Proc. Nat. Acad. Sci. USA
Vol. 72, No. 4, pp. 1263–1267, April 1975

Cyclic AMP Modulates Microvillus Formation and Agglutinability in Transformed and Normal Mouse Fibroblasts

(concanavalin A/dark field microscopy/prostaglandin E_1/cell surface microstructure/immunofluorescence)

MARK C. WILLINGHAM AND IRA PASTAN

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

Communicated by E. R. Stadtman, January 14, 1975

ABSTRACT **We have utilized dark field microscopy to observe the surface microstructure of living cultured cells. Using this method, we have found that dibutyryl cAMP treatment causes regression of the numerous, long cell surface microvilli present on L929 cells. Thirty minutes after removal of dibutyryl cAMP, microvilli reappear. An inhibitor of phosphodiesterase (methylisobutylxanthine) and a stimulator of adenylate cyclase (prostaglandin E_1), both of which raise cAMP levels, cause regression of microvilli in 15 min. Untransformed 3T3 cells show very few microvilli when viewed still attached to their substratum or after removal with EDTA. Treatment of these cells with trypsin causes the formation of numerous microvilli on their surface. When clumps of cells agglutinated by concanavalin A are examined by thin section electron microscopy, the cells are seen to be held together by a "forest" of interdigitating microvilli and only rarely is there apposition of the areas of membrane between microvilli. At the same time the distribution of surface-bound concanavalin A was examined using immunofluorescent light microscopy, and concanavalin A was found to be uniformly distributed over the cell surface. We propose that agglutinability of mouse and rat fibroblasts is regulated through the modulation of cell surface microvilli by cAMP, and that transformed cells are highly agglutinable because their low cAMP levels result in the formation of numerous surface microvilli.**

Adenosine 3′:5′-monophosphate (cAMP) regulates numerous functions of cultured fibroblastic cells. Among these are growth rate (1–5), cell shape (2, 6–8), adhesiveness (9), motility (10), and agglutination by plant lectins such as concanavalin A (Con A) (11–14). Indeed, when transformed cells are treated with cAMP analogues such as dibutyryl cAMP (Bt_2cAMP), their agglutinability is markedly diminished (11–14). Further, a mutant of normally nonagglutinable 3T3 cells that shows a transient fall in cAMP levels with a change in temperature (8) showed increased agglutinability that was prevented by maintaining high cAMP levels (14). The exact mechanism by which cAMP regulates agglutinability has not been established.

While investigating the effects of Bt_2cAMP on the morphology of cultured cells, one of us (M.W.) noted that he could observe microvilli on living cells by means of dark field illumination and visible light. Previously it has only been possible to observe microvilli on fixed cells by transmission or scanning electron microscopy. This new method allows one to observe many cells and readily monitor the appearance and disappearance of microvilli. In the current study we report that two transformed cell lines, L929 and normal rat kidney cells transformed by Moloney murine sarcoma virus (MNRK), have extensive microvilli that cover their surface. When these cells are exposed to Con A the cell clumps that form appear to be held together by interdigitating microvilli. Following treatment of L cells with Bt_2cAMP the microvilli regress, but reappear upon removal of the cyclic nucleotide. We conclude that the agglutinability of transformed cells is due to the presence of microvilli that greatly increase the surface available for the formation of lectin bridges, and that cAMP decreases agglutinability by decreasing the formation of microvilli.

Abbreviations: cAMP, adenosine 3′:5′-monophosphate, cyclic AMP; Bt_2cAMP, N^6,$O^{2'}$-dibutyryladenosine 3′:5′-monophosphate; MNRK, Moloney murine sarcoma virus-transformed normal rat kidney cells; Con A, concanavalin A.

