

# SURFACE MORPHOLOGY AND AGGLUTINABILITY WITH CONCANAVALIN A IN NORMAL AND TRANSFORMED MURINE FIBROBLASTS

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## ABSTRACT

The surface morphology of attached and suspended normal and transformed fibroblasts has been studied with the scanning electron microscope. Normal murine fibroblasts (3T3) grow in vitro with widely extended leading lamellae. During most parts of the cell cycle the surfaces of these cells are practically free of microvilli. When the cells round up for mitosis, their cell surfaces become adorned with many microvilli. In contrast, simian virus 40-transformed fibroblasts (SV3T3) grow more compact, and their cell surfaces remain smooth throughout the life cycle. When confluent 3T3 and SV3T3 cells are suspended with ethylenediaminetetraacetic acid (EDTA) for agglutination assays, similar differences in surface morphology are found: 3T3 cells always bear many microvilli, whereas most SV3T3 cells are essentially free of microvilli. The addition of concanavalin A (Con A) does not influence the surface morphology of the suspended cells. The morphological differences described here may be important for the agglutination process of the normal and transformed 3T3 cells, because they affect the real cell surface area and thus the density of Con A-binding sites.

The plant lectin concanavalin A (Con A) binds specifically to certain carbohydrates of the cell membrane and agglutinates transformed cells generally more easily than their normal counterparts (7, 15). For that reason many investigators have attempted lately to correlate the agglutinability by Con A with the number and distribution of Con A-binding sites on the cell membrane. Although a correlation between agglutination and the amount of cell-bound Con A has sometimes been found (19, 20), other reports indicate that differences and changes in agglutination can occur without concomitant changes in the amount of cell-bound Con A per unit cell surface area (3, 8, 14, 21).

Most recently, differences in the mobility of the

binding sites in the plane of the membrane have been suggested as the cause for differences in agglutination between normal and transformed cells. The theory implies that cross-linking agents, such as antibodies or lectins, can induce clustering of their specific binding sites in the membrane to a degree determined by the fluidity of the membrane and correlated with transformation (4, 16, 17, 18, 25, 26). In spite of the large amount of evidence for the occurrence of clustering and its occasional correlation with transformation, other data suggest that differences in clustering and agglutinability do not always coincide (22, 31, 32).

Thus, additional factors seem to play a role in the agglutination process, and therefore it ap-

peared useful to consider other parameters that change with transformation and agglutinability. One of these parameters is the gross surface morphology of normal and transformed cells (11). Furthermore it is known that, during the cell cycle, agglutinability (29) and surface morphology (23) change considerably. Therefore, we studied changes in surface morphology both during the cell cycle and after suspending cells for agglutination assays in order to find a correlation with changes in agglutination of these cells as determined in previous studies (11, 29). The relevance of our data for the general agglutination theory is discussed.

## MATERIALS AND METHODS

### *Tissue Culture*

The cells used in our experiments were obtained commercially from Flow Laboratories, Inc., Rockville, Md. as research grade normal 3T3 fibroblasts (Swiss albino ATCC [American Type Culture Collection, Rockville, Md.] cell repository no. CCL 92) and the simian virus 40-transformed (SV3T3) cells. In indirect immunofluorescence tests for the presence of T antigen, all SV3T3 cells were found to be positive. The saturation density of 3T3 cells in medium containing 10% newborn calf serum (NBS) is approximately 70,000 cells per cm<sup>2</sup>, and all cells are then resting G<sub>0</sub> cells (9). The midpoint agglutination (mpa) with Con A of these 3T3 cells is reached at approximately 1,000 μg/ml Con A (11, 29). Transformed cells reach a maximum density of approximately 500,000 cells per cm<sup>2</sup>. At higher densities the cells tend to detach from the growth substrate. The transformed 3T3 cells reach mpa at approximately 15 μg/ml Con A (11, 29).

The two cell lines were also used in our laboratory for studies on cell cycle dependent (11, 29) and density dependent (30) Con A-mediated agglutinability and on redistribution of Con A-binding sites on the plasma membrane (32).

Cells were grown in plastic Petri dishes (10 cm) containing 10 ml of Dulbecco's modified Eagle's medium, supplemented with 10% NBS and antibiotics, in a humidified CO<sub>2</sub> incubator at 37°C. The cells were seeded at a density of 50,000 per ml of nutrient medium.

### *Synchronization Procedure*

Cells at the G<sub>1</sub>/S boundary were acquired by synchronization in excess thymidine (7 mM) after stationary SV3T3 or confluent, contact-inhibited 3T3 cells were plated. Cells in S, G<sub>2</sub>, and M were obtained from cultures synchronized with excess thymidine by washing them twice with prewarmed regular medium, and leaving them for 4 h, 8 h, and 10 h, respectively. G<sub>2</sub> cells were also obtained by irradiation with 1800 R of X rays. After 24 h, more than 90% of these cells had accumulated at the

radiation-induced block in G<sub>2</sub>. These synchronization procedures did not specifically alter the cell morphology, as could be ascertained by comparison with asynchronous cultures.

Normal cells were sometimes synchronized by serum stimulation: cells grown to confluency in medium with 3% serum were stimulated with medium containing 25% serum. In this manner, approximately 75% of the cells were induced to go through the complete cell cycle. Samples were taken at different times after serum stimulation.

Samples of all preparations of synchronized cells were prepared for impulse cytophotometry, and the degree of synchronization was checked. Details of the preparation of cells for impulse cytophotometry have been published elsewhere (9). Samples were used for scanning electron microscopy when at least 70–80% of the cells were in the required phase of the cell cycle.

