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Studies on the effect of 8-Cl-cAMP on the proliferation of mammalian cells

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City University of New York, 1993

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STUDIES ON THE EFFECT OF 8-Cl-cAMP ON THE PROLIFERATION OF MAMMALIAN CELLS

BY

ZHIYONG HAN

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1993

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ABSTRACT

Studies on the effect of 8-Cl-cAMP on the proliferation of mammalian cells

by

Zhiyong Han

Adviser: Professor James H. Wyche

The cAMP analogue, 8-Cl-cAMP, was originally thought to inhibit the proliferation of various tumor cell lines via its selective interaction with type II cAMP-dependent protein kinase (type II PKA). In addition, it was proposed that the inhibitory effect of 8-Cl-cAMP on cell proliferation was selective for tumor-derived or transformed cells. In this study, we have confirmed others' observation that 8-Cl-cAMP can effectively inhibit the proliferation of various types of cultured cell lines. However, we have also found that the inhibitory effect of 8-Cl-cAMP on cell proliferation is not limited to tumor cell lines, it also inhibits the proliferation of various normal cell lines. In addition, we have found that the inhibition of cell proliferation by 8-Cl-cAMP is dependent on the presence of serum. Our study has indicated that serum-containing cyclic nucleotide phosphodiesterase and phosphotases which were responsible for catalyzing the breakdown of 8-Cl-cAMP, generate 8-Cl-adenosine which then exerts the inhibitory effect on the proliferation of various cells. Therefore, the inhibitory effect of 8-

Cl-cAMP on cell proliferation is not an effect of 8-Cl-cAMP per se, but that of 8-Cl-adenosine. Our study further suggests that the serum-dependent inhibitory effect of 8-Cl-cAMP on cell proliferation is not associated with potential cytotoxicity of the metabolites of 8-Cl-cAMP. Therefore, this inhibition of cell proliferation by 8-Cl-adenosine may indicate a unique regulatory pathway of cell proliferation.

We have identified and characterized two unique cell systems for future studies on the molecular mechanism of the serum-dependent inhibitory effect of 8-Cl-cAMP on cell proliferation. In this study, we have found that basic fibroblast growth factor (bFGF) could specifically and selectively antagonize the inhibitory effect of 8-Cl-cAMP on the proliferation of endothelial cells. Using endothelial cells as a model system, we have found treatment of endothelial cells with 8-Cl-cAMP in the presence of serum down-regulated the phosphorylation of cellular proteins. Further, the ability of bFGF to selectively antagonize the inhibitory effect of 8-Cl-cAMP on the proliferation of endothelial cells was well correlated with its ability to restore the phosphorylation of proteins in these cells. Thus, it suggests that one aspect of the mechanism of the serum-dependent proliferation inhibitory action of 8-Cl-cAMP involves the negative regulation of phosphorylation of cellular proteins.

Using human promyelocytic leukemia cell line HL-60 as another model system, we have observed that treatment of HL-60 cells with high concentrations of 8-Cl-cAMP resulted in loss of their viability. We have thus used 8-Cl-cAMP and isolated a clone, HCW-2, as a variant of HL-60. The viability of HCW-2 cells is unaffected by 8-

Cl-cAMP and its metabolites, but the proliferation of these cells remained sensitive to 8-Cl-cAMP, 8-Cl-AMP and 8-Cl-adenosine. In addition, we have found that HCW-2 cells do not possess any form of nuclear RI or RII of PKA, suggesting that nuclear RIIb might not be a necessary requirement for 8-Cl-cAMP-induced inhibition of cell proliferation. The noncytotoxic response of HCW-2 cells indicates that these cells should be useful for studying the mechanism of the inhibition of cell proliferation by these agents.

We have also explored the potential therapeutic use of 8-Cl-cAMP as an anticancer agent using HL-60 and HCW-2 cells as experimental systems. We have found that treatment of these cells with 8-Cl-cAMP abolished the tumorigenicity of the cells *in vivo* suggesting that 8-Cl-cAMP might be useful as an anti-leukemia drug in clinical studies.

