

ROLE OF MICROVILLI IN SURFACE CHANGES OF SYNCHRONIZED P815Y MASTOCYTOMA CELLS

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ABSTRACT

The surface morphology of synchronized P815Y mastocytoma cells has been examined by scanning electron microscopy. Early G₁ cells are comparatively smooth or lightly villated, whereas at later stages the surface becomes progressively more villated. In G₁ cells most microvilli have a uniform diameter, whereas in S and G₂, cells, many microvilli show branching and often originate from much larger surface protuberances. Small "blebs" are seen on the surface of many cells but these structures do not appear to be a characteristic feature of cells at any one stage of the cell cycle. The presence of microvilli increases the total surface of the cell to such an extent that the ratio of volume to surface area remains constant throughout the cell cycle. The mechanism of cytokinesis is thus a physical one, involving the unfolding of previously accumulated microvilli.

The surface architecture is an important determinant of cellular growth (10). It is therefore of interest to know whether specific changes accompany the various phases of the cell cycle, and several studies have been directed towards this end (5, 14, 15). The cells used have generally been grown in monolayer culture, as a result of which the changes due to attachment to substratum, to "spreading," or to movement have been superimposed on changes due solely to a doubling of surface components. For this reason we have studied cells in suspension culture that remain spherical throughout interphase. By measuring the composition of the plasma membrane during the cell cycle, we concluded that its components are synthesized and inserted between G₁¹ and G₂ as cells gradually double in volume; the extra surface area required for the production of two daughter cells is presumably provided by some kind of

¹ The usual convention for periods of the cell cycle is used: M, mitosis; G₁, gap 1; S, (DNA) synthesis; G₂, gap 2.

"stretching" or "unfolding" process during cytokinesis (7). The nature of such a process has now been investigated. In a complementary study² it is shown that there is no significant change in the thickness of the plasma membrane, or in the density of intramembranous particles during the cell cycle. In this paper we present data, previously reported in brief (8), showing that during interphase there is an accumulation of microvilli which unfold during cytokinesis.

MATERIALS AND METHODS

The cells used were a cloned derivative of the P815Y line of the mouse mastocytoma originally isolated by Dunn and Potter (3). Cells were grown in suspension culture in Fischer's medium supplemented with 8% horse serum (Tissue Culture Services, Slough, England). Such cells gradually increase in size during interphase and can therefore be separated according to their position in the cell cycle by rate zonal centrifugation (11) as follows (17). Exponentially growing cells (approximately 6×10^5 /ml)

² S. Knutton. Manuscript submitted for publication.

were incubated with radioactive thymidine (Radiochemical Centre, Amersham, Bucks, England; 0.02 $\mu\text{Ci/ml}$) for 30 min, centrifuged, washed in 10 mM Tris-buffered saline, (TBS) pH 7.4, and concentrated in 25 ml of TBS containing 1% Ficoll (Pharmacia, Uppsala, Sweden). The cell suspension (approximately 10^9 cells) was injected at the center of an MSE Type A zonal rotor containing a 2–10% linear Ficoll gradient. Centrifugation was carried out for 15 min at 500 rpm, and 25-ml aliquots were collected into sterile tubes. Cells from the zonal rotor were pooled into eight fractions such that each fraction contained approximately the same number of cells. Fractions were assayed for cell size (Coulter counter model A, Coulter Electronics Inc., Hialeah, Fla.), total DNA (6), and incorporation of [^3H]thymidine into [^3H]DNA.

For growth of synchronous cells, those in fraction 2, corresponding to early G_1 , were diluted with Fischer's medium containing serum (8%) at 37°C to a final concentration of approximately 2×10^5 cells/ml in a 1-liter spinner flask. Cells were incubated for 28 h, 20-ml aliquots being removed every 2 h. After exposure for 15 min to [^3H]thymidine, each sample was assayed for cell number, size, and incorporation of [^3H]thymidine. Synchrony was maintained for the duration of one generation (see Fig. 2).

For time-lapse photography, kindly carried out by Mr. S. A. Buckingham (Dunn School of Pathology, Oxford), cells were suspended at a concentration of 10^6 /ml in 0.65% agar, with 20% serum in Fischer's medium as diluent. A 25-ml Falcon flask was filled with the cell suspension and allowed to stand at 37°C for 1 h, by which time the agar formed a supporting gel. With an inverted phase-contrast microscope, cells near the bottom surface of the flask were photographed at 30-s intervals for the duration of one cell cycle.

