Journal of Cellular Biochemistry 18:515–538 (1982)
Mechanisms of Chemical Carcinogenesis 307–330

Inhibition of Neoplastic Cell Growth by Quiescent Cells Is Mediated by Serum Concentration and cAMP Phosphodiesterase Inhibitors

John S. Bertram, Barbara B. Bertram, and Przemyslaw Janik

Grace Cancer Drug Center, Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York 14263

We have demonstrated that confluent monolayers of the mouse fibroblast cell line C3H/10T1/2 (10T1/2) have the ability to cause reversible growth inhibition of cocultured transformed cells. This was first demonstrated for de novo transformed cells and later extended to established cell lines of proven oncogenicity in vivo. This growth inhibition could be increased by growing the 10T1/2 cells to high density in increasing concentrations of serum or by elevating intracellular concentrations of cAMP using inhibitors of phosphodiesterase (PDE). These manipulations, which in cocultures of nontransformed and transformed cells caused complete inhibition of tumor cell growth, had no effect on growth rate or saturation density of either cell type when cultured alone, demonstrating the cooperative nature of this phenomenon. This cooperation could not be produced by transfer of culture medium, demonstrating the requirement for intimate cell contact. Inhibition of the formation of transformed foci of cells in these mixed cultures was accompanied by a decrease in the incorporation of labeled thymidine into these cultures; the kinetics of this inhibition and recovery suggested a rapidly reversible effect on cell cycle transit times. The potent inhibitor of cAMP PDE, Ro 20-1724 induced dose dependent increases in intracellular cAMP in both nontransformed and in transformed cells. However, at a concentration of 10^{-4}M Ro 20-1724, which inhibited tumor cell growth in mixed cultures, cAMP was elevated 30-fold in nontransformed versus only 3-fold in transformed cells.

The inhibitory effects of PDE inhibitors on tumor growth have been extended to an in vivo model system, utilizing Lewis lung carcinoma cells growing as metastases in the lungs of C57B1 mice. In these mice, inoculated intravenously with a single cell suspension of Lewis lung cells, the formation of lung metastases was dramatically decreased by the twice daily administration of either isobutylmethylxanthine or Ro 20-1724; PDE inhibitors were shown to be active in vitro. The latter compound, which showed highest activity in vitro, was also substantially more potent in vivo as an inhibitor of lung tumor colony for-

Dr. Janik is now at Maria Skoldowska-Curie Memorial Institute of Oncology, Wawelska 15, Warsaw, Poland.

Received June 9, 1981; revised and accepted November 30, 1981.

0730-2312/82/1804-0515$06.50

mation and doubled the life span of the tumor bearing animals. Cell cycle analysis of lung tumor colonies by the labeled mitosis method showed that both phosphodiesterase inhibitors caused a prolonged G_1 phase in the cell cycle but failed to influence other phases. Although detailed analysis of host tissues is not complete, prolonged treatment with these drugs caused no statistically significant weight loss or changes in counts of red or white blood cells indicating a selective growth inhibition of transformed cells at these doses. Studies to determine the mechanism of the cellular communication and the nature of the signal are in progress.

Key words: cell–cell interactions, neoplastic transformation, cAMP, metastasis, phosphodiesterase inhibitors, carcinogenesis, growth control

The process of carcinogenesis is usually considered to consist of several mechanistically distinct phases linked by time and has been successfully explored recently by the use of in vitro model systems. The process is begun by the reaction of an ultimate carcinogen with cellular macromolecules (presumably DNA), followed by the biological fixation of the lesion during cell replication. The chemical lesion appears labile and replication must take place within about 72 hr of lesion formation in order for fixation to occur [1]. Fixation results in the formation of a stable "initiated" cell, which can be expected to be phenotypically normal [2] but under permissive circumstances will progress to become a neoplastically transformed cell. Tumor promoters accelerate or enhance this process [3], while vitamin A and its derivatives inhibit the progression of initiated cells to malignancy [4]. However, a single neoplastically transformed cell is not a tumor, nor in in vitro assays can it be scored as a transformed focus. In both cases this single cell is usually surrounded by quiescent nontransformed cells and a process of clonal expansion is required in order for expression of the cell's malignant potential (Fig. 1). We wish to present data to show that clonal expansion is not an automatic consequence of neoplastic transformation, but that neighboring nontransformed cells can cause reversible growth arrest of both de novo and established neoplastically transformed cells.

METHODS

In Vitro Studies

Cells and cell culture. The C3H/10T1/2 CL8 (10T1/2) cell line was used as a source of nontransformed mouse fibroblasts and as a cell line in which de novo carcinogen-induced neoplastic transformation could be readily induced [5]. Transformation assays were performed as previously described [6]. The reconstruction experiments utilizing mixed cultures of confluent 10T1/2 cells overlayered with transformed cells have also been described [6, 7]. The transformed mouse embryo fibroblast cell line (T10T1/2) was derived from 10T1/2 cells by exposure to methylcholanthrene [6]; the Lewis lung carcinoma (LLCL1) cell line is a cell culture adapted line [8] derived from the original mouse tumor. In this paper, transformation refers to neoplastic transformation of mouse fibroblasts and is characterized by morphological changes, loss of contact inhibition of growth, growth in soft agar, and tumorigenicity. Criteria for neoplastic transformation in 10T1/2 cells have been described [5].

In vitro studies. Mice were injected with a monodisperse suspension of LLCL1 cells by the subcutaneous or intravenous route and treated with phosphodiesterase inhibitors by twice daily intraperitoneal injection as described [9].

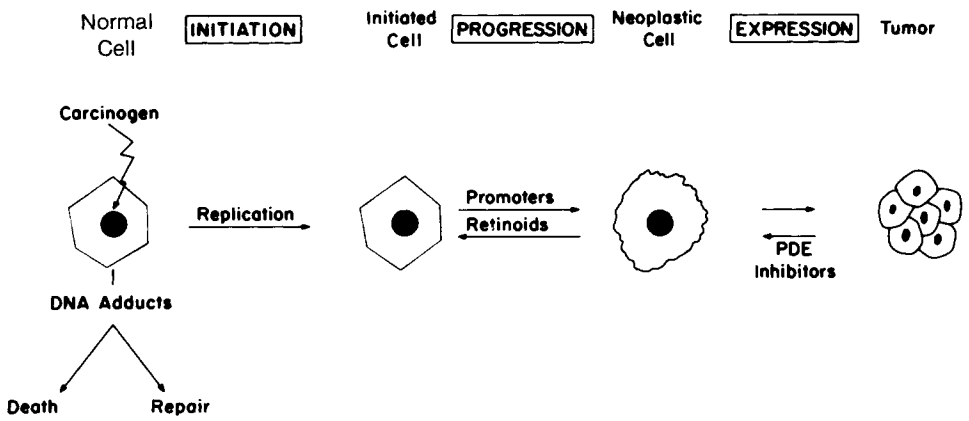


Fig. 1. Schematic representation of processes involved in the chemical induction of cancer.

Quantitation. Foci of transformed cells growing in vitro were fixed, stained, and their number and size assessed by projection of the Petri dish image onto a pressure sensitive digitizer [6]. Cell counts were performed by electronic particle counting [6]. Subcutaneous tumors growing in vivo were measured by calipers. Lung metastases were visualized by inflation of the lungs in situ with India ink, which results in tumors appearing as white areas on a black background [9, 10].

Cyclic nucleotide assays. Both cyclic 3′-5′ adenosine monophosphate (cAMP) and cyclic 3′-5′ guanosine monophosphate (cGMP) were assayed by commercial radioimmunoassay kits (New England Nuclear, Boston, MA) after acetylation to increase sensitivity. Cell extraction and quantitation of assays were performed as described [7].

RESULTS

Effects of Serum Concentration on Expression of De Novo Transformation

A disadvantage of the 10T1/2 system and other established transformable cell lines [11, 12] is that 3 to 5 wk are required from the time of carcinogen treatment to the development of transformed foci. Although the reasons for this long latent period are not known, it seemed possible that the use of a selective medium for the growth of transformed cells might improve this assay system by increasing its speed and sensitivity. We had previously shown that the saturation density achieved by 10T1/2 cells was sensitive to serum concentration, whereas transformed cells were not, that transformed cells exhibited a much higher saturation density than did 10T1/2 cells (Fig. 2), and that the transformation frequency (TF) was inversely proportional to cell density at the time of treatment [5]. It therefore appeared feasible to use a serum concentration suboptimal for nontransformed 10T1/2 cells as a selective medium to aid in the development of transformed cells.

