ANTAGONISM BY DIBUTYRYL ADENOSINE CYCLIC 3',5'-MONOPHOSPHATE AND TESTOSTERONE OF CELL ROUNDING REACTIONS

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ABSTRACT

In an attempt to understand further the mechanism of the morphological and functional "reverse transformation" of CHO-K1 cells induced by dibutyryl adenosine cyclic 3',5'-monophosphate (cAMP) and testosterone, the kinetics of variation in the susceptibility of cells to rounding after the addition or deletion of dibutyryl cAMP and testosterone have been investigated. Changes in susceptibility to cell rounding upon removal of divalent cations or pulse exposure to concanavalin A were complete within 0.5-1 h after addition or deletion of drug. In comparison, the gross conversion of CHO-K1 cells from epithelial- to fibroblast-like morphology after drug treatment or the converse change after drug removal required 8 or 4 h, respectively. The effects on cell rounding are not caused by an effect of dibutyryl cAMP upon cell growth rate. Inhibitor experiments indicate that the changes investigated do not require continued RNA or protein synthesis and are not prevented by agents which depolymerize microtubules.

INTRODUCTION

Treatment of Chinese hamster ovary cells in vitro with dibutyryl cyclic AMP (DBcAMP) supplemented with testosterone or certain prostaglandins causes a readily reversible morphological and functional alteration of the cells from a compact, non-contact-inhibited, epithelial-like form to a spindle-shaped, contact-inhibited, fibroblast-like form (Hsie and Waldren, 1970; Hsie and Puck, 1971; Hsie et al., 1971; Puck et al., 1972). This conversion is in many ways the converse of the transformation of fibroblasts into sarcomas that occurs either spontaneously or after treatment of cells with chemical carcinogens or oncogenic viruses. Because of this, Puck and collaborators have termed the process "reverse transformation." Similar observations with other cell lines have been reported (e.g. Johnson et al., 1971; Sheppard, 1971).

Morphologically, reverse transformation of Chinese hamster ovary cells is blocked by colcemid and cytochalasin B, inhibitors of microtubular and microfilament formation, respectively, but is insensitive to inhibitors of RNA and protein synthesis such as actinomycin D and cycloheximide or puromycin (Hsie and Waldren, 1970; Hsie and Puck, 1971; Patterson and Waldren, 1973). The earliest morphological manifestation of reverse transformation is the disappearance of a set of rapidly extending and retracting, knoblike, pseudopodal structures on the cell surface (Puck et al., 1972). The loss of these knobs may be related to the decreased effect of specific cancer cell agglutinins upon the fibroblast-like form of Chinese hamster ovary cells (Hsie et al., 1971). It was also shown that reverse transformation conditions prevent the characteristic cell rounding which occurs

when concanavalin A (Con A) or specific antibody is added to the culture (Hsie et al., 1971).

In the present work, the kinetics of changes in the susceptibility of cells to rounding upon removal of divalent cations or pulse exposure to Con A have been investigated after addition or deletion of DBcAMP and testosterone, and the effect of inhibitors of microtubule and microfilament formation and of RNA and protein synthesis upon these alterations has been studied.

MATERIALS AND METHODS

Cell Growth

The Chinese hamster ovary clone CHO-K1 (Kao and Puck, 1968) was used in all experiments. Cells were routinely grown in plastic Petri dishes in F12 medium (Ham, 1965) supplemented with 10% fetal calf serum, were harvested by trypsinization (Ham and Puck, 1962), and were plated in 35-mm Nunc Petri dishes (A/S Nunc, Roskilde, Denmark) in F12 medium supplemented with 5% dialyzed fetal calf serum in the absence or presence of the reverse transformation agents, 0.2 mM DBcAMP (Sigma Chemical Co., St. Louis, Mo.) plus 15 μM testosterone propionate (Mann Research Labs. Inc., New York). To determine the effect of reverse transformation conditions on cell multiplication, the total number of cells free in the medium and detached from the substrate by trypsin in duplicate cultures was counted with a Coulter counter (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.). To determine the effect of various agents upon cloning efficiency, duplicate samples of 200 cells each were plated in 35-mm Petri dishes, treated with the agent, and clones counted 7 days after cell plating. The cloning efficiency of cells in F12 medium supplemented with 5% dialyzed calf serum was 80%.