For scanning electron microscopy, a suspension of cells (5×10^6 /ml) from each zonal fraction, or aliquots from continuous culture, were washed and allowed to settle onto a monolayer of SV40/3T3 fibroblast cells grown on carbon-coated glass cover slips; these cells act as a convenient anchor for the small spherical P815Y cells. Immediately afterwards, cells were fixed with 3% glutaraldehyde in TBS for 30 min and postfixed for 1 h with 1% OsO_4 in TBS. Cells were dehydrated through a graded series of alcohols and amyl acetates up to 100% amyl acetate. The cover slips were then transferred into liquid CO_2 and critical point dried (1) with an E3000 CPD apparatus from Polaron Equipment Ltd., Watford, England. The specimens were coated with a thin layer of gold (E5000 Sputter-Coating apparatus, made available by Polaron) and examined in a Cambridge stereoscan Type II electron microscope operated at 30 kV with 40-s scanning periods.

Estimation of the number and lengths of microvilli on individual cells was made by enlarging the scanning electron microscope negative 20 times with a Nikon Profile Projector model 6CT2 (Nikon Inc., Garden City,

N. Y.); the length and number of microvilli in a given area (generally $\sim 20 \mu\text{m}^2$) were assessed. A mean of at least 50 cells was used for calculating data. In some very highly villated cells it is difficult to count individual microvilli; calculations were therefore made from stereo projections or from freeze-fracture replicas as follows.² Microvilli become cross-fractured when fractures travel along the plasma membrane resulting in a fracture face interrupted by numerous holes ($\sim 0.1 \mu\text{m}$ in diameter). The amount of surface area elaborated into microvilli can then be determined from the total area of holes as a percentage of the total area of the fracture face. The results quoted in Table I are the mean of at least 20 measurements for both early G_1 and G_2 cells.

RESULTS

Assessment of Cell Synchrony

After zonal fractionation, cells were allocated to a particular stage of the cell cycle on the basis of: (a) amount of [^3H]thymidine incorporated (i.e. [^3H]DNA synthesis), (b) content of DNA, and (c) cell size. As can be seen from Fig. 1, cell size increased gradually and fractions representing G_1 cells (single complement of DNA, low incorporation of thymidine), S cells (more than a single complement of DNA, high incorporation of thymidine), and G_2 cells (double complement of DNA, low incorporation of thymidine) can be sep-

TABLE I
Comparison of Surface Area and Number of
Microvilli in Early G_1 and G_2 Cells

	Early G_1 cells	G_2 cells
Apparent surface area* (A)	$\sim 225 \mu\text{m}^2$	$\sim 350 \mu\text{m}^2$
% of cell surface elaborated into microvilli†	$\sim 3\%$	$\sim 6\%$
Number of microvilli per cell‡	~ 850	$\sim 2.6 \times 10^3$
Surface area contributed by microvilli (B)	$\sim 110 \mu\text{m}^2$	$\sim 340 \mu\text{m}^2$
Total area of cell surface (A + B)	$\sim 335 \mu\text{m}^2$	$\sim 690 \mu\text{m}^2$

Details of sample size and measurement of cell parameters are given in Materials and Methods.

* Assuming a smooth sphere.

† Calculated from freeze-fracture replicas as described in Materials and Methods.

‡ Calculated assuming that each microvillus has a diameter of $0.1 \mu\text{m}$, and using the figures for apparent surface area and the percent elaborated into microvilli.

|| Calculated assuming that each microvillus is a cylinder of diameter $0.1 \mu\text{m}$ and mean length $0.4 \mu\text{m}$.

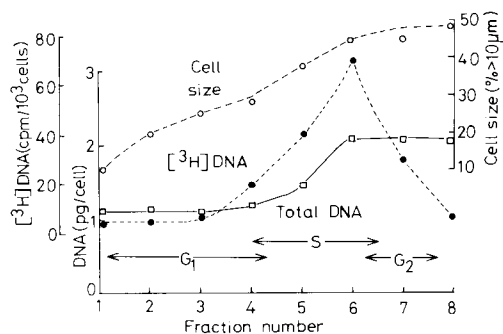


FIGURE 1 Separation of cells by zonal centrifugation. Cell size, $[^3\text{H}]\text{DNA}$, and total DNA in each pooled fraction were measured as described in Materials and Methods. Cell size (percent cells of diameter $> 10 \mu\text{m}$) \circ — \circ ; $[^3\text{H}]\text{DNA}$ (cpm/ 10^3 cells) \bullet — \bullet ; total DNA (pg/cell) \blacksquare — \blacksquare .

arated by this technique. Mitotic cells, which represent $< 5\%$ of an exponentially growing culture (2), tend to move with the G_2 cells, and are not obtained as a discrete fraction by this technique.

