THE EFFECT OF CELL-TO-CELL CONTACT

ON THE SURFACE MORPHOLOGY OF

CHINESE HAMSTER OVARY CELLS

R. W. RUBIN and L. P. EVERHART

From the Department of Molecular, Cellular, and Developmental Biology,
University of Colorado, Boulder, Colorado 80302

ABSTRACT

In the previous report (Porter et al., in this issue) morphological changes in Chinese hamster ovary (CHO) cells during the cell cycle were described. In this report we describe the role of intercellular contact on these changes. We find that intercellular contact is required for cells to exhibit the morphologies Porter et al. described for S and G_2. When cells are synchronized by mitotic selection and plated onto cover slips at very low density such that no intercellular contact occurs, the cells remain in a G_1 configuration (rounded and highly blebbed through G_1, S, and G_2). This G_1 morphology is also observed in nonsynchronized log phase cells plated at low densities and allowed to grow for several generations. The addition of conditioned medium from confluent cultures does not induce low density cells to change morphology during the cell cycle. These results indicate that extensive intercellular contact is required for the complete expression of the morphological changes associated with the cell cycle (as described by Porter et al.). It is concluded that although classic contact inhibition of movement and of growth may be absent in this transformed cell line, some contact-dependent response persists.

INTRODUCTION

Porter et al. (1973) showed that the surface and overall morphology of Chinese hamster ovary (CHO) cells changes during the cell cycle. These changes were observed under conditions of moderate intercellular contact in cultures plated at high cell densities. The question therefore was raised whether the morphological alterations observed were affected by cellular contact or other density-dependent phenomena, such as depletion of the medium or production of some inducer molecule.

In this report we show that direct contact is essential to the development of these morphological changes and that cells in cultures where there is no intercellular contact move through the cell cycle without exhibiting any apparent surface changes except in mitosis.

MATERIALS AND METHODS

Culture Techniques

Stock cultures of CHO cells were maintained as described in the preceding paper (Porter et al., 1973). Nonsynchronous logarithmic cultures were obtained by trypsinization of cells from a glass bottle, dilution with fresh medium, and replating in 35-mm Falcon Plastics Petri dishes containing 25 mm diameter, low background glass cover slips (Arthur H. Thomas Co., Philadelphia, Pa.). These cover slips were not carbon coated.

For synchronous cultures, mitotic cells were

selected by the procedure of Tobey et al. (1967). They were plated out directly at low density (approximately 20,000 cells/dish) or concentrated by a single centrifugation at 100 g. To determine their position in the cell cycle, cells from replicate cover slip cultures were pulse labeled at intervals after the shake-off for 15 min with [^{3}H]thymidine (Schwartz, 17.6 Ci/mmol) at a final concentration of 1 μCi/ml. Cover slips were washed, fixed, hydrolyzed, and dehydrated and then counted in a low background gas flow planchet counter (Everhart, 1972).

These same cover slips were prepared for autoradiography by dipping in Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, N. Y.).

In some experiments cover slips were dehydrated through an acetone series, and a portion of the cover slip was then critical-point dried. The remainder of the cover slip was prepared for autoradiography as described above.

Microscopy

Cultures were prepared for scanning electron microscopy by the procedures described previously (Porter et al., 1972). After critical-point drying, all preparations were coated with carbon and then with gold, and cells were photographed at random on a Kent Cambridge S4 scanning electron microscope operating at 20 kV.

RESULTS

Initially we examined the surface morphology of nonsynchronized log phase cells. Cells were removed from a late log phase culture by trypsinization and plated onto cover slips at two densities. One group was plated at 1.6 $\times$ 10^4 cells/dish (1.8 $\times$ 10^3 cells/cm^2) and the other at 8 $\times$ 10^4 cells/dish (8.8 $\times$ 10^3 cells/cm^2). The cells were allowed to progress through two to three generations and were then fixed and prepared for scanning electron microscopy. The results clearly showed that in the low density cultures most of the cells (95%) possessed numerous surface blebs (Figs. 1 and 2). The vast majority of these cells were also spherical or oblong in overall morphology and were distributed on the cover slip in clones of four closely adhering cells. These cells were nonsynchronous, had gone through at least two generations on the cover slip, and were in some degree of contact with one to three other cells. Nevertheless, 95% of these cells possessed surface blebs. Thus, under these conditions cells remain blebbed throughout the greater part of the cell cycle.