### *Scanning Electron Microscopy*

For scanning electron microscopy, 3T3 and SV3T3 cells were grown on cover slips, rinsed in PBS, and fixed for 30 min in 2.5% buffered glutaraldehyde. The cover slips with attached cells were put in a holder that had been specially designed to fit the pressure vessel of a critical point drying apparatus. For dehydration, this holder was left in each of a graded series of ethanol solutions (50%–70%–90%–100%) for 5 min and twice in 100% amyl acetate for 10 min. After the holder was quickly transferred to the pressure vessel, the cells were dried according to the method of Anderson (2), using liquid CO<sub>2</sub>. The dried cells on glass were mounted on stubs and covered with gold in a Balzers freeze-etching apparatus.

Suspended cells were obtained by detaching cells growing in Petri dishes with 1 × 10<sup>-5</sup> M ethylenediaminetetraacetic acid, EDTA, in Ca<sup>++</sup>- and Mg<sup>++</sup>-free PBS and washing them twice in PBS containing Ca<sup>++</sup> and Mg<sup>++</sup>. The cells were layered on cover slips with attached confluent 3T3 cells by adding 2 ml of the suspension (1 × 10<sup>6</sup> cells/ml). After 10-min incubation at 37°C in PBS, most of the suspended cells had become attached to the underlying monolayer, and the cover slips were fixed and dried as described above.

Treatment of cells with Con A at room temperature occurred in 1 ml of cell suspension (2 × 10<sup>6</sup> cells/ml) by adding 1 ml of Con A to reach the desired final concentration (up to 1000 μg/ml). After 20-min incubation, the cells were layered on cover slips. All material was studied in a Cambridge Stereoscan microscope.

## RESULTS

### *Cells in Situ*

Recently, Porter et al. (23) have shown changes in surface morphology of CHO cells during the cell cycle, and a cell cycle dependent agglutinability

with Con A has been found in transformed fibroblasts (29). Therefore, we studied changes in surface morphology of 3T3 and SV3T3 cells during the cell cycle in an attempt to find differences that might influence the agglutination process of these cells.

To that purpose, asynchronous and synchronized normal and transformed 3T3 cells were prepared for scanning electron microscopy. The degree of synchronization was checked with an impulse cytophotometer. Fig. 1 shows a representative example of impulse cytophotometric graphs of SV3T3 cells, indicating the degree of synchronization routinely obtained with both cell types.

Normal subconfluent 3T3 cells in late  $G_1$ , S, and  $G_2$  had widely spread, flat leading lamellae (1). At these stages, all cells had practically no microvilli or zeiotic blebs (Fig. 2 *a* and *b*), and the cells were morphologically indistinguishable from one another. Only impulse cytophotometry showed that the cells were synchronized as indicated in the legend. In late  $G_2$  (just before mitosis) the cells began to retract the leading lamellae, and their surfaces showed many microvilli of varying length (Fig. 2 *c*) and sometimes zeiotic blebs (not shown). Slender extensions at the sides of the cells were also found and considered to be developing retraction fibrils (12) (Fig. 2 *c*, arrow). With the decreasing size of the cell surfaces, the number of

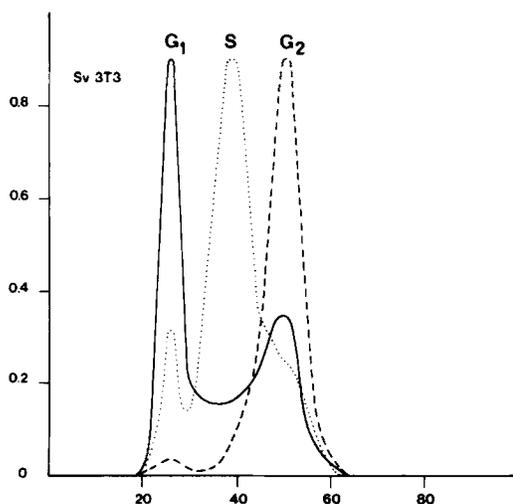


FIGURE 1 Impulse cytophotometric graph of distribution of SV3T3 cells in the cell cycle. Abscissa: channel number (increasing amount of DNA/cell). Ordinate: cells per channel ( $\times 10^4$ ). (—), asynchronous culture; (····), thymidine-synchronized culture in S; (----), culture in  $G_2$  after X-ray irradiation.

microvilli seemed to increase, and the retraction fibrils on the sides of the cells became longer. After completely rounding up for mitosis, the cells remained attached to the growth substrate by a number of retraction fibrils only (Fig. 2 *d*). After cytokinesis, the two daughter cells immediately began to spread over the substrate along the retraction fibrils. This process continued in early  $G_1$ , and during that phase of the cell cycle the number of microvilli gradually decreased (Fig. 2 *e*). If zeiotic blebs had been present, they also tended to disappear during or after mitosis, and by late  $G_1$  the cells had become fully expanded with smooth surfaces. Resting confluent 3T3 cells ( $G_0$ ) did not differ markedly from subconfluent 3T3 cells in late  $G_1$ , S, or  $G_2$  with respect to the size of the leading lamellae. Their surfaces were not always completely free of microvilli (Figs. 2 *f* and 4 *a*; Fig. 6, underlying cells), but, if present, the microvilli were much shorter than on subconfluent 3T3 cells in mitosis.

