Effects of Dibutyryl Cyclic Adenosine Phosphate Plus Theophylline on Murine Sarcoma Virus Transformed Non-Producer Cells (36930)

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(Introduced by P. S. Sarma)

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Adenosine 3′,5′-cyclic monophosphate (cAMP) and its derivative N^6,$O^{2'}$-dibutyryl cyclic adenosine phosphate (dbcAMP) have considerable effects on the morphology and other characteristics of various contact inhibted and transformed cell cultures (1–5). These actions are potentiated by theophylline (Th). It has been suggested that cAMP is an important factor in the maintainence of normal cell morphology and growth rate of fibroblasts, and that these functions may be lost or altered during transformation, perhaps due to depletion of endogenous levels of cAMP (3, 6, 7).

The studies reported herein were performed to study the normalizing effects of cAMP on murine cells transformed by the Kirsten strain of murine sarcoma virus (MSV).

Methods and Results. Cell lines. The clonal lines BALB/3T3, clone A31, (B-3T3) (8), and a Kirsten MSV transformed subclone of this line, K-234 (Ki-3T3) (9) were obtained from Dr. Stuart Aaronson, National Cancer Institute, Bethesda, Md. They were maintained in McCoy's 5a modified medium (GIBCO, Grand Island, NY) with 10% heat inactivated fetal calf serum and antibiotics. The B-3T3 line is contact inhibited and of low tumorigenicity when transplanted into severely immunosuppressed BALB/c mice (Dr. Peter Wright, personal communication). The Ki-3T3 line is not contact inhibited and is highly oncogenic in immunocompetent BALB/c mice (10). Ki-3T3 cells are non-producer cells, *i.e.,* they lack evidence of virus replication, but contain the murine sarcoma genome, which can be rescued by superinfection of the cells with murine leukemia virus. The RBL-5 line (11), a Rauscher virus-induced leukemia, was maintained in ascites form by serial intraperitoneal passage in C57BL/6 mice. S+L− cells and 3T3FL cells, a subline of the original 3T3 Swiss mouse cell line, were obtained from Dr. Robert Bassin, National Cancer Institute.

Chemicals. Adenosine 3′,5′-cyclic monophosphate (cAMP) and N^6,O^2-dibutyryl 3′, 5′-adenosine monophosphate (dbcAMP) were obtained from P-L Biochemicals, Milwaukee, Wisc. Residual butyric acid was removed from the dbcAMP by acid treatment and ether extraction. The ether was removed by exhaustive treatment in a vacuum evacuator, and the pH adjusted to 7.2. Theophylline and ethylene diamine tetracetic acid disodium dihydrate (EDTA) were purchased from Schwarz/Mann, Orangeburg, NY. Trypsin solution (casein digestive action 1:250) was obtained from GIBCO, Grand Island, NY.

Mice. C57BL/6N male mice were obtained from the Rodent and Rabbit Production Section, Division of Research Services, National Institutes of Health, Bethesda, Md.

Effects on cell morphology and growth. Varying concentrations of cells were seeded into 75 cm^2 flasks. After 24 hr cAMP, dbcAMP, Th, or cAMP + Th were added. The medium was changed every 48 hr. Morphological changes were observed in the cells 12–24 hr after cAMP, dbcAMP or dbcAMP + Th treatment, but not with Th alone. The changes were most consistent and uniform after treatment with 1 mmole/ml of dbcAMP + 1 mmole/ml of Th. Less uniform but otherwise similar results could be obtained after treatment with 0.4 mmole/ml of both dbcAMP and Th, 8 mmole of cAMP, or

3 mmole of both cAMP and Th. All of the results that follow were obtained after treatment with 1 mmole/ml of dbcAMP + 1 mmole/ml of Th.