Cells from cultures plated at high density, on the other hand, showed a wide range of morphologies. Some possessed smooth surfaces while others had greatly flattened onto the glass substratum (Fig. 3). It seemed likely that these changes in surface morphology were the result of increased cell contact and that changes in surface morphology during the cell cycle, described by Porter et al. (1973), are observed only when a considerable amount of cell contact exists.

In order to define more precisely the effect of cellular contact on cell morphologies at various stages in the cell cycle we examined cells synchronized by the shake-off procedure. Throughout most of the cell cycle the cells in low density cultures were rounded, blebbed, and showed little or no flattening. Figs. 4 and 5 show cells with this morphology. When these cells were fixed at 12 h, a time when most of the population is in S or G$_2$, the blebbing phenomenon was still apparent (Fig. 6). However, at this time the cells had begun to flatten onto the surface. Throughout the cell cycle the cells remained in pairs derived from the original cell division after shake-off. We did not continue to observe these cells, for beyond this point synchrony had seriously deteriorated. The results from log cultures, reported above, indicate that cells remain blebbed through at least two generations at this low cell density.

To examine further the role of cell density in the expression of the morphological changes during the cell cycle, cells were synchronized by shake-off, concentrated by centrifugation, and plated out at densities such that most cells were in contact with surrounding cells. This density is comparable to that observed in unsynchronized cultures (see Fig. 3). At intervals after shake-off, replicate cover slip cultures were pulse labeled with tritiated thymidine ([^{3}H]TdR), washed, fixed, and critical-point dried. A portion of each cover slip was then examined in the scanning electron microscope while the remainder was prepared for autoradiography. Fig. 8 shows the rate of entry into S by cells thus treated. There was little DNA synthesis until after 4 h, and by 10 h approximately 36% of the cells had entered S (see autoradiogram, Fig. 9). During those first 4 h after shake-off all cells were in G$_1$ and possessed a blebbed surface, even though they had spread out on the substrate and were in contact (Fig. 7). This spreading appeared to be facilitated by contact since low density cells show no spread-
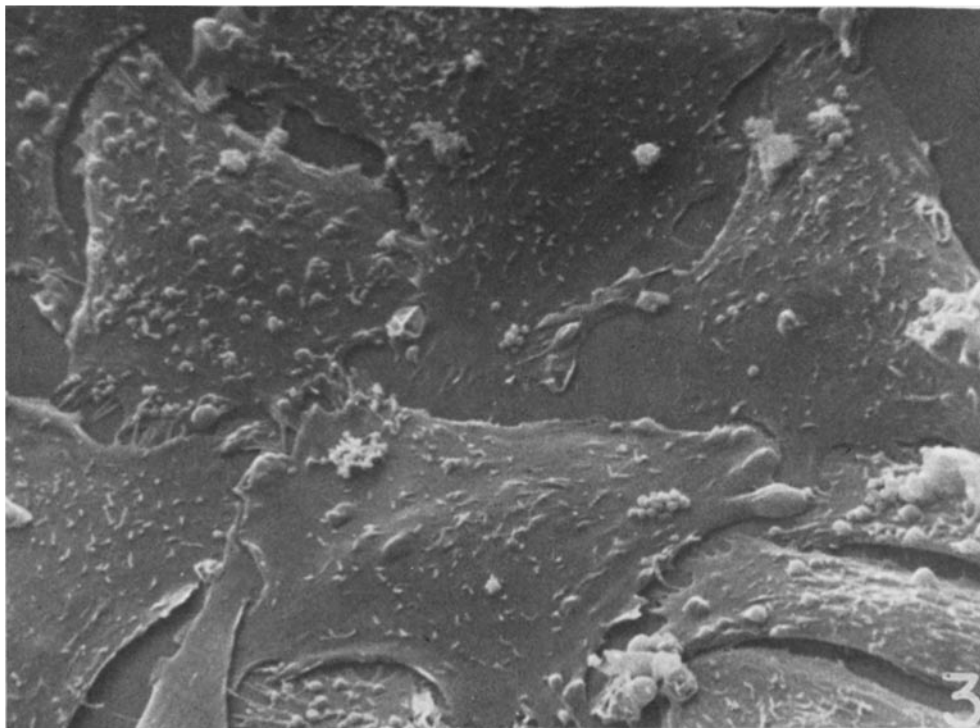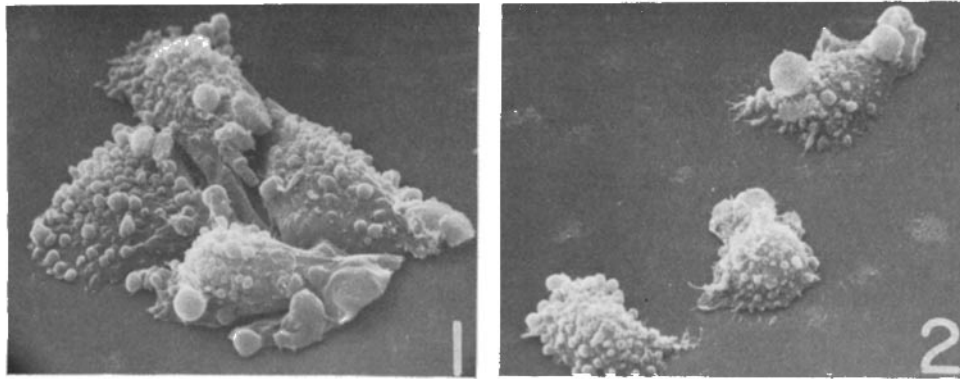
FIGURES 1 and 2   Nonsynchronized low density log phase culture. Fig. 1 shows daughter pairs from two divisions. × 1,100.