SV3T3 cells synchronized in late  $G_1$ , S, and  $G_2$  resembled their normal parent cells somewhat, especially in subconfluent and confluent cultures, but they were smaller and less widely spread over the underlying glass (Fig. 3 *a* and *b*). The differences in morphology and in growth pattern between normal and transformed 3T3 cells became more clearly visible after the cells had reached confluent and superconfluent densities (compare Figs. 4 *a* and 5 *a*). The surfaces of SV3T3 cells in late  $G_1$ , S, and  $G_2$  were completely smooth. In late  $G_2$ , the cells began to contract, but, in contrast to 3T3 cells, the SV3T3 cells did not develop many microvilli on the upper surfaces (Fig. 3 *c*, arrow 1). Sometimes, zeiotic blebs could be found, but in most cases the upper cell surfaces were free of extensions (Fig. 3 *c*, insert). Only shallow ridges and small folds were often present, perhaps as traces of withdrawn leading lamellae. With the decreasing size of the cells, retraction fibrils at the periphery of the cells increased in number and length. During mitosis, the cell surfaces remained practically free of microvilli (Fig. 3 *c*, arrow 2). After cytokinesis, the cells began to spread, and during early  $G_1$  the number of retraction fibrils decreased with the progressive flattening of the cells over the underlying surface.

Thus, the scanning electron micrographs of 3T3 and SV3T3 cells *in situ* revealed two major differences between normal and transformed cells: (a) transformed cells are less widely extended over the growth substrate than normal cells and (b)

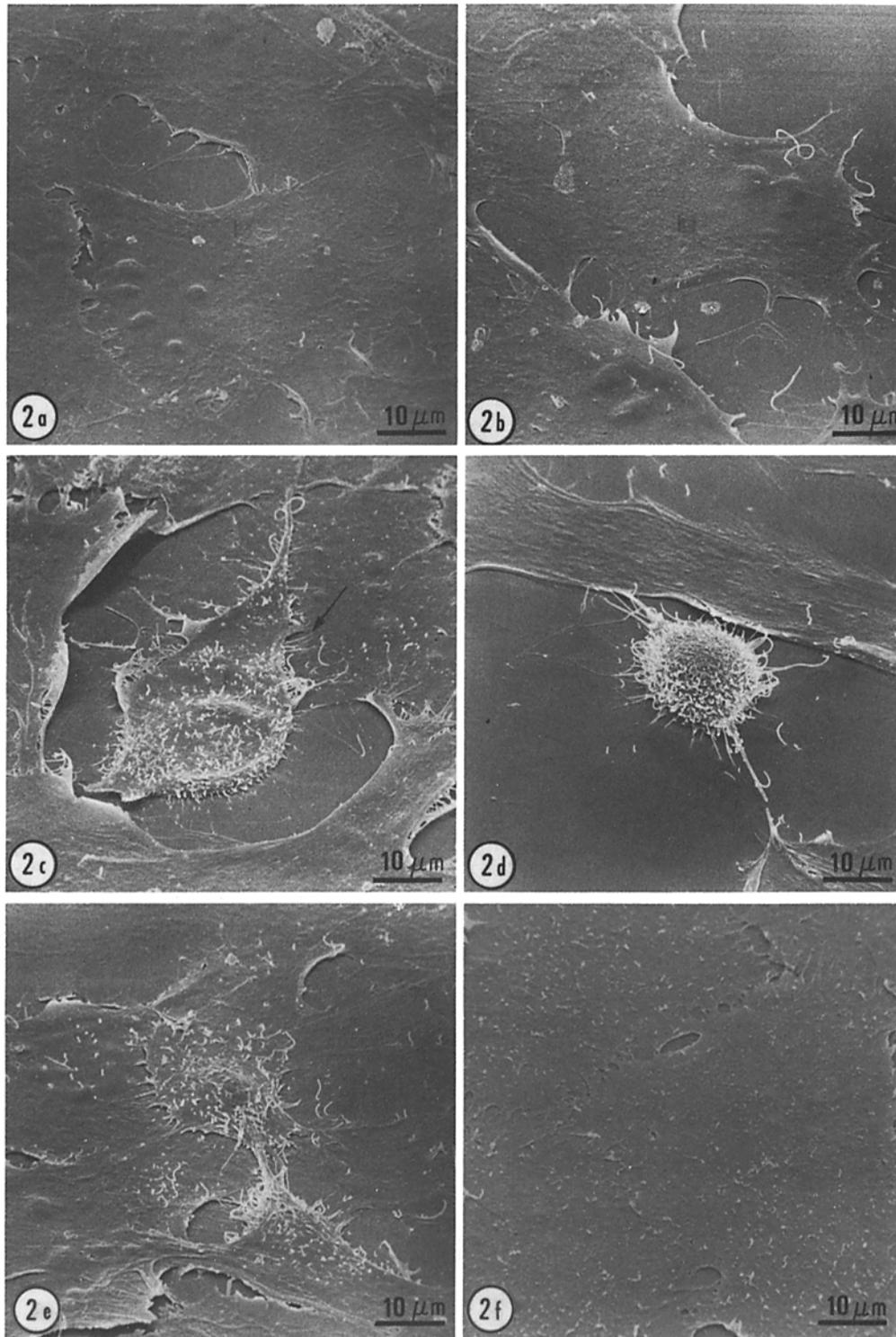


FIGURE 2 Subconfluent, normal 3T3 cells *in situ*. (a) Late G<sub>1</sub> cells at thymidine block; (b) S cells grown for 4 h after release of thymidine block; (c) Late G<sub>2</sub> cell in field of G<sub>2</sub> cells, grown for 8 h after release of thymidine block; (d) Mitotic cell in culture grown for 10 h after release of thymidine block; (e) Early G<sub>1</sub> cells in same culture as (d); and (f) Confluent culture (G<sub>0</sub> cells).