Although the surface changes to be documented in the next section are clearly related to cell size (e.g., Table I), it is desirable to confirm that they are also dependent on progression through the cell cycle. Synchronous growth was therefore initiated as described in Materials and Methods. Fig. 2 shows that when a fraction of early G_1 cells (fraction 2) was returned to spinner culture, the cell number remained constant for approximately 14 h and then proceeded to increase as cells divided. Cell size increased gradually until division, after which small cells were produced and the percentage of large cells decreased. Incorporation of $[^3\text{H}]\text{thymidine}$ was low during the first 12 h but reached a maximum just before cell division. These data are consistent with synchronous growth of the G_1 cells. With other fractions corresponding to S and G_2 periods, cells also grew synchronously for the duration of one cell cycle.

Measurement of Cell Volume

Volumes were calculated (a) from phase-contrast micrographs of cells in agar, by measuring the internal diameter to the edge of the dark halo (Fig. 3) (note that P815Y cells remain spherical whether suspended in liquid medium or in soft agar); (b) from scanning electron micrographs, by measuring the diameter of the spherical portion only, the volume occupied by microvilli being ignored; (c) from mode volumes from Coulter counter measurements (calibrated against latex

spheres [17]). Mean values ($\pm 10\%$ in each case) for the volume of an early G_1 cell and a G_2 cell are: (a) 660 and $1,315 \mu\text{m}^3$, (b) 320 and $690 \mu\text{m}^3$, (c) 490 and $1,000 \mu\text{m}^3$. In each case, therefore, an approximate doubling is observed to occur. Differences in actual values are due to factors such as shrinkage during preparation, inclusion of area "swept" by microvilli, etc.

Scanning Electron Microscopy

The following results were obtained with cells separated by zonal centrifugation and with syn-

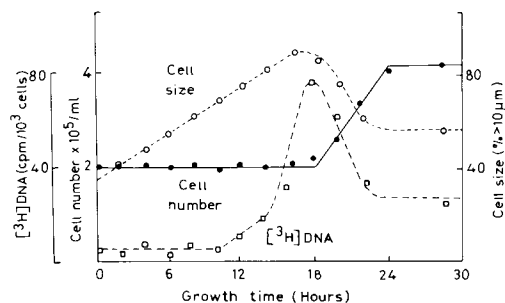


FIGURE 2 Synchronous growth of early G_1 cells (Fraction 2 of Fig. 1). Cell number $\times 10^5/\text{ml}$, \bullet — \bullet ; cell size (percent of cells of diameter $> 10 \mu\text{m}$) \circ — \circ ; and $[^3\text{H}]\text{DNA}$ (cpm/ 10^3 cells) \blacksquare — \blacksquare .