MATERIALS AND METHODS

Examination of Cells by Light Microscopic Methods. L929 or 3T3-4 cells were grown on 22 × 40 mm sterile glass coverslips in 20 cm^2 plastic dishes in Dulbecco-Vogt's modified Eagle's medium supplemented with 10% calf serum and penicillin–streptomycin (50 units/ml each) at 37° in 95% air–5% CO_2. Preparation and addition of Bt_2cAMP, 1-methyl-3-isobutylxanthine, and prostaglandin E_1 were performed as previously described (8). Cells on coverslips were viewed either under phase contrast microscopy or dark field, visible light microscopy on a microscope stage in a plastic enclosure containing 95% air, 5% CO_2. The plastic enclosure was kept in a constant temperature room at 37°. Coverslips were removed from their dishes and overlaid with a second sterile coverslip. This sandwich with cells in between was placed on the stage of a Zeiss RA or inverted phase contrast microscope. For dark field observation, a dark field oil condenser (Zeiss ultracondenser) and a 40× oil immersion lens with an integral iris diaphragm were used. The light source for this observation was a dc-powered mercury vapor light source (HBO 200W/4) using a green (546 nm) excitor filter for visible light. With this method, objects below the limit of resolution of refractile light microscopy can be viewed, particularly surface microvilli. Polaroid photographs were taken of these cells under phase contrast or dark field illumination. The micrographs presented here were taken after 6% buffered glutaraldehyde fixation, but accurately represent the appearance of living cells. The cells were fixed to preserve their structure during the 15–30 min required to obtain photographs in each experiment.

Examination of Cells During Agglutination by Electron Microscopy. L929 or MNRK cells (15) were placed in an agglutination assay as previously described (14). These cells were grown in 75 cm^2 Falcon plastic flasks in Dulbecco-Vogt's modified Eagle's medium with 10% calf serum at 37°.