Assay of Cell Morphologies

Cells growing on solid substrate were fixed for 15–30 s with 10% Carnoy's fixative and stained for 1–1.5 min with 1:50 crystal violet stain. Cells with a ratio of longest to shortest dimension of ≥ 3.0 were scored as fibroblast-like. Cells with ≥ 3 knoblike, pseudopodal projections were scored as knobby. Spherical, ball-like cells with no visibly recognizable nucleus were scored as rounded. A minimum of 500 cells in total were scored for each determination.

Removal of Divalent Cations and Treatment of Cells with Con A

1×10^5 cells were plated in F12 medium with 5% dialyzed fetal calf serum in the absence or presence

of 0.2 mM DBcAMP plus 15 μM testosterone and were challenged 16–20 h later by removal of divalent cations or by addition of Con A (grade IV, Sigma Chemical Co.). Removal of divalent cations was effected by rinsing and replacing the medium with saline D (Ham and Puck, 1962) containing DBcAMP and testosterone as appropriate. Con A stock solutions were freshly prepared for each experiment in F12 with 5% dialyzed fetal calf serum minus or plus DBcAMP and testosterone as required. The frequency of rounded cells was scored after fixing and staining cells exposed to saline D or Con A for 30 min (unless otherwise indicated) in a humidified CO_2 incubator. Specificity of Con A binding was tested by exposing cells to hapten (Hirano et al., 1972).

For kinetic studies of the effect of addition or removal of reverse transformation conditions on cell rounding, the frequency of rounded cells was assayed at various times after the appropriate medium change, which occurred 16–20 h postplating. An inoculum of 2.5×10^4 cells per dish was used in experiments assaying cell rounding at periods greater than 20 h postmedium change. In inhibitor experiments, cells were pretreated for 30 min with cycloheximide, puromycin, or actinomycin D and for 15 min with colcemid or cytochalasin B before changing the medium.

Solvent controls were performed to ensure that none of the effects were due to traces of the solvent in which the drugs were dissolved.

RESULTS

Cell Growth in the Presence of Reverse Transformation Agents

Cells plated in either the absence or presence of the reverse transformation agents 0.2 mM DBcAMP plus 15 μM testosterone, in F12 medium supplemented with 5% dialyzed calf serum go through appreciably less than one cell doubling in the first 18 h after plating. In the interval 18–42 h postplating, the interval of concern in most of the experiments reported here, cells (epithelial-like or E form) maintained in basal medium multiply with an average generation time of 16 h; cultures (E $\rightarrow$ fibroblast-like or F form) plated in basal medium with a change of medium to F12 plus reverse transformation agents $\sim$18 h postplating grow with an 18 h generation time during this interval; and cells (F form) maintained in the continuous presence of DBcAMP plus testosterone have an average generation time of 28 h under the specific conditions employed here. Further studies on the effects of these agents on cell multiplication will be published elsewhere.

TABLE I

*Effect of Saline D Treatment on Cell Rounding and
its Antagonism by DBcAMP plus
Testosterone (T)*

Treatment	Rounded cells in basal medium	Rounded cells in basal medium + (DBcAMP + T)
	%	%
None	14	9
Saline D	96	12
Saline D + Ca^{++} Mg^{++}	26	14

Cells were plated in basal medium in the absence or presence of DBcAMP plus testosterone (T). 20 h postplating the medium was removed and replaced with basal medium, saline D, or saline D containing Ca^{++} Mg^{++} at the same concentration as in basal medium. Reverse transformation agents were present as appropriate in these solutions.

Cells plated in the presence of the reverse transformation agents (F form) show a relative cloning efficiency of 60% of control while cells (E → F form) initially plated in basal medium and then exposed 18 h postplating to medium containing DBcAMP plus testosterone have a relative cloning efficiency of 86%.