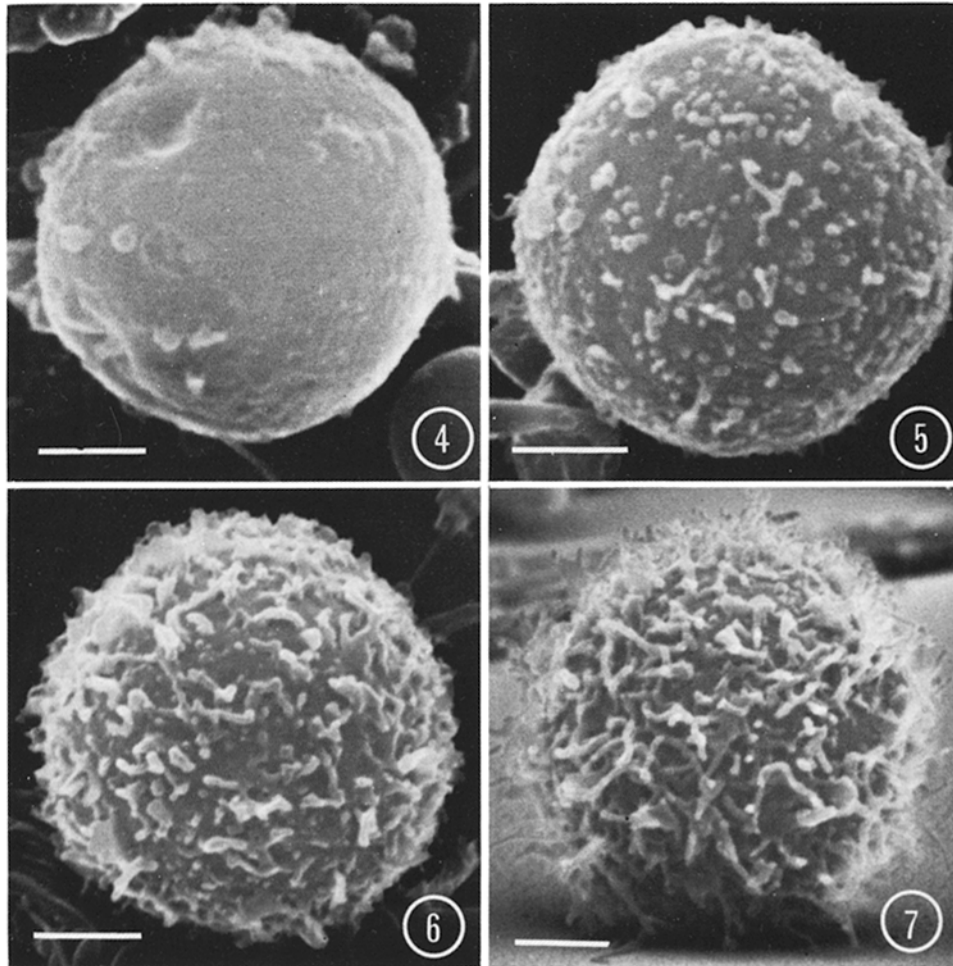
Time (min)	Volume (μm^3)
0	695
150	735
300	860
450	905
600	930
750	1205
940	1315
960	---
967	---
975	660

FIGURE 3 Time-lapse photograph of a P815Y mastocytoma cell through one division cycle. 2 frames/min were taken, 10 of which are illustrated. Volumes were calculated from the measured diameter (inside edge of dark halo), assuming a smooth sphere.

chronously growing cultures. In no case were any discrepancies observed.

All early G_1 cells have a fairly uniform diameter of $\sim 8.5 \mu\text{m}$ but other characteristics differ. Some cells ($< 5\%$) appear comparatively smooth and

devoid of any clearly defined surface protuberances, apart from an occasional small "bleb" on some cells (Figs. 4 and 10). Such "blebs" ($0.3\text{--}1.5 \mu\text{m}$ in diameter) are seen on the surface of many cells (Figs. 4, 5, 10, 14, 16) and are not characteris-



FIGURES 4-16 All illustrations are scanning electron micrographs of P815Y mastocytoma cells (see Materials and Methods). The scale marker in all micrographs equals $2 \mu\text{m}$.