After 12–24 hr of treatment of Ki-3T3 cells, their processes began to elongate and subdivide. After 24–36 hr the cells began to spread, their apparent surface area was increased, and processes were no longer prominent. The nuclei increased in size and became more vesicular with a speckled chromatin pattern while the nuclei of untreated cells remained small with a dense chromatin pattern. These changes were maximal 48–60 hr after treatment. At this stage the treated Ki-3T3 cells closely resembled B-3T3 cells. Removal of dbcAMP and Th resulted in a complete reversal of the changes after a similar time period and sequence. These changes and their reversal are illustrated in Figs. 1–4.

As illustrated in Fig. 5, dbcAMP + Th treatment considerably slowed the growth rate of Ki-3T3 cells, and increased their doubling time from 16 hr to 7 days. The untreated cells reached saturation density after 6.5 days, but the treated cells had not reached saturation density after 14 days.

Treatment with dbcAMP + Th also induced reversible spreading of B-3T3 cells and slowed their growth rate, but to a lesser extent than Ki-3T3 cells.

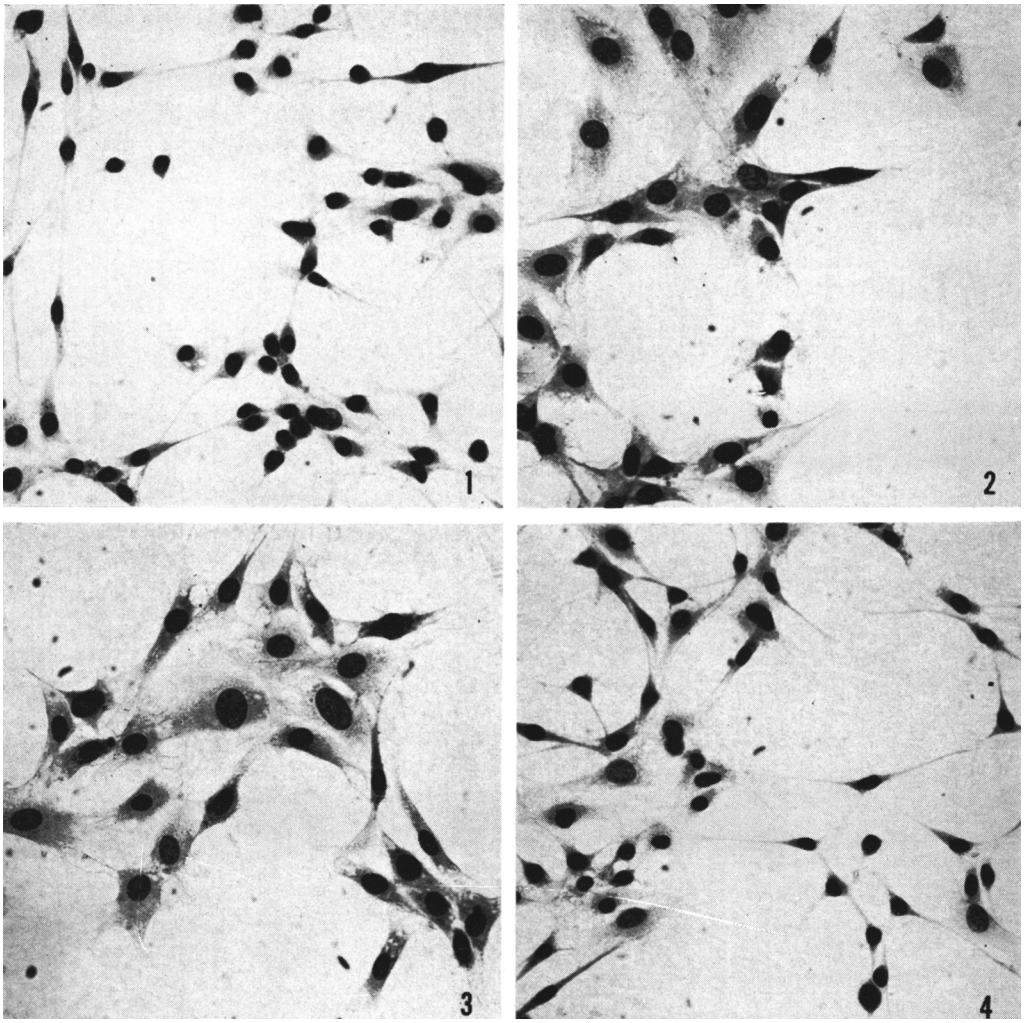
Cell adhesion studies. To determine whether cells treated with dbcAMP + Th treatment were more adherent to the substratum than untreated cells, 2×10 cells were seeded into 25 cm^2 flasks and treated for 3 days. The medium was removed, the cells washed with phosphate-buffered saline (Ca- and Mg-free), and trypsin or EDTA solution added. The flasks were agitated vigorously. The time for detachment of 95–100% of the cells was determined by observation with an inverted microscope.