Effect of (Ca^{++} and Mg^{++}) Removal on Cell Rounding and Its Antagonism by DBcAMP plus Testesterone

Attachment of cells to solid substrate requires divalent cations such as Ca^{++} and Mg^{++} (Fisher et al., 1958). Removal of divalent cations forces CHO-K1 cells to round, assuming a spherical shape, even when grown upon a solid substrate. Cells (epithelial form) grown in basal medium alone are almost 100% rounded by a 30 min exposure to saline D, a calcium- and magnesium-free phosphate-buffered saline solution (Table I). Upon rounding these cells lose the characteristic knoblike, pseudopodal projections of the epithelial form of CHO-K1 cells. This rounding of the epithelial form upon treatment with saline D is almost completely obliterated if calcium and magnesium are restored to the solution. The fibroblast-like form of CHO-K1 cells is rounded little, if any, above the basal level of ~10% (some of which may be mitotic cells) by a 30 min treatment with saline D containing DBcAMP plus testosterone.

In the conversion of CHO-K1 cells from the epithelial or E form to fibroblast or F form induced by DBcAMP plus testosterone, the change in susceptibility of cells to rounding upon treatment with saline D occurs very rapidly (Fig. 1 *a*). Within 1 h after addition of reverse transformation agents, the number of cells rounded by the saline D challenge is decreased by 90%. In comparison, elongation of the cells from epithelial to fibroblast-like morphology required ~8 h for completion. Conversely during conversion from the F to E cell morphologies after removal of DBcAMP plus testosterone, the sensitivity of cells to removal of divalent cations increases rapidly (Fig. 1 *b*). Within 30 min after drug removal, 70% of the cells become rounded by saline D treatment and in the next 2–3 h this number approaches the maximum exhibited by control cells. Little condensation to the E form occurs during the first 30 min after drug removal, but 4 h later cells have converted almost completely.

Effect of Con A on Cell Rounding and Its Antagonism by DBcAMP plus Testosterone

Con A has been reported to preferentially round up or partially detach from a solid substrate the epithelial form of CHO-K1 cells with a resultant loss of the knoblike projections (Hsie et al., 1971).

Fig. 2 *a* shows the dependence on Con A concentration of rounding of cells grown in the absence (E form) or presence (F form) of DBcAMP plus testosterone for 16 h and exposed to Con A for 30 min in the presence of the reverse transformation agents as appropriate. The epithelial form of CHO-K1 cells is four- to fivefold more easily rounded (10% → 45–60% rounded) than the fibroblast form during a pulse exposure to Con A concentrations between 10–100 µg/ml. This difference is even greater if the basal level of ~10% rounded cells of either form in the absence of Con A is subtracted. The relative difference between cells cultured in the absence or presence of reverse transformation agents is decreased to threefold at 200 µg/ml Con A and is almost completely effaced at a Con A concentration of 2,000 µg/ml. The maximum proportion of the epithelial form rounded during a 30 min exposure to 100–2,000 µg/ml Con A is 60–70%. About 54% of fibroblast form cells are rounded by pulse treatment with 2,000 µg/ml Con A.