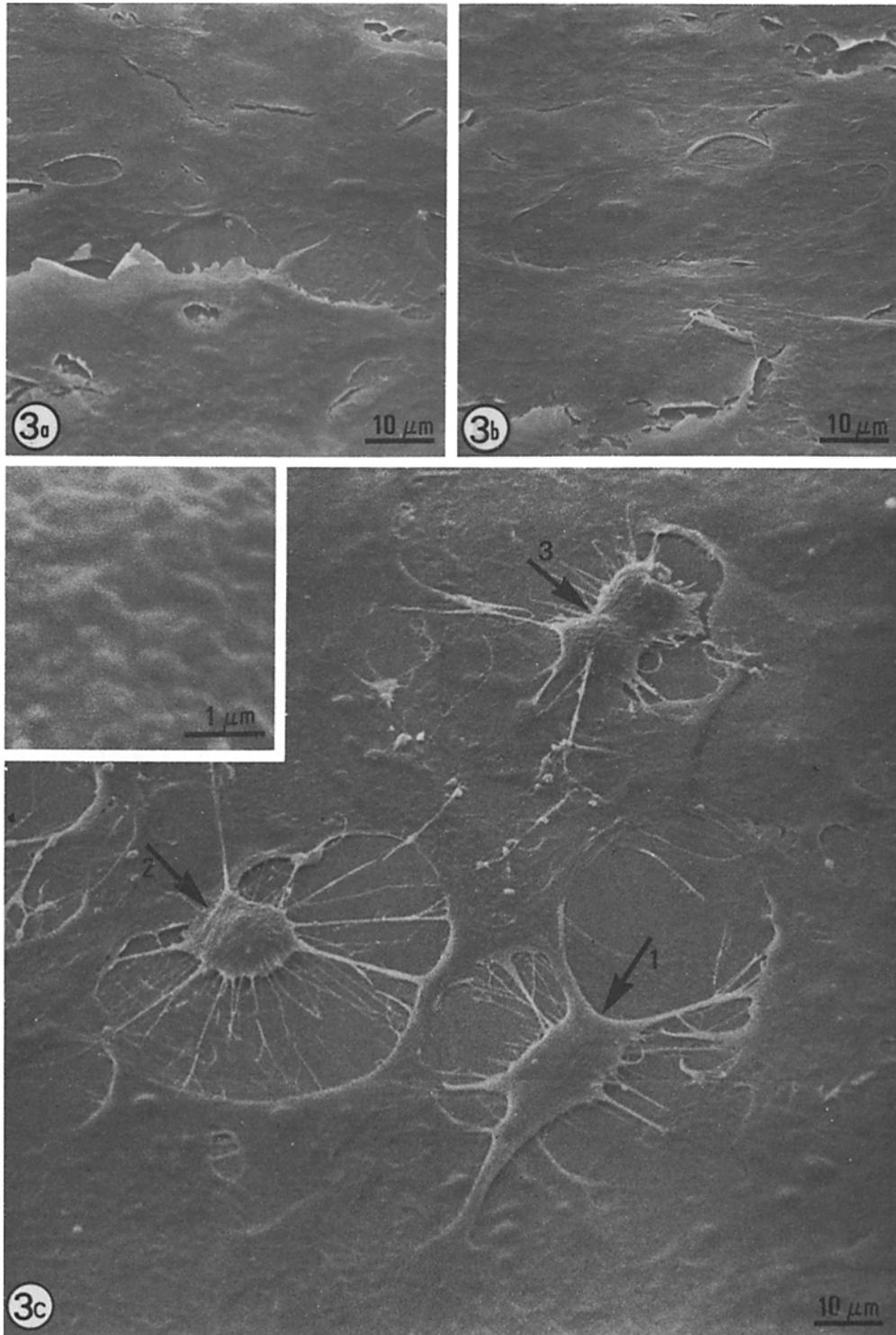


FIGURE 3 Almost confluent, transformed 3T3 cells *in situ*. (a) Late G<sub>1</sub> cells at thymidine block; (b) S cells grown for 4 h after release of thymidine block; and (c) Late G<sub>2</sub> cell (arrow 1), mitotic cell (arrow 2), and early G<sub>1</sub> cell (arrow 3) in culture grown for 10 h after release of thymidine block; the *insert* shows part of the cell surface of a mitotic cell at higher magnification.

whereas 3T3 cells have a great number of microvilli on the upper cell surfaces during late G<sub>2</sub>, M, and early G<sub>1</sub>, SV3T3 cells remain free of microvilli throughout their entire life cycle.

#### *Cells Detached with EDTA*

To determine whether the morphological differences between 3T3 and SV3T3 cells *in situ* are relevant for the agglutination process, we investigated the same cells in suspended form since suspended cells are used in the agglutination assays.

Fig. 4 *a-d* show the changes in surface morphology of confluent 3T3 cells during detachment from the growth substrate with EDTA. As mentioned above, these cells were widely spread and the surfaces occasionally bore few short microvilli (Fig. 4 *a*). The edges of adjacent cells were in complete contact with one another, except where slight shrinkage during the drying procedure had broken the contact locally. Impulse cytophotometry of these cells indicated that no S and G<sub>2</sub> cells were present (9). After incubation with EDTA for 5 min at 25°C, the cells began to withdraw their leading lamellae (Fig. 4 *b*). At this stage, no changes on the cell surface were yet observed compared to untreated control cells. After incubation with EDTA under the same conditions for 7 min, the leading lamellae were almost fully withdrawn. Many microvilli of different lengths began to appear on the cell surface (Fig. 4 *c*). A few short retraction fibrils became also visible at this stage. After treatment with EDTA for 10 min, the cells were practically rounded up (Fig. 4 *d*). Many microvilli were seen on the surfaces of these cells. Contact with the underlying substrate was maintained by slender retraction fibrils resembling morphologically some of the longer microvilli. Sometimes, blebs might also appear on the surfaces of some cells during rounding up. Fig. 4 *e* and *f* show the cells brought into suspension as described and layered on cover slips with attached confluent 3T3 cells. Most suspended 3T3 cells had surfaces adorned with many microvilli. The microvilli might vary in length and, to a certain extent, in number, but they were always numerous.