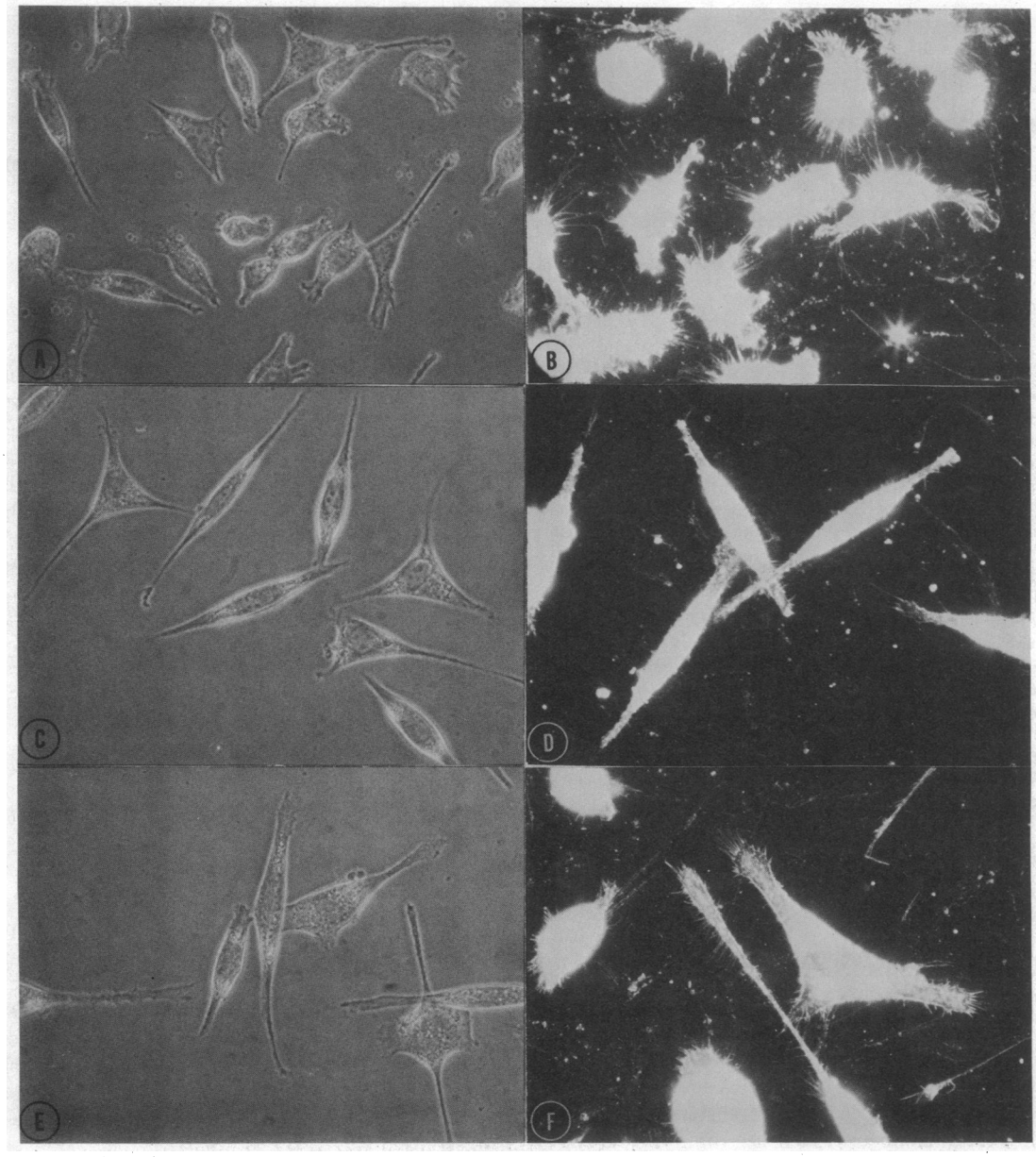

FIG. 1. L929 cells viewed with phase contrast or dark field microscopy with or without Bt_2cAMP treatment. L929 cells were grown on coverslips and observed under phase contrast or dark field microscopy as described in *Materials and Methods*. In normal medium, cells with round or ovoid shape can be seen with phase contrast (A) or dark field (B) microscopy. With this latter technique, numerous surface microvilli are evident over the entire cell surface (B). After treatment with 1 mm Bt_2cAMP for 24 hr, these cells become spindly in shape (C, D) and lose their microvilli (D). Removal of Bt_2cAMP from these cells results in retraction of these spindly processes evident here at 30 min after medium removal (E, F). In addition, surface microvilli have begun to reappear at the tips of and along the retracting processes (F). (A, C, E = ×287; B, D, F = ×357).

They adhere weakly to the surface of the flask and were removed from the surface by spraying them with the overlying medium. In these cells, mechanical removal has no effect on viability. The cells were placed in a 3 ml agglutination swirling assay dish (20 cm^2) at 25 rpm in the presence of Con A (100 μg/ml). After 10 min, extensive agglutination had occurred and the cells were immediately fixed in 6% cacodylate-buffered glutaraldehyde (pH 7.4). After gentle centrifugation and post-fixation in 2% OsO_4, the cells were dehydrated in graded alcohols and propylene oxide, and embedded in Epon. Thin sections of these cell pellets were viewed with a Hitachi HU-12A electron microscope after staining with uranyl acetate and lead citrate.

Immunofluorescence Localization of Con A and Cell Surface Myosin on L929 Cells. L929 cells grown at 37° in normal 10% calf serum medium were removed from culture by mechanical agitation and placed in an agglutination assay at 23° with Con A (100 μg/ml). Extensive agglutination was evident after 5–10 min. After 15 min incubation in Con A, the cells were washed by centrifugation, and incubated for 5 min in goat anti-Con A antibody (Miles Labs) that had been absorbed with L cells, washed in phosphate-buffered saline, and finally incubated for 5 min in rabbit anti-goat globulin conjugated with fluorescein (Schwarz/Mann). Under dark field illumination (UG-5 exciter filter) on a Zeiss RA microscope with mercury vapor light source, these cells showed

intense, continuous ring fluorescence (500 nm barrier filter). L929 cells incubated with goat anti-L cell myosin antiserum (15), an antiserum known to induce patching of surface label, were incubated under similar conditions for 15 min, and labeled with the same rabbit anti-goat fluorescein conjugate. This experiment was performed to demonstrate the ability of this technique to detect the presence of large surface patches. Con A antiserum preabsorbed with cells showed no significant surface localization in the absence of Con A. Rabbit anti-goat gamma globulin (fluorescein conjugated) also failed to bind to the cell surface in the absence of anti-myosin serum.

