reaction pattern was very much like that described on other cells *in vitro* (13, 14). The patches seemed to be loosely attached to the cell membrane and consisted of more or less clearly discernible globules of varying electron density but relatively uniform size (Figs. 1 and 5). On the adjacent plasma membranes of partly overlapping cells the patches looked compressed, and separate globules were

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FIGURES 1-4 Normal 3T3 cells stained with RR after different treatments.  $\times 50,000$ .

FIGURE 1 Normal medium, unrinsed.

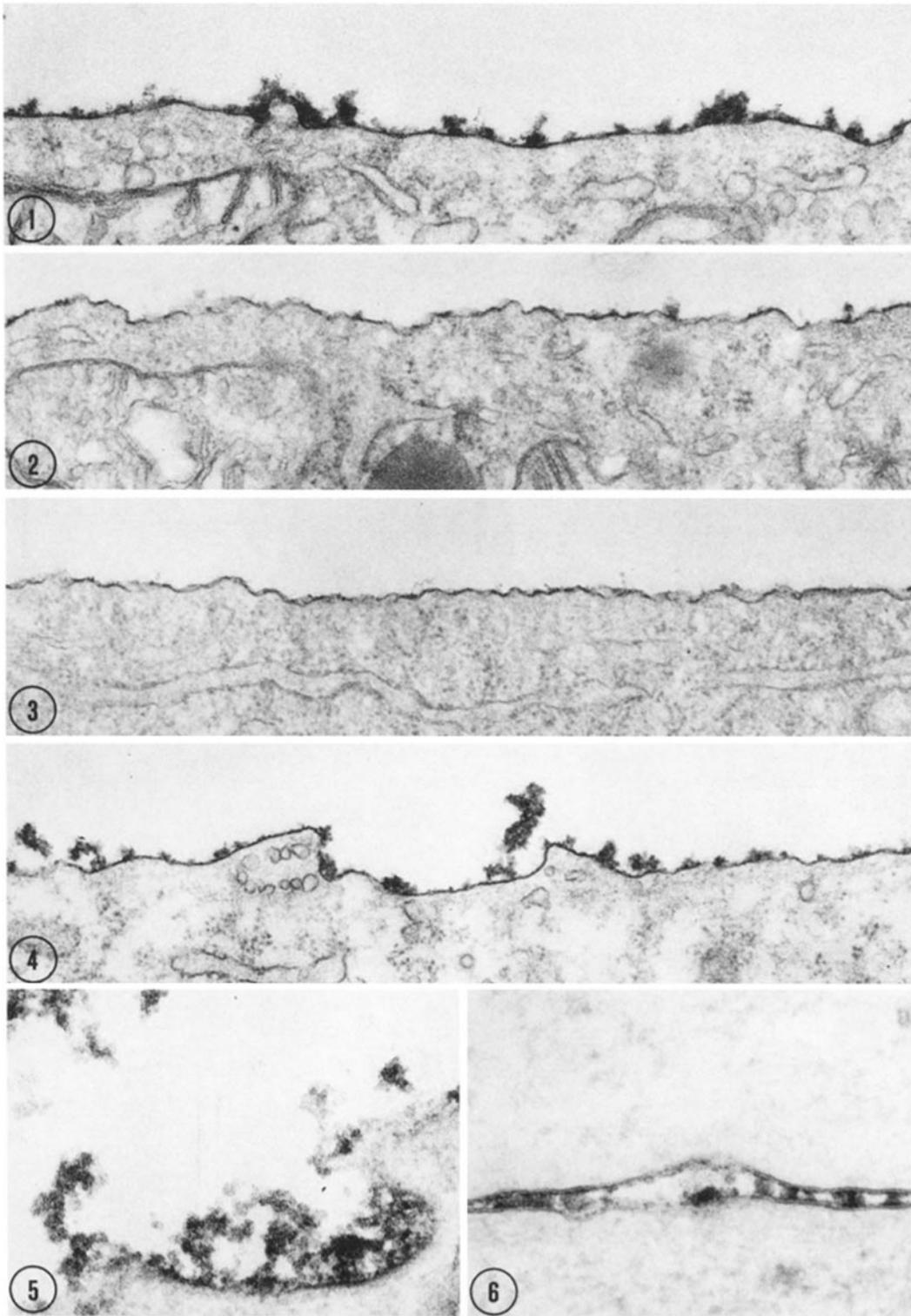
FIGURE 2 Normal medium, rinsed.