Fig. 5 *a-d* illustrate the detaching of superconfluent SV3T3 cells with EDTA. Untreated SV3T3 cells had no extensions on the cell surfaces (Fig. 5 *a*). After treatment with EDTA for 3 min at 25°C, the small leading lamellae began to withdraw, but the surface structure remained unaltered (Fig. 5 *b*). The leading lamellae were almost completely with-

drawn after incubation with EDTA for 5 min, and a number of retraction fibrils became visible (Fig. 5 *c*). Practically no microvilli were visible on the surfaces of most cells, but the cell surfaces showed some shallow folds and ridges. Incubation with EDTA for 10 min resulted in rounded cells attached to the substrate with some retraction fibrils (Fig. 5 *d*). Most of the cells had relatively smooth surfaces free of microvilli but with folds and creases. On some other cells, varying numbers of rather short microvilli were present. When SV3T3 cells were suspended and layered on cover slips with confluent 3T3 cells, the transformed cells showed a range of appearances (Fig. 5 *e* and *f*). Most cells were smooth without microvilli, but cells with varying numbers of microvilli were also found. The smooth surface in most cases had some folds and ridges. These were less clearly seen on cells with microvilli.

Because of the variation in surface morphology of individual cells and the resulting difficulty in demonstrating this in a few micrographs, we introduced a double-blind test to determine the composition of each sample: suspended 3T3 and SV3T3 cells were investigated under code numbers, and approximately 25 pictures were randomly taken of each sample of four different experiments. The coded photographs of several samples were put together, and a number of persons were asked to sort the cells into three categories according to the number and length of the microvilli on the surfaces (rough, intermediate, and smooth). The results are shown in Fig. 7. A marked difference was found consistently in the average surface morphology between 3T3 and SV3T3 cells, that is, between cells with low and high agglutinabilities.

#### *Suspended Cells Treated with Con A*

Experiments on lymphoblasts had shown that their surface morphology may be greatly influenced by the addition of Con A in concentrations necessary for agglutination. Upon addition of Con A, the microvilli present on these cells tend to flatten over the cell surfaces and seem to be resorbed by the cells (Collard, J. G., and J. H. M. Temmink. Submitted for publication.). Because of our interest in the relation between surface morphology and agglutinability, we investigated the effect of Con A on the surface morphology of suspended 3T3 and SV3T3 cells after incubation with different concentrations of Con A.

