DIBUTYRYL CYCLIC AMP TREATMENT OF 3T3 AND SV40 VIRUS-TRANSFORMED 3T3 CELLS IN AGGREGATES

Effects on Mobility and Cell Contact Ultrastructure

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ABSTRACT

The random cell movement of BALB/c 3T3 and SV40 virus-transformed BALB/c 3T3 cells within homogeneous aggregates was studied by observing the degree of penetration of newly attached [^{3}H]thymidine-labeled cells into the interior of the aggregates. The 3T3 cells penetrated into 3T3 aggregates an average of 0.89 cell diameter in 1.5 days, whereas the SV40-3T3 cells penetrated into SV40-3T3 aggregates an average of 3.20 cell diameters in the same time. Treatment of the aggregates with theophylline, theophylline plus prostaglandin E_1, or theophylline plus dibutyryl cyclic AMP all decreased the penetration of the SV40-3T3 cells into SV40-3T3 aggregates (2.36, 1.22, and 0.79 cell diameters, respectively). The same treatments had little effect on 3T3 aggregates.

The ultrastructure of 3T3 and SV40-3T3 cells in aggregates was examined by transmission electron microscopy. The 3T3 cells in aggregates were surrounded by microvilli and lamellipodia which were in contact with neighboring cells, whereas SV40-3T3 cells were nearly devoid of microvilli and lamellipodia and made contact at broader, less regular surface undulations. Treatment with theophylline plus dibutyryl cyclic AMP resulted in the appearance of microvilli on SV40-3T3 cells and also appeared to increase the area of intercellular contacts in both 3T3 and SV40-3T3 cells. These observations were supported for the surface cells of the aggregates by scanning electron microscopy.

The increased mobility of SV40-transformed 3T3 cells relative to their nontransformed counterparts has been demonstrated in monolayer culture (8) and postulated to be a general property of transformed cells (25). Previous work from our laboratory (9) has indicated that the ability of BALB/c 3T3 cells to penetrate into aggregates or masses of the same cell type was much less than that of SVT-2 cells, an SV40 virus-transformant of the BALB/c 3T3 line.

Cyclic AMP, some of its analogues, and agents that increase cyclic AMP levels appear to increase cell-dish adhesiveness (11, 12, 22) by decreased detachability. In addition, dibutyryl cyclic AMP (dbcAMP) or prostaglandin E_1 (PGE_1) decrease the mobility of L-929 fibroblasts in monolayer cultures (13). Since cells in tissues move over the surfaces of other cells, rather than over artificial surfaces, we thought that it was important to examine the effects of these agents on mobility in an

all-cellular system. The effects of dbcAMP, PGE_1, and theophylline on mobility of 3T3 and SV40 virus-transformed 3T3 cells in cellular aggregates were therefore tested. The assay method (9, 28) consists of "seeding" a few radiolabeled cells onto the surfaces of aggregates of unlabeled cells of the same type. Since the newly adhering radiolabeled cells and the unlabeled cells of the aggregates are otherwise identical, the penetration of the radiolabeled cells into the unlabeled aggregates occurs by a process of random intermixing. Cell penetration is monitored by autoradiography of histological section of the aggregates. The degree of penetration of the radiolabeled cells is taken to be a function of the extent of random movement of the cells in the aggregate. The data presented below indicate that a cAMP analogue (dbcAMP), a drug that inhibits cAMP phosphodiesterase (theophylline), and an agent that stimulates adenylate cyclase activity (PGE_1) all result in decreased mobility of transformed cells but have little effect on their normal counterparts.

We have also attempted to correlate enhanced mobility and supression of this enhancement with morphological changes in these cells. To this end, we have examined aggregates of 3T3 and SV40-transformed 3T3 cells by transmission and scanning electron microscopy in both the presence and absence of dbcAMP and theophylline.

MATERIALS AND METHODS

Chemicals and Radiochemicals

All chemicals were purchased from commercial sources and were either biological grade, if available, or else the highest grade available.

[*methyl*-^{3}H]Thymidine was purchased from Amersham/Searle Corp. (Arlington Heights, Ill.) and had a specific activity of 18-20 Ci/mmol. dbcAMP (sodium salt) was obtained from P-L Biochemicals, Inc. (Milwaukee, Wis.). PGE_1 was a generous gift of Dr. John Pike of Upjohn Co. (Kalamazoo, Mich.). Theophylline was obtained from Sigma Chemical Co., (St. Louis, Mo.).

Culture Media

Cells were grown in Eagle's Minimum Essential Medium (MEM) with fourfold increased concentrations of vitamins and amino acids, plus 10% fetal calf serum and 100 U/ml penicillin and 100 μg/ml streptomycin (MEM × 4 plus fetal calf serum).

The medium was purchased from Grand Island Biological Co. (Grand Island, N. Y.) as the dry mix, and stored at 4°C. Serum was purchased from the same source, stored at −20°C and used within 2 mo.

Growth of Cell Lines

Mouse fibroblast BALB/c 3T3 and SV40-transformed BALB/c 3T3 (designated SVT-2) were originally derived from the A31 clone isolated by Aaronson and Todaro (1) and grown as described previously (9). Cells were kept as frozen stocks at liquid N_2 temperature and were used between the 8th and 12th passage. Lines were examined periodically for mycoplasma contamination but none was detected. Cells were routinely grown in 32-ounce bottles and subcultured, using trypsin-ethylenediaminetetraacetic acid (-EDTA) solutions (0.05% trypsin (Difco Laboratories, Detroit, Mich.) 1:250 and 0.5 mM EDTA). Before use in an experiment, cells were plated into 100-mm plastic culture dishes in 15 ml of medium and grown as subconfluent cultures for 3-4 days (three to five generations). Medium was changed 24 h after plating and every 48 h thereafter. Cells were removed from the dishes with ethylene glycolbis[β-aminoethylether]-*N*,N′-tetraacetic acid (EGTA) (0.5 mM in Ca^{++}-Mg^{++}-free, phosphate-buffered saline). These cells were used to form aggregates as described below.

Formation of Aggregates

Aggregates were formed by the same procedure as described previously (9). Briefly, this involves pelleting a suspension of single cells at low speed, incubating the pellet for 1.5 h, and cutting it up with microscalpels, into aggregate-sized pieces. The fragments are cultured overnight in individual 20-μl hanging drops, then pooled, and used as described below. For experiments with drug addition, 12 h before seeding, one quarter of the aggregates was placed in medium containing either 1.2 mM dbcAMP plus 1 mM theophylline, 50 μg/ml PGE_1 plus 1 mM theophylline, 1 mM of theophylline alone, or in control medium. Fresh medium containing these drugs was used during all subsequent manipulations.

Labeling of Cells with [^{3}H]Thymidine

Cells were labeled by addition of [^{3}H]thymidine to the medium at a concentration of 0.33 μCi/ml. The cells were plated into 100-mm dishes in nonradioactive medium and the [^{3}H]thymidine was added 6 h later, after attachment of the cells. Cells were grown in the medium for 2 days. On the 3rd day, the radioactive medium was replaced with nonradioactive medium containing either 1.2 mM dbcAMP plus 1 mM theophylline, 50 μg/ml PGE_1 plus 1 mM theophylline, 1 mM theophylline alone, or control medium.

Seeding of Aggregates with Radioactive Cells

Aggregates were collected and transferred to a 10-ml flask in about 3 ml of medium containing the various drugs in the same concentrations as previously noted, and radioactive cells in suspension (50–100 unlabeled aggregates and about 10^6 cells). The flasks were left

stationary at 37°C in an atmosphere of 5% CO_2 in air for 2 h, but every 15–30 min they were gently agitated to resuspend the single cells and disrupt any contacts between aggregates. The aggregates were then rinsed three times in fresh medium to remove unattached radioactive cells. This procedure produced aggregates each with about 30 radiolabeled cells adhering to the surface. This corresponds to about 1–2% of the total surface of the aggregate. No changes in number of adhering cells were noted as a function of cell type or drug treatment. To insure against fusion of aggregates, each aggregate was cultured in a separate container. The containers used were cylindrical 5-ml glass vials (1.3 × 4 cm) containing 1 ml of complete medium and capped with a modified Bellco closure (Bellco Glass, Inc., Vineland, N. J.). 40 aggregates were used for each treatment. The vials were agitated at 180 gyrations per minute on a gyratory water bath shaker in an atmosphere of 5% CO_2 in air 1.5 days.

Aggregates were fixed in Bouins fluid, dehydrated, and embedded in Paraplast Plus (Fisher Scientific Co., Cleveland, Ohio). Slides and autoradiographs were prepared and processed by the methods described previously (9).