RESULTS

Effect of Bt_2cAMP Treatment on Cell Surface Microvilli. In Fig. 1, L929 cells grown on glass coverslips are shown by both phase contrast and dark field microscopic techniques. This dark field method allows the observation of surface microstructure in living cells. In normal medium L cells appear round or have short, stubby process (Fig. 1A). Under dark field, numerous long thin structures are visible on the cell surface (Fig. 1B), identifiable ultrastructurally as microvilli (Fig. 3A). After Bt_2cAMP treatment for 24 hr, the cells have assumed a spindly, extended shape (Fig. 1C). Accompanying this shape change, the surface microvilli have regressed (Fig. 1D). Pretreatment of these cells for only 15 min with a potent phosphodiesterase inhibitor (methylisobutylxanthine, 1 mM) or an activator of adenylate cyclase (prostaglandin E_1, 50 μg/ml) caused a similar regression of microvilli, even though the overall cell shape had not changed (results not shown). To evaluate the reversibility of the effect of Bt_2cAMP, new medium without Bt_2cAMP was added to these cells. Thirty minutes later, cells had begun to retract their spindly processes and approach their normal shape (Fig. 1E). At the same time, many new microvilli began to appear on the surface of the cells (Fig. 1F). This experiment provides direct evidence that cell surface microstructure can be modulated by cAMP, and raises the possibility that the decreased agglutinability of L929 cells with Con A after Bt_2cAMP treatment may be mediated by loss of surface microvilli.

In Fig. 2, 3T3-4 cells are shown under dark field microscopy. These cells have few visible microvilli under normal culture conditions (Fig. 2A). We did observe occasional mitotic 3T3 cells, and these showed numerous surface microvilli (Fig. 2B). After only 5 min exposure to 0.25% trypsin, many microvillar structures were visible on the surface of almost all cells (Fig. 2C). Microvilli were also observed in $3T3cAMP^{tcs}$ cells (8) 15 min after a fall in cAMP levels had been induced by lowering the temperature to 33° (results not shown).

To evaluate surface microvilli during an agglutination assay, Bt_2cAMP treated or untreated L-cells were removed from their substratum by EDTA treatment (14) and observed with dark field microscopy in suspension before and after the addition of Con A. After treatment with 1 mM Bt_2cAMP for 24 hr, very few microvilli were noted on the cell surface, whereas numerous, long microvilli were present on the untreated cells (results not shown). As previously reported, the untreated cells formed large clumps upon addition of 100 μg/ml of Con A, whereas the cells treated with Bt_2cAMP did not (14).

Ultrastructural Appearance of Cell Surface Microvilli During Agglutination. To investigate whether surface microvilli actually could participate in the agglutination process, L929 and MNRK cells, both highly agglutinable transformed cells, were examined during an agglutination assay as follows: The cells were removed from the substratum with EDTA, exposed to Con A at 100 μg/ml for 10 min at 23°, and then fixed with glutaraldehyde. The fixed cells were observed by transmission electron microscopy to evaluate the participation of surface microvilli in agglutination. Fig. 3A shows a single L cell and illustrates the numerous long microvilli that cover L cells during an agglutination assay. The presence of Con A has no effect on the presence of microvilli. When a cell clump is examined, the microvilli are seen to interlace and occupy the space between agglutinated cells (Fig. 3B). Clumps of MNRK cells are also held together by microvilli, although their microvilli are shorter and stubbier (Fig. 3C). Only rarely are the areas of membrane between microvilli in close apposition. From this we conclude that microvilli provide large surfaces that easily come in intimate contact and allow Con A molecules to bind the microvilli between cells together.