The data, presented in Fig. 6, indicates that dbcAMP + Th treatment considerably increased the time for detachment of both B-3T3 and Ki-3T3 cells by trypsin and EDTA. The increased times required for detachment of the treated cells were magnified at low concentrations of trypsin and EDTA.

Replication of leukemia virus and rescue of sarcoma genome. The strain of murine leukemia virus (MuLV) used was the helper virus associated with Gz-MSV, a recently isolated strain of MSV (12). The MuLV, passage LTV 820, obtained by end point dilution of Gz-MSV, induces lymphatic leukemia when inoculated into newborn mice. MuLV assays were performed by plaque formation on S+L− cells (13). One day after plating 1.2×10^5 cells into 25 cm^2 dishes, the dishes were infected with 0.3 ml of various dilutions of virus for 1 hr at 37°. Four milliliters of medium were then added. An additional 4 ml of medium were added 3 days after infection, and a medium change performed the next day. Plaques were enumerated 5 days after infection.

For MSV assays (14), 1×10^5 3T3FL cells were plated onto 25 cm^2 dishes. The next day the cells were pretreated with DEAE-dextran (25 μg/ml, 4 ml/dish, 1 hr) and infected with 0.3 ml of virus. After incubation at 37° for 1 hr, 4 ml of medium were added. Because the foci obtained from MSV rescued from Ki-3T3 cells were unusually small in size, they were enumerated 6–7 days after infection.

The effects of dbcAMP + Th treatment on the replication of MuLV in Ki-3T3 cells and the rescue of the MSV genome from the cells were studied by seeding 3×10^5 cells/75 mm^2 flask. The cells were infected the next day with 10^6 plaque-forming units of LTV 820. After 1 hr at 37°, 25 ml of medium were added to half of the flasks, and medium containing dbcAMP + Th to the remainder. The medium was changed daily. The supernatant fluids were collected, clarified, passed through a millipore filter (22 nm) and assayed for MuLV and MSV. Because the dbcAMP + Th-treated cells grew much slower than untreated cells, the data was expressed as virus titers/10^6 cells instead of the customary method of virus titers/ml. MuLV titers from dbcAMP + Th-treated cells were 0.5–0.9 logs lower than from untreated cells on days 2–4, but were the same 5 days after infection. At this time the untreated cells were approaching saturation density of approximately 2.1×10^7 cells/flask, and cell division was considerably reduced. The titers of the MSV



FIGS. 1–4. Cells were grown on glass coverslips, fixed in ethanol and stained with hematoxylin and eosin. Original magnification ×240. Areas of sparse cellularity were deliberately selected for photography in order to demonstrate individual cell morphology.

FIG. 1. Ki-3T3 cells.

FIG. 2. B-3T3 cells.

FIG. 3. Ki-3T3 cells after 5 days of dbcAMP + Th treatment.

FIG. 4. Ki-3T3 cells treated for 5 days with dbcAMP + Th followed by 4 days of growth in normal medium.

rescued from dbcAMP + Th-treated cells were consistently slightly lower (0.2–0.4 logs) than those from untreated cells.