Assay for Mobility

Mobility of 3T3 and SVT-2 cells was measured by an autoradiographic assay first described by Wiseman and Steinberg (28). Specific modifications and a detailed description of this assay have been published previously (9). In this assay, homologous radiolabeled cells are allowed to adhere to the surface of the aggregates, cultured for varying periods (in this study 0 or 1.5 days), then fixed, and sectioned. The positions of the radiolabeled cells relative to the nearest edge of the aggregate section are measured optically, using a microscope with a × 40 objective and a × 10 eyepiece equipped with an ocular micrometer. The cell locations are converted to cell diameters and tabulated. Positions are therefore expressed as distance from the nearest edge of the section in cell diameters. While this assay does not measure the movements of individual cells, or their actual paths, it provides a statistical estimate of how much random motion the cells of the aggregate are undergoing. Data are expressed either as a histogram plot of number (or frequency) of radiolabeled cells vs. distance from the edge of the section, or else as mean position (of the entire population of radiolabeled cells) from the edge of the aggregate section. The latter method of expressing position has the advantage of brevity, although the former method gives considerably more information about the actual distributions of cell locations.

Transmission Electron Microscopy

Cells were grown on dishes and removed with EGTA as described above. During the last 12 h of growth, the medium was replaced with either fresh control medium or fresh medium containing 1.2 mM dbcAMP plus 1.0 mM theophylline. After rinsing in culture medium, they were resuspended in complete culture medium with no additions or the same medium containing 1.2 mM dbcAMP plus 1.0 mM theophylline. The suspension was centrifuged and the pellet cut up to form aggregates as described above. All of these procedures were carried out in either complete medium or complete medium plus dbcAMP theophylline. Aggregates were fixed immediately after cutting into fragments (1.5 h after centrifugation) or cultured for 0.5, 1.0, or 2.0 days in the same medium and then fixed. Aggregates were rinsed briefly in medium lacking serum and fixed in 4% glutaraldehyde and 2% paraformaldehyde plus 0.5 mg/ml $CaCl_2$ in 0.2 M cacodylate buffer (pH 7.5) for 30 min (modified Karnovsky fixative [14]) at room temperature. They were then rinsed several times with buffer at room temperature and stored for several hours in buffer at 4°C. Aggregates were stained en bloc with 1% OsO_4 in 0.2 M cacodylate buffer and then 2% uranyl acetate in ethanol. After dehydration, embedding in Spurr plastic, and sectioning, the sections were picked up on copper grids and restained with lead citrate and uranyl acetate. Grids were examined in a modified RCA RMU-3 electron microscope.

Each experimental treatment was repeated at least three times. 50–100 aggregates were examined in each experiment, and several hundred cells were examined in detail for each treatment. Most of the cells studied in detail and shown in Figs. 3-12 were equatorial sections, as judged from the nuclear cross section.

Scanning Electron Microscopy

Aggregates were formed and treated as described above, then fixed for 60 min at room temperature in 2% paraformaldehyde, 2% glutaraldehyde in MEM 4 × culture medium with no fetal calf serum added. Aggregates were rinsed three times in phosphate-buffered saline, dehydrated in graded water-ethanol mixtures, and then in graded ethanol-Freon 113 (E. I. du Pont de Nemours & Co., Wilmington, Del.) mixtures, and critical point dried with Freon 13. The aggregates were retained in stainless steel mesh chambers during this procedure. Before viewing, the aggregates were affixed to the stainless steel mesh with carbon conductive cement, then sputter-coated with gold-palladium to provide a conductive surface. The coated aggregates were viewed with a Cambridge Stereoscan S4-10 scanning electron microscope.

RESULTS

Mobility of Cells

In agreement with previous studies (9), BALB/c 3T3 cells were found to be less mobile than SVT-2 cells. Fig. 1*a* shows the distribution of radiolabeled 3T3 cells in cell aggregates as measured in cell diameters from the nearest edge of the aggre-

gate section. Immediately after the 2-h attachment period, the bulk (87%) of the radiolabeled cells as on the surface and the remainder (13%) was within one cell diameter of the surface. After 1.5 days of culture, there was very limited penetration of radiolabeled cells into the interior of the aggregate; 49% of the radiolabeled cells were still on the surface and 97% of the cells were within three cell diameters of the surface. The mean cell positions corresponding to the data in Fig. 1*a*, and those of a replicate experiment, are given in Table I (lines 1–4). These mean cell positions were 0.075 diameter immediately after incubation and 0.89 diameter after 1.5 days.

Treatment with theophylline or PGE_1 plus theophylline, or dbcAMP plus theophylline, had only a minimal effect on the already low mobility of 3T3 cells. These data are shown in Fig. 1*b–d* and Table I (lines 5–16). The equivalent data for a replicate experiment are also given in Table I. The largest difference was obtained by treatment with dbcAMP plus theophylline which reduced the mean position after 1.5 days from 0.89 cell diameter from the surface to 0.65 diameter from the surface. These differences are probably within experimental error.

Similar experiments conducted with radiolabeled SVT-2 cells seeded onto unlabeled SVT-2 aggregates are shown in Fig. 2*a–d* and Table II. As reported previously, the mobility of SVT-2 cells in aggregates was significantly greater than that of 3T3 cells. Immediately after seeding, 99% of the SVT-2 cells were located one cell diameter or less from the surface; however, by 1.5 days, only 13% of the cells were on the surface and 35% of the radiolabeled SVT-2 cells had penetrated four or more diameters into the aggregate. The mean cell positions immediately after and 1.5 days after incubation were 0.32 and 3.20 cell diameters from the surface, respectively. Treatment of the aggregates with theophylline alone resulted in a modest suppression of mobility compared to untreated SVT-2 aggregates (from 3.20 to 2.36 diameters). Treatment with PGE_1 plus theophylline was more effective, resulting in a reduction from 3.20 to 1.22 cell diameters. The most effective treatment, as with the 3T3 aggregates, was the combination of dbcAMP and theophylline, which reduced the 1.5-day mean cell position from 3.20 to 0.79 cell diameters. The histograms depicting these data are shown in Fig. 2*a–d* while the mean values for this experiment, along with those for two other replicate series of experiments, are given in Table II.

The sizes of individual SVT-2 and 3T3 cells in aggregates were also measured in order to convert optical micrometer measurements to distances in cell diameters. These sizes are given in Table III. No difference in size was observed with any drug treatment.

TABLE I

Movement of Radiolabeled BALB/c 3T3 Cells into Aggregates of BALB/c 3T3 Cells

Treatment	Days in culture	Cells measured	Mean cell position (in cell diameters)	
Control	0	255	0.13	(0.075)*
	0	36	0.02	
	1.5	488	0.79	(0.89)
	1.5	388	0.99	
Theophylline	0	157	0.13	(0.09)
	0	31	0.05	
	1.5	286	0.61	(0.71)
	1.5	373	0.81	
PGE_1 + theophylline	0	111	0.43	(0.26)
	0	61	0.08	
	1.5	198	0.64	(0.81)
	1.5	81	0.98	
dbcAMP + theophylline	0	254	0.24	(0.16)
	0	37	0.08	
	1.5	282	0.45	(0.65)
	1.5	335	0.84	

* Numbers in parentheses are the means of replicate experiments (the numbers to their immediate left).

Ultrastructural Observations

As described in Materials and Methods, aggregates were formed by pelleting cells together and, after a 1.5-h incubation period, cutting the pellet up into fragments which were then cultured individually. The aggregates were therefore homogeneous masses consisting of a single cell type. Untreated (control medium) 3T3 and SVT-2 aggregates were examined either immediately after the pellets were cut (i.e., 1.5 h after centrifugation), or 0.5, 1.0, and 2.0 days later. As indicated below, few changes were noted with increasing time of culture except for a general increase in area of cell-cell contact. Essentially all of this increase occurred before 1.0 day of culture. The observations were therefore repeated using 3T3 and SVT-2 aggregates formed and cultured in control medium or in medium containing 1.2 mM dbcAMP and 1.0 mM theophylline, and these aggregates were examined immediately after cutting and 1.0 day later. To facilitate comparison, the observa-