FIGURE 3 Serum-free medium, unrinsed.

FIGURE 4 Normal medium, rinsed, 1 min in normal medium, unrinsed.

FIGURE 5 High magnification of RR-positive reaction product on unrinsed, normal 3T3 cells.  $\times 110,000$ .

FIGURE 6 High magnification of RR-positive reaction product between partly overlapping 3T3 cells.  $\times 110,000$ .



seldom seen (Fig. 6). Endocytic vesicles that were open to the cell surface showed the same reaction with RR as the free plasma membrane (Fig. 11).

When 3T3 cells were rinsed before fixation the RR-positive patches were completely absent from the free cell surface (Fig. 2). However, the cell membrane itself was stained as in unrinsed cells and occasionally very few threadlike remnants of extracellular material were found. Closely apposed membranes of partly overlapping cells were not free of the electron-dense patches, probably because PBS could not penetrate these areas sufficiently to wash away the material (Fig. 6).

The differences in amount of RR-stained material on the free surface of rinsed and unrinsed 3T3 cells suggested that this RR-positive, extracellular material consisted of serum components from the culture medium (15, 16). To prove this hypothesis two experiments were carried out. In the first experiment 3T3 cells were exposed for 16 h to serum-free medium and subsequently fixed and stained without previous rinsing. No RR-stained patches of material were found on the free surface of these cells, and they looked like rinsed 3T3 cells grown in serum-containing medium (Figs. 2 and 3). The absence of RR-positive patches could most easily be explained by postulating that all serum components originally present on the cell surface had either been taken up by endocytosis during the growth in serum-free medium, or been released by diffusion into the serum-free medium (17). However, the possibility still existed that the patches were cell products that had been released in the serum-free medium without the cells being able to replace them under the unfavorable growth conditions. Therefore in a second experiment the cells were first rinsed and then exposed to medium containing 10% serum for less than 1 min. After fixing and staining, the same

electron-dense patches were found on the surface of these cells as on normally grown, unrinsed 3T3 cells (Figs. 1 and 4). Thus the possibility was excluded that the patches were cell products, because the cells could not have synthesized and excreted them so quickly.

These experiments proved that RR-positive serum components are responsible for the electron-dense patches on unrinsed 3T3 cells. This implies that an investigation of possible differences in amount and distribution of acid mucopolysaccharides between normal and transformed cells *in vitro* should be carried out on rinsed cells in order to prevent loosely attached serum components from obscuring these differences. The necessity for this requirement became clear when unrinsed SV-3T3 cells were fixed and stained. The free surface of these cells as well as the surface of overlapping SV-3T3 cells looked exactly like that of unrinsed 3T3 cells (Figs. 1 and 7).

When SV-3T3 cells were rinsed before fixation and staining, their free cell surface looked different from that of rinsed 3T3 cells (Figs. 2 and 8). Although most of the big, RR-stained patches were absent, a fuzzy electron-dense layer was still present on the outside of the cell membrane. Rinsing did not affect the amount of RR-positive material between overlapping cells. In order to determine whether the material of the fuzzy layer was medium derived like the more readily removable RR-positive patches, the SV-3T3 cells were treated in the same way as the 3T3 cells and left in serum-free medium for 16 h before fixation. This resulted in a free cell surface practically without the electron-dense, fuzzy layer, exactly like the surface of similarly treated 3T3 cells (Figs. 3 and 9), suggesting that the RR-positive substances left on SV-3T3 cells after rinsing might also be serum components. To exclude completely that the fuzzy material was

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FIGURES 7-10 SV-40-transformed 3T3 cells stained with RR after different treatments.  $\times 50,000$ .