FIGURE 3   Nonsynchronized high density culture. Not quite confluent. Note high degree of cellular flattening and absence of blebs. × 1,400.

ing at this time. At a time when cells were entering S, some cells began to flatten, and cells lacking blebs began to appear. By 10 h when the labeling curve indicated that 36% of the cells had entered S, about the same number of cells were seen in a highly flattened conformation possessing smooth surfaces or surfaces covered with microvilli (Fig. 10). These observations are similar to those described by Porter et al. (1973).

To make sure that these results were not affected by the nature of the substrate on which the cells were spreading, the high and low density shake-off
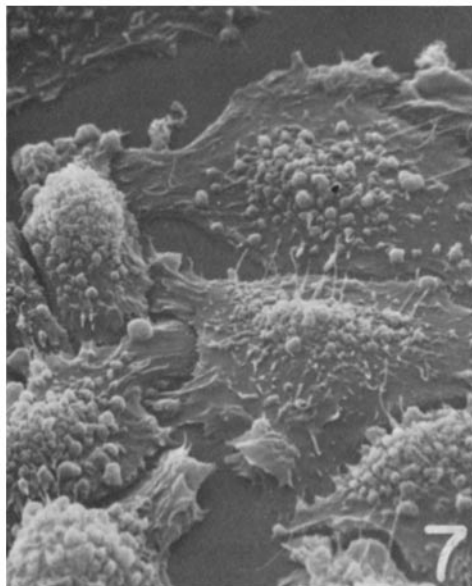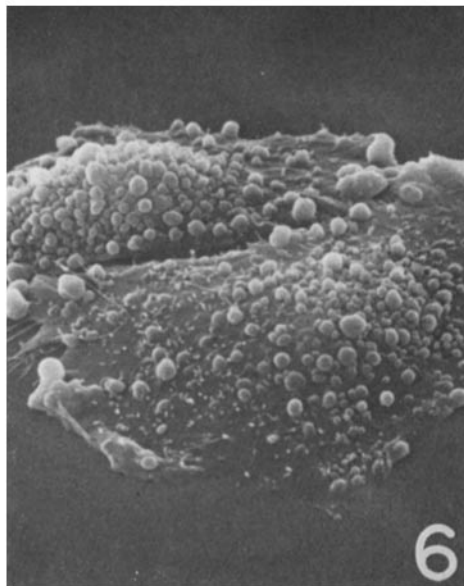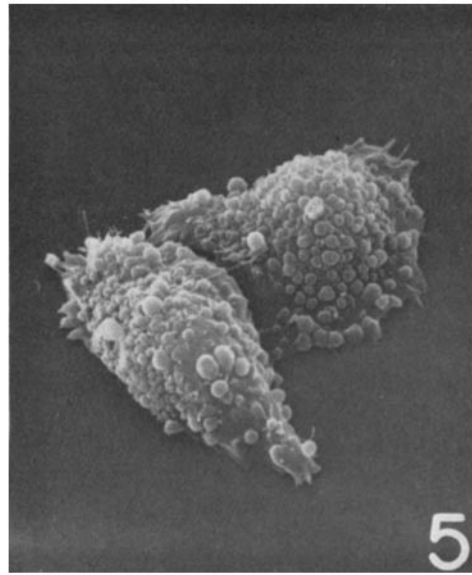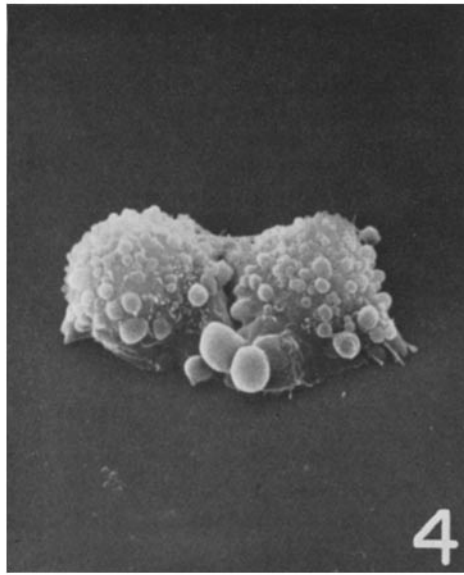
FIGURES 4 and 5   Low density shake-off synchronized daughter pairs 8 h after shake-off. Note rounded blebbed appearance and presence of intercellular bridge (Fig. 5). × 2,000.