To test this, cultures of 10T1/2 cells were plated in basal Eagle's medium (BME) supplemented with the standard 10% concentration of heat-inactivated fetal calf serum (HIFCS) and exposed to 7,12-dimethylbenz(α)anthracene (DMBA) (1 μg/ml) for 24 hr. Seven days after removal of the carcinogen, during which time

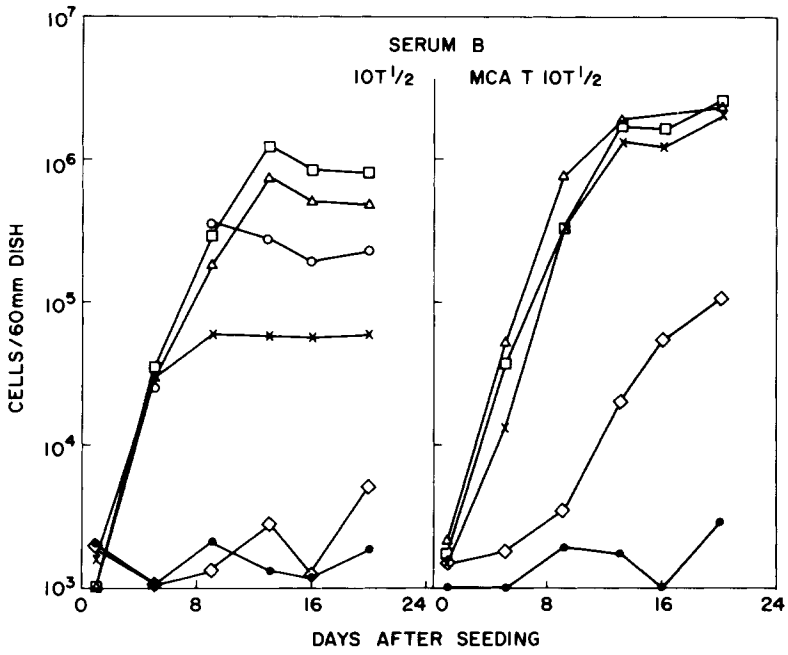


Fig. 2. Growth curves for cells cultured in HIFCS. On day 0, 5×10^3 10T1/2 cells (left panel) or MCA-transformed cells (right panel) were seeded in 60 mm Petri dishes in BME supplemented with the following concentrations of HIFCS, Lot A750318: 20%, ■; 10%, △; 5%, ○; 2.5%, ×; 1%, ◇; and 0.1%, •. Cultures were refed with the appropriate serum every 3 to 4 days. Total cells per dish were determined starting 24 hr after seeding. Results represent the mean of four dishes. From Cancer Res, with permission.

the chemical damage is fixed as stable biological damage (ie, initiation has occurred), cultures were randomized and refed with BME supplemented with 2.5, 5.0, 10.0, or 20.0% HIFCS. Cultures were refed with the appropriate serum concentration at weekly intervals for 4 wk, at which time they were fixed with methanol, stained with Giemsa and the numbers of transformed foci counted. As shown in Table I, the maximum number of transformed foci were found in dishes cultured in 2.5 or 5.0% serum; further increases in serum concentration led to a decrease in the number of foci, such that at a 20% serum concentration no foci were observed. This enhancement of transformation has been demonstrated for all serum samples and for all carcinogens so far examined (Table II). For MNNG the use of this enhancement system allows for the expression of transformants after treatment of logarithmic growth phase cultures, previously not attained [13].

Reversibility of the Serum Concentration Effect

To test whether the inhibition of neoplastic transformation by serum was owing to selective cytotoxicity to initiated cells and would thus be irreversible, replicate cultures were plated as previously in 10% HIFCS exposed to 0.5 μg/ml DMBA, and 8 days posttreatment refed with BME with 5, 10, or 15% HIFCS. In cultures given 15% HIFCS in which the transformation frequency was zero as expected, a portion

TABLE I. Modulation by Serum of the Expression of DMBA-Induced Neoplastic Transformation in 10T1/2 Cells*

Serum concentration	Saturation density (cells/60mm dish)	Transformation frequency %
2.5	8×10^4	3.0
5.0	1.1×10^5	3.0
10.0	4×10^5	1.9
15.0	8×10^5	0.0
20.0	9×10^5	0.0

*For experimental protocol see text. From [6].

TABLE II. Enhancement of Transformation With Diverse Carcinogens*

Carcinogen	Final serum concentration	Plating efficiency percent control	Transformation frequency	Enhancement factor
Acetone control	10	100	0	0
MCA (1 μg/ml)	10	95	0.5	2.0
	5		1.0	
DMBA (1 μg/ml)	10	53	1.6	2.4
	5		3.8	
X-ray (400 rad)	10	33	0.2	4.2
	5		1.4	

*Cells were exposed to the stated carcinogen 24 hr after plating and received fresh medium containing 10% serum 16 hr later. Eight days after treatment all cultures received medium containing the stated serum concentration, and this concentration was maintained with weekly refeeding until day 36 after treatment when they were fixed, stained, and assessed for transformed foci. No transformation was observed in solvent-treated or sham-irradiated controls (from [6]).

were then refed with 5% serum concentration and allowed a 4 wk period to express latent foci. In these dishes, 1.6 ± 0.4 latent foci/dish developed in the presence of a 5% serum concentration; the number of these foci was not statistically different from the number of foci in controls treated from day 8 with 5% serum concentration (2.0 ± 0.3 foci/dish). In other experiments the elevated saturation densities induced by 20% HIFCS were also found to be reversible upon shifting confluent monolayers of 10T1/2 cells to a 5% serum concentration and vice versa (Fig. 3).

Growth Inhibition of Established Transformed Cells

The experiments described above demonstrated effects on the capacity for de novo transformed cells to express their transformed phenotype. To investigate whether a similar phenomenon could be shown in established transformed cells of known oncogenic capacity, reconstruction experiments were set up utilizing confluent monolayers of 10T1/2 cells grown to confluence in serum concentrations of 2.5, 5.0, 10.0, and 20.0%, then seeded with 100 cells of neoplastically transformed 10T1/2 cells. As shown in Figure 4 the colony size of these transformed cells decreased progressively to zero in cocultures of 10T1/2 cells grown in progressively

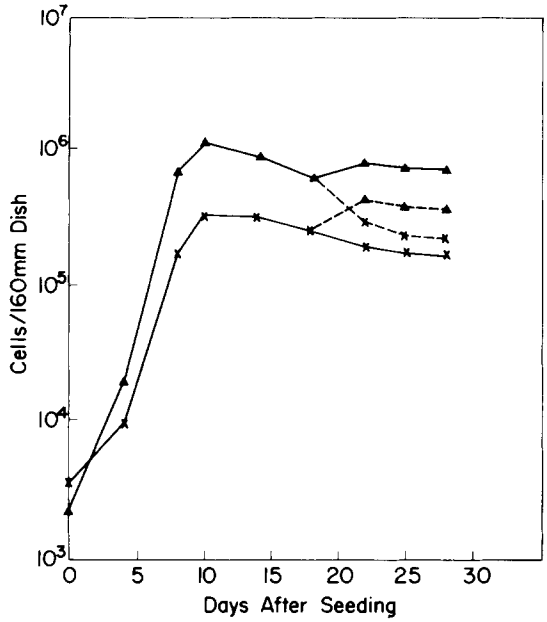


Fig. 3. Variation in saturation density with serum concentration. Cultures of 10T1/2 cells were grown to confluence in either 5% HIFCS (x—x) or 20% HIFCS (▲—▲); half of the cultures were then switched to the high (▲--▲) or low (x--x) serum concentration, respectively, and the others were maintained at the original concentration. Cell counts were performed at the indicated times. From Cancer Res, with permission.

higher serum concentrations. In contrast, when these same cells were placed in medium containing these same serum concentrations in otherwise empty dishes (ie, without the 10T1/2 cells), no such inhibition of growth was observed (upper panels). Because colony size need not accurately reflect cell number/colony, we took replicate cocultures, dispersed them with trypsin, and seeded these cells into soft agarose, a selective growth medium for transformed cells. Analysis of colony formation in these cell suspensions showed that as the serum concentration increased, the number of transformed colonies decreased. Of interest is the finding that whereas in a 20% serum concentration, no foci were observed on the 10T1/2 monolayers, yet a few transformed colonies grew in soft agarose indicating again the reversible nature of growth inhibition. To test this, cocultures established in a nonpermissive serum concentration of 20% were shifted after 1 wk to 5% serum concentration. Transformed colonies rapidly appeared in numbers comparable to those growing in cultures exposed to a 5% serum concentration throughout (data not shown).

Thus, confluent nontransformed cells have the ability to reversibly suppress the growth of neoplastically transformed 10T1/2 cells and this suppression can be modulated by serum concentration. We believe serum is acting by increasing the number of 10T1/2 cells available for interaction with the transformed cells. This view is supported by the reversible nature of the action of serum on saturation density (Fig. 3), as well as on growth inhibition.