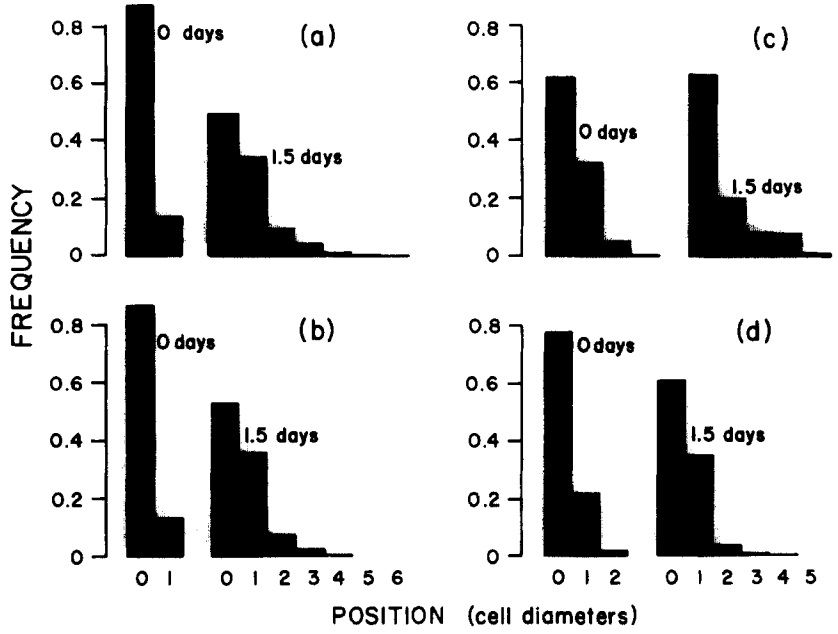


FIGURE 1 Location of radiolabeled 3T3 cells in sections of unlabeled 3T3 aggregates. Aggregates were fixed immediately (0 days) or 1.5 days after incubation with radiolabeled cells. Ordinate, frequency of occurrence of radiolabeled cells. Abscissa, position of radiolabeled cells relative to the nearest edge of the section, measured in cell diameters. (*a*) aggregates cultured in control medium; (*b*) aggregates cultured in medium containing 1.0 mM theophylline; (*c*) aggregates cultured in medium containing 50 μg/ml PGE_1 and 1.0 mM theophylline; (*d*) aggregates cultured in medium containing 1.2 mM dbcAMP and 1.0 mM theophylline.

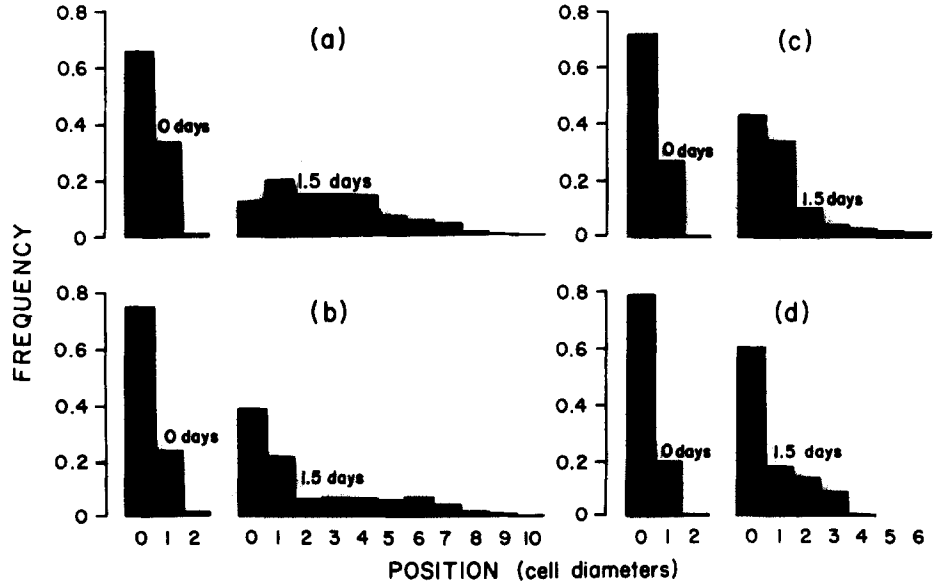


FIGURE 2 Location of radiolabeled SVT-2 cells in sections of unlabeled SVT-2 aggregates. Aggregates were fixed immediately (0 days) or 1.5 days after incubation with radiolabeled cells. Ordinate, frequency of occurrence of radiolabeled cells. Abscissa, position of radiolabeled cells relative to the nearest edge of the section, measured in cell diameters. (*a*) aggregates cultured in control medium; (*b*) aggregates cultured in medium containing 1.0 mM theophylline; (*c*) aggregates cultured in medium containing 50 μg/ml PGE_1 and 1.0 mM theophylline; (*d*) aggregates cultured in medium containing 1.2 mM dbcAMP and 1.0 mM theophylline.

tions in this report are organized so that specific ultrastructural features of all combinations of cell type, times of culture, and drug treatments are described together. All the observations below were made on aggregates prepared for transmission electron microscopy. In addition, the data on microvilli were confirmed for cells on the surface of aggregates by scanning electron microscopy.

TABLE II

Movement of Radiolabeled SVT-2 Cells into Aggregates of SVT-2 Cells

Treatment	Days in culture	Cells measured	Mean cell position (in cell diameters)
Control	0	243	0.36
	0	269	0.28 (0.32)*
	0	251	0.32
	1.5	201	2.90
	1.5	501	2.65 (3.20)
	1.5	488	4.06
Theophylline	0	211	0.27
	0	257	0.25 (0.29)
	0	257	0.36
	1.5	275	2.06
	1.5	382	1.92 (2.36)
	1.5	462	3.11
PGE_1 + theophylline	0	230	0.28
	0	255	0.30 (0.31)
	0	255	0.34
	1.5	148	1.04
	1.5	182	1.02 (1.22)
	1.5	212	1.59
dbcAMP + theophylline	0	180	0.21
	0	255	0.31 (0.27)
	0	272	0.28
	1.5	82	0.71
	1.5	295	0.74 (0.79)
	1.5	139	0.91

* Numbers in parentheses are the means of replicate experiments (the numbers to their immediate left).

GENERAL FEATURES OF CELLS IN AGGREGATES: Both 3T3 and SVT-2 cells were generally spherical when examined 1.5 h after aggregate formation. Except in the immediate vicinity of cell-cell contacts, cells were separated from each other by 0.5–3.0-μm gaps (e.g. Fig. 3). Nuclei of both cell types were large and well defined and often irregular. As reported by McNutt et al (17), the nuclei of SVT-2 cells often display deep invaginations of the membrane. In contrast to the observations of McNutt et al. (17), and our own observations on confluent monolayer cells (unpublished observations), both SVT-2 and 3T3 cells in aggregates displayed shallow nuclear invaginations. The SVT-2 cells appeared to be slightly less rounded than the 3T3 cells after 1.5 h, and some cells appeared to have contours fitted to those of adjacent cells (e.g. the two upper cells in Fig. 5). With increasing time in culture, both 3T3 and SVT-2 cells become more elongated, although, in the case of the 3T3 cells, this change in shape was not very great. The SVT-2 cells, on the other hand, often became quite extended and tightly applied to the contours of neighboring cells. This effect was pronounced 0.5 day after aggregate formation and at a maximum after 1.0