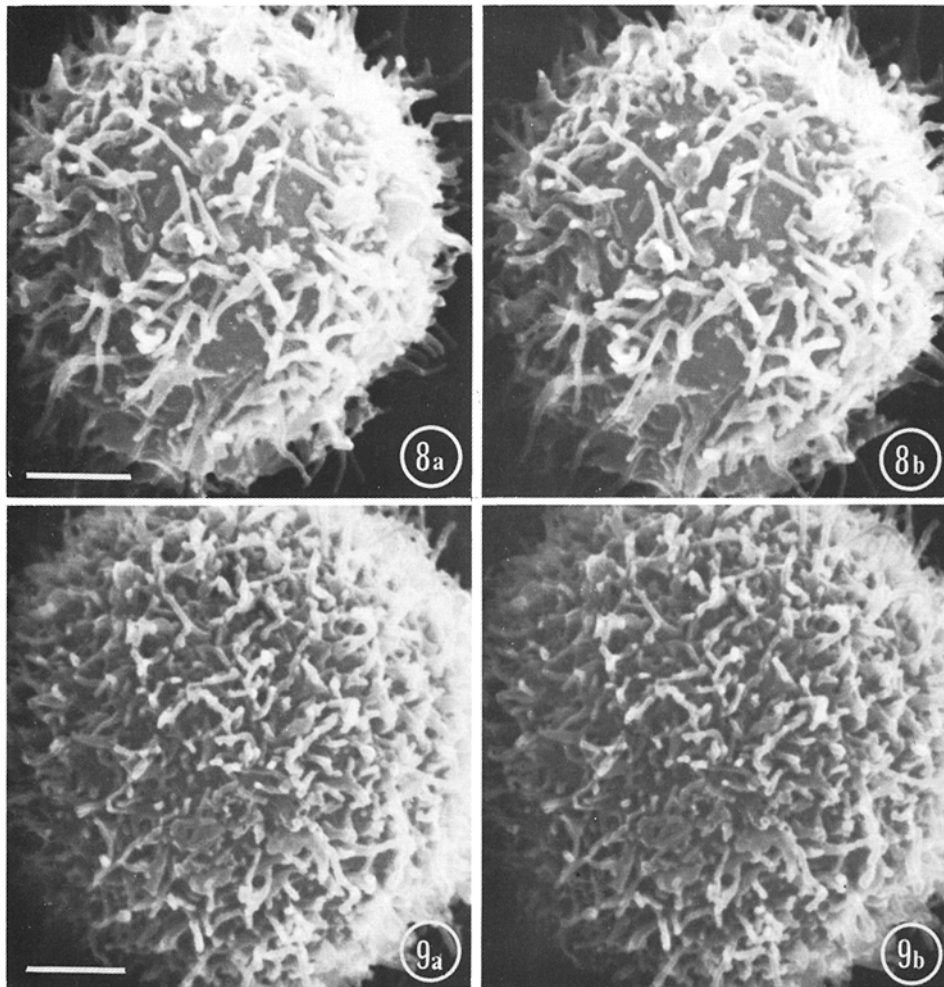
FIGURES 4-6 Examples of early G_1 cells.

FIGURE 4 A small percentage ($< 5\%$) of early G_1 cells are comparatively smooth. A few small blebs are often seen on these smooth cells. $\times 7,000$.

FIGURE 5 A further $\sim 5\%$ of early G_1 cells have part of their surface covered with many $\sim 0.1\text{-}\mu\text{m}$ diameter "nodules." A few small blebs and $\sim 0.1\text{-}\mu\text{m}$ diameter microvilli can also be seen. $\times 7,500$.

FIGURE 6 The majority of early G_1 cells have part of their surface (up to 3%) elaborated into numerous $\sim 0.1\text{-}\mu\text{m}$ diameter microvilli of varying lengths. $\times 7,000$.

FIGURE 7 Lateral view of a typical late G_1 cell. By this stage much of the surface is obscured by microvilli; branching of some microvilli is apparent, and many microvilli are seen to emerge from small flat protuberances of the cell surface. $\times 6,200$.



FIGURES 8 and 9 Examples of S and G₂ cells. All cells at these stages have a large part of their surface obscured by microvilli; the major difference between S and G₂ cells is one of size and the extent to which the surface is elaborated into microvilli.