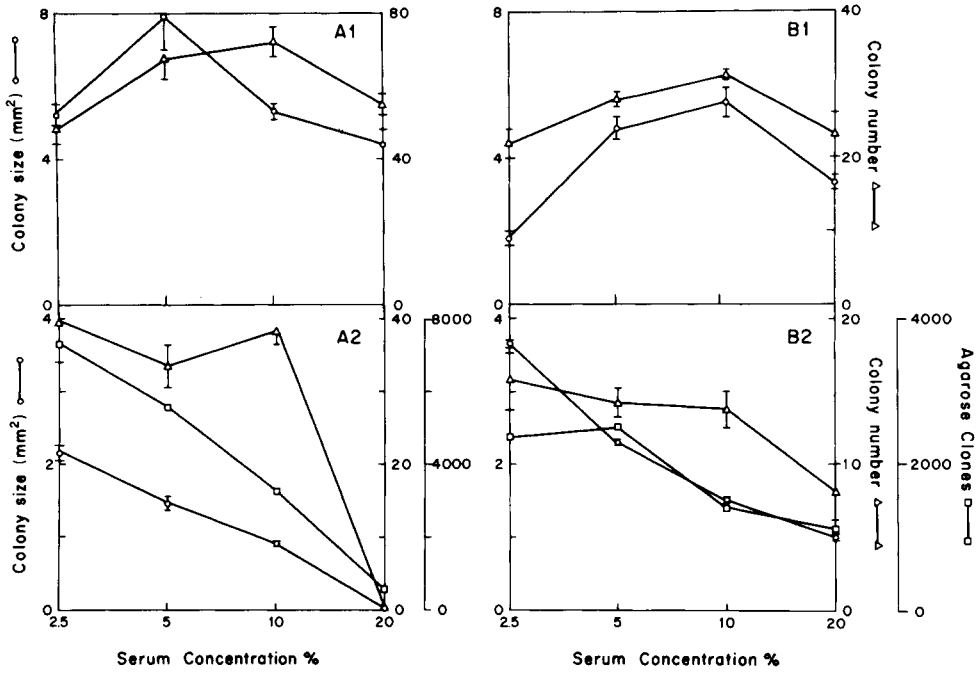


Fig. 4. Effect of serum concentration on the growth of transformed cells in the presence and in the absence of 10T1/2 cells. Confluent cultures of 10T1/2 cells were prepared by seeding 10^4 cells in the stated serum concentrations. When confluent, as judged microscopically, 5 ml of fresh medium supplemented with the appropriate serum concentration were added to the confluent monolayers and to an equal number of empty dishes. All dishes were seeded 24 hr later with transformed cells. Dishes were incubated for about 8 days without further medium change. At this time dishes were scored for colony number (△) and colony size (○), and in the case of the mixed cultures, the contents of two dishes were seeded into agarose for determination of the number of clonogenic cells (□). Clone A (left panels) was a malignantly transformed MCA line at passage 72 when used; Clone B (right panels) was a recently isolated MCA-transformed line. Upper panels (A1,B1) show cells seeded in the absence of 10T1/2 cells; lower panels (A2,B2) show cells seeded onto confluent monolayers of 10T1/2 cells. Results represent the mean ± SE for colony number and area and the mean of two cultures each for the clonogenicity in agarose. From Cancer Res, with permission.

Role of Cyclic Nucleotides in Cell/Cell Communication

Because of the persuasive evidence for the participation of cyclic nucleotides, especially cAMP, in cell growth control processes [14, 15], and because of reported elevations of cAMP levels in confluent versus logarithmic growth phase of mouse fibroblasts [16], the effects of cAMP and dibutyryl cAMP in the 10T1/2 system were examined. When these drugs were applied to mixtures of confluent 10T1/2 $\times 16$ and T10T1/2 cells under permissive concentrations of serum, growth inhibition of transformed cells could only be achieved at concentrations (10^{-4}–10^{-3} M) that caused growth inhibition of cells plated alone, ie, the effect was independent of the cocultured 10T1/2 cells [7].

Action of Inhibitors of Cyclic Nucleotide Phosphodiesterase

Methylxanthines. A more physiological method of causing cyclic nucleotide elevations is to inhibit the enzymes responsible for their degradation. When methylxanthine inhibitors of cyclic nucleotide phosphodiesterase (PDE) were tested for effects in the 10T1/2 system, it was found that these would mimic the effects of high serum concentrations in causing the reversible inhibition of growth of transformed cells but without altering the saturation density of 10T1/2 cells. Addition of the methylxanthines, caffeine, theophylline, or isobutylmethylxanthine (IBX) to cultures of 10T1/2 cells treated 7 days previously with a transforming concentration of methylcholanthrene (2.5 μg/ml), as described above, caused complete inhibition of transformation at a minimum concentration of 10^{-3} M. IBX (Table III) was active at a minimum concentration of 10^{-4} M, which by itself had no effect on growth rates of 10T1/2 or T10T1/2 cells (Fig. 5). Caffeine and theophylline were active only at 10^{-3} M and are correspondingly less active as PDE inhibitors. To test for the reversibility of this effect, cultures were first exposed to MCA, then 4 wk of treatment with either 10^{-4} or 10^{-5} M IBX, which decreased the observed transformation frequency to 0 or 35% of control, respectively. After this time drug treatment was withdrawn, and cultures were maintained for an additional 4 wk, then fixed and stained. Removal of drug led to the expression of latent transformed foci in numbers comparable to those observed in MCA-treated controls (Table IV). Reconstruction experiments utilizing confluent monolayers of 10T1/2 cells overlaid with T10T/2 cells also demonstrated that IBX between 10^{-5} and 10^{-3} M would cause progressive inhibition of the colony size of the T10T1/2 cells, but that this inhibition was not potentiated by exogenous cAMP or dibutyryl cAMP (data not shown).

TABLE III. Inhibition by Methylxanthines of MCA-Induced Transformation*

		Caffeine		Theophilline		IBX			
	Acetone	10^{-3} M	10^{-4} M	10^{-3} M	10^{-4} M	10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M
Number foci	2.1	0	1.3	0.1	1.9	0	0	1.5	1.8
Percent control	100	0	63	5.2	90	0	0	70.1	80.5

*10T1/2 cells were exposed to MCA 1 μg/ml for 24 hr, 7 days later replicate cultures were treated with the appropriate PDE inhibitor and for the remaining 28 days duration of the experiment. Fixed and stained cultures were then assessed for number of transformed foci (From [7]).

TABLE IV. Reversibility of Inhibition of Expression of the Neoplastic Phenotype by IBX*

Treatment (duration, days)	Time fixed (d)	Foci/dish	% Control
Acetone 0.5% Control	35	1.5 ± 0.3	100
IBX 10^{-4}M (9–35)	35	0	0
IBX 10^{-4}M (9–35)	63	1.3 ± 0.3	87
IBX 10^{-5}M (9–35)	35	0.4 ± 0.2	27
IBX 10^{-5}M	63	1.9 ± 0.4	127

*10T1/2 cells were exposed to 1 μg/ml of MCA for 24 hr, 7 days later cultures were treated with the stated concentration of IBX for 28 days. At this time half the cultures were fixed and stained, the remainder were maintained on drug-free medium for an additional 28 days to allow expression of the neoplastic phenotype (From [7]).

Action of Ro 20-1724. The methylxanthines are poor pharmacological agents because of their actions on many enzymes including cGMP phosphodiesterase, poly-ADP-ribosylase, adenylate cyclase, and postreplication DNA repair. A search for a more specific inhibitor of cAMP PDE, yielded the imidazolidinone derivative Ro 20-1724 [17], which has high specificity for cAMP PDE and no documented interactions with adenylate cyclase [18]. Its structure is shown in Figure 6.

When acetone solutions of Ro 20-1724 were added to reconstruction experiments, set up as described above, both the size and numbers of transformed colonies were found to decrease in a dose dependent manner as the concentration of drug increased (Fig. 7). Total suppression of growth occurred at 10^{-4} M, which had no effect on the growth rate or saturation density, as measured by serial cell counting, of 10T1/2 or T10T1/2 cells when cultured separately (data not shown). Thus, as with IBX, the inhibitory effects of Ro 20-1724 on T10T1/2 growth are mediated only in mixed cultures and without influencing saturation density.

Modulation of Cyclic Nucleotide Levels

Confluent cultures of 10T1/2 cells and cultures of T10T1/2 cells in logarithmic or plateau growth phase were treated with various concentrations of Ro 20-1724 for

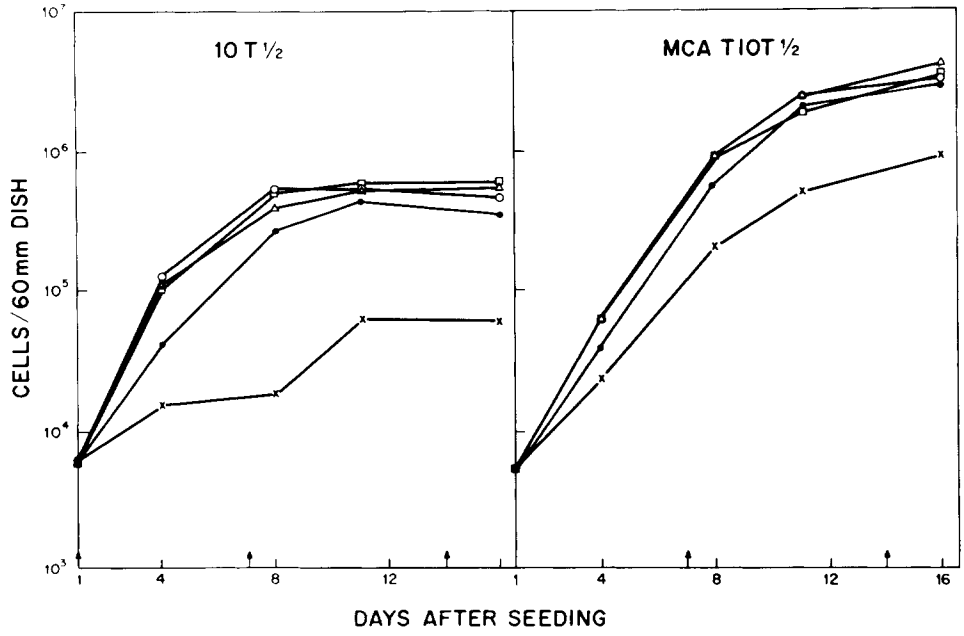
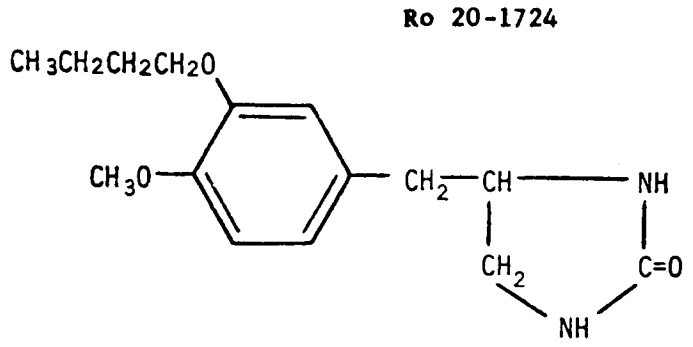


Fig. 5. Growth curves of 10T1/2 cells and MCA-transformed 10T1/2 cells in the presence of agents that modify cyclic nucleotide metabolism. On day 0, 10^4 10T1/2 cells (left) or MCA T10T1/2 cells (right) were seeded in 60 mm dishes containing BME plus 10% HIFCS. On day 1 cultures were treated with the following compounds: ×, 10^{-3} M IBX; △, 10^{-4} M IBX; □, 5×10^{4} M cAMP; •, 5×10^{-4} M dbcAMP; ○, 0.9% NaCl control. Cultures were refed and retreated at the times indicated by the arrows, and total cells/dish were determined as described. Results represent the mean of four dishes. From Cancer Res, with permission.



d,l-4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone

$C_{15}\ H_{22}\ N_2\ O_3$

Fig. 6. Chemical structures of d,l-4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone. Ro 20-1724 kindly supplied by Hoffmann-La Roche, Nutley, NJ.