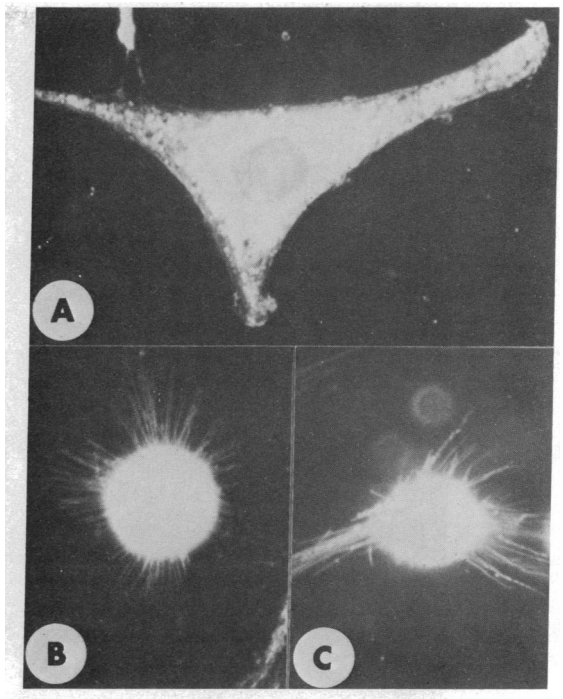

FIG. 2. The appearance of 3T3-4 cells with dark field microscopy. 3T3-4 cells were grown in 10% calf serum medium on glass coverslips as described in *Materials and Methods*. The lack of surface microvilli is evident in (A). Occasional mitotic cells (B) showed numerous "retraction fibers" which may or may not be the origin of microvilli seen on these mitotic cells in suspension (results not shown). Treatment with 0.25% trypsin for 10 min (C) also caused numerous "retraction fiber" structures, and showed many long microvilli when removed from this substratum and observed in suspension (not shown). (×510).

Immunofluorescent Localization of Con A and Cell Surface Myosin. Nicolson has suggested that redistribution of Con A binding sites on the surface of cells is responsible for the high agglutinability of transformed cells (16). Therefore, we also investigated the distribution of Con A on the surface of agglutinating L cells by immunofluorescent techniques. Incubation of L cells with Con A for only 10 min produces marked agglutination. Therefore, if gross cell surface redistribution of Con A attached to Con A binding sites induces agglutinability, fluorescent patches should certainly be evident