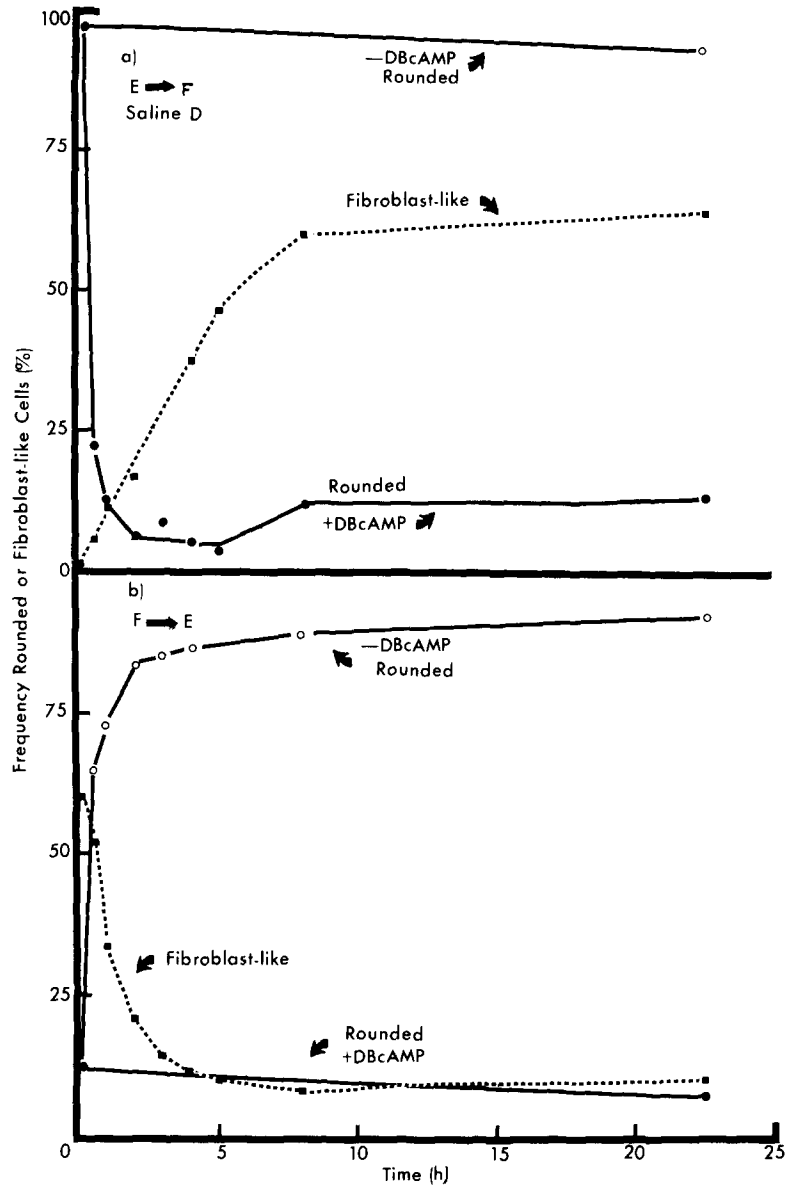Fig. 2 *b* shows the time dependence of Con A

FIGURE 1   Kinetics of DBcAMP plus testosterone (*T*) induced changes in the susceptibility of CHO-K1 cells to rounding upon exposure to saline D. In (*a*), cells were plated in basal medium and the medium changed to plus reverse transformation agents 17 h postplating. The direction of morphological conversion is epithelial (E) to fibroblast (F). In (*b*), cells were plated in basal medium containing DBcAMP plus testosterone and 18 h postplating the medium was changed to basal medium. The direction of morphological conversion is fibroblast (F) to epithelial (E). ○——○, frequency of cells rounded in absence of DBcAMP plus testosterone. ●——●, frequency of cells rounded in presence of DBcAMP plus testosterone. ■---■, frequency of cells of fibroblast-like morphology.

cell rounding at 10–100 μg/ml Con A of cells grown in the absence (E form) or presence (F form) of reverse transformation agents for 17 h. Again at all concentrations a four- to fivefold difference in

cell rounding is found between epithelial and fibroblast form cells in a 30 min Con A treatment. However, this difference decreases greatly after a time lag with longer Con A exposure even though
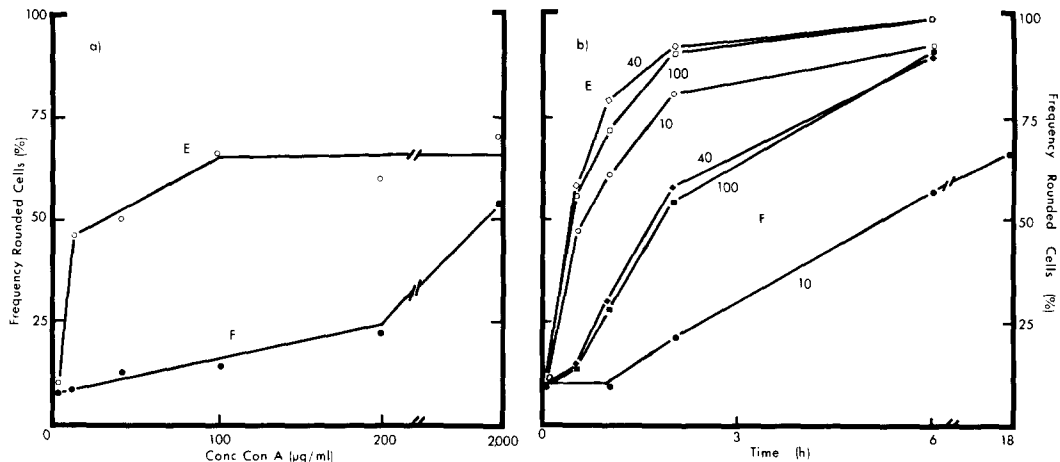
FIGURE 2   Concentration (a) and time (b) dependence of Con A cell rounding of CHO-K1 cells grown in the absence (E or epithelial form) or presence (F or fibroblast form) of DBcAMP plus testosterone for 18 h. In (a) O———O, frequency of E form cells rounded; ●———●, frequency of F form cells rounded. In (b) Open symbols (10, 40, 100); frequency of E form cells rounded by 10, 40, or 100 μg/ml Con A, respectively. Closed symbols (10, 40, 100); frequency of F form cells rounded by 10, 40, or 100 μg/ml Con A, respectively.