However, the surface morphology of both 3T3

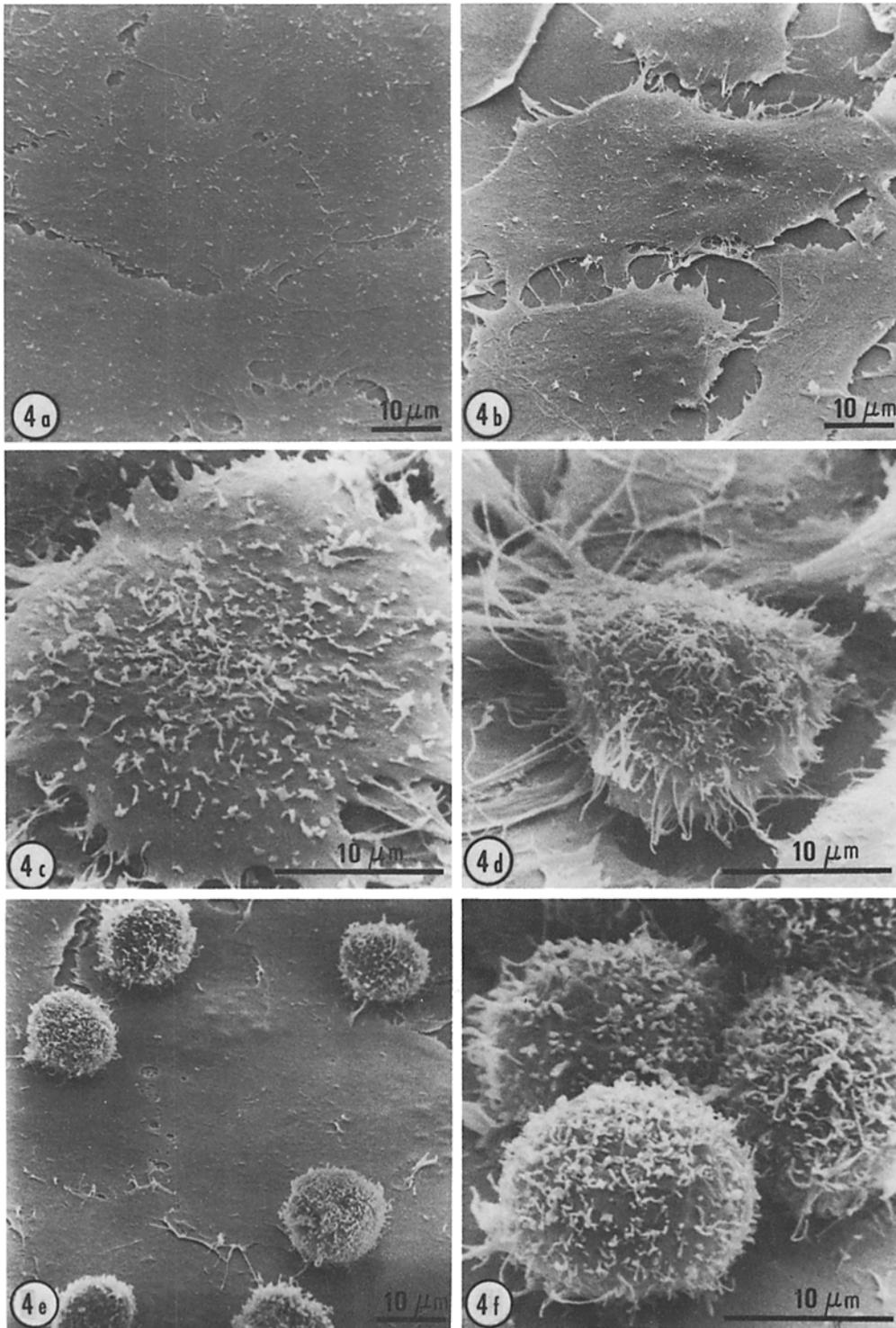


FIGURE 4 Confluent, normal 3T3 cells detached with EDTA. (a) Confluent culture before EDTA addition; (b) Culture 5 min after EDTA addition; (c) Culture 7 min after EDTA addition; (d) Culture 10 min after EDTA addition; and (e and f) EDTA-detached cells layered on confluent 3T3 cells.

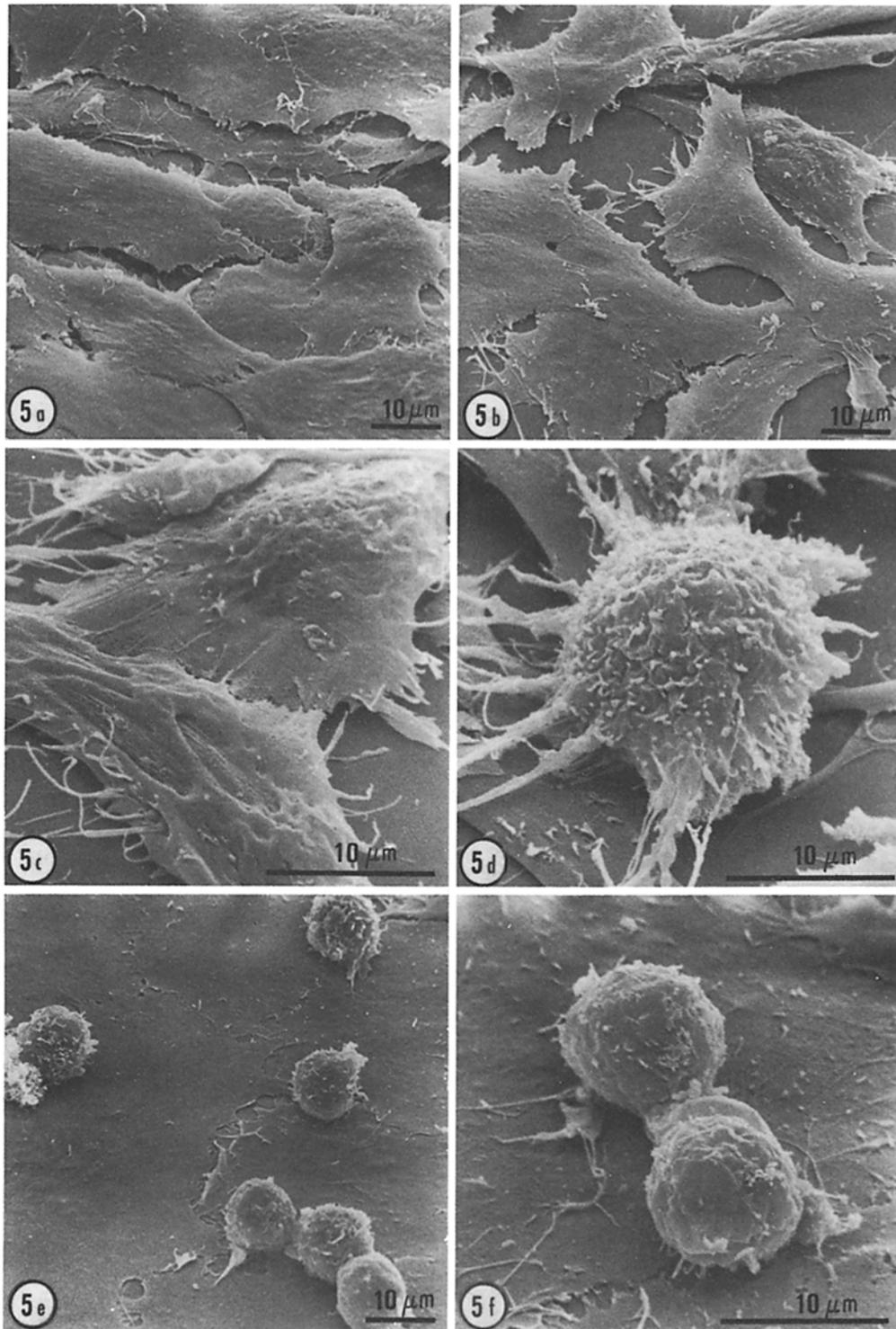


FIGURE 5 Superconfluent, transformed 3T3 cells detached with EDTA. (a) Superconfluent culture before EDTA addition; (b) Culture 3 min after EDTA addition; (c) Culture 5 min after EDTA addition; (d) Culture 10 min after EDTA addition; and (e and f). EDTA-detached cells layered on confluent 3T3 cells.

and SV3T3 cells incubated with different concentrations of Con A (up to 1,000  $\mu\text{g}/\text{ml}$ ) was not affected (Figs. 6 and 7). In number and length, the microvilli were similar to those on suspended cells not exposed to Con A.