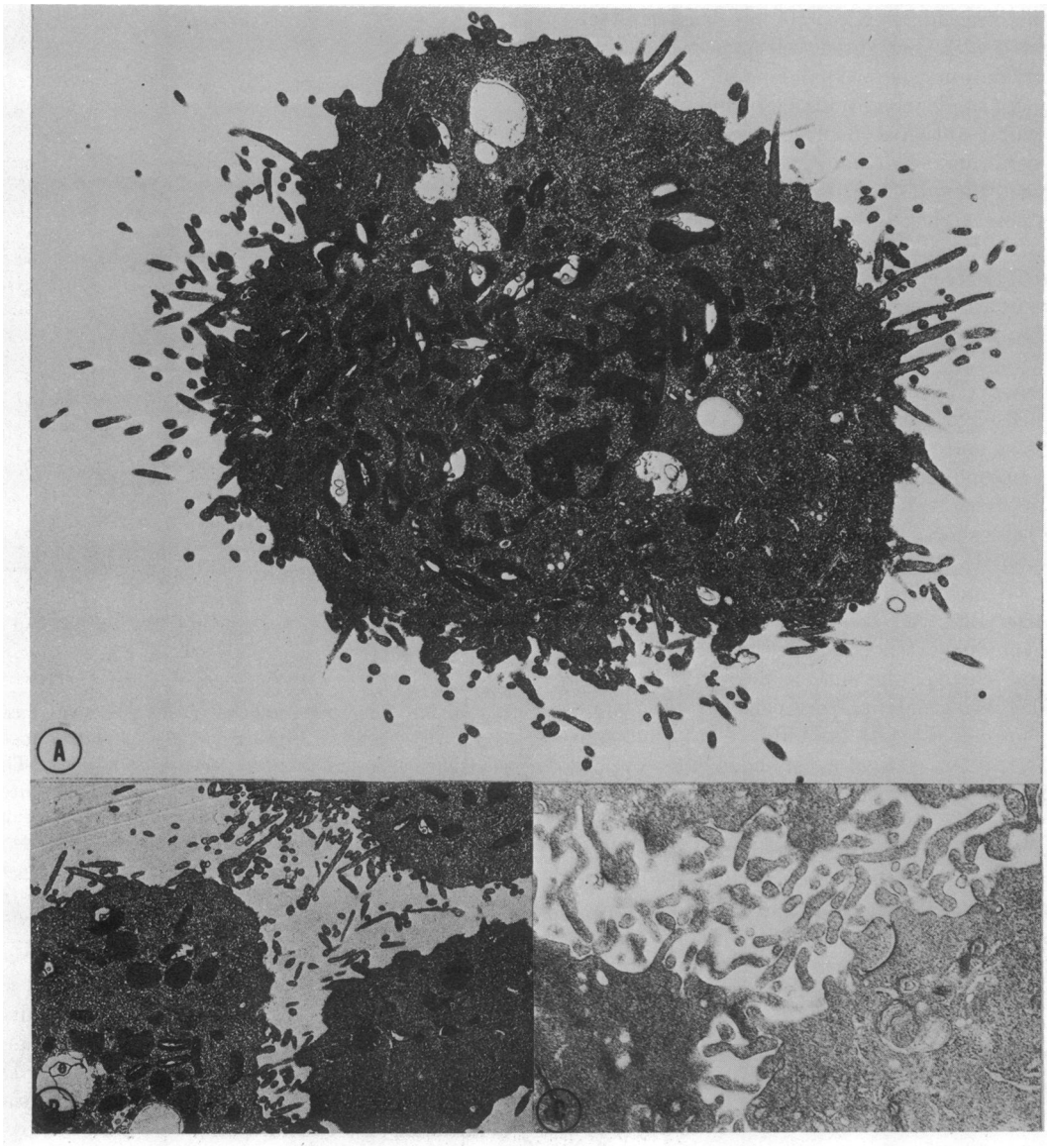

FIG. 3. Ultrastructural appearance of L929 and MNRK cells during agglutination with concanavalin A. L929 and MNRK cells were processed for transmission electron microscopy after an agglutination procedure as described in *Materials and Methods*. Numerous cell surface microvilli are evident in section (A) around the entire cell periphery. Agglutinated clumps of cells show many microvilli interdigitating between the L929 (B) or MNRK (C) cells in these clumps. (A = ×12,750; B = ×9,750; C = ×18,000).

after 30 min. In Fig. 4A, it is clear that the Con A surface distribution pattern studied by indirect immunofluorescence is quite uniform at this level of resolution. As a result we feel that the proposal that agglutination is due to patches of Con A binding sites visible by light microscopy (16) is unlikely. To demonstrate that gross surface patching could be induced in these cells, we incubated L cells with anti-L cell myosin antiserum under the same conditions (Fig. 4B). Subsequent to the binding of this antibody to cell surface myosin (15), patch formation is plainly evident after 30 min at 23°.