the absolute frequency of epithelial form cells rounded increases.

Con A (40 μg/ml) rounding of cells grown in basal medium (E form) was completely prevented in the presence of 0.15 M D-mannose, a competitive inhibitor of Con A binding whereas 0.15 M β-D-galactose, not an inhibitor, had no effect. During a 6 h incubation with 40 μg/ml Con A, cells grown in basal medium plus DBcAMP and testosterone (F form) rounded in the presence of 0.05 M β-D-galactose but not in the presence of 0.05 M D-mannose. Treatment of cells in the absence or presence of reverse transformation agents with Con A concentrations as great as 100 μg/ml for either 30 min or 6 h followed by removal of the Con A did not affect the cloning efficiency of either cell form.

Neither 15 μM testosterone nor 0.2 mM DBcAMP individually confers appreciable resistance to Con A cell rounding (Table II). In combination they provide complete resistance. Growth of cells in the presence of 1 mM DBcAMP alone provides comparable resistance. The action of DBcAMP and testosterone in conferring resistance to rounding resembles their effects in producing reverse transformation (Hsie and Puck, 1971).

Fig. 3 shows the effect of addition or deletion of DBcAMP plus testosterone on the susceptibility of cells to rounding during a 30 min pulse exposure to 40 μg/ml Con A. Cultures to which the reverse

TABLE II

Synergistic Antagonism by Testosterone and DBcAMP of Con A Cell Rounding

Agents added	Frequency of cells rounded by 30 min challenge with 40 μg/ ml Con A
	%
None	50
Testosterone (15 μM)	46
DBcAMP (0.2 mM)	37
DBcAMP (1.0 mM)	12
DBcAMP (0.2 mM) plus testosterone (15 μM)	11

Cells were plated in basal medium in the absence or presence of agents as shown. 18 h postplating Con A was added to the preexisting medium.