Cell surface antigen studies. Assay for cell surface antigen by absorption of cytotoxic antibody was performed by a method previously described (15). An antiserum pool was obtained from C57BL/6 mice 30–50 days after inoculation of Moloney MSV. One-tenth milliliter of ^{51}Cr-labelled RBL-5 target cells (1×10^5) cells was added to 0.1 ml of serial twofold dilutions of antiserum. After incubation at 37° for 30 min, 0.1 ml of rabbit serum was added as the source of complement and the incubation continued for a further 30 min. Antibody studies were per-

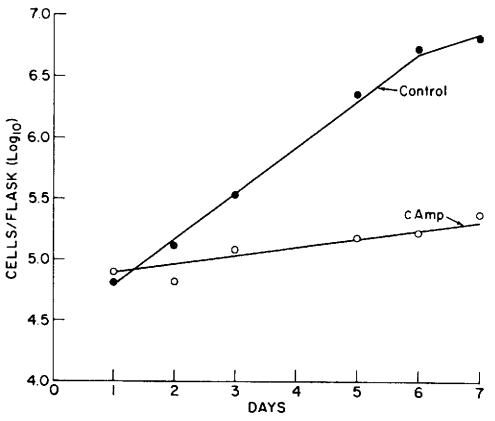


FIG. 5. Effects of dbcAMP + Th treatment on growth of Ki-3T3 cells. 10^5 Ki-3T3 cells were seeded into 25 cm^2 flasks.

formed with the final dilution (1:400) of antiserum producing 70% lysis. Serial twofold dilutions of 0.1 ml of the cells to be tested for antigenicity (the first tube contained 10^8 cells) were incubated with 0.2 ml of antiserum for 30 min at 37°. Labelled target cells were added and the mixtures tested for residual cytotoxic activity. One unit of antigen was defined as the number of cells needed to reduce cytotoxic activity by 50%. The method for assay of cell surface antigen by inhibition of cell-mediated cytotoxicity has been described (16). Immune spleen cells (1×10^7) from C57BL/6 mice, 14 days after inoculation of Moloney MSV, were mixed with 5×10^4 ^{51}Cr-labelled RBL-5 target cells. Inhibitor cells (2×10^6) were added to give a final attacker:target cell ratio of 5:1. The assay for cytotoxicity was then performed in the usual manner, with incubation at 37° for 4 hr. Inhibitor cells were considered to contain antigen if there was significant reduction ($p < 0.05$ by Student's t test) from the positive control group.

The results of the cell-mediated cytotoxicity and antibody cytotoxicity experiments are presented in Table I. While virus-coded cell surface antigens were not present on B-3T3 cells, they could be detected on Ki-3T3 cells by both cell mediated and antibody cytotoxicity tests. Treatment with dbcAMP + Th had no detectable effect on the antigens measured by antibody cytotoxicity. With the cellular assay there appeared to be increased antigen in one experiment (Table I) after dbcAMP + Th treatment. However, in a repeat experiment, there was no significant difference from the untreated Ki-3T3 cells.

Sugar uptake studies. Assay of sugar uptake by cultured cells was performed by a previously described method (17). The cells were treated with dbcAMP + Th for varying periods of time, and washed with glucose-free Hank's solution several times. ^{14}C labelled D-glucose was used for the uptake studies and labelled 2-deoxy-D-glucose was used for kinetic measurements (18). The cells were incubated with the radioactive sugars for 20 min at 37°, washed quickly with water, homogenized and samples taken for radioactive determination in a liquid scintillation counter