FIGURE 6   Same treatment as Fig. 5 but these cells were fixed after 12 h. × 2,000.

FIGURE 7   High density shake-off synchronized culture after 3 h. Note flattening. × 2,000.

experiment was repeated on carbon-coated cover slips. The results were identical.

In an attempt to discern whether the effects noted with increasing cell density were caused by substances added to or deleted from the medium by the cells themselves, medium from confluent cultures was added to synchronized cells plated at low densities. When samples were prepared for

microscopy throughout the cell cycle, it was apparent that the conditioned medium had no morphological effect (Fig. 11). The cells remained blebbed throughout the cell cycle and were spherical, showing little peripheral flattening through 8 h.
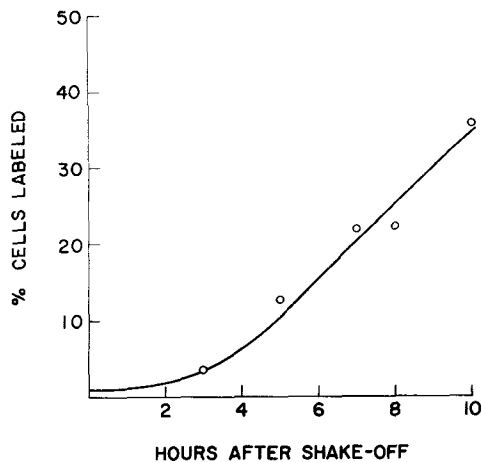

FIGURE 8 Plot of the percentage of cells labeled with [³H]TdR through the cell cycle. These were synchronized by the shake-off.

DISCUSSION

The results described here indicate that the morphological changes observed during the cell cycle, described in the preceding paper (Porter et al., 1973), are dependent upon cellular contact. We have demonstrated here that at a low cell density cells can move through the cell cycle without expressing the changes. We conclude that at low cell density there is insufficient contact to induce the changes. Furthermore, these results were obtained without the use of thymidine for synchronization as was done in the previous paper (Porter et al., 1973).