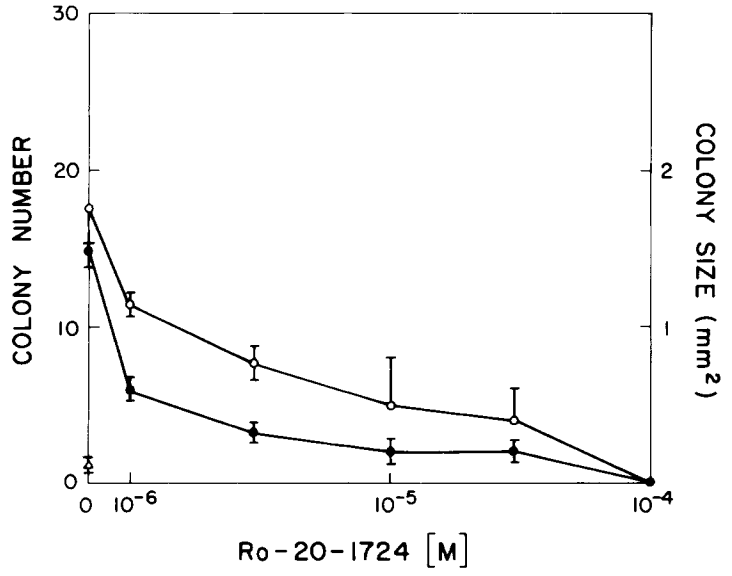


Fig. 7. Effects of Ro 20-1724 on growth of T10T1/2 cells cultured on confluent monolayers of 10T1/2 cells. T10T1/2 cells (100/dish) were seeded onto confluent 10T1/2 cells grown in BME + 5% HIFCS. After 24 hr cultures were treated with the stated concentration of Ro 20-1724, which was not removed for the 8 day duration of the experiment. Cultures were then stained and the number (●—●) and size (○—○) of transformed foci determined. Mean of three separate dishes ± SE.

3 days, then extracted for determinations of cAMP and cGMP. As seen in Figure 8 intracellular cAMP levels in 10T1/2 cells increased progressively with doses of Ro 20-1724 from a basal level of about 5.0 pmoles to 150 pmoles/10^6 cells when exposed to 10^{-4} M Ro 20-1724, a 30-fold elevation. Conversely, levels of cGMP, which in control cultures were 100-fold lower than cAMP levels, decreased about 5-fold at the lowest drug concentration, then recovered slightly to show a final decrease of about 2-fold from control levels. In T10T1/2 cells control cAMP levels were 10- to 200-fold lower than in 10T1/2 cells, according to whether cells were assayed when in logarithmic or plateau growth phase, respectively. Addition of Ro 20-1724 resulted in moderate (2–5-fold) increases in cAMP levels (Table V). Analysis of cGMP was not possible since control levels were below the sensitivity limits of the assay system (about 10^{-16} M/10^6 cells). Thus, cAMP levels were elevated in both normal and transformed cells, with the former showing the most striking increases, and a better dose/response correlation with the growth inhibition studies (Fig. 7). However, it cannot yet be determined in which cell type (nontransformed or transformed) the crucial elevations of cAMP occur. The data strongly suggest, though clearly do not prove, that cAMP is involved in the response, and the enhanced potency of Ro 20-1724 over IBX in the growth inhibition studies is reflected in the ability of the former compound when tested at 10^{-5} M, to cause comparable elevations of cAMP levels requiring 10^{-4} M of IBX (eg, about 60 pmoles/10^6 cells) (Fig. 8, [7]). At these dosage levels the compounds are about equipotent in inhibiting the growth of transformed cells (Fig. 7, [7]).

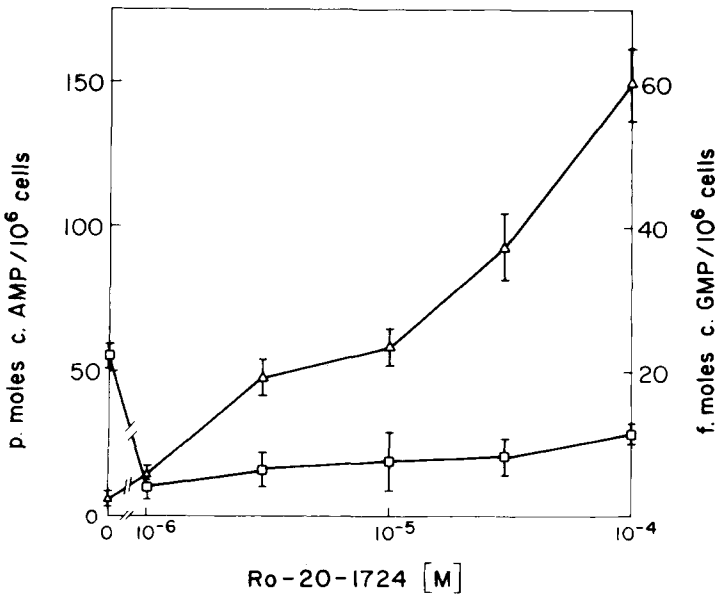


Fig. 8. Elevation of cyclic nucleotides by Ro 20-1724. The indicated concentration of Ro 20-1724 was added to confluent monolayers of 10T1/2 cells. After 3 days intracellular concentrations of cAMP (△—△) and cGMP (□—□) were determined by radioimmunoassay.

TABLE V. Elevation of cAMP in Transformed 10T1/2 Cells*

Concentration Ro 20-1724	cAMP pmoles/10^6 cells	
	Logarithmic	Plateau
Control	.5 ± .1	.010 ± .003
10^{-6} M	1.5 ± .1	.015 ± .004
10^{-5} M	1.5 ± .1	.025 ± .005
10^{-4} M	2.5 ± .5	.020 ± .004

*Cultures were treated with the stated concentration of drug for 4 days, after which intracellular concentrations of cAMP were assayed by radioimmunoassay as described in the text.
Means ± S.D.

Kinetics of Growth Inhibition

To study the time course of inhibition of DNA synthesis and of recovery after removal, cultures were labeled with tritiated thymidine and the uptake into acid insoluble material measured. In pure cultures of logarithmic growth phase T10T1/2 cells or of confluent 10T1/2 cells addition of Ro 20-1724 had little effect on thymidine incorporation. Incorporation, expressed as CPM/culture, increased exponentially in T10T1/2 cells as would be expected in a logarithmically growing cell population. This increase was slightly decreased by 10^{-4} M Ro 20-1724, whereas 10^{-5} M produced no change from acetone treated controls (Fig. 9, left panel). In confluent 10T1/2 cells, which initially incorporated very little thymidine, the addition of 0.5% acetone as control caused a small burst of DNA synthesis that was complete by 24 hr. Drug addition reduced the peak height of this response, but again essentially no thymidine incorporation could be detected at 24 hr or later (Fig. 9, right panel). Thus, in mixed cultures of confluent 10T1/2 and T10T1/2 cells virtually the entire incorporation of thymidine can be attributed to uptake by T10T1/2 cells. In mixed cultures of 10^3 T10T1/2 cells overlaid as before on about 10^5 confluent 10T1/2 cells, control cultures showed a progressive rise in thymidine incorporation with time, while addition of 10^{-5} or 10^{-4} M Ro 20-1724 induced a dose dependent decrease in this rate of increase. At 10^{-4} M incorporation was only slightly elevated over control (Fig. 10). To study time of onset and reversal of the drug effects, 10^4 T10T1/2 cells were seeded on confluent monolayers as before, and treated 24 hr later with 10^{-4} M Ro 20-1724. Within the first 4 hr posttreatment thymidine incorporation was below control levels, and this difference widened as incorporation into the treated cultures decreased, while that into controls increased (Fig. 11). Drug removal after 48 hr of treatment led to a dramatic and rapid increase in incorporation within 8 hr. The increase in incorporation between 0 and 24 hr posttreatment was as rapid as the initial increase observed in control cultures.