Acknowledgement

I should like to thank my thesis adviser Dr. James H. Wyche for all the advise, help, and encouragement he has given to me during all these years. He has not only been an academic mentor but also a great friend to me. I thank Dr. Wyche for all the academic freedom he has given to me ever since I joined his laboratory. It allowed me to experience something that rarely occurs to a graduate student. I shall always treasure this experience.

I should also like to thank Drs. Andrew Mendelsohn and Devasis Chatterjee, and Mr. Leonard W. Erickson for their help. Our friendship has meant a great deal to me.

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Zhiyong Han

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TABLE OF CONTENTS

Page No.

List of Tables

List of Figures

INTRODUCTION

The regulation of the proliferation of mammalian cells is a very complicated process involving coordinated functions of many cellular components. Due to this complexity, it has been very difficult to identify cellular factors whose activities are specifically required for the regulation of cell proliferation. In the past, the identification of regulatory factors of cell proliferation has been, more or less, an empirical process in which a potential regulatory factor was initially identified by its effect on the proliferation of cells. The specificity of this effect was then further investigated. Currently, many factors are thought to be potential cell proliferation regulatory factors. However, one of the main problems in studying the regulation of cell proliferation by a particular factor has been the difficulty in assessing whether or not an observed effect of this factor on the proliferation of cells could be physiologically meaningful.

There have been several general experimental approaches in the process of identifying potential regulatory factors of the cell proliferation. One of the approaches has been the study on how the proliferation of cells is positively regulated, e.g., what are the factors that stimulate the proliferation of cells? In the past two decades, many hormonal factors, termed growth factors such as interleukens or ILs (Metcaf, 1985); nerve growth factor or NGF (Cohen et al., 1954; Greene and Shooter, 1980); fibroblast growth factor or FGF (Gospodarowicz et al., 1986); epidermal growth factors or EGF (Cohen, 1962; Carpenter1981; Carpenter et al., 1982, 1983), transforming growth factor alpha or TGFa (Todaro et al., 1980), insulin-like growth factor-I and II or IGF-I and IGF-II (Zapf et al., 1984) and platelet-derived growth factor or PDGF (Ross, 1981), that stimulate the proliferation of various types of cells have been identified, purified, characterized, and cloned.

The second approach involves studies on how the proliferation of cells is negatively regulated, e.g., what are the factors that can specifically inhibit the proliferation of cells? Unfortunately, progress in this area has not been as rapid as those studies on growth factors. However, a few cell-produced inhibitory factors have been identified . For instance, transforming growth factor beta (TGFβ), initially identified as a peptide growth factor for transformed fibroblast cells (Anzano et al., 1982, 1983), was also found to negatively regulate the proliferation of some cell types (Roberts et al., 1985; Sporn et al., 1987). Tumor necrosis factor or TNF (Old, 1985) and interferon or IFN (Langer and Pestka, 1985; Sagar et al, 1982) were identified by their ability to inhibit the proliferation of certain tumor cells.

It is now well known that growth factors stimulate the proliferation of their target cells by interacting with specific receptors in the plasma membrane. The receptors for EGF, PDGF, insulin, IGF-I, colony-stimulating factor (CSF), and FGF have been cloned and found to be endowed with tyrosine kinase activities that phosphorylate tyrosine residues in their target proteins. The interaction between growth factors with their corresponding receptors result in the activation of the receptor tyrosine kinase activity, which in turn phosphorylates specific tyrosine residues of its target proteins in the membrane and cytoplasm (Ushiro and Cohen, 1980; Kasuga et al., 1982; Sherr et al., 1982; Westermark et al., 1982; Jacobs et al., 1983; Rozengurt, 1983; Hunter and Cooper, 1984; Huang and Huang, 1986; Kadowaki et al., 1987; Williams, 1989; Ullrich and Schlessinger, 1990). Through molecular studies it has been demonstrated that the tyrosine kinase activity of various growth factor receptors is absolutely essential for the receptor to mediate the stimulus of the growth factor, such as EGF, insulin and PDGF, in various cells (Chen et al, 1987; Chou et al, 1987; Honegger et al, 1987; McClain et al 1987; Russell et al, 1987; Williams, 1989). Like growth factors, peptide growth inhibitors, such as TGFb, TGF and IFN also interact with specific receptors on the cell surface (Cheifetz et al., 1987; Massague et al., 1986; Sporn et al., 1987; Old, 1985; Sagar et al., 1982). However, unlike the growth factors, the question of whether or not receptors for these growth inhibitors also possess intrinsic enzymatic activity remained to be seen.