FIGURE 7 Normal medium, unrinsed.

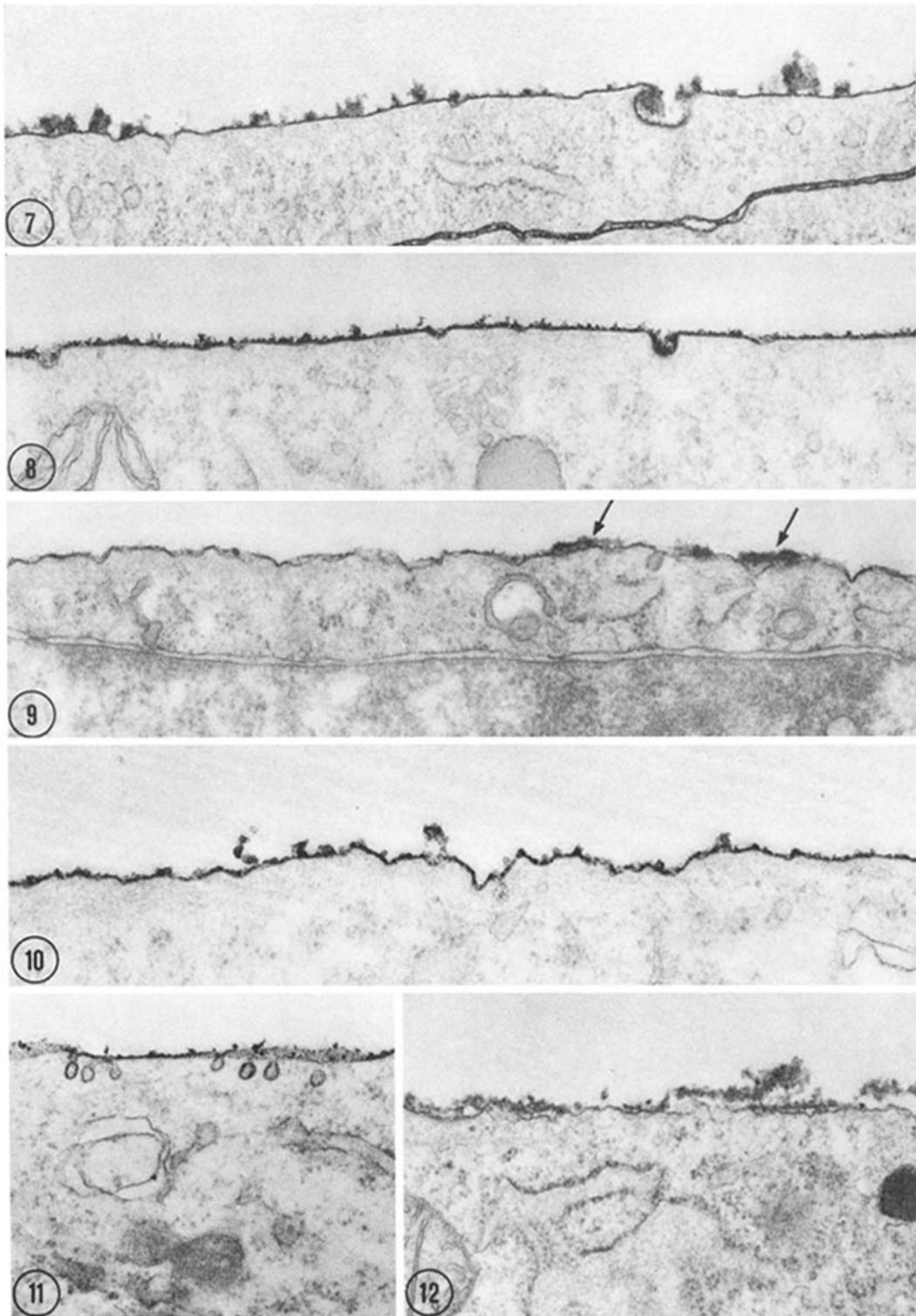
FIGURE 8 Normal medium, rinsed.