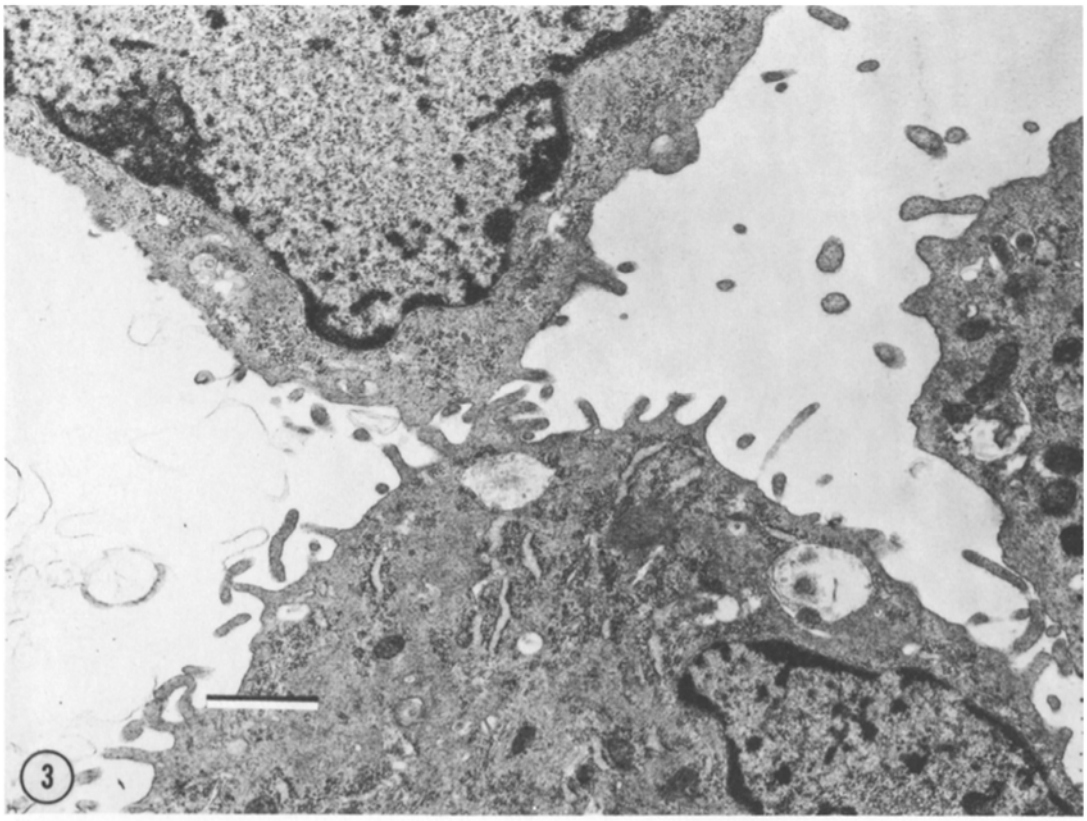
TABLE III

Diameters of Cells Cultured in Aggregates and Aggregate Sections

Cell line	Treatment	Cell size (μm)*	Aggregate section diameter (μm)‡
3T3	None	8.44 ± 0.82; 8.44 ± 0.82	153.9 ± 12.7; 163.6 ± 13.3
	Theophylline	8.39 ± 0.77; 8.28 ± 0.93	145.6 ± 10.1; 163.0 ± 14.0
	PGE_1 + theophylline	8.23 ± 0.75; 8.39 ± 0.79	142.6 ± 10.8; 150.7 ± 5.3
	dbcAMP + theophylline	8.23 ± 0.67; 8.28 ± 0.53	148.1 ± 7.8; 149.5 ± 6.2
SVT-2	None	6.25 ± 0.52; 6.25 ± 0.68	149.2 ± 14.8; 175.8 ± 17.7
	Theophylline	6.30 ± 0.63; 6.20 ± 0.53	167.3 ± 12.5; 175.6 ± 13.4
	PGE_1 + theophylline	6.20 ± 0.53; 6.20 ± 0.98	156.5 ± 13.2; 159.4 ± 13.3
	dbcAMP + theophylline	6.36 ± 0.75; 6.41 ± 0.78	147.7 ± 15.2; 156.4 ± 10.3

* First value ± SD was measured in sections fixed immediately after seeding. Second value ± SD was measured in sections fixed 1.5 days after seeding with radiolabeled cells. n = 20 for each measurement. In no case was there a significant difference between the two values. Measurements of cell size were made using a × 100 oil immersion objective and an eyepiece equipped with an ocular micrometer. Measurements included representatives of both surface cells and interior cells selected on a random basis. Measurements of aggregate section size were made with a × 40 objective and an ocular micrometer. In the case of noncircular sections, the mean of the long and short axes was taken. n was between 100 and 200 for each measurement.

‡ First value ± SD was measured in sections fixed immediately after seeding. Second value ± SD was measured in sections fixed 1.5 days after seeding. In no case was there any significant effect of drug treatment or time on section diameter.



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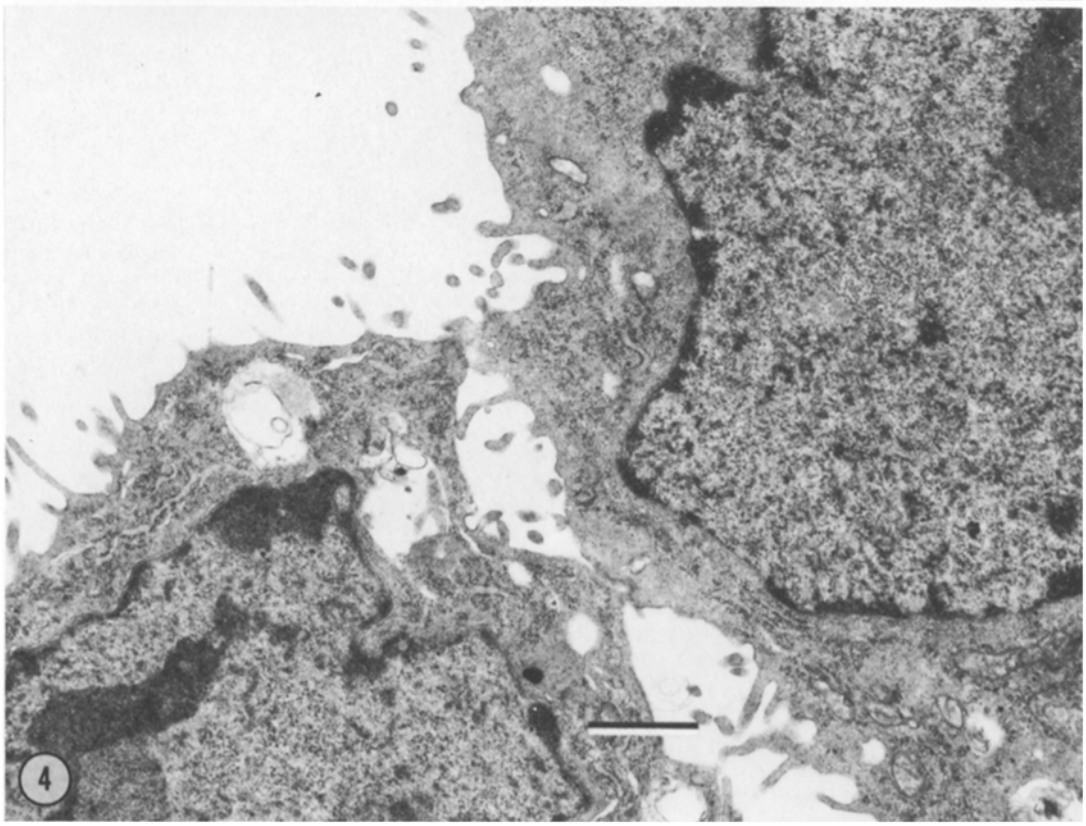

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day of culture. No additional alterations were seen between 1.0 and 2.0 days of culture. No differences in the shape of SVT-2 cells were observed in different regions of the aggregate. In the case of 3T3 aggregates, some cells in the outer one to two cell layers tended to be slightly more flattened than the interior cells. The ratio of the long to the short dimensions of these slightly flattened cells did not exceed 2:1.

MICROVILLI: The most striking morphological difference between 3T3 and SVT-2 cells was the number of microvillar extensions (long cylindrical cytoplasmic processes with a regular diameter of about 0.1 μm). 3T3 cells projected numerous microvilli out into the free space between cells while SVT-2 cells in aggregates displayed many fewer such extensions. For example, note the many cytoplasmic extensions surrounding the 3T3 cell shown in Fig. 3. These microvilli had a constant diameter of about 0.1 μm (0.12 $\pm$ 0.016 μm, n = 44) and were often more than 3.0 μm in length. 3T3 cells also displayed irregular broad cytoplasmic extensions 1–3 μm in diameter which often bore microvilli. The large number of extensions with circular (0.1 μm in diameter) profiles relative to those with rod-like (0.1 $\times$ 1.0 μm or longer) profiles suggested that many of these extensions were finger-like microvilli, rather than sheet-like lamellipodia. The number of microvilli and lamellipodia present was somewhat decreased by 0.5 and 1.0 day after aggregate formation (e.g. Fig. 7) although large numbers of extensions were present at all times examined.

In marked contrast to 3T3 cells, SVT-2 cells cultured as aggregates in control medium had many fewer cytoplasmic processes, and these tended to be heteromorphic; only a very few of these extensions could be classified as microvilli (cf. Figs. 5 and 6). While an occasional microvillus was visible, especially at 1.5 h, the surfaces of SVT-2 cells were generally covered with broader undulations or extensions. For example, only one or two microvilli are present on the SVT-2 cells in Fig. 5. These varied greatly in size, but were usually 0.2–0.8 μm across at the base and no more than 1.0–2.0 μm in length.