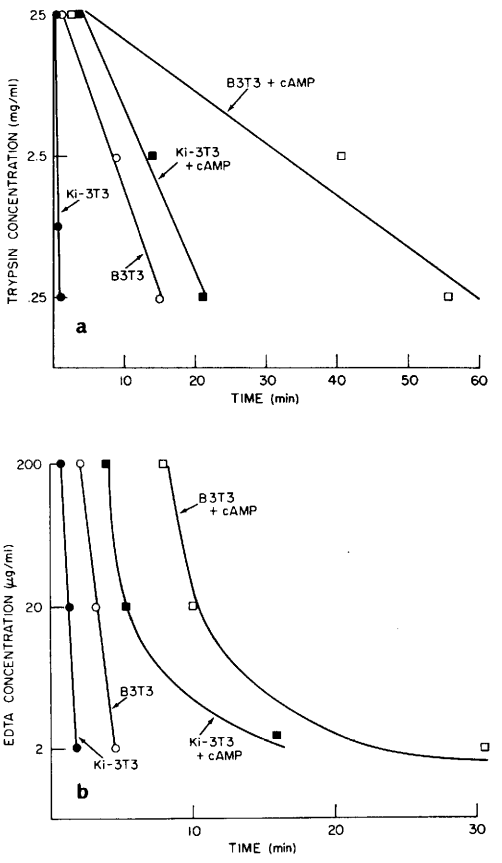


FIG. 6. Effects of dbcAMP + Th treatment on cell adhesiveness. The times required for detachment of 95–100% of the cells by trypsin (Fig. 6a) and EDTA (Fig. 6b) were determined after 3 days of treatment.

TABLE I. Assay for Cell Surface Antigen.

Inhibitor cell	Cell-mediated cytotoxicity[a] (% inhibition of lysis)	Antibody cytotoxicity (antigen units/10^8 cells)
Non-immune C57BL/6 spleen cells	—17	<1
RBL-5	68[b]	80
B-3T3	9	Not done
Ki-3T3	56[b]	320
Ki-3T3 + dbcAMP	76[b]	320

[a] % lysis of RBL-5 target cells produced by immune C57BL/6 spleen cells above background control.

[b] Significant inhibition of lysis by inhibitor cells ($p < 0.05$).

and for protein determination by Lowry's method (19). Over the 1000-fold range of substrate concentrations used, a linear relationship was found between the reciprocal of the velocity and the reciprocal of the substrate concentration (Lineweaver-Burk plot), thus allowing representation of each culture by single K_m and V_{max} values.

Table II indicates that treatment of B-3T3 and Ki-3T3 cells with dbcAMP + Th increases sugar uptake compared to untreated cells. The increased sugar uptake commences after one day of treatment, and becomes more pronounced with time. In another experiment Ki-3T3 cells were treated with dbcAMP + Th for 6 days. Glucose uptake in the treated cells was more than 10 times greater than in untreated cells. The considerable increase in sugar uptake in B-3T3 and Ki-3T3 cells after dbcAMP + Th treatment was observed over a wide range of substrate concentration, from 10^{-2}–10^{-5} M. The increased uptake is also reflected in the increased V_{max} of both cell lines after dbcAMP + Th treatment (Table III). Despite the great increase in glucose uptake, the apparent K_m was not altered after dbcAMP + Th treatment.

TABLE II. Enhanced Glucose Uptake in Cells After dbcAMP + Th Treatment.[a]

Cells	Glucose uptake ratio of treated to untreated cells: Number of days treated 1	2	3	4
B-3T3	1.1	1.2	4.9	7.9
Ki-3T3	1.9	2.1	3.0	6.9

[a] Cells were treated with dbcAMP + Th for a varying number of days as indicated. Washed cells were incubated with 2 ml of ^{14}C-labelled D-glucose (9×10^{-7} M) for 20 min at 37°. The values shown are the ratios of glucose uptake/mg protein in treated cells compared to untreated cells.