## DISCUSSION

The present investigation on the surface morphology of normal and transformed 3T3 cells revealed two conspicuous differences. (a) Normal cells have widely spread leading lamellae in subconfluent and confluent cultures, whereas transformed cells in subconfluent cultures have relatively small leading lamellae that decrease further in size when the cultures reach confluent and superconfluent densities. This difference in size has been described

before in light microscope studies (5) and in studies on replicas of these cells (11). It partially agrees with a recent scanning electron microscope study on attached normal and transformed BALB/3T3 cells (24), although the SV40-transformed BALB/3T3 cells are much more spindle-shaped than our SV40-transformed Swiss 3T3 cells. In addition, the SV40-transformed BALB/3T3 cells, but not the spontaneously transformed BALB 3T3 cells, resemble our SV40-transformed Swiss 3T3 cells in the virtual absence of microvilli from the surfaces (24).

(b) Synchronized cultures of 3T3 and SV3T3 cells differ clearly in the morphological changes that occur around the mitotic process. Whereas most SV3T3 cells have practically no microvilli

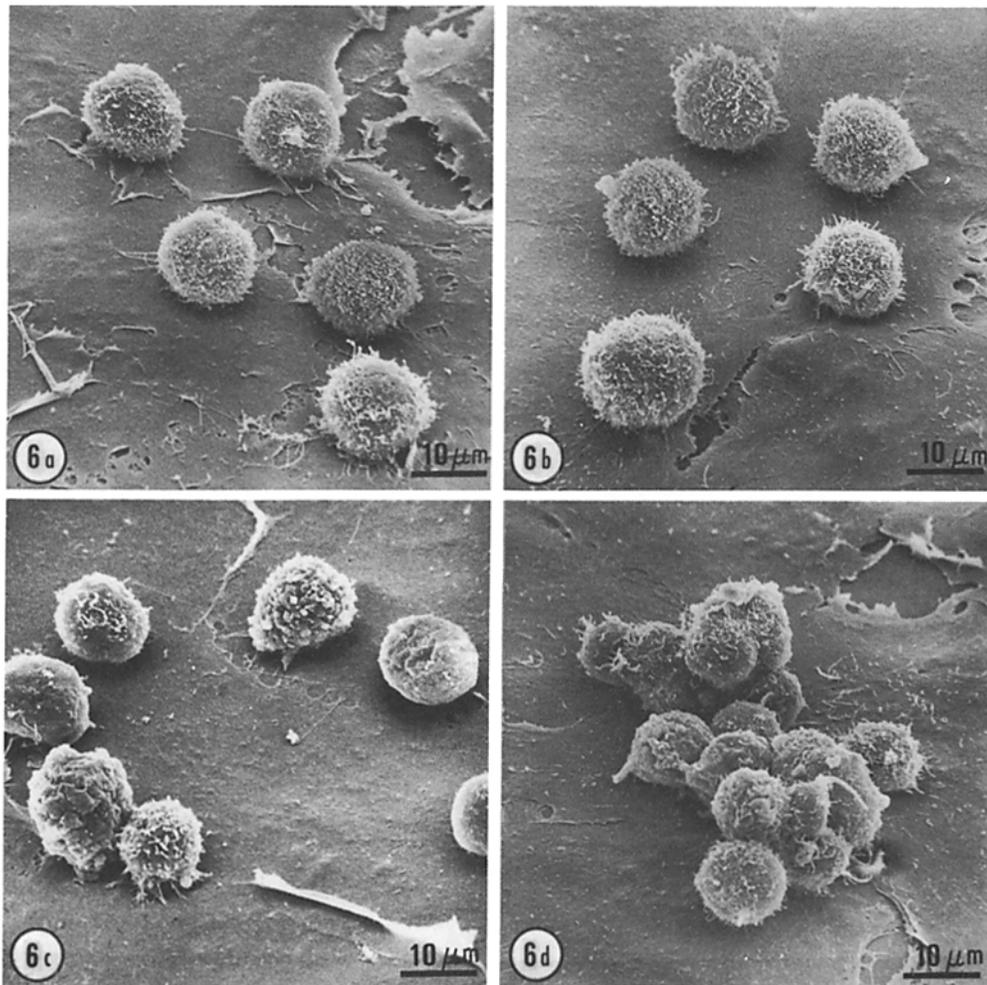


FIGURE 6 Suspended 3T3 and SV3T3 cells with and without addition of Con A (500  $\mu\text{g}/\text{ml}$ ). (a) 3T3 cells without Con A; (b) 3T3 cells with Con A; (c) SV3T3 cells without Con A; and (d) SV3T3 cells with Con A.

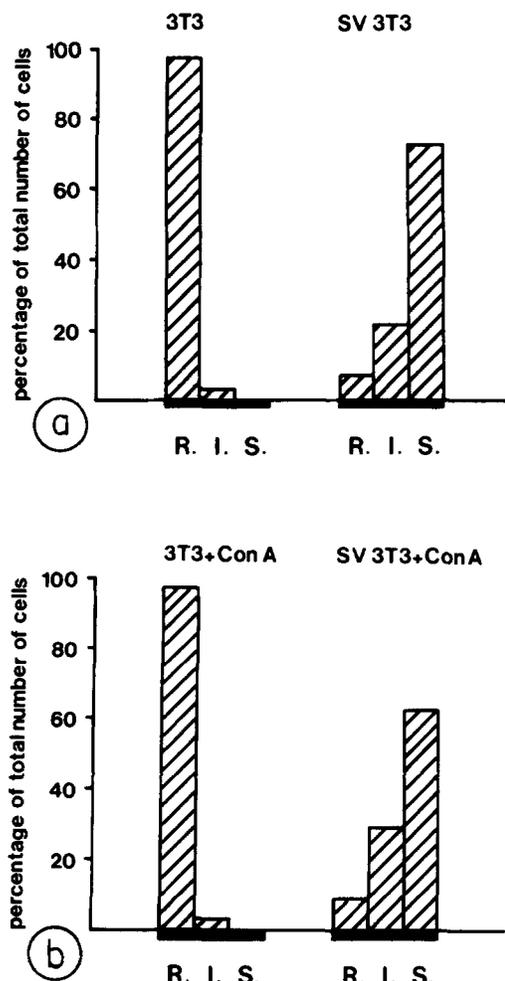


FIGURE 7 (a) Histogram of differences in surface morphology between 3T3 and SV3T3 cells. R, rough (with many microvilli); I, intermediate; and S, smooth (without microvilli). (b) Histogram of differences in surface morphology between 3T3 and SV3T3 cells at 20 min after Con A addition (500  $\mu\text{g}/\text{ml}$ ). R, rough (with many microvilli); I, intermediate; and S, smooth (without microvilli).