Effects on Cell Morphology

We have shown above that in IBX-treated cultures, containing de novo transformed cells, expression of the transformed phenotype was inhibited (Table III). We had also been unsuccessful in attempting to recognize by phase contrast microscopy individual transformed cells seeded on confluent monolayers of 10T1/2 cells in PDE inhibitor-treated cultures. In control cultures T101/2 cells can easily be

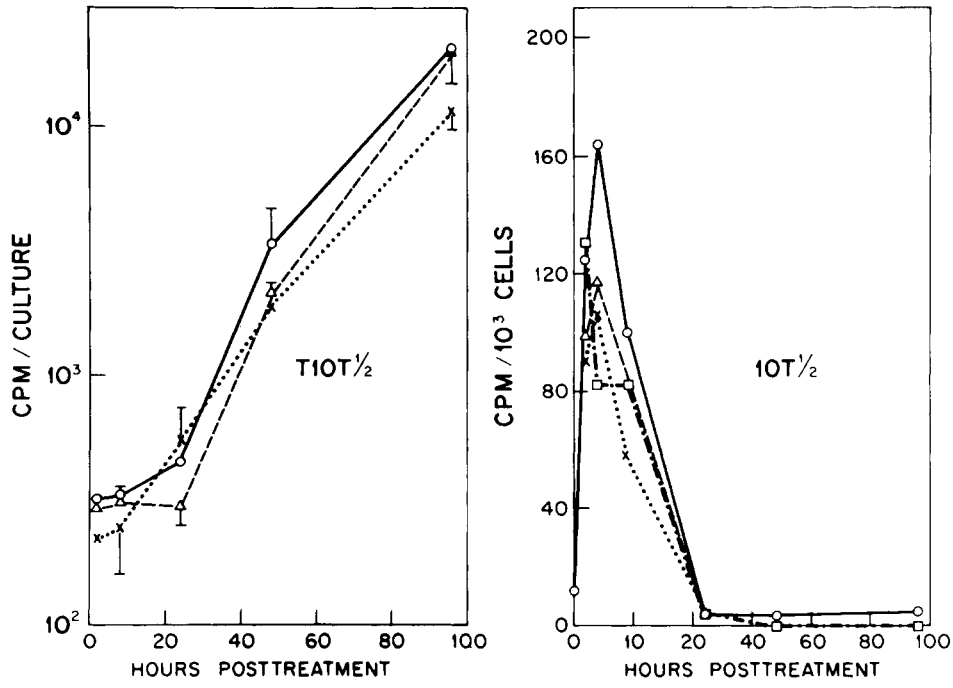


Fig. 9. Effects of Ro 20-1724 on tritiated thymidine incorporation into transformed and non-transformed 10T1/2 cells. T10T1/2 cells (10^4/dish) or confluent monolayers of 10T1/2 cells (about 2 × 10^5/dish) were treated with acetone 0.5% (○—○), Ro 20-1724 10^{-4} M (x—x), 10^{-5} M (△—△), or 10^{-6} M (□-□). At the indicated times, cultures were labeled for 1 hr with tritiated thymidine (1 μCi/ml) and uptake into acid insoluble material measured.

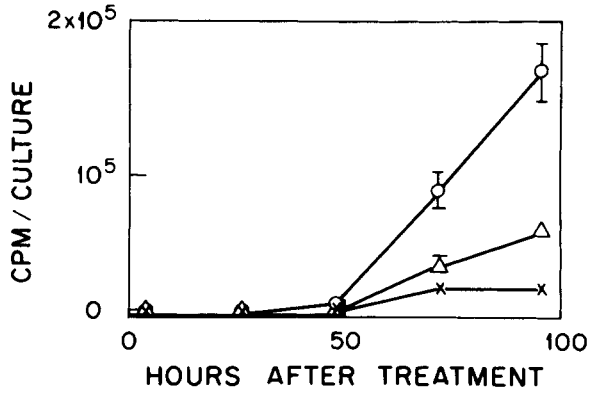


Fig. 10. Dose response for inhibition by Ro 20-1724 of thymidine incorporation into mixed cultures of 10T1/2 and T10T1/2 cells. Confluent monolayers of 10T1/2 cells were seeded with 10^3 T10T1/2 cells and were treated for 24 hr with acetone 0.5% (○—○), Ro 20-1724, 10^{-5} M (△—△), or 10^{-4} M (x—x). Labeled thymidine incorporation was determined after a 3 hr pulse as described above. Results show the mean ± SD of triplicate cultures after subtraction of the radioactivity taken up by confluent monolayers of 10T1/2 cells (see Fig. 9).

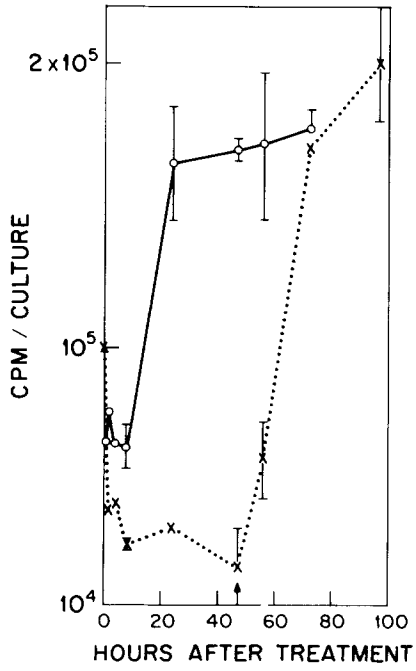


Fig. 11. Effects of Ro 20-1724 on thymidine incorporation into mixed culture of 10T1/2 and T10T1/2 cells. Twenty-four hours after seeding 10^4 MCA cells onto confluent monolayers of 10T1/2 cells, cultures were treated with acetone as control (○—○) or Ro 20-1724 (x—x). Incorporation of tritiated thymidine after a 1 hr pulse was determined as described above. Results show the mean ± SD of triplicate cultures after subtraction of the radioactivity taken up by confluent monolayers of 10T1/2 cells (see Fig. 9). These background values were about 5,000 cpm/culture 24 hr posttreatment and fell to about 1,500 cpm/culture 72 hr posttreatment.

recognized by their refractile morphology. To investigate drug effects on morphology of T10T1/2 cells, we therefore allowed transformed colonies to grow for 4–5 days in the absence or presence of 10T1/2 cells, then phase contrast micrographs were taken before and after drug treatment. Cultures were also fixed for scanning electron micrography but the data from this study are not yet available. In control cultures, transformed colonies were composed of a moderately dense central disc of transformed cells surrounded by a less dense outer zone clearly containing mixtures of 10T1/2 and T10T1/2 cells, the later having a random orientation (Fig. 12a,c). In contrast, treated colonies were smaller than controls, the central region appeared more compact, and cells in the outer zone were highly orientated, radiating away from the central cell mass (Fig. 12b,d).

In contrast, T10T1/2 cells cultured in the absence of 10T1/2 cells and treated with 10^{-4} M Ro 20-1724, exhibited no alterations in clonal morphology, (data not shown) in growth rate, or in thymidine incorporation (see above).

Mechanism of In Vitro Cellular Interactions

It is clear that PDE-mediated growth inhibition of transformed cells requires coculturing with 10T1/2 cells. Of the possible interactions between the transformed