transformation agents are added become fully resistant to Con A cell rounding within 1 h after drug addition; about 8 h are again required for complete reconversion from E form to F form (Fig. 3 a). After removal of DBcAMP plus testosterone, cells within 30 min become as sensitive to Con A rounding as cells never exposed to reverse transformation agents (Fig. 3 b). 30 min after drug removal, the cell population has become only somewhat less fibroblastic.
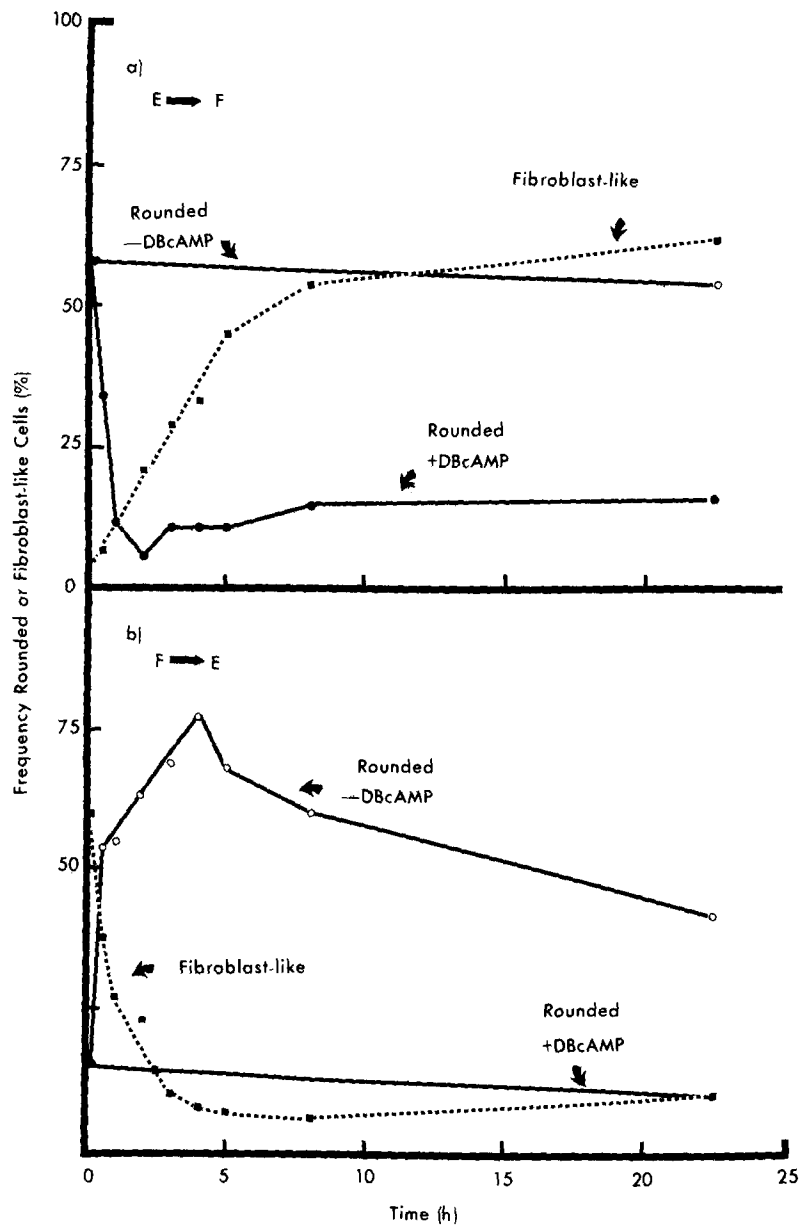
FIGURE 3 Kinetics of DBcAMP plus testosterone (T) induced changes in the susceptibility of CHO-K1 cells to rounding upon pulse exposure to 40 μg/ml Con A. In (a), the direction of morphological conversion is epithelial (E) to fibroblast (F) and in (b) fibroblast (F) to epithelial (E). Cells were cultured as described in the legend of Fig. 2. Cells were exposed to 40 μg/ml Con A for 30 min. O———O, frequency of cells rounded in absence of DBcAMP plus testosterone. ●———●, frequency of cells rounded in presence of DBcAMP plus testosterone. ■---■, frequency of cells of fibroblast-like morphology.

It should be noted that during the conversion from fibroblast to epithelial-like morphology that occurs after deletion of reverse transformation agents, the cells actually become temporarily more sensitive to Con A rounding than cells never exposed to DBcAMP plus testosterone (Fig. 3 b). This "overshoot" may be related to the less pronounced "undershoot" or greater resistance to cell

rounding that occurs in cultures after addition of DBcAMP plus testosterone (Figs. 1 a, 3 a).

Effect of Inhibitors on Alterations in Con A Cell Rounding

Tables III and IV show the effect of inhibitors of microtubule and microfilament formation and of protein and RNA synthesis on alterations in Con A cell rounding 1 h after addition or deletion of the reverse transformation agents. For comparison, the effect of each inhibitor on a second, rapidly occurring change affecting the cell surface, the presence or absence of knoblike pseudopodal projections of the cell membrane (Puck et al., 1972) has been scored.