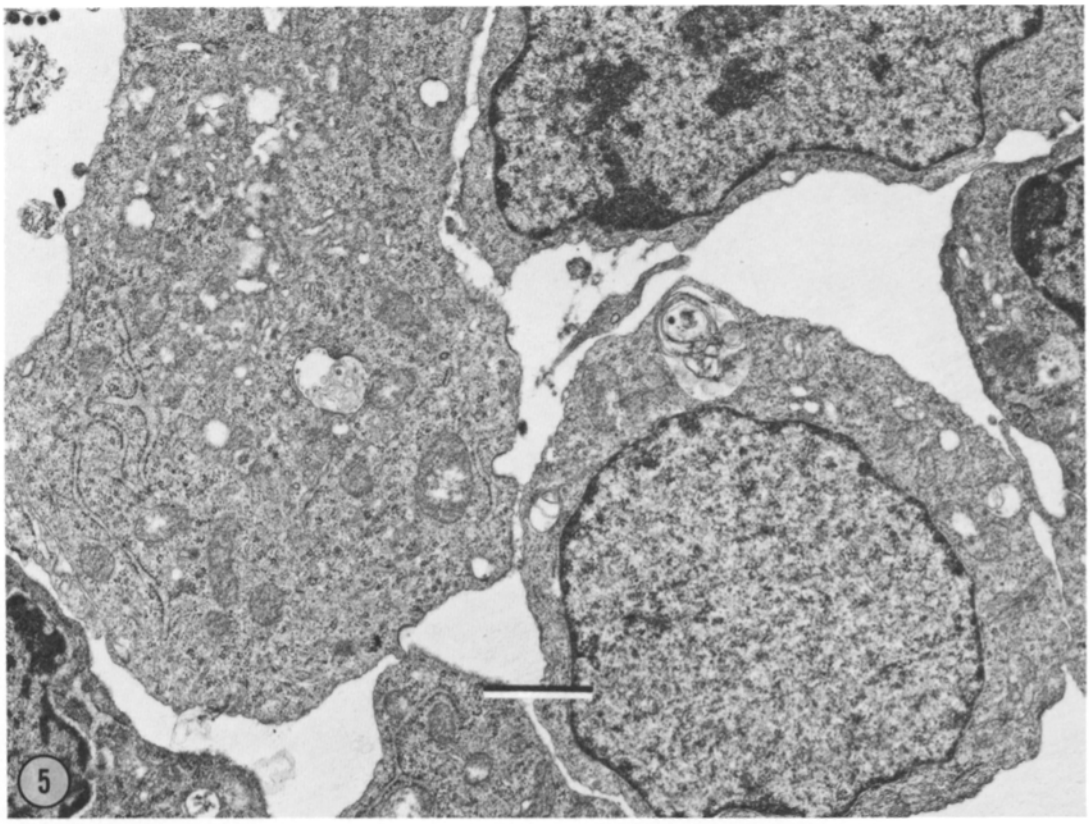
The effect of dbcAMP and theophylline on the presence of microvilli in SVT-2 cells was dramatic: at 1.5 h after aggregate formation, the surfaces of treated SVT-2 cells were covered with large numbers of cytoplasmic extensions, including many microvilli (compare the SVT-2 cells in Fig. 5 with the dbcAMP plus theophylline-treated SVT-2 cells in Fig. 6). These microvilli were about the same diameter as those seen on control 3T3 cells (0.11 $\pm$ 0.019 μm, n = 20) but usually shorter (usually 0.2–0.6 μm long). They were rarely seen in regions where cells made close contact, but were common at free borders where cells were separated by more than 1.0 μm. Microvilli were considerably less common in treated aggregates after 1.0 day.

The effect of dbcAMP plus theophylline on 3T3 cells was not striking. There appeared to be an increase in the number of microvilli; however, there were already so many microvilli present in control aggregates that it was difficult to be certain (compare the untreated 3T3 cells in Fig. 3 with the treated 3T3 cells in Fig. 4). There was no significant change in microvillus diameter (0.13 $\pm$ 0.019 μm, n = 48) as a function of drug treatment. The number of microvilli were decreased at 1.0 day compared to 1.5 h of culture, as with control 3T3 aggregates and dbcAMP-treated SVT-2 aggregates.

These observations were supported by some preliminary observations made by scanning electron microscopy. Fig. 13*b* indicates that the surface cells of whole BALB/c 3T3 aggregates displayed numerous microvilli, while SVT-2 cells bore only a few microvilli and were characterized by broader projections (0.23 $\pm$ 0.038 μm in di-

FIGURE 3 3T3 cells cultured as aggregates for 1.5 h. Portions of three 3T3 cells in the interior of an aggregate. Note the large number of cytoplasmic extensions surrounding the cells and several microvillar contacts between them. Compare this micrograph to Figs. 4–6, all at the same magnification. Bar, 1 μm.

FIGURE 4 3T3 cells treated with dbcAMP plus theophylline and cultured as aggregates for 1.5 h. Portions of two 3T3 cells in the interior of an aggregate. In number and type, the cytoplasmic extensions on cells of treated aggregates are quite similar to those of cells in untreated aggregates (Fig. 3). Note that several microvillar contacts as well as two more extensive contacts are visible in this micrograph. Bar, 1 μm.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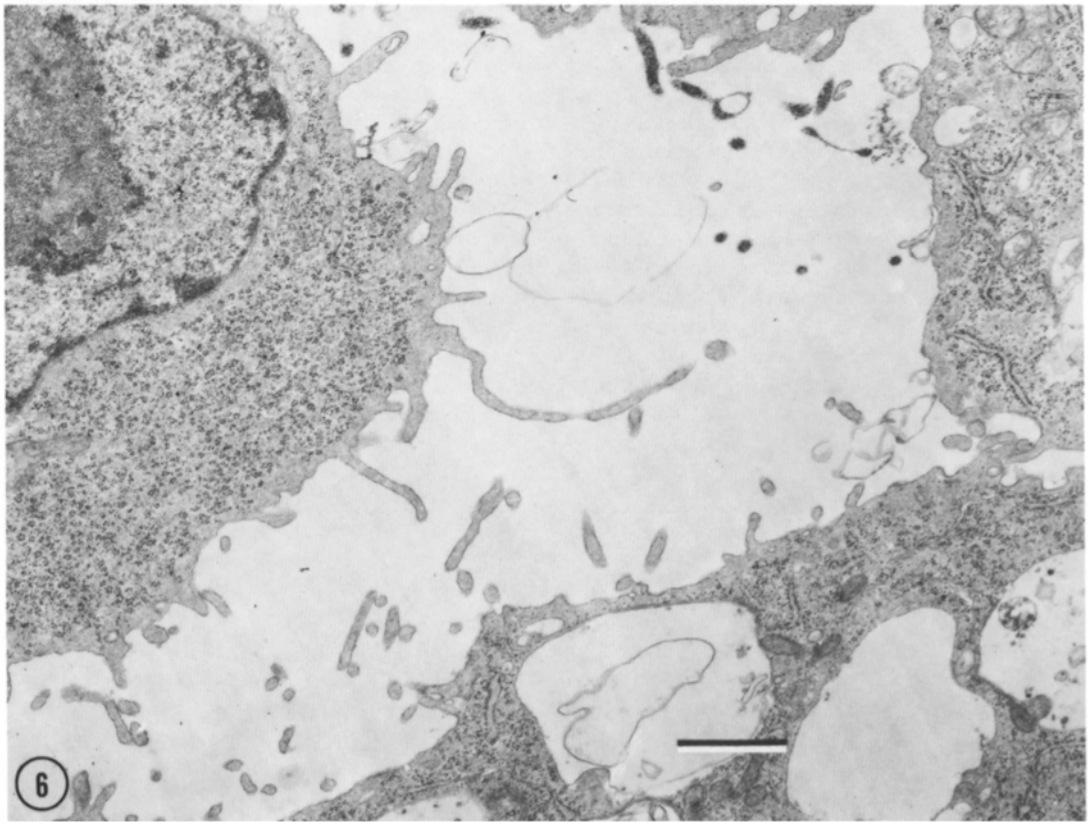
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ameter, n = 20), most of which were no more than 0.5 μm in length (Fig. 13*e*). As observed with transmission electron microscopy, dbcAMP induced the appearance of numerous cytoplasmic processes, including microvilli, on the surfaces of SVT-2 but had little obvious effect on 3T3 cells (Fig. 13, *c* and *f*).

CELL-CELL CONTACTS: The area of contact between 3T3 cells 1.5 h after aggregate formation was rather small. Contact was made almost exclusively between a microvillus of one cell and the surface of an adjacent cell, or between two microvilli. The total length of apposed surface rarely exceeded 0.4 μm. In most cases, substantial separations between the cells were present, often in excess of 0.5 μm (e.g., Fig. 3). By 0.5–1.0 day of culture, many of the contacts were those between broad cytoplasmic extensions, usually a matching extension from each cell, although contacts by means of microvilli were still common. A typical area of cell-cell apposition including both microvillar and nonmicrovillar contacts is shown in Fig. 7. Junctions were of the "adherens" type (6). That is, an electron-transparent gap between the apposed membranes is usually visible, roughly equal to the membrane thickness (i.e., 90–120 Å). Electron-dense depositions in the cytoplasm adjacent to the contact zone were not observed in 3T3 aggregates fixed 1.5 h after formation, and only very rarely in those fixed after 1.0 day of culture.