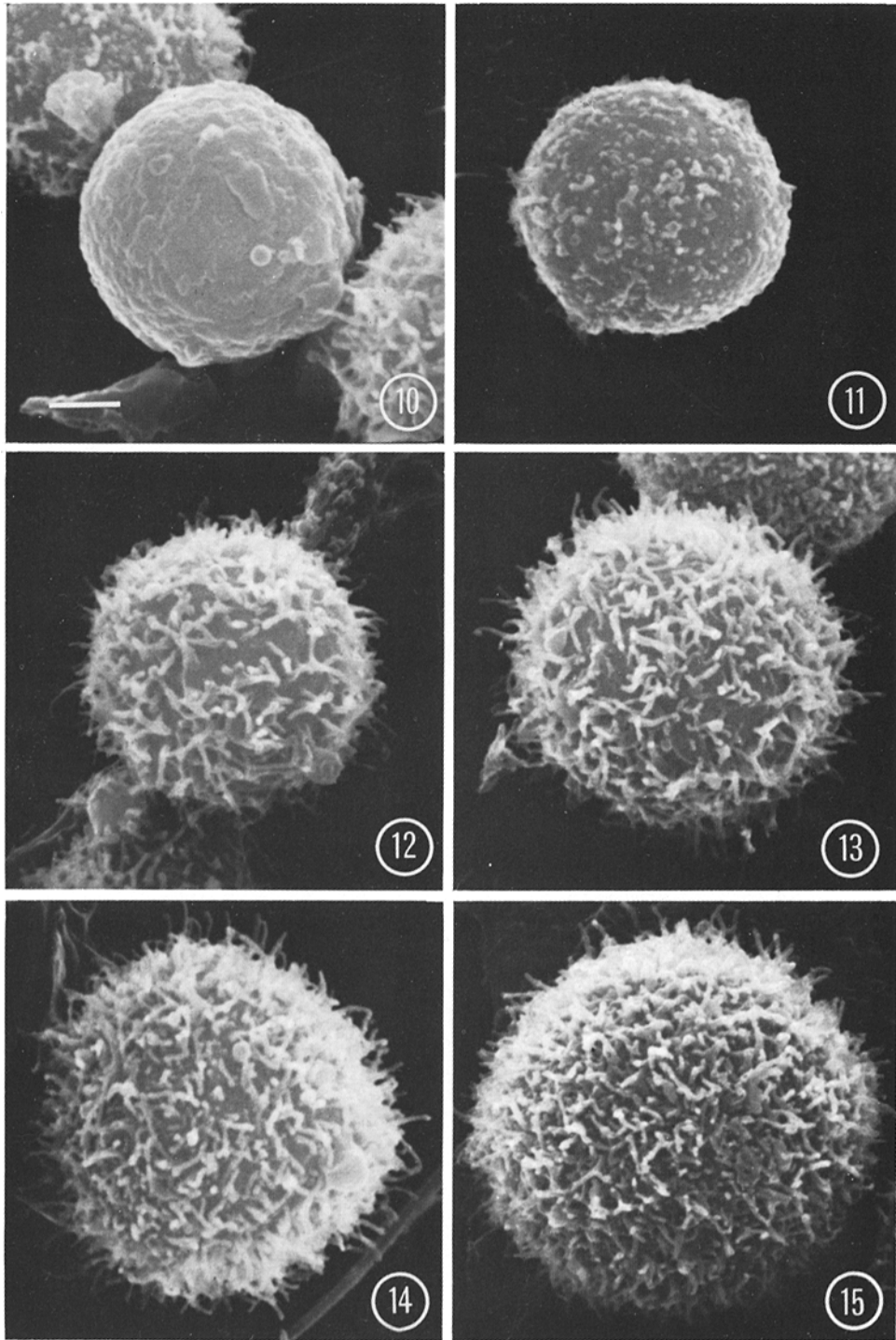
FIGURE 8 *a* and *b* Stereo views of a typical S cell. Examination with a stereo viewer shows that microvilli range from 0.1 to 2.0 μm in length, stand erect from the surface, frequently emerge from much larger (usually flat) protuberances of the cell surface, and are often branched. $\times 6,700$.

FIGURE 9 *a* and *b* Stereo views of a G₂ cell. By this stage virtually all of the cell surface is obscured by microvilli. As with S cells, stereo images reveal the complex structure of many microvilli. $\times 6,300$.

tic of a particular stage of the cell cycle. Approximately 5% of early G₁ cells have part of their surfaces covered with numerous small "nodules," each $\sim 0.1 \mu\text{m}$ in diameter (Figs. 5, 11). A few 0.1- μm diameter microvilli are also seen on some of these cells (Figs. 5, 11). Stereo images suggest that these nodules are, in fact, very short microvilli. Since nodules have the same diameter as

microvilli (i.e. $\sim 0.1 \mu\text{m}$) and since cells at later stages of the cell cycle have fewer nodules relative to microvilli, it seems possible that nodules represent an early stage in microvillus extension.

The remaining early G₁ cells ($\sim 90\%$) all have part of their surface elaborated into numerous microvilli (Figs. 6, 12). The percentage of the surface area elaborated into microvilli ranges from



FIGURES 10-15 Further examples of early G_1 cells (Figs. 10-12), late G_1 (Fig. 13), S (Fig. 14), and G_2 cells (Fig. 15), all printed at the same magnification. A direct comparison of these cells clearly illustrates the gradual increase in cell size and the extent to which the surface area is elaborated into microvilli. $\times 5,000$.

zero in the smooth cells to ~3% of the cell surface area in the most highly villated cells (Fig. 12). Considerable variations exist from cell to cell in both the number and length of individual microvilli. A comparison of Figs. 6 and 12 illustrates this point. Fig. 6 shows an early G_1 cell in which very few microvilli exceed $0.4 \mu\text{m}$ in length, whereas Fig. 12 shows a cell at the same stage of the cell cycle with numerous microvilli $0.5 \mu\text{m}$ in length; the mean length of microvilli in early G_1 cells in approx. $0.4 \mu\text{m}$.