We are not certain what amount of contact may be necessary to induce these surface changes, but the following experiment provides some insight on the problem. Nonsynchronous cells were plated at low density and allowed to proceed through two cell divisions to produce compact clones of four cells. Because these cells appear to be close together (see Fig. 1) one assumes that there is some contact between the cells. Nevertheless this amount of contact is not sufficient to bring about surface changes. Since these cells were nonsynchronous, approximately 50% must be in S and
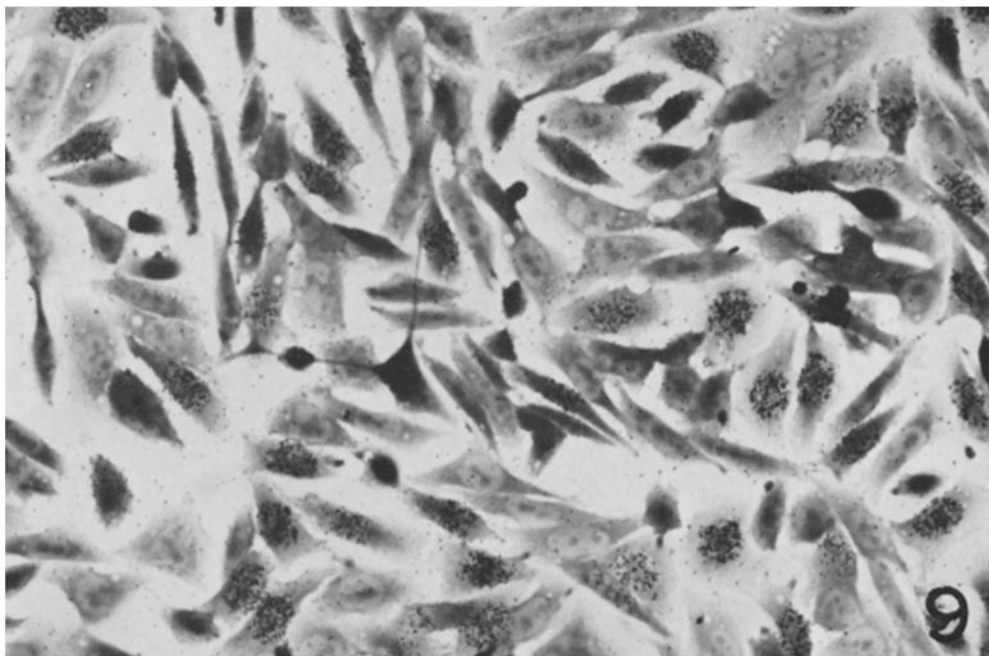

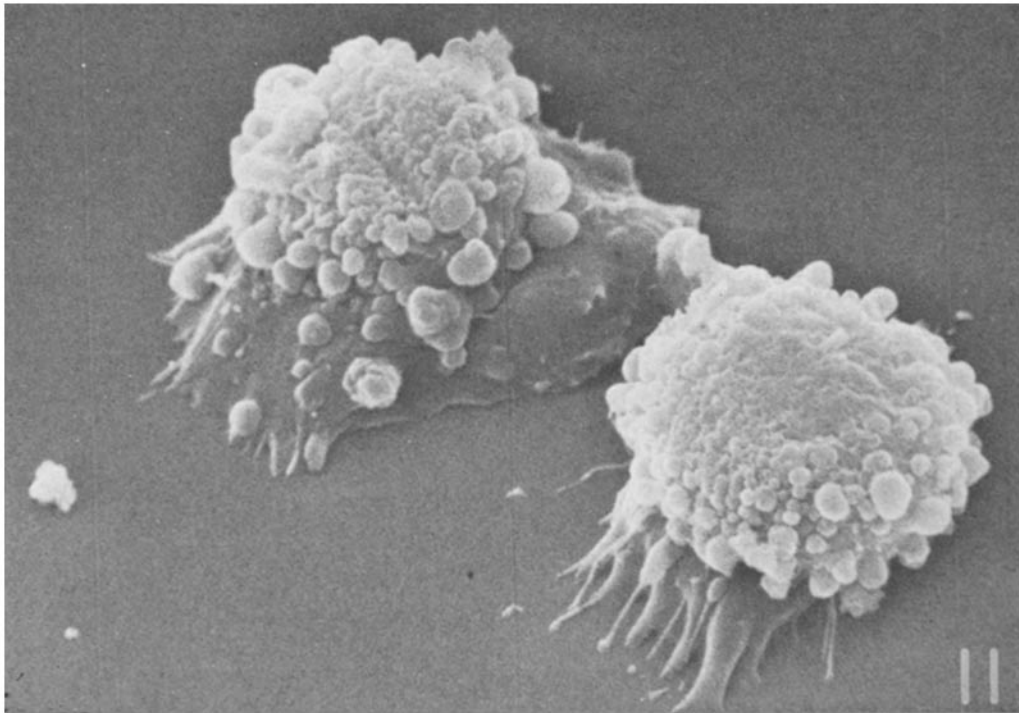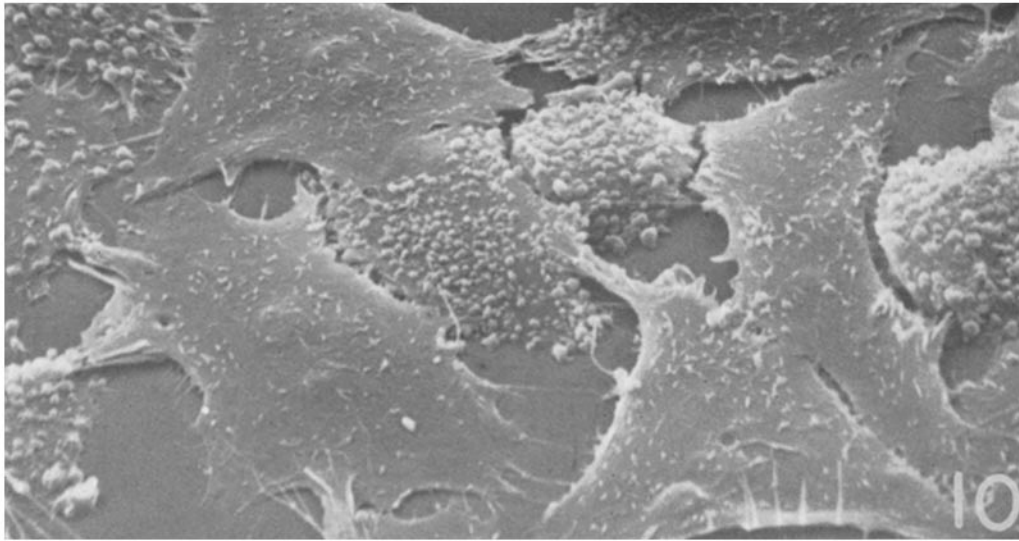FIGURE 9 Autoradiogram of synchronized cells pulsed with [³H]TdR and then fixed at 10 h after shake-off. × 400.