DISCUSSION

The reason that transformed cells are more susceptible than normal cells to agglutination by Con A and other plant lectins has remained obscure despite numerous investigations (reviewed in ref. 14). One idea has been that transformed cells bind more Con A than normal cells but many studies showed that there was no difference in the amount of lectin bound by normal or transformed cells (17, 18). Another proposal was that Con A induced the clustering of Con A binding sites into discrete patches (16). This clustering, demonstrated in transformed but not normal cells, was thought to enhance the ability of these cells to stick together. Porter *et al.* (19) have pointed out that cells that have microvilli are more agglutinable by lectins, and suggested that the presence of microvilli somehow enhances agglutination (19).

The agglutination of cells by Con A is modulated by cAMP (14). The ability of high concentrations of cAMP to suppress the agglutinability of transformed cells (11–14) and the appearance of agglutinability in normal cells after lowering cAMP levels (14), suggests that the low cAMP levels in transformed cells (3, 4) are at least in part responsible for their agglutinability.

In this paper we have described a new method of observing

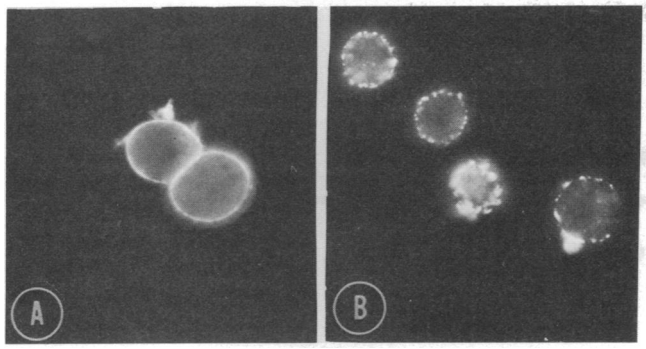

FIG. 4. Immunofluorescent localization of concanavalin A and cell surface myosin on L929 cells. L929 cells were incubated with Con A (A) or anti-myosin antiserum (B) and these substances were localized by immunofluorescence as described in *Materials and Methods*. The absence of large patches of surface label is evident with Con A localization (A). The presence of surface patching of label is seen with anti-myosin antiserum (B). (A = ×444; B = ×357).

living cells that allows the visualization of surface microvilli and other structures below the conventional limit of resolution of refractile light microscopy. This method provides the means for observing cell surface microstructure similar to scanning electron microscopy but, in addition, allows observation of these structures in a cell under continuous culture conditions. The length and shape of the microvilli observed in this way agree with their appearance with electron microscopic techniques.

Living transformed L929 cells and MNRK cells possess numerous microvilli. Bt_2cAMP treatment of these cells causes regression of these microvilli. Removal of Bt_2cAMP results in restoration of microvilli on the cell surface in less than 30 min. 3T3-4 cells normally have few microvilli on their surface. During mitosis, or after trypsin treatment, when cAMP levels are thought to be low (20–22), numerous microvilli appear on the cell surface. With low cAMP levels due to a temperature change-sensitive mutation (8), microvilli appear on the cell surface. Thus, it is clear that cAMP modulates the presence of cell surface microvilli. The presence of surface microvilli may be useful as a new criterion of transformation. If so, dark field microscopy would be a convenient screening method to differentiate transformed from normal cells. Removal of cells from their substratum by EDTA does not change the pattern of formation of microvilli. Thus, during an agglutination procedure, transformed L cells have numerous surface microvilli and these are relatively absent in cells treated with Bt_2cAMP or in normal cells.

At the ultrastructural level we have shown that microvilli are present in agglutinated cell clumps, interdigitating between adjacent cells. In almost all of the clumps we have examined, the cell bodies appear to be held apart by a "forest" of microvilli in between. Thus, it is evident that *most* of the interaction of such cells during agglutination must involve their surface microvilli.

Thirty minutes after the addition of Con A, when agglutination of cells is easily demonstrated, Con A appears by light microscopy to be evenly distributed over the cell surface (Fig. 4). Apparently it requires 1.5–2 hr to observe the clustering of Con A binding sites into the visible patches observed by Nicolson (16). Further, to be seen by light microscopy these patches would have to be present on the cell body, and not on microvilli where the principle agglutination interaction occurs. Thus these large patches do not seem to have anything to do with the agglutination of cells.