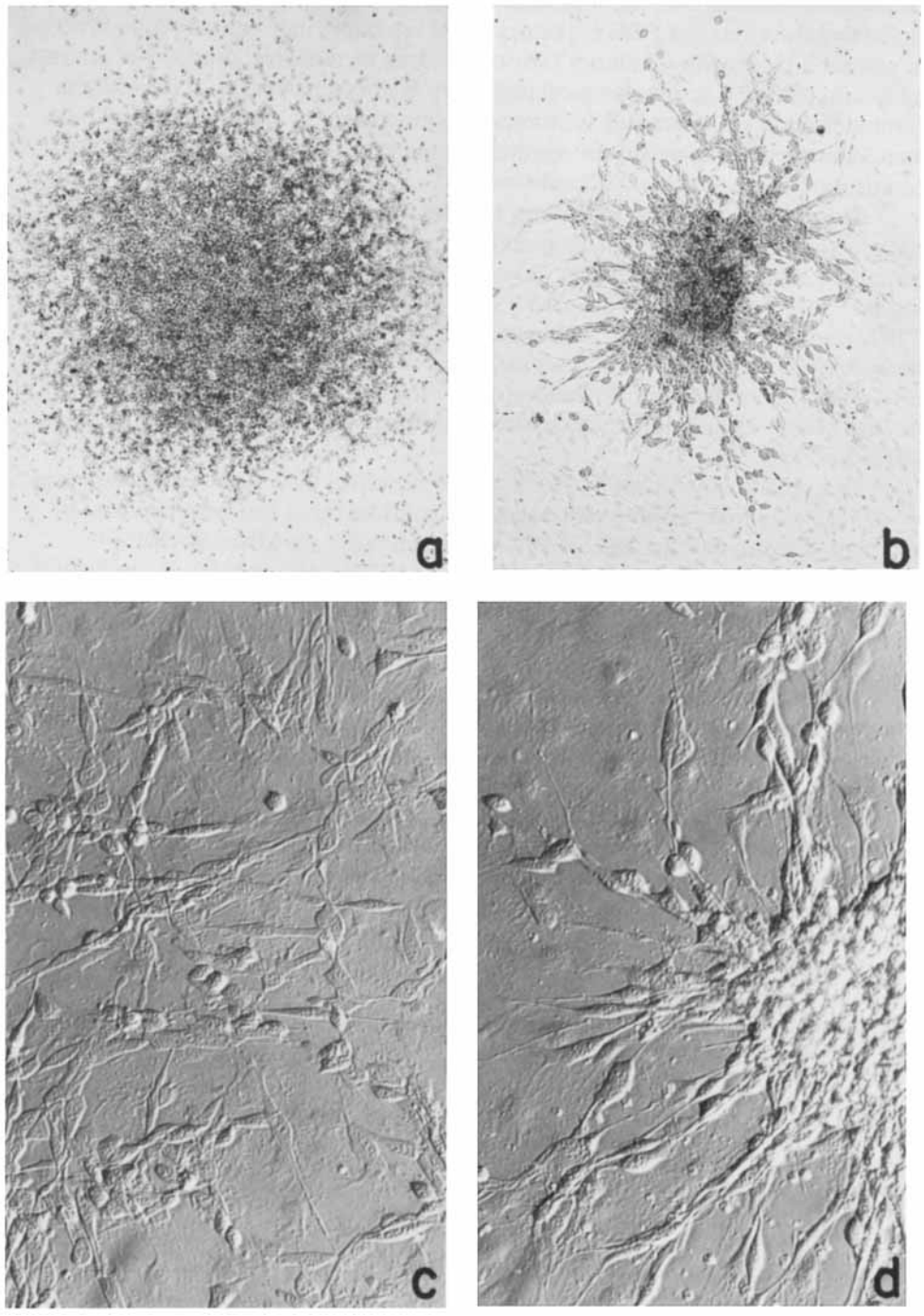


Fig. 12. Clonal morphology of T10T1/2 cells grow on confluent monolayers of 10T1/2 cells. Fig. 12a,c acetone-treated control cultures × 30 and × 250, respectively; Fig. 12b,d Ro 20-1724 10^{-4} M-treated cultures × 75 and × 250, respectively.

and nontransformed cells the following hypotheses appear the most attractive in explaining the effects of PDE inhibitors. PDE inhibitors may enhance a) production of extracellular growth inhibitory factors (eg, drug metabolites, products of enzyme inhibition (cAMP), or physiological inhibitory factors [chalones]); b) intercellular communication via permeable junctions (gap junctions); c) membrane/membrane interactions triggering messenger synthesis in the target (transformed) cell; and d) competition for available plastic substratum.

Extracellular growth inhibitory factors. No evidence confirms the existence of such factors in supernatant medium obtained under nonpermissive concentrations of serum [6]. IBX or Ro 20-1724. As shown in Table VI, conditioned medium containing 10^{-4} or 10^{-5} M IBX removed after 7 days incubation with mixtures of 10T1/2 and T10T1/2 cells failed to inhibit the replication of T10T1/2 cells when plated in the absence of 10T1/2 cells. On the contrary, growth was stimulated in comparison with cells plated in new medium. Therefore, unless the inhibitory factor is highly labile, or very poorly diffusable, no extracellular inhibitory factor is being secreted by 10T1/2 cells.

In a second experiment T10T1/2 cells were seeded onto confluent monolayers of 10T1/2 cells from which a central zone of confluent cells had been removed by scraping. Control and Ro 20-1724 10^{-4} M-treated dishes were then labeled with tritiated thymidine and the labeling indexes of T10T1/2 cells growing in this central zone were compared. Both control and treated populations showed essentially the same labeling index, again demonstrating the requirement for intimate cell contact (data not shown).

Membrane and/or gap junctional communication. Experiments are in progress to determine the possible role of communication by gap junctions and/or direct membrane interactions in this response. No conclusions have yet been reached.

Substratum competition. We do not feel that competition for available substrate caused by either high serum (cell crowding) or increased adhesion due to cAMP can explain the observed response since i) preliminary studies using scanning electron microscopy have shown that both control and treated cells rapidly penetrate

TABLE VI. Comparative Effects of New Medium Containing IBX Versus Used Medium Containing IBX on the Growth and Plating Efficiency of MCA T10T1/2 Cells*

Medium	Plating efficiency	Size (mm^2)
New	60 ± 2.8[a]	1.7 ± 0.1
New + IBX, 10^{-4}M	58 ± 2.2	1.7 ± 0.1
Used + IBX, 10^{-4}M	53 ± 4.4	2.4 ± 0.2
New + IBX, 10^{-5}M	52 ± 2.7	1.6 ± 0.1
Used + IBX, 10^{-5}M	61± 2.8	2.8 ± 0.2

*Seven-day-old culture medium containing either 10^{-4} or 10^{-5} M IBX was aspirated from cultures of 10T1/2 cells grown to confluence in BME plus 5% HIFCS. This used medium was centrifuged and poured into empty Petri dishes; identical dishes were prepared using fresh BME plus 5% HIFCS, and to some of these dishes 10^{-4} or 10^{-5} M IBX was added. One hundred MCA T10T1/2 cells were then added to all dishes, and growth was measured after 6 days without medium change.

[a]Mean ± S.E. of four dishes (from [7]).

the cell monolayer and spread on the substrate (Eilenberg and Bertram, in preparation), ii) the growth inhibitory effect is completely reversible upon drug withdrawal in spite of medium changes suggesting firm adhesion of transformed cells, and iii) both light and electron microscopy have shown that treated transformed cells spread extensively on the substrate in contrast to control cells, again suggesting at the single cell level at least that substrate is not limiting. While this spreading is most evident in solitary cells, some can be seen in malignant cells on the periphery of the colony shown in Figure 12d.

Effects of PDE Inhibitors In Vivo

Because of our belief that in vitro findings should, where appropriate, reflect events occurring in the whole animal, we have studied the actions of PDE inhibitors in a mouse tumor model. The in vitro model suggests that cell-cell interaction is a critical requirement for growth inhibition in tumors. Thus, a model in vivo system was chosen that, in the early growth stages at least, allowed the extensive interaction of single tumor cells with host tissue. The Lewis lung carcinoma (a highly malignant mouse tumor) line has such properties [19]. It metastasizes, apparently by single cell seeding, to the lung of the syngeneic C57B1 mouse and readily produces quantifiable tumor nodules. To test its responsiveness in vitro to inhibitors of PDE, a cell line adapted to in vitro conditions was first derived [8]. This line, designated LLCL1, was then used in the reconstruction experiments described above. Both IBX [9] and Ro 20-1724 (data not shown) caused partial inhibition of colony growth of LLCL1 cells when cocultured with confluent 10T1/2 cells, and on this basis the compounds were tested in vivo.

C57B1/6 mice received twice daily IP injections of IBX ranging from 2.5 to 20.0 mg/kg on days 1 through 21. On the second day of the experiment they were given IV tail vein injections of a monodisperse suspension of 10^5 LLCL1 cells. After 21 days all mice were killed and their lungs visually examined for metastases. As shown in Table VII, mice injected with 5.0 mg/kg or more of IBX had significantly ($P < 0.05$) fewer lung tumors than did solvent treated controls. The larger drug dose resulted in progressively fewer lung nodules.

TABLE VII. Dose-Response Effects of IBX on Formation of Lung Tumor Nodules in C57BL/6J Mice Given I.V. Injections of Lewis Lung Carcinoma Cells*

IBX (mg/kg)	No. of lung nodules/ mouse	No. of mice with nodules/ total no. of mice
0	5.69 ± 2.6[a]	13/13
2.5	8.7 ± 3.7	11/11
5.0	2.5 ± 2.46[b]	10/12
10.0	0.9 ± 0.87[b]	7/10
20.0	0.5 ± 0.7[b]	4/10

*Two days prior to tumor inoculation, mice were treated with a twice daily i.p. injection of the stated dose of IBX given in 0.2 ml PBS (0.4 ml at the 20 mg/kg level). Controls received 0.2 ml PBS, and treatments continued throughout the experiment. LL1 cells were injected into treated and control groups as an i.v. injection of 10^{-5} cells into the tail vein. After 21 days, mice were killed, and tumor nodules were counted, as described in the text.

[a]Mean ± S.D.

[b]Significantly different ($P < 0.05$) from PBS-treated controls.

(From [8]).

These observations are not due to differences in the initial seeding of lungs, or in the rate of release of tumor cells from the lungs over the first 24 hr postinjection, as shown using radiochromium labeled cells. Furthermore, treatment delayed until after lung seeding had occurred [9] still was effective. Thus, the lung appears to contain cells that may act in an analogous manner to cultured 10T1/2 cells.