In addition to the activation of tyrosine kinases of the receptors, interaction between the growth factor and its receptor also lead to a series of changes in other intracellular biochemical processes including (1) activation of non-tyrosine kinases, such as the activity of protein kinase C (Blackshear et al 1985; Rodriguez-Pena and Rozengurt, 1985; Rozengurt, 1986; Nishizuka, 1988; Pardee, 1989), activity of calmodulin-dependent protein kinase (Ober and Pardee , 1987), activity of raf oncogenic protein (Morrison et al, 1989; Ullrich and Schlessinger, 1990), and phosphotidylinositol 3-kinase (Varticovski et al, 1989); (2) activation of phospholipase C, resulting in a rapid metabolism of cytoplasm membrane-associated phospholipids and accumulation of diacylglycerol (DAG) and triphosphate inositol (IP-3) inside cells (Brown et al, 1984; Farrar and Anderson, 1985; Moolenaar et al, 1988; Margolis et al, 1989, 1990; Meisenhelder et al, 1989); (3) activation of adenylate cyclase and production of cAMP were seen with PDGF and NGF in fibroblast and neuroblast cells respectively (Nikodijevic et al., 1975; Rozengurt et al, 1983a, 1983b); (4) activation of other cellular events such as activation of Na^+/H^+ antiport, and Na^+/K^+ pump, resulting in alkalinization of cytoplasm of the cells (Smith and Rozengurt, 1978; Rozengurt, 1981; Boonstra et al., 1983; Inoue et al., 1988; Ober and Pardee, 1987); (5) release process of calcium ions into cytoplasm from intracellular storage compartments, or entry of calcium ions from extracellular environment (Lopez-Rivas and Rozengurt, 1983; Owen and Villereal, 1983; Berridge et al, 1984; Lopez-Rivas and Rozengurt, 1984; Moolenaar et al, 1984; Mcneil et al, 1985; Majerus et al, 1986; Mendoza et al, 1986; Berridge, 1987; Housey et al, 1988; Wahl et al, 1989); and (6) rapid production of other molecules such as prostaglandins and polyamines (Russell and Snyder, 1968; Kay and Lindsay, 1973; Means and Rasmussen, 1988; Pegg, 1988; Tannock and Rotin, 1989).

Small molecules whose cellular levels are regulated by hormonal regulators including growth factors are now called signal molecules. These signal molecules can specifically interact with specific target proteins intracellularly and induce intracellular biochemical changes that are required for the initiation of the process of cell proliferation. For instance, (1) DAG and Ca^{++} are activators of protein kinase C (PKC) (Kaibuchi et al, 1981; Nishizuka, 1986, 1988). Activation of PKC is a very important step in the initiation of the growth factor-stimulated cell proliferation (Rozengurt, 1986: Nishizuka, 1986, 1988) and as well as to mediate the transforming effect of various viral oncogenes such as *ras*, *sis*, *fms*, *fes* and *src* (Gould et al., 1985; Jackoswski et al., 1986; Preiss et al., 1986); (2) the growth factor-induced activation of the activities of ion pumps such as that of Na^+/H^+ was demonstrated to be responsible for growth factor-induced alkalinization of cytoplasm of the cells (Rozengurt, 1985; Smith and Rozengurt, 1978). Such alkalinization was shown to be required for cells to enter a proliferating state (Pousseyur et al., 1984; Pouyssegur et al., 1986); and (3) inhibition of the cellular synthesis of polyamine has been shown to inhibit the proliferation of various cell lines (Heby and Jane, 1981).