Using electron microscopic techniques, a number of workers have observed that 10–30 min after the addition of Con A, small patches of Con A molecules about 1 μm in diameter are observed (17, 23, 24). Whether these small clusters affect agglutinability is unknown.

In summary, our evidence indicates that transformed cells are more agglutinable than normal cells because of the presence of large numbers of microvilli on the surface of transformed cells. We also suggest that the presence of microvilli on transformed cells is due to their low cAMP levels (3, 4), since Bt_2cAMP can cause these microvilli to regress, and high cAMP levels in normal cells are accompanied by few microvilli.

Our data also indicates that the ability of cAMP to decrease agglutinability (14) is mediated through its ability to cause the regression of microvilli. Similarly, the increased agglutinability of cells with falling cAMP levels (14) is due to the appearance of new microvilli on the cell surface. We recognize that cells without microvilli can agglutinate with plant lectins under a variety of conditions, which include high lectin concentrations, high cell numbers, long agglutination times, or vigorous agitation. The difference in agglutinability between normal and transformed cells is obscured under all these conditions.

1. Johnson, G. S. & Pastan, I. (1972) *J. Nat. Cancer Inst.* **48,** 1377–1387.
2. Hsie, A. W. & Puck, T. T. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 358–361.
3. Otten, J., Johnson, G. S. & Pastan, I. (1971) *Biochem. Biophys. Res. Commun.* **44,** 1192–1198.
4. Sheppard, J. R. (1972) *Nature New Biol.* **236,** 14–16.
5. Smets, L. A. (1972) *Nature New Biol.* **239,** 123–124.
6. Johnson, G. S., Friedman, R. M. & Pastan, I. (1971) *Ann. N.Y. Acad. Sci.* **185,** 413–416.
7. Johnson, G. S., Friedman, R. M. & Pastan, I. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 425–429.
8. Willingham, M. C., Carchman, R. A. & Pastan, I. (1973) *Proc. Nat. Acad. Sci. USA* **70,** 2906–2910.
9. Johnson, G. S. & Pastan, I. (1972) *Nature New Biol.* **236,** 247–249.
10. Johnson, G. S., Morgan, W. D. & Pastan, I. (1972) *Nature* **235,** 54–56.
11. Hsie, A. W., Jones, C. & Puck, T. T. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 1648–1652.
12. Sheppard, J. R. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 1316–1320.
13. Kurth, R. & Bauer, H. (1973) *Nature New Biol.* **243,** 243–245.
14. Willingham, M. C. & Pastan, I. (1974) *J. Cell. Biol.* **63,** 288–294.
15. Willingham, M. C., Ostlund, R. E. & Pastan, I. (1974) *Proc. Nat. Acad. Sci. USA* **71,** 4144–4148.
16. Nicolson, G. L. (1973) *Nature New Biol.* **243,** 218–220.
17. Nicolson, G. L. (1971) *Nature New Biol.* **233,** 244–246.
18. Sharon, N. & Lis, H. (1972) *Science* **177,** 949–958.
19. Porter, K., Prescott, D. & Frye, J. (1973) *J. Cell Biol.* **57,** 815–836.
20. Burger, M. M., Bombik, B. M., Breckenridge, B. McL. & Sheppard, J. R. (1972) *Nature New Biol.* **239,** 161–163.
21. Otten, J., Johnson, G. S. & Pastan, I. (1972) *J. Biol. Chem.* **247,** 7082–7087.
22. Sheppard, J. R. & Prescott, D. M. (1972) *Exp. Cell Res.* **75,** 293–296.
23. Rosenblith, J. Z., Ukena, T. E., Yin, H. H., Berlin, R. D. & Karnovsky, M. J. (1973) *Proc. Nat. Acad. Sci. USA* **70,** 1625–1629.
24. Weller, N. K. (1974) *J. Cell Biol.* **63,** 699–707.