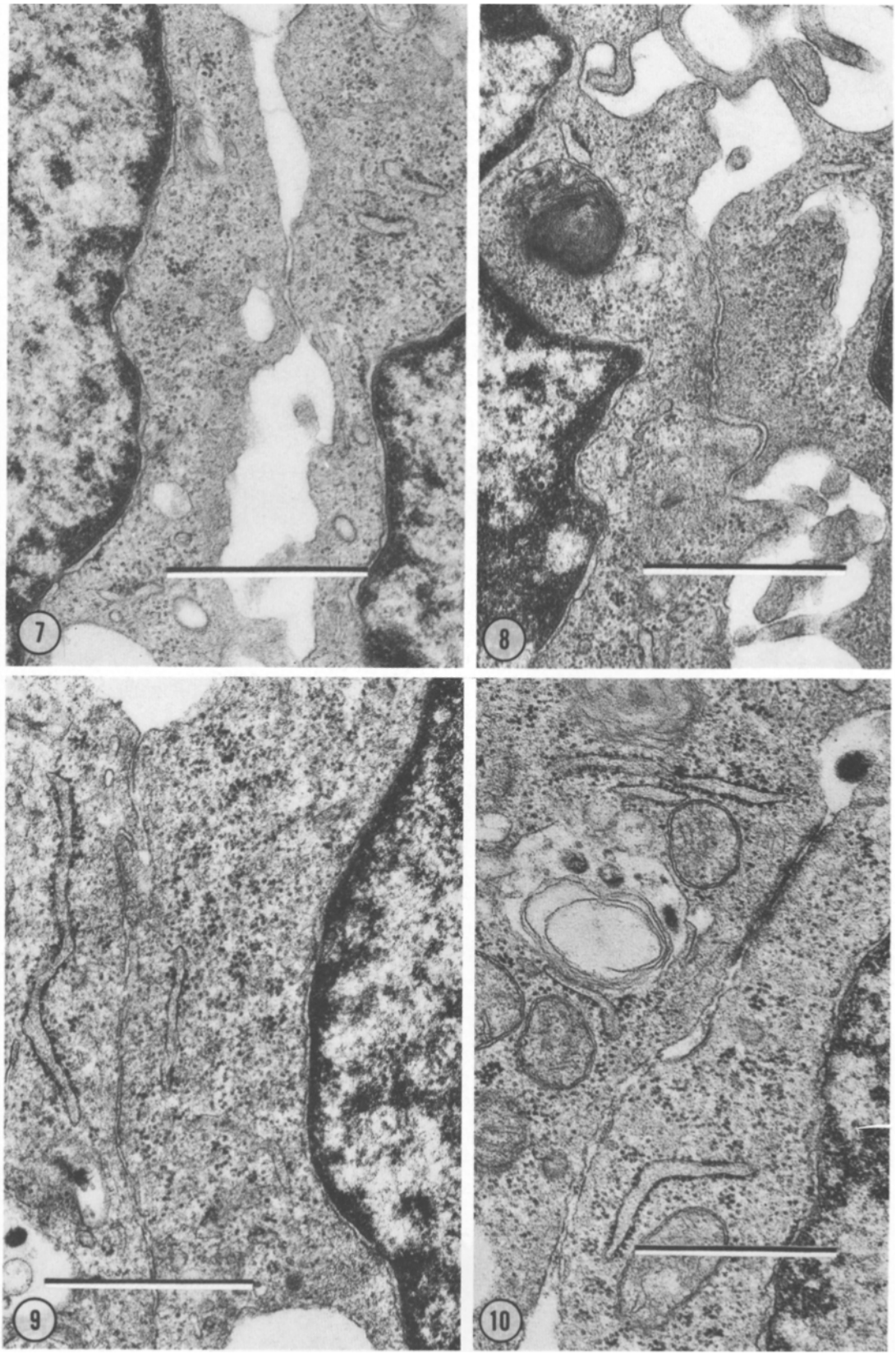
In contrast to 3T3 cells, SVT-2 cells made extensive areas of contact at broad cytoplasmic extensions even as early as 1.5 h after formation (as indicated in Fig. 5). An extensive region of cell-cell apposition is also shown at higher magnification in Fig. 11. Contact zones, 5 μm or more in length, were often seen in which adjacent surfaces were in close proximity (adherens junctions) over 0.5-1.0-μm stretches, while the remainder of the surfaces remained within 0.02 μm of each other. These areas of close approach were increased in aggregates cultured for 1.0 day after formation, and a larger proportion of the contact zone was classified as an adherens junction. Fig. 9 shows an extensive interdigitated contact between two SVT-2 cells over 3 μm in length. Electron-dense plaques were common after 1.0 day. These always consisted of symmetrical electron-dense depositions in both apposed cells, extending about 0.03 μm into the cytoplasm. The length observed varied from 0.1 to 0.5 μm.

Contacts between dbcAMP-treated 3T3 cells cultured for 1.5 h were similar to those observed in untreated 3T3 cultured for the same time. The contacts were usually those between a microvillus and a cell, or those between two microvilli. The total length of apposed surface per contact appeared to be slightly increased in dbcAMP-treated 3T3 cells relative to the controls. Occasionally, an area of contact between broader cytoplasmic extensions was seen. By 1.0 day after aggregate formation, these broad contact zones were fairly common and also contained electron-dense depositions similar to those seen in control SVT-2 aggregates. Fig. 8 shows two broad contacts, one with electron-dense depositions. Contacts at microvilli were still common 1.0 day after aggregate formation, but did not include electron-dense depositions. Fig. 8 also shows a number of such microvillar contacts.

SVT-2 aggregates treated with dbcAMP and theophylline also displayed more intercellular contact than control SVT-2 aggregates 1.5 h after aggregation. Contacts between treated SVT-2 cells were by means of both broad extensions and microvilli. Extensive areas of close surface apposition of the "adherens" type were seen, as in untreated cells. Electron-dense depositions were occasionally observed 1.5 h after aggregate formation. After 1 day of culture in the presence of dbcAMP and theophylline, areas of contact had increased somewhat, often comprising stretches of adherens-type junctions 1 μm or more in length (Figs. 10, 12). Electron-dense depositions were also more common than in aggregates fixed 1.5 h

FIGURE 5 SVT-2 cells cultured as aggregates for 1.5 h. Portions of six SVT-2 cells in the interior of an aggregate. Note that only one or two microvilli are visible, in contrast to 3T3 aggregates (Fig. 3) and to SVT-2 aggregates treated with dbcAMP plus theophylline (Fig. 6). Bar, 1 μm.

FIGURE 6 SVT-2 cells treated with dbcAMP plus theophylline and cultured as aggregates for 1.5 h. Portions of four SVT-2 cells in the interior of the aggregate. Numerous microvilli are present (compare to Fig. 5), and at least one microvillar contact is visible. Bar, 1 μm.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after formation (e.g., well-defined electron-dense depositions in Figs. 10 and 12).

DISCUSSION

The data presented here indicate that the mobility of SV40-transformed 3T3 cells within cellular aggregates can be inhibited by treatment with dbcAMP plus theophylline. A less pronounced inhibition was also seen in aggregates treated with PGE_1 plus theophylline or theophylline alone. Theophylline is an inhibitor of the phosphodiesterase that hydrolyzes cAMP; and PGE_1 stimulates adenylate cyclase activity. It therefore seems reasonable to conclude that these agents inhibit mobility by increasing intracellular cAMP levels. These data are in agreement with previous observations indicating that mobility of cells on solid substrata is also inhibited by agents that increase intracellular cAMP levels (13).