Discussion. The effects of cAMP and dbcAMP on transformed cells include reversion of morphology towards normal, slower growth rate, diminution of motility, restoration of contact inhibition, and increased adhesion to the substratum (1–5). The suggestion that cAMP is an important factor in maintaining the normal appearance and growth rate of cells was investigated using MSV transformed non-producer cells as a model. Treatment of Ki-3T3 cells with dbcAMP + Th was found to have a profound effect on reducing their growth rate, reversibly restoring the morphological characteristics of the parent B-3T3 cell line, and in-

TABLE III. Effect of dbcAMP + Th Treatment on Sugar Transport.[a]

Cell line	K_m ($\times 10^{-4}$ M): Control	Treated	V_{max} (nanomoles/mg protein/min): Control	Treated
B-3T3	20	20	18	25
Ki-3T3	2	2	45	80

[a] The cells were treated with dbcAMP + Th for 2 days. D-glucose was used for the uptake studies and 2-deoxy-D-glucose for kinetic studies.

creasing the adhesion of the cells to the substratum. However, the treatment resulted in only a slight reduction in the ability of MuLV to replicate in Ki-3T3 cells. The MSV genome persisted in the cells after treatment, and could readily be rescued from them. The slight reduction in replicating MuLV and rescued MSV titers from treated cells may reflect their slower growth rate, as type C virus replication is dependent, to a large extent, on cell division. While earlier studies were unable to demonstrate the presence of virus-directed surface antigens on these cells (10), we have been able to detect their presence on Ki-3T3 cells and their absence in the parent B-3T3 cells with the use of sensitive humoral and cellular cytotoxicity tests. Treatment of Ki-3T3 cells with dbcAMP + Th did not diminish or abolish the expression of these antigens.

The effects of dbcAMP + Th treatment on glucose transport are of particular interest. It is well established that transformation of cells by avian and murine sarcoma viruses is accompanied by severely reduced K_m values for glucose transport, while values for V_{max} are variable and follow no obvious pattern (17, 18, 20). Morphologic transformation and increased sugar upake occur concomitantly (21), and spontaneous or temperature-dependent reversal to the nontransformed state is accompanied by a return of the sugar uptake to normal (20, 22, 23). Ki-3T3 cells have a K_m for glucose transport approximately tenfold lower than the parent B-3T3 line. Treatment of both cell lines with dbcAMP + Th did not alter these K_m values, indicating a discrepancy between the normal morphologic appearance of the treated Ki-3T3 cells and the persistence of their low K_m value. Treated Ki-3T3 and B-3T3 cells show a marked increase in the V_{max} values for glucose transport. Cells transformed by polyoma and SV40 viruses, and lymphocytes exposed to phytohemagglutinin show similar changes in glucose transport (*i.e.*, altered V_{max} but no change in K_m) (24). It has been suggested that alterations in V_{max} reflect quantitative changes in the membrane transport system whereas decreases in K_m reflect qualitative changes in the affinity of the substrate for the carrier (24).

Treatment of Ki-3T3 cells with dbcAMP + Th causes changes in morphology, cell growth and adhesiveness, suggesting a change toward the non-transformed state, but an important biochemical marker of sarcoma virus-induced transformation, the K_m for glucose transport, remains low. These findings suggest that an intracellular lack of cAMP is not the sole cause of transformation. In keeping with this idea is the finding that transformation by murine and avian sarcoma viruses is not prevented by dbcAMP + Th treatment (A. G. and Y. I., unpublished observations, and H. E. Varmus and I. Pastan, personal communication).

Summary. Treatment of Kirsten sarcoma virus-transformed non-producer cells (Ki-3T3) with dibutyryl cyclic adenosine phosphate and theophylline (dbcAMP + Th) resulted in marked changes in morphology, reduction in growth rate, and increased adhesiveness, suggesting a reversal towards the non-transformed BALB/3T3 (B-3T3) parent cell line. The morphologic changes were reversible by removal of the dbcAMP + Th. However, treatment of Ki-3T3 cells had little or no effect on the replication of leukemia virus, the presence and rescuability of the sarcoma genome, and the expression of virus-directed cell surface antigens. Sarcoma virus-transformed cells, including Ki-3T3 cells, characteristically have lowered K_m values for glucose transport. The treatment did not affect the K_m value, indicating that at least one important biochemical marker of the virus transformed state is unaffected by dbcAMP + Th treatment. Treated Ki-3T3 and B-3T3 cells had markedly increased V_{max} values for glucose transport.

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