Adenosine 3',5'-cyclic monophosphate (cAMP) is a well known signal molecule. It was initially identified as the signal molecule for mediating the regulatory effect of epinephrine and glucagon on glucose metabolism (Sutherland and Rall, 1958; Sutherland and Butcher, 1965; Robinson, Butcher, and Sutherland, 1968). Currently, cAMP is also considered as a regulator of cell proliferation. In general, it appears that the proliferation of some epithelia and fibroblasts is positively regulated by cAMP (Boynton and Whitfield, 1979; Dumont et al, 1989).

On the other hand, a cAMP-induced inhibitory effect on the proliferation of various cells has also been observed. It has been reported that treatment of different cell lines with cAMP analogues such as N^6, 2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (DBt-cAMP), N^6-monobutyryladenosine 3':5'-cyclic monophosphate (MBt-cAMP) and 8-Br-adenosine 3':5'-cyclic monophosphate (8-Br-cAMP) could greatly inhibit the proliferation of various cell types (Ryan and Heidrick, 1968; Hsie and Puck, 1971; Froehlich and Rachmeler, 1972; Blat et al., 1973; Grimes and Schroeder, 1973; Hsie et al., 1975; Nose and Katsuta, 1975; Weidman and Gill, 1976; Dipasquale and McGuire, 1977; Tupper et al., 1978; Boynton and Whitfield, 1983; Johnson et al, 1988). Therefore, these studies suggest that cAMP might be a bifunctional signal molecule for the regulatory process of cell proliferation.

The primary mechanism for cAMP-induced stimulation or inhibition of the cell proliferation is the activation of cellular cAMP-dependent protein kinase or PKA (Robison, Butcher and Sutherland, 1968; Krebs and Beavo, 1979). The holoenzyme of PKA consists of 4 subunits, 2 regulatory subunits (R) and 2 catalytic subunits (C); the R subunits are further divided into type I and type II (RI and RII), and RI and RII could be further classified as RII alpha and beta (RIIα and RIIβ) and RI alpha and beta (RIα and RIβ) respectively (Krebs, 1972; Rosen et al, 1977; Krebs and Beavo, 1979; Taylor, 1989). Two major isoforms of PKA have been described as a results of the association between C subunits and a particular type of R subunits. 2RII/2C is a type II PKA and 2RI/2C is a type I PKA (Krebs and Beavo, 1979:

Taylor, 1989). It is clear that these isoforms of PKA contain identical C subunits, but the R subunit differs (Taylor, 1989). The function of these R subunits was shown to inhibit the catalytic kinase activity of the C subunits (Hoffman et al., 1975; Flockhart and Corbin, 1982). The activation process of PKA by cAMP involves the binding of cAMP to R subunits of the holoenzyme, resulting in the disassociation between R and C subunits. The freed C unit subsequently phosphorylates its target proteins at serine or threonine residues (Hoffman et al, 1975; Krebs and Beavo, 1979; Rannel and Corbin, 1980; Flockhart and Corbin, 1982).

In general, there is no evidence to suggest that there might be differential biological functions between type I and type II PKA. However, during the course of numerous studies on the inhibitory effect of cAMP on cell proliferation, cAMP-resistant mutants have been isolated from different cell lines. These mutants have been shown to possess lower than normal cellular levels of PKA (Insel et al., 1975; Schimmer et al., 1977; Cho-Chung and Clair, 1977c; Evain et al., 1979; Abraham et al., 1986; Gottesman and Fleischmann, 1986; Gottesman et al, 1980). In addition, characterization of a DBt-cAMP-resistant mutant of Walker rat mammary tumor cell line demonstrated that a specific lack of type II PKA in the nucleus of those cells confirmed their resistance to DBt-cAMP (Cho-Chung et al., 1977a, 1977b). Therefore, this study suggested that type II PKA in the nucleus is primarily responsible for mediating the inhibitory effect of cAMP on cell proliferation.