FIGURE 10   High density shake-off synchronized culture fixed at 10 h. × 2,000.

FIGURE 11   Low density shake-off synchronized culture fixed at 9 h. These cells had been in the presence of conditioned media (media from confluent cultures) for 8 h. × 4,000.

should show S-phase morphology, i.e., be flattened and nonblebbed. Yet all the cells we observed under these conditions were blebbed and rounded. At high cell density, surface changes related to the cell cycle were seen (see Fig. 10). Thus the critical amount of contact necessary to induce these changes must occur at densities intermediate to those used in the two experiments described here.

The CHO line is a spontaneously transformed noncontact-inhibited cell line isolated by Puck et al. (1958). These cells grow to compact colonies in which the cells are nonoriented and piled up. If one pulse labels such a colony with [³H]TdR, cells which incorporate [³H]TdR are found distributed throughout the colony (Harkins and Gamow, unpublished observations). Despite the *absence* of classic contact inhibition we find that when these cells come into contact with each other they exhibit two kinds of cell-to-cell interactions: (*a*) inhibition of membrane ruffling (Porter et al., 1973); (*b*) surface morphology changes. The loss of contact inhibition therefore does not reflect a total loss of intercellular communication. In spite of their transformation they are still susceptible to contact effects.

O'Neill and Follett (1970), using the scanning electron microscope, observed that in BHK21 cells the number of microvilli per cell was related to cell density. While cells from sparse cultures had an average of 18.4 microvilli/cell, cells from confluent cultures had only 4.6. The authors conclude that the number of microvilli is determined by the number of cell contacts. O'Neill and Follett do not mention the presence of blebs on BHK21 cells. While blebs are the conspicuous surface structure on CHO cells at low density, if one closely examines a blebbed cell, microvilli are also present. This difference is not surprising because the surface morphology of different cell types is vastly diverse (Porter et al., 1973). It should also be pointed out that it is necessary to take certain precautions in the preparation of samples for the scanning electron microscope to avoid artifacts. Some of these artifacts, described by Porter et al. (1972), include removal of microvilli and the formation of ridges such as observed by O'Neill and Follett (1970). Nevertheless we agree with O'Neill and Follett that the morphology of the cell surface is dependent upon cell contact and that in general the more the contact there is, the less active is the cell surface in terms of extending structures such as blebs or microvilli.