around mitosis, normal 3T3 cells always have many microvilli in late  $G_2$ , M, and early  $G_1$ . These changes in surface morphology during the cell cycle of 3T3 cells are in general agreement with the observations of Porter et al. on CHO cells (23). However, in contrast with the observations on CHO cells (27), the variation in the number of microvilli during the cell cycle of SV3T3 cells was greater in subconfluent than in confluent cultures. SV3T3 cells in confluent and superconfluent cultures had only retraction fibrils, and microvilli were absent from the cell surfaces during mitosis.

The difference in size between spread 3T3 and SV3T3 cells *in situ* seems to be reflected in detached cells in differences in the number of microvilli similar to the differences in number of microvilli in spontaneously contracting cells *in situ* during mitosis. This might perhaps be considered to indicate that microvilli develop on cells as a means of "storing" surplus membrane material, as suggested before (13).

The presence or absence of zeiotic blebs has been mentioned in the present paper without much emphasis. We do not know what conditions during growth or handling of the cells determine whether zeiotic blebs arise. Since in our material some mitotic or suspended cells had zeiotic blebs only occasionally, we considered the blebs to be artifacts. Because the formation of blebs is not known to interfere with the formation of microvilli and because blebbing does not influence the viability of the cells, we have neglected the phenomenon as irrelevant for the subject of this paper.

The variation in morphological appearance, especially of individual suspended transformed cells, as demonstrated in the histograms (Fig. 7), is probably due to a variable effect of EDTA on the cells, and is also, perhaps, a result of the different phases of the cell cycle in which the cells are suspended.

Treatment of suspended cells with Con A as in agglutination assays did not influence markedly the morphological appearance of suspended normal and transformed 3T3 cells. Our data indicate that the described morphology of our suspended fibroblasts reflects the surface morphology of these cells in agglutination assays.

The data presented here show a large difference in surface morphology between normal and transformed cells, probably as a result of the large difference in size between these cells *in situ* and the difference in capacity to absorb excess membrane. This difference in morphology affects the cell surface area determinations made in order to correlate the density of Con A-binding sites with agglutinability. As yet, cell surface areas of suspended normal and transformed 3T3 cells have been determined on the assumption that both cell types are perfect spheres in suspension (5, 15, 19). However, our data indicate that the cell surface areas of normal suspended cells are much larger than those of transformed cells, due to the microvilli. Because equal numbers of Con A molecules are bound to normal and transformed cells (3, 5, 8, 21), and because our preliminary data on Con A-binding site distribution on suspended cells

indicate that microvilli have essentially the same number of Con A-binding sites as cell membrane areas between microvilli, the density of Con A-binding sites must be much greater on transformed than on normal cells. The quantitative aspects of the influence of surface morphology on the number of Con A-binding sites have been treated in a separate paper (10). The difference in the density of Con A-binding sites may also play a role in the difference in degree of clustering of these sites on normal and transformed cells, as originally observed by Nicolson (16) and Singer and Nicolson (28), and may be the main cause of the different agglutination responses of normal and transformed 3T3 cells, as originally suggested by Burger (6).

We are presently investigating whether the described morphological changes in attached cells during the cell cycle are reflected in the surface morphology of synchronized suspended cells in correlation with described changes in the agglutinability (29). Furthermore, it is of interest how brief proteolytic treatment affects the surface morphology of suspended normal cells in relation to the described increase in agglutinability (6).

#### ADDENDUM

After submission of this manuscript, a paper by Willingham and Pastan<sup>1</sup> appeared with data that seemed to completely contradict the results presented in our report. The cells of their transformed murine fibroblast line (L 929), *in situ* as well as in suspension, were adorned with many microvilli, whereas their normal cells (3T3-4) were free of these surface extensions when *in situ* but developed them when rounding off spontaneously (mitosis) or by trypsin treatment. The L 929 could be made free of microvilli by dibutyryl cyclic AMP, a drug that also converts the growth pattern and agglutinability of these cells to those of nontransformed cells.

However, additional research may prove the above discrepancies to be due to differences in the cell material used. This seems likely when the results of Porter et al. (24) are also taken into account. The absence of microvilli from their SV40-transformed BALB/3T3 resembles that on our transformed Swiss 3T3, whereas the results on their spontaneous transformants (e.g. S2-4) would

<sup>1</sup> Willingham, M. C., and Ira Pastan. 1975. Cyclic AMP modulates microvillus formation and agglutinability in transformed and normal mouse fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **72**:1263.

seem to substantiate Willingham and Pastan's data.

In addition, we agree with Nicolson<sup>2</sup> that the presence or absence of microvilli alone does not explain the differences in agglutinability: our smooth SV3T3 cells agglutinate very well with Con A, but so do our lymphoblasts (Raji and EB<sub>3</sub>) that have many microvilli, whereas normal peripheral lymphocytes with many microvilli do not agglutinate with Con A (Collard, J. G., and J. H. M. Temmink. Submitted for publication.).

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