This view that type II PKA was responsible for mediating the inhibitory effect of cAMP on cell proliferation seemed to be further supported by some recent studies on the inhibitory effect of 8-chloro-adenosine 3':5'-cyclic monophosphate (8-Cl-cAMP). First, it was shown that the 8-halogen substituted cAMP analog, at low concentrations, selectively bound to R II and activates type II PKA *in vitro* (Robinson-Steiner and Corbin, 1983; Ogreid et al, 1985) and secondly, unlike many other cAMP analogues, 8-Cl-cAMP, at very low concentrations (10-50 uM), exerted an extremely potent inhibitory effect on the proliferation of various tumor cell lines *in vitro* (Katsaros et al., 1987; Tortora et al., 1988; Ally et al., 1988; Tagliaferri et al, 1988a, 1988b; Tortora et al, 1989a, 1989b, 1990). In addition, it has also been reported that treatment of breast carcinoma cell lines (MDA-MB-231 and MCF-7), colon carcinoma cell line (LS-174T), and leukemia cell line (HL-60) with 8-Cl-cAMP induced 5-10 fold increases in the expression of nuclear RIIβ and concomitant decreases in RIα in these cells (Katsaros et al., 1987; Tagliaferri et al., 1988; Cho-Chung et al., 1989). Interestingly, it was found that the proliferation of those cells was resistant to DBt-cAMP-treatment and DBt-cAMP did not cause changes in the expression and the levels of nuclear RIIβ in these cells. These experimental observations have led to the hypothesis that 8-Cl-cAMP selectively activates type II PKA in the cell, which then directly induces high Levels of nuclear ratio of RIIβ/RI. This change of nuclear RIIβ/RI ratios is responsible for mediating the inhibitory effect of 8-Cl-cAMP on cell proliferation (Cho-Chung et al., 1989;

Cho-Chung, 1989). A recent report has provided additional evidence that support this hypothesis. In that study, human promyelocytic leukemia cell line, HL-60, was treated with synthetic anti-sense oligonucleotides against mRNA encoding human RIIβ proteins and it was shown that this treatment dramatically reduced the cellular RIIβ at the protein level and completely abolished the inhibitory effect of 8-Cl-cAMP on the proliferation of these cells (Tortora et al., 1990).

However, there were reports that demonstrated that the observed inhibition of cell proliferation by certain cAMP analogs, such as DMt-cAMP (Schroder and Plagemann, 1971; Granner et al., 1975: O'Neill et al., 1975; Beavers et al., 1976; Hargrove and Granner, 1980), 8-Br-cAMP (Niles et al., 1979; Martin and Ronning, 1971), and Ara-cAMP (LePage and Hersh, 1972), may not be mediated by PKA. These studies suggested that metabolites of these cAMP analogs exerted the observed inhibitory effect on the proliferation of cells. In addition, 8-Cl-adenosine has been found to inhibit the proliferation of cells (Tagliaferri et al., 1988a). Therefore, whether or not the inhibition of cell proliferation by 8-Cl-cAMP treatment was due to specifically an effect of 8-Cl-cAMP or its metabolites remains to be investigated.

SPECIFIC AIMS OF THIS STUDY

1. TO STUDY THE MECHANISM OF INHIBITION OF THE PROLIFERATION OF VARIOUS CELL LINES BY 8-Cl-cAMP: It has been concluded that the inhibitory effect of 8-Cl-cAMP on cell proliferation is limited to tumor-derived and transformed cell lines (Cho-Chung, 1989; Cho-Chung et al., 1989). But there has no apparent explanation to why 8-Cl-cAMP does not inhibit the proliferation of normal cell lines. However, based on the proposed mechanism of how 8-Cl-cAMP inhibit the proliferation of tumor cells (Cho-Chung et al., 1989), the inability of 8-Cl-cAMP to inhibit the proliferation of normal cell lines suggests several possibilities including (1) that normal cells do not possess type II PKA; (2) that normal cells possess type II PKA, but only in the cytosolic compartment, and 8-Cl-cAMP could not induce translocation of type II PKA from the cytosolic compartment into the nucleus; and (3) that besides type II PKA, additional cellular factors are required for mediating the inhibitory effect of 8-Cl-cAMP, and these factors are missing in normal cells It appears that to study the mechanism of how 8-Cl-cAMP inhibits the proliferation of tumorigenic cells, the use of normal cell lines could serve as a appropriate control system for studying the mechanism of normal cell resistance to 8-Cl-cAMP. Therefore, one of the objectives in this study was to study the mechanism of normal cell-associated resistance to 8-Cl-cAMP.