In another study using a replica technique to study the cell surface, Follett and Goldman (1970) observed the occurrence of microvilli during spreading and growth of BHK21 fibroblasts. Microvilli were only found on round, dividing, or partially spread cells and rarely seen on fully spread cells. They concluded that microvilli are storage organelles for excess cell membrane when the cell rounds up. There is agreement here between the behavior of microvilli and the behavior of the blebs we have described, for we generally see blebs only on cells that are not fully spread. However, at this time we cannot equate the functions of these two structures.

It is possible that the disappearance of blebs is a consequence of cell flattening and that this process of flattening is promoted by the increased cell contacts in dense cultures. The time required for cells to flatten out in response to increased contact may be coincidentally related to the time required for cells to initiate DNA synthesis. In this respect it should be pointed out that no direct correlation between flattening and initiation of S has been demonstrated. We are attempting to develop a technique which will combine autoradiography and scanning electron microscopy on the same preparation to help establish this correlation.

Otten et al. (1972) have demonstrated that there is an inverse relationship between growth rate and endogenous levels of cyclic AMP in mouse fibroblast cell lines. They suggested that some of the properties that are characteristic of contact-inhibited cells might be mediated through cyclic AMP. It is significant that the addition of cyclic AMP to CHO cell cultures produces surface alterations similar to those reported here when cells come into contact, including loss of blebs (Puck et al., 1972; Porter, unpublished observations). Furthermore, cyclic AMP has been shown to increase adhesions between the cell and the substratum perhaps by promoting spreading and attachment such as we have observed here upon increasing cell contact (Johnson and Pastan, 1972). It seems likely that many of the surface phenomena which occur when cells come into contact with one another result from the local production of cyclic AMP.

There is yet no clear understanding of the interrelationship between the cell surface, cell contact, and cyclic AMP production in the control of cell reproduction and how this control is lost in situations such as neoplasia and transformation. Nevertheless it is clear from the work reported here that the cell surface is modulated by cell-to-cell interactions, even in transformed cells.

REFERENCES

EVERHART, L. P. 1972. The effects of deprivation of two essential amino acids on DNA synthesis in Chinese hamster cells. *Exp. Cell Res.* **74**:311.

FOLLETT, E. A. C., and R. D. GOLDMAN. 1970. The occurrence of microvilli during spreading and growth of BHK21/C13 fibroblasts. *Exp. Cell Res.* **59**:124.

JOHNSON, G. S., and I. PASTAN. 1972. Cyclic AMP increases the adhesion of fibroblasts to the substratum. *Nat. New Biol.* **236**:247.

O'NEILL, C. H., and E. A. C. FOLLETT. 1970. An inverse relation between cell density and the number of microvilli in cultures of BHK21 hamster fibroblasts. *J. Cell Sci.* **7**:695.

OTTEN, J., G. S. JOHNSON, and J. PASTAN. 1971. Cyclic AMP levels in fibroblasts: Relationship to growth rate and contact inhibition of growth. *Biochem. Biophys. Res. Commun.* **44**:1192.

PORTER, K. R., D. KELLEY, and P. M. ANDREWS. 1972. The preparation of cultural cells and soft tissues for scanning electron microscopy. *In* Froceedings of the Fifth Annual Stereoscan Scanning Electron Microscope Colloquium, Kent Cambridge Scientific, Inc., Morton Grove, Ill.

PORTER, K. R., D. PRESCOTT, and J. FRYE. 1973. Changes in surface morphology of Chinese hamster ovary cells during the cell cycle. *J. Cell Biol.* **57**:815.

PUCK, T. T., S. J. CIECIURA, and A. ROBINSON. 1958. Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects *J. Exp. Med.* **108**:945.

PUCK, T. T., C. A. WALDREN, and A. W. HSIE. 1972. Membrane dynamics in the action of dibutyryl adenosine 3':5'-cyclic monophosphate and testosterone on mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* **69**:1943.

TOBEY, R. A., E. C. ANDERSON, and D. F. PETERSEN. 1967. Properties of mitotic cells prepared by mechanically shaking monolayer cultures of Chinese hamster cells. *J. Cell. Physiol.* **70**:63.