Carter (3) has reported that, when cells are the most extensively flattened on a solid substratum, they move the least. On this basis, he has proposed that mobility and adhesion are inversely related. Ultrastructural observations of 3T3 and SVT-2 cells in aggregates have revealed no such inverse correlation between the amount of intercellular contact and the degree of mobility of these cell lines. On the contrary, 3T3 cells had a much smaller area of intercellular contact than did SVT-2 cells. Even more striking, SVT-2 cells, but not 3T3 cells, commonly displayed electron-dense, desmosome-like junctions of the type usually associated with strong adhesions. However, even if the strength of adhesion is the major determinant of mobility of aggregates, it may not be morphologically apparent. For example, the strength of adhesion per unit area of contact may be different for these two cell lines. It is noteworthy that 3T3 cells sort out (self-segregate) internally to SVT-2 cells in mixed aggregates (10). Interpreted according to Steinberg's differential adhesiveness hypothesis (23), this would also indicate that 3T3 cells are more adhesive than SVT-2 cells. The rate at which adhesions are formed and broken (the rate of contact turnover) may also play a role in controlling mobility. Another possibility is that mobility is promoted when the total number of intercellular contacts is at a minimum, regardless of their total area. SVT-2 cells, with a few broad contacts, may thus translocate more freely than 3T3 cells with multiple small intercellular contacts. Finally, it is possible that the mobility differences between these two cell lines are mainly under the control of other factors not morphologically apparent, such as the availability of metabolic energy, or the presence or absence of serum factors that affect migration of cells on solid substrata (2, 16). Treatment of both cell types with dbcAMP and theophylline did have an observable effect on cell contacts more consistent with Carter's (3) hypothesis: both 3T3 and SVT-2 cells displayed an increase in total area of contact after treatment (cf. Figs. 7 and 8). The number of electron-dense plaques also increased, as did the degree to which cell conformed to each other's configurations.

Several contradictions exist in the current literature concerning the occurrence of microvilli on the surfaces of normal and transformed cells, and their alteration by dbcAMP. Porter et al. (21), who examined BALB/c 3T3 cells and SVT-2 cells on plastic surfaces, and Collard and Temmink (4),who studied Swiss 3T3 cells and their SV40

FIGURE 7 3T3 cells cultured as aggregates for 1 day. Area of contact between two 3T3 cells is shown in this micrograph. The cells appear to make one or two microvillar contacts and a broader area of contact. Separation between the cells varies from 0.5 μm to less than 150 Å. Compare this micrograph to Figs. 8–10, all at the same magnification. Bar, 1 μm.

FIGURE 8 3T3 cells treated with dbcAMP plus theophylline and cultured as aggregates for 1 day. Area of contact between two interior 3T3 cells. Note the extensive area of contact in the center as well as a number of microvillar contacts. Compare this micrograph to Fig. 7. Bar, 1 μm.

FIGURE 9 SVT-2 cells cultured as aggregates for 1 day. An extended area of contact between two SVT-2 cells is shown in this micrograph. Although the contact area extends at least 3 μm, no electron-dense depositions are visible. Compare this micrograph to the one shown in Fig. 10. Bar, 1 μm.

FIGURE 10 SVT-2 cells treated with dbcAMP plus theophylline and cultured as aggregates for 1 day. An area of contact between two SVT-2 cells is shown. The contact area is about 3 μm in length and includes a well defined area of electron-dense deposition about 0.75 μm in length. Compare to Fig. 9. Bar, 1 μm.

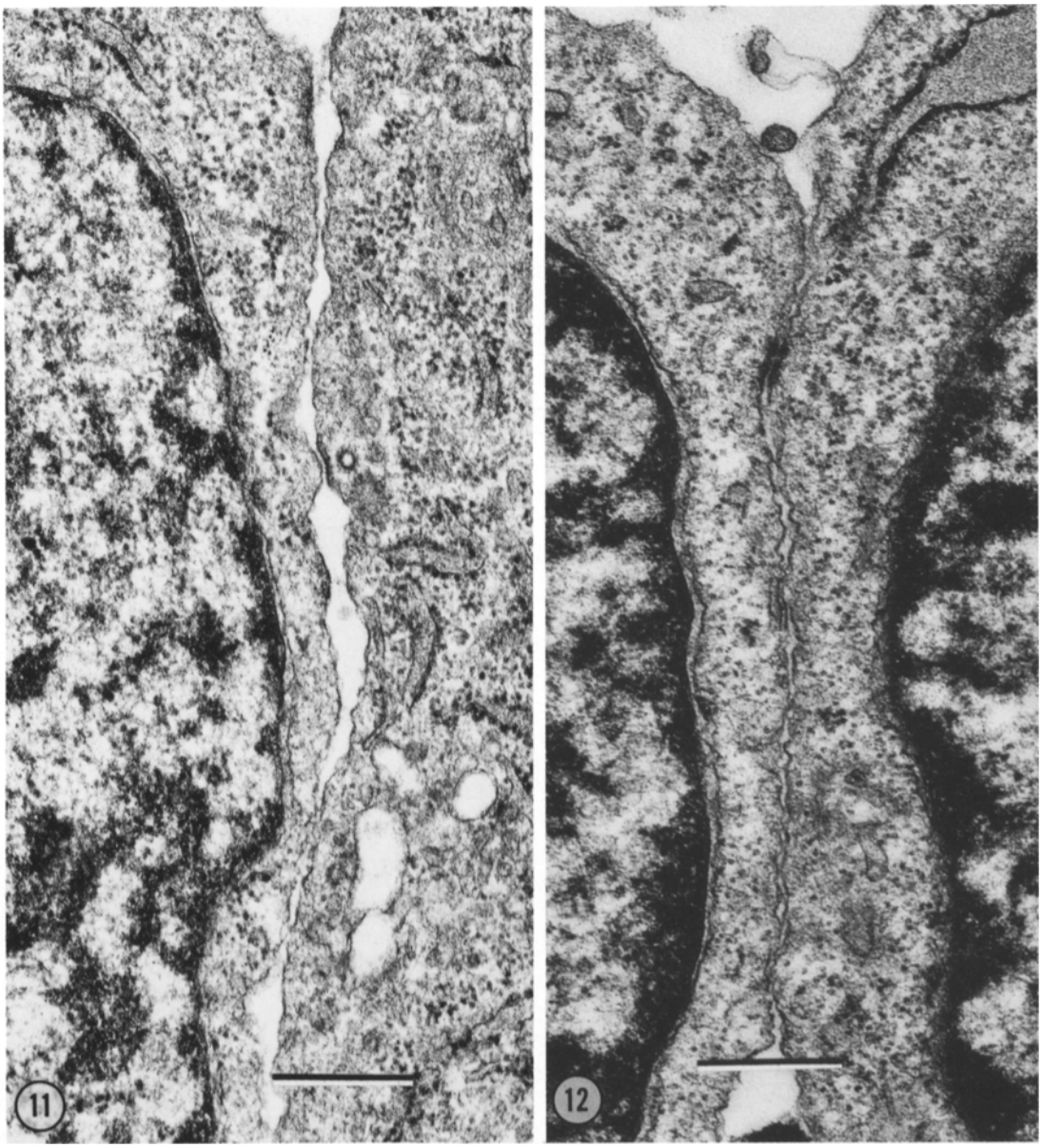


FIGURE 11 Contact between two SVT-2 cells cultured as aggregates. A contact between two SVT-2 cells cultured 1.5 h is shown at higher magnification. Although the length of the contact is extensive (over 4 μm), the gap between cells is quite variable. Bar, 0.5 μm.

FIGURE 12 Contact between two SVT-2 cells treated with dbcAMP plus theophylline and cultured as aggregates. A contact between the treated SVT-2 cells cultured for 1 day is shown at higher magnification. The contact extends over 3 μm and includes a small, well-defined electron-dense deposition. Note that the cells approach very closely over much of the contact area. Bar, 0.5 μm.