Cells at later stages of the cell cycle, e.g., late G_1 (Figs. 7, 13), S (Figs. 8, 14), and G_2 (Figs. 9, 15), all have part of their surfaces elaborated into numerous microvilli. Thus the gradual increase in cell size (from approximately $9 \mu\text{m}$ diameter in late G_1 to approximately $11 \mu\text{m}$ in G_2 , measured from scanning electron micrographs, Figs. 12-15) is accompanied by an increase in the extent to which the surface is elaborated into microvilli; by the

time the cells have reached the G_2 period, virtually the entire cell surface is obscured by microvilli (Figs. 12-15).

As cells become highly villated, many of the microvilli are seen to emerge from much larger surface protuberances; branching of microvilli is also common. Although these features can be seen at early stages of the cycle also (Fig. 7), they are much more prominent in S (Fig. 8) and G_2 (Fig. 9) cells. The larger surface protuberances usually have the same thickness at the tip as a typical microvillus (i.e. $\sim 0.1 \mu\text{m}$), but at the base where they emerge from the cell surface they can be as wide as $0.5 \mu\text{m}$. Cross sections of these structures are readily seen in freeze-fracture preparations;² other details are most clearly seen in stereo images (Figs. 8, 9).

A mitotic cell in the later stages of cytokinesis is depicted in the stereo images in Fig. 16. Part of the surface is smooth and part is elaborated into

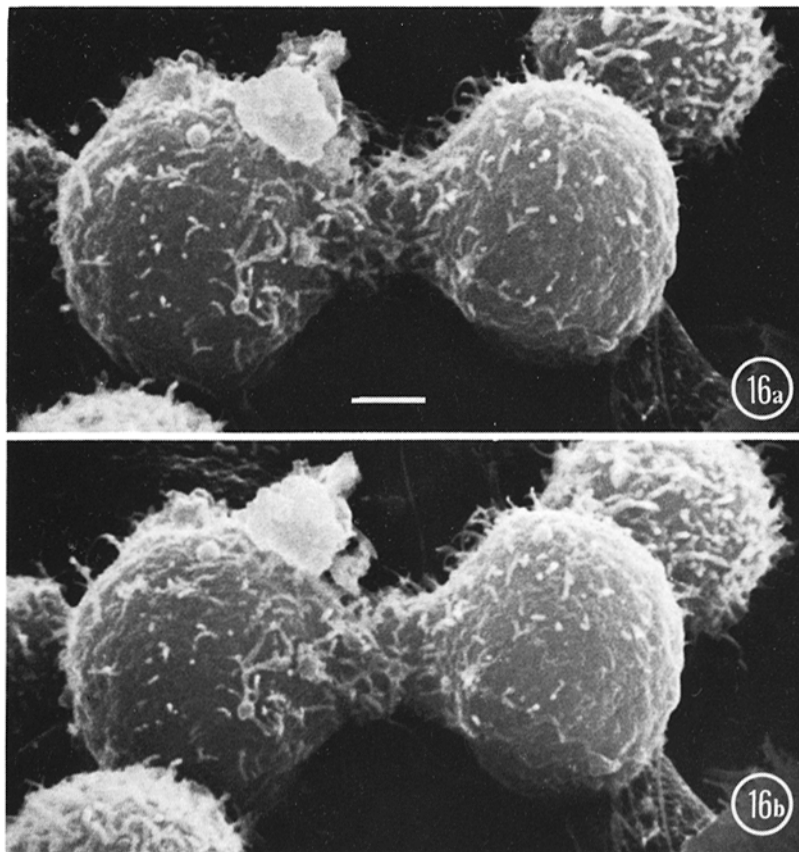


FIGURE 16 *a* and *b* Stereo images of an M cell in the latter stages of cytokinesis. Most microvilli are concentrated in the region around the division plane, while the remainder of the cell surface is comparatively smooth and typical of many early G_1 cells. $\times 4,600$.

numerous microvilli; a few small blebs are also apparent. At this stage of cytokinesis the majority of microvilli are in the region around the division plane. Away from the cleavage plane the surface of the two daughter cells becomes increasingly devoid of microvilli, typical of many early G_1 cells. Those microvilli which are present stand erect from the surface and show little evidence of branching or of attachment to larger surface protuberances. Fig. 16 thus provides evidence that the comparatively smooth and lightly villated early G_1 cells arise by division of highly villated G_2 cells, rather than being representative of a discrete type of cell.