In addition, an effort was made to isolate and characterize 8-Cl-cAMP-resistant revertants of tumorigenic cell lines. It was our hope that comparative studies on the mechanism of resistance to 8-Cl-cAMP between normal cell lines and 8-Cl-cAMP-resistant revertants will be helpful in understanding the mechanism of how 8-Cl-cAMP inhibits the proliferation of tumorigenic cells.

2. TO STUDY THE POTENTIAL REGULATORY EFFECT OF 8-Cl-cAMP ON THE MITOGENIC ACTION OF DIFFERENT GROWTH FACTORS IN VARIOUS CELL LINES: In the case of 8-Cl-cAMP-induced inhibition of the proliferation of v-Ki-*ras*-transformed normal rat kidney (NRK) cell line, it was reported that 8-Cl-cAMP inhibited the production of TGFa which was an autocrine growth factor for those cells (Tortora et al., 1989a). However, 8-Cl-cAMP-induced inhibition of the production of TGFa alone might not be the reason that the proliferation of those cells was inhibited by 8-Cl-cAMP, because addition of purified TGFa to the culture medium of those cells did not reverse the inhibition of their proliferation by 8-Cl-cAMP (Tortora et al., 1989a). Therefore, it appears that the mechanism of 8-Cl-cAMP-induced inhibition of the proliferation of v-Ki-*ras*-transformed NRK cells would involves more than an inhibition of TGFa production.

TGFa is known to be a mitogen for both normal and transformed NRK cell lines (Derynck, 1988). However, It has been suggested that unlike v-Ki-*ras*-transformed NRK cells, the proliferation of normal NRK cells is not inhibited by 8-Cl-cAMP (Chu-Chong, 1989; Chu-Chong et al., 1989). Therefore, this view seems to suggest that 8-Cl-cAMP only selectively inhibit the mitogenic function of growth factors including TGFa in transformed cells. Currently there is no evidence to suggest that the signal transduction mechanism of a growth factor in a normal cell line differs from that in its tumorigenic variant(s). Thus the above conclusion poses a question that needs to be answered. That is, does 8-Cl-cAMP differentially regulate the growth stimulatory function of a growth factor, such as TGFa, between a normal cell line and its tumorigenic variant(s)? And if so, how ?. For this reason, part of this study was thus focused on an investigation as to whether or not 8-Cl-cAMP may differentially regulate the growth stimulatory activity of a particular growth factor between a normal cell line and its tumorigenic variants.

3. TO STUDY AND EVALUATE THE ANTI-CANCER POTENTIAL OF 8-Cl-cAMP USING THE HUMAN PROMYELOCYTIC LEUKEMIA CELL LINE, HL-60, AS A MODEL SYSTEM: It has been reported that inoculation of nude mice with L1210 leukemia cells plus 8-Cl-cAMP significantly increased the life span of the animals in comparison to those animals inoculated with L1210 cells alone (Avery et al., 1988). In addition to this study, it has been reported that treatment of various leukemia cell lines including HL-60, K526, and MOLT-4 with 8-Cl-cAMP *in vitro* not only inhibited the proliferation of these cells, but also induced differentiation of these cells (Tortora et al., 1988, 1990). Because the development of leukemia has been believed to be caused by the inability of leukemia cells to differentiate into mature non-proliferative cells (Gallo, 1975; Greaves, 1982; Sachs, 1982; Bloch, 1984; Sartorelli, 1985; Sachs, 1987), it thus appears that 8-Cl-cAMP might have anti-leukemia potential. For this reason, we also plan to use the HL-60 cell lines as a system for an initial study on the anti-leukemia potential of 8-Cl-cAMP.