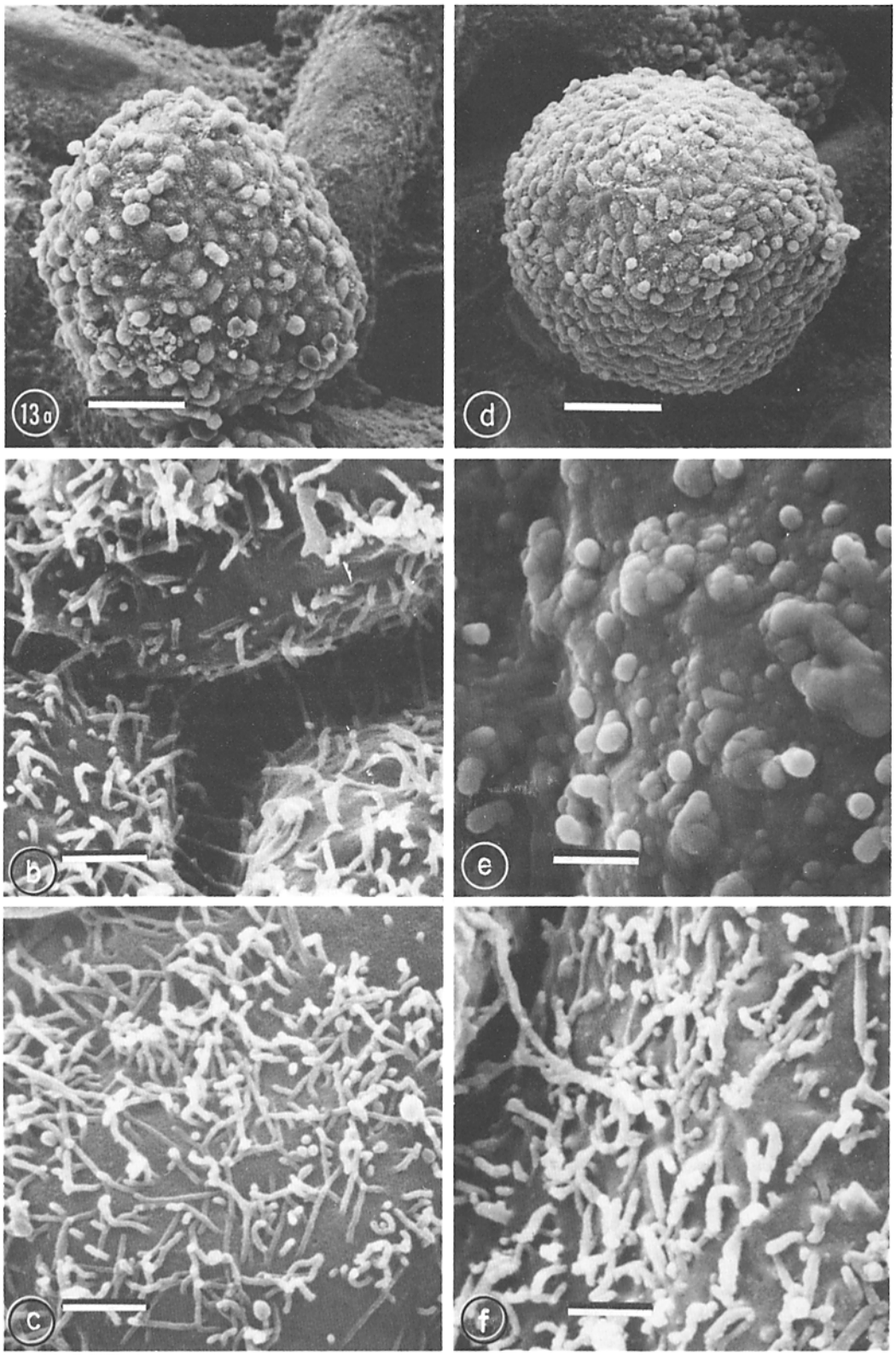
transformant on plastic dishes and in suspension, report that the two 3T3 lines displayed numerous microvilli, while the two SV40-transformed 3T3 lines lacked microvilli. Willingham and Pastan (26), in contrast, have reported that 3T3-4 cells lack microvilli. Our observations of BALB/c 3T3

and SVT-2 cells in aggregates are in agreement with the results of Porter et al., and Collard and Temmink: BALB/c 3T3 cells in aggregates displayed numerous microvilli, while SVT-2 cells in aggregates did not. Porter et al. (20) noted an increase in the number of microvilli on Chinese hamster ovary cells upon treatment with dbcAMP. On the other hand, Willingham and Pastan (26) have reported that dbcAMP treatment of L929 and MNRK cells results in a disappearance of microvilli and that, in a 3T3 variant sensitive to temperature changes, a fall in cAMP levels is accompanied by the appearance of surface microvilli. Our results are consistent with those of Porter et al. (20). Treatment of SVT-2 cells in aggregates with dbcAMP resulted in an increase in the number of microvilli. BALB/c 3T3 cells in aggregates, which already displayed many such projections, were relatively unaffected by this treatment. No unequivocal explanation for these contradictions is apparent to us. Collard and Temmink (4) have suggested that they may be due to differences among cell strains. Alternatively, these contradictions might be the result of differing methodologies. For example, Porter et al. (21), Collard and Temmink (4), and Porter et al. (20) utilized scanning electron microscopy; this current study utilized transmission and scanning electron microscopy, and Willingham and Pastan (26) relied mainly on darkfield microscopy and also used transmission electron microscopy. It also seems likely that the state of attachment of cells is an important determinant of their surface topography. Follett and Goldman (7) and Trinkaus and Erickson (24) have postulated that microvilli may represent a storage form for membrane which disappears during cell spreading. Since cAMP treatment results in either more or less spreading of cells on solid substrates, depending on the cell line (discussed by Willingham and Pastan, 27), the appearance of microvilli might also be expected to very, not only among cell lines but also as a function of the surface to which the cells are attached (e.g., cell-dish attachment versus cell-cell attachment). This may, in turn, account for some of these discrepancies, particularly since the cells in these various studies were in three distinctly different adhesive states: attached to plastic, in suspension, or attached to other cells in aggregates. Microvilli have also been shown to vary as a function of different phases of the cell cycle (19) even in suspension-grown cells (15). The effects of dbcAMP on the occurrence of microvilli on cells in aggregates may therefore be an indirect result of its effect on cell growth. The effect of aggregate culture on anchorage-dependent 3T3 and anchorage-independent SVT-2 cells has not yet been well enough studied to allow this last hypothesis to be critically tested.

The dimensions previously reported for microvilli on CHO cells (0.1 × 3–4 μm [20]), BHK cells (0.1 × 5 μm [7]), P815Y cells (0.1 × 0.1–2 μm [15]), and 3T3 and SVT-2 cells grown on solid substrates (0.1 μm diameter [21]) are similar to those observed here for 3T3 and SVT-2 cells in aggregates.

In this study, differences in general morphology were noted between 3T3 and SVT-2 cells cultured as aggregates as compared to conventional monolayer culture. First, SVT-2 cells on dishes have been described as highly flattened and polygonal (5, 18, 21). In aggregates, in contrast, 3T3 cells are highly rounded although they display numerous surface projections, while SVT-2 cells tend to elongate and flatten on the surfaces of adjacent cells. This difference in morphology is presumably due to that fact that cells on dishes are presented with a potentially adhesive surface on one side only, while in aggregates they are surrounded three dimensionally with other cell surfaces. If 3T3 cells are actually more adhesive than SVT-2 cells, this effect would then account for the slight flattening of peripheral 3T3 cells as compared to cells in the interior of the aggregate. In this context, one caution should be emphasized regarding the interpretation of the scanning electron micrographs presented in this report. Only the surface cells of the aggregate are visualized by this technique, and these may not be fully representative of cells in the aggregate interior. In their type and numbers, cytoplasmic processes on the free borders of cells may be quite different from those extended toward other cells, as in the interior of these aggregates.

The results of this study indicate that the enhanced mobility of SV40-transformed 3T3 cells in aggregates, as compared to cells of the nontransformed 3T3 line, is inhibited by agents that increase intracellular cAMP levels. The same agents induce the transformed cells to form numerous surface projections, including microvilli, as visualized by transmission and scanning electron microscopy. We have suggested that this striking modification of surface topography may be related to the inhibition of mobility. Studies now in progress, utilizing cells of other established lines and of


13a
d
b
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primary tumors, may provide additional evidence on this point.

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FIGURE 13 Scanning electron micrographs of 3T3 and SVT-2 cells in aggregates. (*a*) Aggregate of 3T3 cells cultured in control medium for 24 h. Bar, 50 μm. (*b*) Portions of three 3T3 cells on the surface of an aggregate cultured in control medium for 24 h. Note the large numbers of microvilli on the surface of the cells. The cells are separated from each other by a gap of 0.5–1.0 μm into which numerous microvilli extend. The cells appear to be making contact by means of these microvilli. Bar, 1 μm. (*c*) Portion of a 3T3 cell on the surface of an aggregate cultured for 24 h in medium containing theophylline and dbcAMP. Note the similarity in numbers and distribution of microvilli between this cell and the ones shown in Fig. 10*b*. Bar, 1 μm. (*d*) Aggregate of SVT-2 cells cultured in control medium for 24 h. Bar, 50 μm. (*e*) Portions of two SVT-2 cells on the surface of an aggregate cultured in control medium for 24 h. No microvilli are present in this field although numerous broader shorter protrusions or blebs are evident. The protrusions are 0.25–0.4 μm in diameter. Bar, 1 μm. (*f*) Portions of two SVT-2 cells on the surface of an aggregate cultured for 24 h in medium containing theophylline plus dbcAMP. A large number of long surface projections are present, including some typical microvilli. Many of the thicker projections appear to consist of bundles of several microvilli. Bar, 1 μm.

Magnification of 13*a* equals × 300. Magnification of 13*d* equals × 250. Magnification of 13*b*, 13*c*, 13*e*, 13*f* equals × 13,000.

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