Pretreatment of cultures with 0.25 μg/ml colcemid, an inhibitor of microtubule polymerization (Weisenberg et al., 1968), or 0.4 μg/ml cytochalasin B, a presumed inhibitor of microfilament

TABLE III

Effect of Inhibitors on Alterations Induced by the Addition of DBcAMP plus Testosterone (T) in Cell Knobbiness and in Susceptibility of Cells to Con A Rounding

Treatment	Frequency of knobbed cells	Frequency of cells rounded by 30 min challenge with 40 μg/ml Con A*
	%	%
Continuous exposure		
No addition	34	54
+ DBcAMP + T	5	11
1 h postaddition of DBcAMP + T		
No inhibitor	3	6
+ colcemid (0.25 μg/ml)	8	17
+ cytochalasin. B (0.4 μg/ml)	49	12
+ cycloheximide (20 μg/ml)	7	7
+ puromycin (100 μg/ml)	3	7
+ actinomycin D (1 μg/ml)	4	8

* Corrected for small differences in basal level of rounded cells caused by inhibitor treatment alone by subtraction of the frequency of rounded cells above basal level found in parallel cultures treated with inhibitor only.

TABLE IV

Effect of Inhibitors on Alterations Induced by the Deletion of DBcAMP plus Testosterone (T) in Cell Knobbiness and in Susceptibility of Cells to Con A Rounding

Treatment	Frequency of knobbed cells	Frequency of cells rounded by 30 min challenge with 40 μg/ml Con A*
	%	%
Continuous exposure		
No addition	42	59
+ DBcAMP + T	4	14
1 h postdeletion of DBcAMP + T		
No inhibitor	20	51
+ colcemid (0.25 μg/ml)	31	60
+ cytochalasin B (0.4 μg/ml)	77	51
+ cycloheximide (20 μg/ml)	22	59
+ puromycin (100 μg/ml)	19	53
+ actinomycin D (1 μg/ml)	21	52

* Corrected for small differences in basal level of rounded cells caused by inhibitor treatment alone by subtraction of the frequency of rounded cells above basal level found in parallel cultures treated with inhibitor only.

function (Schroeder, 1969; Spudich and Lin, 1972), causes a very minor block of the increased resistance to Con A rounding induced by addition of DBcAMP plus testosterone (Table III). In control experiments, colcemid and cytochalasin B at these concentrations completely block the morphological transformation from epithelial to fibroblast-like cells induced by reverse transformation agents. In time lapse cine-photomicrographic experiments, colcemid was found to cause accumulation of cells in mitosis without lag (see also Puck, 1964) and cytochalasin B was found to elicit knob formation within 1–2 min after addition. Cycloheximide and puromycin, inhibitors of cytoplasmic and total cell protein synthesis, respectively, and' actinomycin D, an inhibitor of RNA synthesis, at concentrations sufficient to inhibit >95% of either process (Patterson and Waldren, 1973), do not affect the decrease in cell rounding induced by DBcAMP plus

testosterone. Cytochalasin B alone, it should be noted, inhibits the retraction of the knoblike projections of the cell membrane induced by DBcAMP plus testosterone. Pretreatment of cultures with inhibitors of microtubule polymerization, microfilament function, protein synthesis, or RNA synthesis has no effect on the increased Con A cell rounding after removal of reverse transformation agents (Table IV). The only significant effect of inhibitors on the increased frequency of knobbed cells found 1 h after deletion of DBcAMP plus testosterone is an accentuated frequency of knobbed cells in cultures treated with colcemid and cytochalasin B, as reported previously (Puck et al., 1972).

DISCUSSION

Brief exposure of CHO-K1 cells (epithelial form) growing on solid substrate to Ca^{++}- and Mg^{++}-free medium, results in the rounding of 90% of the cells. Cells (fibroblast form) growing in the presence of DBcAMP plus testosterone are not rounded to any significant extent by this treatment. This result provides a quantitative assay for at least one parameter of the response of CHO-K1 cells to DBcAMP plus testosterone treatment and may be a reflection of differences in cells binding to substratum. Other authors have reported transformed cells to adhere less strongly to substrate than untransformed cells (Sanford et al., 1970).