In all the cells examined, the diameter of microvilli is uniform ($\sim 0.1 \mu\text{m}$), whereas the length varies from 0.1 to 2.0 μm , with a mean of 0.4 μm . Although most of the cell surface is obscured by microvilli in the largest G_2 cells, calculations indicate that, on average, only $\sim 6\%$ of the surface is elaborated into microvilli; even this, however, as much as doubles the total surface area of the cell (see below). In most early G_1 cells, $\sim 3\%$ of the surface is elaborated into microvilli. From these figures may be calculated the number of microvilli per cell, and hence the surface area contributed by microvilli (Table I). Because of the complex and irregular shapes of many of the microvilli, especially in the larger cells, these figures should be considered as approximations only; more detailed analysis is required in order to obtain more accurate values. Nevertheless, the figures do show that an approximate doubling in total surface area is achieved as P815Y cells progress from early G_1 to G_2 .

DISCUSSION

When spherical cells, like P815Y that double in volume before mitosis (reference 17, Fig. 3), progress from G_1 to G_2 , the *apparent* surface area increases by a factor of $2^{2/3}$ or 1.6-fold (7). The evidence presented here (e.g., Table I) confirms the suggestion that the *actual* surface area increases twofold, and that cytokinesis is a physical unfolding or stretching process. In other words, the ratio of surface area to volume remains approximately constant throughout the cell cycle. In order to maintain such constancy, there must be some mechanism whereby a cell controls the proportion of new surface membrane to be elaborated into microvilli. The extent to which microtubules and microfilaments, the availability of which may regulate the degree of cell spreading during the cell cycle (12), are involved in such a process remains to be seen.

In the case of cells growing in monolayer culture, the sequence of events is different. During early G_1 , cells spread out and there is a large increase in surface area. Before mitosis, cells round up, with a consequential decrease in surface area. Assuming that the volume of a monolayer cell before mitosis is, like that of a suspension cell, double that in early G_1 , and that the mechanism of cytokinesis is the same, then such a cell will likewise undergo a net doubling of its actual surface area during interphase. The important point is that changes in surface area due to spreading during early interphase greatly exceed those due to rounding up during mitosis. Hence, changes involved specifically with a doubling of surface membrane constituents are likely to be obscured. Note that a P815Y cell in early G_1 has, on average, some 33% of its plasma membrane in the form of microvilli (Table I), which indicates the potentiality of this cell line for spreading and moving across surfaces without recourse to membrane synthesis.

The elegant results of Porter et al. (14), who showed a variety of changes in the surface architecture of Chinese hamster ovary (CHO) cells during the cell cycle, are relevant here. Although these authors (14) were unable to draw definitive conclusions about the role of microvilli, blebs, and ruffles, their results are entirely consistent with the present suggestions. For, if microvilli are a source of extra surface membrane, then the observation that the number of microvilli diminishes as cells spread thinly over the substratum during S but increases again as they round up before mitosis in G_2 (14) is explained. Thus, scanning electron microscopy of CHO (14) and P815Y cells (present results) during the cell cycle provides further evidence for the suggestion (4) that microvilli may be a source of extra surface membrane in a variety of situations. The present experiments, albeit with tumor cells, also confirm the prediction (18) that an unfolding of previously accumulated membrane may be involved in the cleavage of sea urchin eggs and perhaps of animal cells in cytokinesis in general.

The accumulation and growth of microvilli during the cell cycle of P815Y cells may be related to the observed change in fragility to immune and nonimmune lysis which is minimal in late interphase (7, 16). For if microvilli confer on cells the ability to stretch without rupture during cytokinesis, the same may be true during exposure to hypotonicity or to complement-mediated attack by antibody. Other surface phenomena, such as the

binding of concanavalin A (9, 13) or the activity of Na^+/K^+ -ATPase (7), that change dramatically as cells approach mitosis, may likewise depend on the relative abundance of microvilli.

It has been observed that transformed, malignant cells in monolayer are generally richer in microvilli than their normal counterparts (documented in reference 14). The extent to which this reflects the fact that normal cells spend more time in G_1 (or G_0) relative to the rest of the cell cycle has now to be established. For if the mechanism of cytokinesis by an unfolding of previously accumulated microvilli is a general one, it clearly applies to any cell that doubles its surface constituents before mitosis.

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