Comparative activities of IBX and Ro-1724. We has shown in vitro that Ro 20-1924 was more potent than IBX in inhibiting the growth of T10T1/2 cells. A comparative experiment was set up in vivo where mice were randomized into groups receiving IBX 20 mg/kg, Ro 20-1724 20 mg/kg, or vehicle as control. They were injected with LLCL1 cells as before and their survival time determined. The results plotted in Figure 13 show that both drugs extend the life span of the mice. In the control groups 50% of mice were dead 24 days after tumor inoculation, whereas IBX caused an increase in lifespan of 52% and Ro 20-1724 an increase of 101%. Thus in vivo as well as in vitro, the latter compound has the greatest activity. The most pronounced effect of Ro 20-1724 is seen as an increase in time to first death, whereas the death rate curves were similar between treated and control groups. This indicates that once tumors have grown to a size capable of killing some of the mice, drug treatment has little effect. This could be predicted from the model since large tumors can have only limited interactions with adjacent normal tissue. It should also

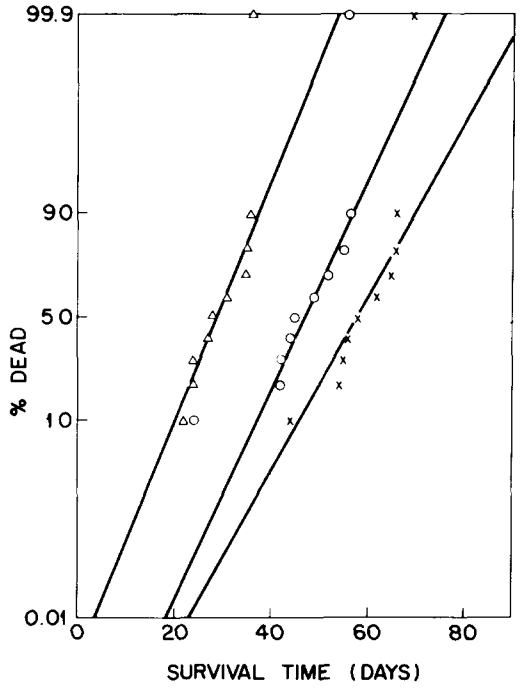


Fig. 13. Increased survival times of tumor-bearing C57B1 mice treated with phosphodiesterase inhibitors. Mice were injected with 2 × 10^5 LLCL1 cells, 2 days after beginning treatment with IBX 20 mg/kg (○—○), Ro 20-1724 20 mg/kg (x—x), or with vehicle as control (△—△). Drugs were given twice daily by IP injection until death. Curves represent computer fitted linear regression analyses of all respective data points except the 10% and 100% death frequencies.

be noted that all mice eventually died of tumor; thus the effects of drug treatment are to slow the tumor growth rate, not to exert a cytotoxic effect. This again could be predicted from the in vitro experiments, which demonstrated reversibility of growth inhibition.

Nature of tumor growth inhibition. While it was apparent in vitro that inhibition of growth of T10T1/2 cells by PDE inhibitors was caused by cell cycle arrest (Figs. 9 and 10), it was not clear in the in vivo studies whether such mechanisms were operating. We thus utilized the frequency of labeled mitosis (FLM) [20] method to determine the distribution of tumor cells throughout the cell cycle in treated and control mice. Since this method requires the identification and counting of a large population of tumor cells, we first allowed tumors to grow in the lungs for about 14 days, then began treatment of mice with IBX 20 mg/kg or Ro 20-1724 20 mg/kg on a twice daily basis. After the third injection of IBX, mice received 25 μCi of 50 Ci/mmole labeled thymidine. After 45 min, and at regular intervals thereafter, two mice from each treated and control group were killed, their lungs removed, fixed, and processed for autoradiography. The percent labeled mitoses with time after H-3-thymidine injection for IBX and control-treated mice are shown in Fig. 14. It is clear that both control groups are indistinguishable during the first half of the cell cycle (ie, G_2+M and S phase), clearly indicating that drug treatment does not influence entry or progression through these positions of cycle. However, a clear delay in transit through the subsequent G_1 phase was observed in tumor cells from treated mice. Whereas, control cells traversed G_1 within about 3 hr, as was previously reported [21], the treated cells showed an approximate 6 hr delay. Concomitant with

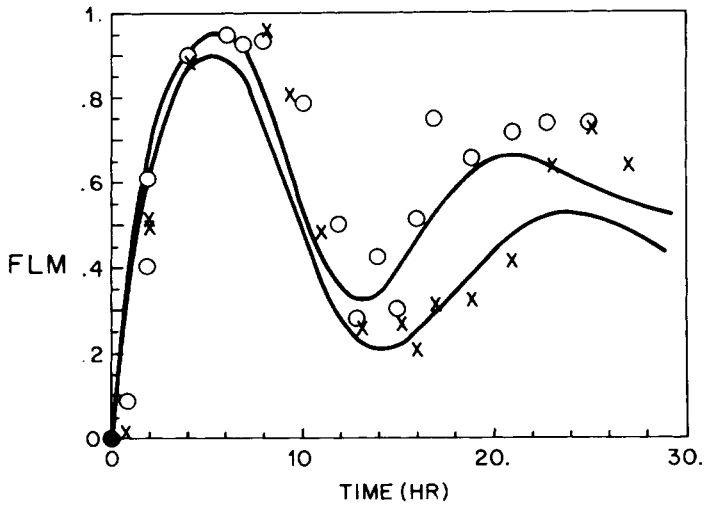


Fig. 14. Cell cycle distribution analysis of Lewis lung tumor cells as micrometastases in the lungs of C57Bl mice. Mice were injected IV (tail) with 2×10^5 LLCL1 cells; after 14 days they were randomized and treated with vehicle control (○—○) or IBX 20 mg/kg (x—x) twice daily, IP. After the third injection mice received 25 μCi of tritiated thymidine IP. Two treated and two control mice were killed at intervals thereafter, their lungs removed and processed for autoradiography. Data represent the frequency of labeled mitoses (FLM) as a percent of total mitoses with time after labeling; a minimum of 50 mitoses was scored/time point.

this cell cycle delay, the labeling index (ie, percent S phase cells) was decreased from 36% in controls to 18% in the treated population. This 100% decrease is in excess of the theoretical 30% decrease in labeling index predicted from the observed G_1 extension. Therefore, the data suggest that an additional population of cells, comprising about 7% of the original treated population of cycling cells, is effectively out of cycle during the measured labeling period.

Drug effects on host cells. Cell cycle analysis of host cells (eg, intestinal crypts) has not yet been performed, however the failure of IBX or of Ro 20-1724 to cause weight loss or significantly affect red and white blood cell counts suggests strongly that only minimal effects are produced in proliferating host cells over this time period (Table VIII).

DISCUSSION

These studies have shown that for transformed mouse fibroblasts and a lung carcinoma model system, the expression of transformation is conditional upon the permissive behavior of neighboring cells. The permissive behavior could be owing to either a failure to communicate or, conversely, to a failure on the part of the neoplastic cell to receive or process the signal. Failure to receive the signal could be owing to a defect in the cell itself or to the presence of a barrier or buffer to signal transmission, caused perhaps by the presence around such a transformed cell of other transformed cells that are themselves not transmitters. These alternatives will be dealt with below and implications to the carcinogenic process will be discussed.

In the 10T1/2 cell line we have shown, and Thilly's group has confirmed [22], that expression of neoplasia depends upon both cell density and clone size. In the latter study in which carcinogen-treated cells were trypsinized and reseeded at different plating densities, it was clearly shown that in order for a colony of potentially transformed cells to express that transformation it must achieve a minimum size of

TABLE VIII. Effects of IBX (20 mg/kg/injection) Given Twice Daily by I.P. Injection on RBC and WBC Counts*

Treatment duration (days)	RBC/ml × 10^{-6}	WBC/ml × 10^{-3}
0	6.3 ± 0.7[a]	9.1 ± 2.3
2	4.9 ± 1.1	7.2 ± 1.2
5	6.4 ± 1.0	8.4 ± 2.1
9	5.4 ± 0.8	13.4 ± 3.1
12	5.8 ± 0.2	11.5 ± 7.3
16	7.1 ± 1.1	6.2 ± 1.2
19	5.6 ± 0.8	6.8 ± 1.7
19 (PBS control)	5.9 ± 0.8	5.6 ± 1.6

*Groups of 10 treated and 10 control C57BL/6J mice were treated with IBX in PBS or with an equal volume of 0.2 ml PBS for the duration of the experiment. At the indicated times, 10 μl of blood were removed from tail veins of 6 mice for each time period, and RBC and WBC counts were performed as described in the text. Mice weighed 19.5 ± 1.0 g in the treated group and 19.9 ± 0.9 g in the control group prior to treatment and 20.4 ± 1.1 and 21.6 ± 1.1 g, respectively, after treatment.

[a]Mean ± S.D. of 6 mice.

(From [8]).

from 32–256 cells before it comes into contact with expanding colonies of non-transformed cells [22]. This interaction was suggested in our original studies on the inverse relationship between plating and transformation frequency [5]. In the studies described above (Table I), for a constant plating density an increase in serum concentration was shown to cause a dose responsive decrease in transformation frequency. This increase in serum concentration produces an increase in saturation density of confluent cells (Fig. 2); thus, at confluence, each colony of potentially transformed cells would occupy the same area regardless of serum concentration but these colonies would contain increasing numbers of cells. This is achieved by a progressive decrease in the spreading of cells on the plastic substrate. It is apparent that a colony cannot be viewed simply as a two dimensional plate, but as a disc of finite thickness. Our model predicts that as the depth of this disc increases (owing to decreased spreading), so the effective area for interaction with neighboring cells also increases, as does the potential for informational flow. We are currently constructing mathematical models for this phenomenon.

In our view, the most likely mechanism for informational transfer between contacting cells is via gap junctions, and this view is strengthened by our failure to detect extracellular growth inhibitory factors (Table 6). Gap junctions consist of hydrophilic channels between cells that have been positively correlated in many cell types with the ability to transfer ions [23], small biologically active molecules [24], and dyes [25] between contacting cells. The molecular weight limit to passage is about 1,000. For review of the relationship between these structures and the phenomenon of metabolic cooperation between contacting cells see [26]. This model would therefore call for the passage via gap junctions of small molecular weight growth inhibitory substances between contacting cells. It is presumed that this substance would require a critical concentration for activity. If factors such as diffusion constant or half-life of the molecule were constant, it is clear that the size of the clone would be directly related to signal intensity. This would provide a means for an overall control of the cell density in a particular cellular compartment. Bell has proposed a model to integrate this concept with the demonstrated ability of tumor cells, after a prolonged latency, to overcome population density effects [27]. Transformed cells could either fail to respond, fail to secrete, or would destroy that signal at a higher rate than normal cells. In the latter two cases, growth of the transformed cells could only occur after a critical clonal size had been reached. A third possibility, not considered by Bell, would be the failure of these cells to conduct the signal. In all cases, except in the case of a failure to respond, autonomous growth would occur through attentuation of signal intensity and thus, would be conditional upon factors controlling signal strength. We believe that our studies have identified two such factors operative in vitro (ie, cell density and cAMP concentration). Furthermore, the demonstrated ability of transformed cells to be growth inhibited under conditions of high signal transfer (cell crowding) or high signal intensity (elevated cAMP) suggests that in our system autonomous growth is due to signal attenuation rather than to a failure to respond.