FIGURE 9 Serum-free medium, unrinsed.

FIGURE 10 Serum-free medium, 1 min in normal medium, rinsed.

FIGURE 11 Normal 3T3 cell stained with RR after rinsing in PBS, note reaction product in endocytic vesicles.  $\times 40,000$ .

FIGURE 12 Protocollagen-like material on normal 3T3 cells  $\times 40,000$ .



of cellular origin the following experiment was done. Cells left in serum-free medium for 16 h were given a 1-min exposure to medium with 10% serum and rinsed in PBS immediately afterwards. When these cells were fixed and stained with RR the large electron-dense patches were essentially absent from the cell surface, but in most areas the fuzzy, RR-stained material was present again (Fig. 10). The amount of RR-positive, extracellular material was smaller than in rinsed SV-3T3 cells that had been grown in serum-containing medium (Figs. 8 and 10). This is probably because a 1-min exposure to complete medium is too short for a full covering of the cell membrane by serum components. Thus, all RR-stained material on the cell membrane of SV-3T3 cells is apparently serum derived, but in contrast to 3T3 cells part of this material can not easily be removed by rinsing in PBS.

For the sake of completeness it should be added that fibroblasts are known to excrete protocollagen-like substances (18). These substances were found to be RR positive and were encountered in all our experiments (Figs. 9, arrows, and 12). This did not interfere with the interpretation, however, because the protocollagen-like substances could easily be recognized by the fibrillar nature and the orientation parallel to the cell surface.

#### DISCUSSION

Our experiments have shown that RR staining can help to visualize differences between cell surfaces of normal and transformed 3T3 cells. These differences pertain to the affinity of the cells for certain serum components present in the culture medium. To detect these differences cells should first be rinsed so as to remove, as much as possible, serum components that are less firmly attached to both normal and transformed 3T3 cells. Although phosphate seems to inhibit the reaction of RR when added with the stain (19), no such effect was found in our experiments when phosphate was used for rinsing the cells before treatment with cacodylate-buffered RR and glutaraldehyde (Figs. 6 and 11). In addition, results of experiments where we used (low phosphate) Tyrode's for rinsing did not differ from those where PBS was used, and lymphosarcoma cells rinsed with PBS had essentially the same pattern of RR-stained material on their cell membrane as unrinsed lymphosarcoma cells (Dr. Feltkamp, Department of Electron Microscopy, The Netherlands Cancer Institute, personal communication).

Absolute specificity of RR for cell membrane

components has never been claimed. Nevertheless it has been argued that RR could still serve as a relatively specific cytochemical marker for membrane mucopolysaccharides, thanks to the absence of other RR-positive substances in or on the membrane (20). Our experiments show that this is not always the case. Serum contains substances that are RR positive and that adhere more or less firmly to the cell membrane, and extracellular protocollagen-like substances are also RR positive. Whether this lack of specificity makes RR completely useless for investigating normal and transformed cells on differences related to growth control is a different matter.

It is well known that cell growth *in vitro* as well as cell movement are influenced by serum factors (21, 22) and that transformed cells are less serum dependent for growth than normal cells (23, 24), perhaps because of a greater affinity for serum components. Therefore it would seem logical to include in the study of the cell surface the more or less firmly attached serum components. This would necessitate a clear terminological distinction between the morphologically defined cell membrane (25) and a functionally defined cell surface complex that might include extracellular components as well as microfibrillar structures of the cell cortex. If it should prove to be possible to consistently detect differences between normal and transformed cells in the cell surface complex by means of RR, the stain would be valuable in cytological studies on growth control. That it fails to differentiate between the cellular part and the extraneous part of this complex would be of minor importance and seems to be true of other cytochemical markers as well (15).

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