Treatment of either the E or F forms of the CHO-K1 cell with Con A causes complete cell rounding. However, in the latter case, which is produced by DBcAMP plus testosterone, the rounding requires either a much higher concentration or a much longer exposure to Con A. Con A rounding is blocked by the addition of the appropriate hapten and is not a result of cell killing. The differential susceptibility to rounding of E and F cells arises from an apparently physiological interaction between DBcAMP and testosterone and can be produced as a response to DBcAMP alone.

The simplest explanation for this phenomenon is that differential Con A cell rounding directly reflects a quantitative or qualitative alteration in Con A binding. No data are presented in the present work which exclude the possibility that secondary effects of Con A binding on intracellular levels of cAMP or other small molecules may be the immediate causal agent(s) of cell rounding.

Differences in the binding of Con A and other lectins have been reported for chemically and virally transformed cells (for review see Burger, 1973) and for normal cells during mitosis (Fox et al., 1971). The increased agglutination of malignant cells and of normal cells during mitosis by lectins is correlated with a decrease in cAMP level (Otten et al., 1971; Sheppard, 1972; Burger et al., 1972). The modulation by DBcAMP plus testosterone of cell rounding reported here further supports the hypothesis that cAMP may be a causative agent in controlling certain surface properties of cells and may provide a model system for studying changes associated with the mitotic cell, a physiological example of a rounded cell. The rounding of cells by Con A or other agents may also be useful as a titration method with which to compare different cell lines.

The rapidity of the DBcAMP plus testosterone induced alteration in Con A response is in contrast to the case of viral transformation where at least one traverse of the cell cycle is required after viral infection before any change in lectin binding is detected (Eckhart et al., 1971; Sheppard et al., 1971; Noonan et al., 1973) and suggests that new RNA and protein synthesis is not required for the alteration. Experiments with RNA and protein inhibitors confirm this suggestion. The rounding is not blocked by inhibitors of microtubule or microfilament organization at concentrations sufficient to inhibit the gross morphological transformation of CHO-K1 cells by reverse transformation agents. The change does not arise from an effect of the reverse transformation agents on cell growth rate. The change studied appears to arise from an altered state of receptor or binding molecules on the cell surface.

A plausible mechanistic explanation of Con A cell rounding based on Con A itself being the causal agent of rounding can be suggested. The almost complete rounding of either cell form upon prolonged Con A exposure suggests that sufficient Con A receptors are present to round CHO-K1 cells grown in either the absence or presence of the reverse transformation agents. At a given Con A concentration the chief difference in rounding of cells grown in the absence or presence of DBcAMP plus testosterone is in the rapidity of the response. The lag in Con A rounding of cells treated with agents suggests that cell rounding results from either a cooperative process during binding of Con A to the cell or a cooperative interaction between multivalent (Wang et al., 1971)

Con A molecules. Continuous exposure to native multivalent Con A kills Chinese hamster cells (Wright, 1973); continuous exposure of 3T3 to monovalent Con A is without toxicity (Burger and Noonan, 1970). In a possibly analogous system, aggregation of ferritin-labeled divalent but not monovalent antibody on the surface of lymphocytes is reported to occur (dePetris and Raff, 1973). All these observations suggest that the antagonism by DBcAMP plus testosterone of cell rounding may result from a change in number or kind of available cell binding sites for Con A that restricts the ability of multivalent Con A molecules to interact. This possibility is presently being investigated. Presumably the previously reported rounding of CHO-K1 cells by antibody (Hsie et al., 1971), a bivalent molecule, results from a similar mechanism.

Johnson and Pastan (1972) have recently reported somewhat related observations on cell adhesion assaying detachment from substrate by EGTA or trypsin of L929 and 3T3SVC1X cells before and after cAMP treatment. They also find a rapid change and no requirement for new protein synthesis. The observations reported here are consistent with the finding that continued RNA and protein synthesis is not required for the gross morphological transformation of CHO-K1 cells by reverse transformation agents (Patterson and Waldren, 1973). The lack of effect of inhibitors of microtubule and possibly microfilament function suggests that changes in cell rounding are unrelated to the retraction or extension of knoblike, pseudopodal structures on the cell surface.

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