What factors could cause this signal attenuation? The most satisfactory explanation at present is that transformed cells are deficient in their ability to form communication channels with themselves or with adjacent normal cells. Deficiencies in ionic coupling have been demonstrated in human tumor cells (HT) in comparison with normal human fibroblasts (HF). Communication between these cell types was

in the HT:HT< HT:HF< HF:HF [28]. Reduced coupling has also been demonstrated to occur between neoplastic liver cells [29]. In the original observation by Stoker's group [30] of the ability of nontransformed cells to inhibit growth of transformed cells, metabolic coupling, presumably mediated via gap junctions, was found to be operative [31]. Of interest to the present studies is the report that addition of dibutyryl cAMP and theophilline to a transformed Syrian hamster fibroblast cell line caused a partial restoration of a gap-junctional defect in these cells [32]. Deficient communication in neoplastic cells may be expected to be a consequence of membrane alterations that occur in neoplasia [33-34] and/or the known disorganization of the cytoskeletal system [15,35]. Elegant studies by Lawrence et al have shown that the transfer of cAMP can occur between communicating cells and that cells receiving this messenger react in appropriate ways [24]. Although not yet shown, it is conceivable that the compartmentalization of signal made possible by localized transmission could result in specific responses not observable with treatment protocols that expose the whole cell to high drug levels. Restoration of signal intensity in transformed cells in this model could therefore occur by the formation of more junctional complexes/cell (cell crowding effect) or by increasing signal intensity (PDE inhibitor effect). From the studies of Wright et al [32] it appears possible that an initial stimulation of communication could lead to further restoration through correction of the defect.

Our studies in vivo have shown that the major effect of PDE inhibitors is to increase the latent period between tumor injection and the appearence of that tumor. This has been shown both for a SC growing tumor [9] and for lung metastases (Fig. 13). In these experiments, once tumors had grown to a sufficient size to be palpable, or to cause death in the most susceptible animals, drug therapy had little effect on the subsequent growth or death rate. This again would be predicted from the model of Bell [27] as owing to attentuation of signal. This same effect was shown in the studies by Haber et al [22] in the 10T1/2 system.

It is clear from this discussion that agents decreasing intercellular communication should effectively reduce the minimum clone size for tumor autonomy and thus lead to an acceleration of the carcinogenic process. Tumor promoters such as TPA act by increasing the probability that a carcinogen-initiated cell can express its neoplastic potential within the life span of the individual. While it is clear that the action of TPA is multifaceted, one intriguing possibility is that they act on the terminal phase of neoplastic progression by inhibiting metabolic cooperation between initiated cells and neighboring normal cells. Trosko's group has recently shown that TPA and other promoters indeed inhibit metabolic cooperation between wild-type and mutant CHO cells, presumably by inhibition of gap-junctional communication [36]. Furthermore, early studies by Sivak et al [37] demonstrated that TPA could inhibit the ability of nontransformed 3T3 cells to inhibit growth of transformed cells. We have recently been able to repeat these studies in the 10T1/2 cell line and have shown that growth arrest of neoplastic cells, produced by coculturing with 10T1/2 cells in high serum (see Table I), can be inhibited by treatment with TPA (unpublished results).

Most normal cells become arrested in G_1 and may enter a state of profound cell cycle arrest known as G_0 [38]. The model proposed here is that growth inhibitory signals normally passing between normal cells, and which cause G_1 arrest in these cells, are now allowed to enter neoplastic cells in sufficient concentrations and

to cause cell cycle arrest. It therefore follows that these latter cells should also become arrested at this physiological growth restriction point (ie, G_1). In the studies reported here, using the labeled mitosis method to measure cell cycle parameters, we found that both PDE inhibitors caused an extended G_1 interval in Lewis lung carcinoma cells growing as lung metastases in C57B1 mice (Fig. 13). Furthermore, the reduced labeling index in treated tumors suggested that in some cells this G_1 extension was sufficiently long so as to effectively put them out of cycle during the observation period. Similar studies have not yet been conducted in the in vitro system but the time course of inhibition and restoration of thymidine labeling (Fig. 10) suggests that a similar mechanism may be operative. The nature of the transmitted signal is unknown. The large elevations of cAMP observed in treated 10T1/12 cells (Fig. 7) and the demonstration by Lawrence [24] that cAMP can pass between coupled cells makes this a prime candidate, especially since cAMP serves as a messenger in many other systems. It is clear however that there exist many other candidates, eg, Ca^{++} [39,40]. Also, until junctional communication can be unequivocally demonstrated to be implicated in the transfer of growth control, other possibilities such as direct membrane/membrane interactions triggering receptor activation [41,42] or the production by serum or PDE inhibitors of competition for available substrate as discussed above cannot be ignored. We hope that these studies will clarify the role of cell/cell communications in the process of malignant transformation and lead to new modalities of cancer therapy.

ACKNOWLEDGMENTS

This work was supported by grant CA21359 and core grant CA24538.

REFERENCES

1. Kakunaga T: Cancer Res 35:1637, 1975.
2. Berenblum I, Shubik P: Br J Cancer 3:384, 1949.
3. Boutwell RK: Exp Tumor Res 4:207, 1964.
4. Merriman RL, Bertram JS: Cancer Res 39:1661, 1979.
5. Reznikoff CA, Bertram JS, Brankow DW, Heidelberger C : Cancer Res 33:3239, 1973.
6. Bertram JS: Cancer Res 37:514, 1977.
7. Bertram JS: Cancer Res 39:3502, 1979.
8. Bertram JS, Janik P: Cancer Lett 11:63, 1980.
9. Janik P, Assaf A, Bertram JS: Cancer Res 40:1950, 1980.
10. Wexler H: J Nat Cancer Inst 36:641, 1966.
11. Chen TT, Heidelberger C: Int J Cancer 4:166, 1969.
12. Kakunaga T: Int J Cancer 12:463, 1973.
13. Bertram JS, Heidelberger C: Cancer Res 34:526, 1974.
14. Chlapowski FJ, Kelly LA, Butcher RW: Adv Cyclic Nucleotide Res 6:245, 1975.
15. Puck TT: Proc Natl Acad Sci USA 74:449, 1977.
16. Rudland PS, Seeley M, Seifert W: Nature (London) 251:417, 1974.
17. Sheppard H, Wiggan G: Mol Pharmacol 7:111, 1971.
18. Londos C, Cooper DF, Schlegel W, Rodbell M: Proc Natl Acad Sci USA 75:5362, 1978.
19. Mayo JG: Cancer Chemother Rep 3:325, 1972.
20. Gilbert CW: Cell Tissue Kinet 5:53, 1972.
21. Simpson-Herren L, Sanford AH, Holmquist JP: Cell Tissue Kinet 7:349, 1974.
22. Haber DA, Fox DA, Dynan WS, Thilly WS: Cancer Res 37:1644, 1977.
23. Corsaro CM, Migeon BR: Nature (London) 268:737, 1977.

24. Lawrence TS, Beers WH, Gilula NB: Nature (London) 272:501, 1978.
25. Flagg-Newton J, Simpson I, Loewenstein WR: Science 205:404, 1979.
26. Hooper ML, Subak-Sharpe JH: Int Rev Cytol 69:45, 1981.
27. Bell GI: Science 192:569, 1976.
28. Corsaro CM, Migeon BR: Proc Natl Acad Sci USA 74:4476, 1977.
29. Borek C, Higashino S, Loewenstein WR: J Membr Biol 1:274, 1969.
30. Stoker MGP, Shearer M, O'Neill C: J Cell Sci 1:297, 1966.
31. Stoker MGP: J Cell Sci 2:293, 1967.
32. Wright ED, Slack C, Goldfarb PSG, Subak-Sharpe JH: Exp Cell Res 103:79, 1976.
33. Warren L, Buck CA, Tuszynski GP: Biochim Biophys Acta 516:97, 1978.
34. Hakomori S: Biochim Biophys Acta 417:55, 1975.
35. Pollack R, Osborn M, Weber K: Proc Natl Acad Sci USA 72:994, 1975.
36. Yotti LP, Chang CC, Trosko JE: Science 206:1089, 1979.
37. Sivak A, Van Duuren BL: Science 157:1443, 1967.
38. Pardee AB: Proc Natl Acad Sci USA 71:1286, 1974.
39. MacManus JP, Boynton AL, Whitfield JF: Adv Cyclic Nucleotide Res 9:485, 1978.
40. Paul D, Ristow HJ: J Cell Physiol 98:31, 1979.
41. Ali IU, Mautner V, Lanza R, Hynes RO: Cell 11:115, 1977.
42. Yamada KM, Yamada SS, Pastan I: Proc Natl Acad Sci USA 73:1217, 1976.