4. TO INVESTIGATE WHETHER THE INHIBITION OF CELL PROLIFERATION BY 8-Cl-cAMP TREATMENT IS MEDIATED BY ITS METABOLITES: A recent study indicated that treatment of cells with 8-Cl-cAMP or 8-Cl-adenosine at the same concentrations inhibited the proliferation of cells to similar degrees (Tagliaferri et al., 1988a). In addition, other studies also demonstrated that the inhibitory effect of cAMP analogs, such as DBt-cAMP (Schroder and Plagemann, 1971; Granner et al., 1975; Bevers et al., 1976; Hargrove and Granner, 1980), 8-Br-cAMP (Niles et al., 1979; Martin and Ronning, 1981), and Ara-cAMP (LePage and Hersh, 1972), was actually mediated by their metabolites. Therefore, we want to investigate whether or not the inhibitory effect of 8-Cl-cAMP on cell proliferation might be mediated by its metabolites.

MATERIALS AND METHODS

Materials

All tissue culture dishes, flasks or plates (Falcon brand); liquified phenol, and hypochlorite used in this study were obtained from Fisher Scientific Inc. (Springfield, N.J.). Fetal bovine serum; 1X trypsin-EDTA solution; RPMI 1640, Coon's modified F-12, DME (4.5 grams glucose/L), MEM, McCoy's 5a media; N6,O2'-dibutyryl adenosine 3',5'-monophosphate; N^6-monobutyryl 3':5'cAMP; 8-Br-adenosine 3':5'-monophosphate; Adenosine 3':5'-monophosphate; 8-Br-adenosine; 2-Cl-adenosine; adenosine; butyric acid; forskolin; dimethylformamide; 3-isobutyl-1-methylxanthine; adenosine deaminase of calf intestinal mucous (200 units/mg protein); 3':5'-cyclic nucleotide phosphodiesterase of bovine heart (0.28 units/mg protein); phenylmethylsufunyl fluoride; dipyridamole; NP-40; Triton X-100; zinc-free insulin; and ion-saturated transferrin were obtained from Sigma Chemical Cop. (St. Louis, MO). 2,8-[^{3}H]adenosine (25 Ci/mmol); 8-Cl-cAMP; and [^{32}p]8-azido-cAMP (50 Ci/mmol) were purchased from ICN Biochemicals, Inc. (Costa Meca, CA). [^{32}p]phosphoric acid (disodium salt) was obtained from Du Pont-New England Nuclear (Boston, MS). Recombinant human basic and acidic fibroblast growth factor, and mouse epidermal growth factor were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Recombinant human insulin-like growth factor I was purchased from Mallincrodt Chem. Co. (St. Louis, MO). N,N'-methylene-bis-acrylamide; acrylamide; N,N,N'',N'-tetramethyl-ethylenediamine;

ammonium persulfate; sodium dodecyl sulfate; 2-mercaptoethanole; SDS-PAGE molecular weight standards; and protein assay Kit were obtained from BioRad Laboratories Ltd. (Melville, NY). 8-Cl-cAMP; 8-Cl-AMP; 8-Cl-adenosine; 8-Cl-IMP; 8-Cl-inosine; 8-Cl-hypoxanthine; and 8-Cl-xanthine were obtained from BioLog Life Science Institute (La jolla, CA). Bovine bFGF was supplied by Dr. Michael Klagsburn (Harvard University, Medical School). Recombinant human insulin-like growth factor II was supplied by Eli Lilly Co. (Indianapolis, IN). Pig platelet-derived growth factor was supplied by Dr. Harry Antoniades (Blood Research Center, Harvard University, Boston).

Cell Lines

(1) 18-54,SF is a rat tumor epithelial cell line that is capable of growing in serum-free Coons' modified F-12 medium (Wyche and Noteboom, 1979). It has been shown to form tumors in nude mice (Wilson et al., 1987).

(2) BRL-3A is an epithelial cell line derived from Buffalo rat liver (Nissley et al., 1975). This cell line was purchased from American Type Culture Collection (ATCC, Rockville, Maryland) and was cultured in Coon's modified F-12 medium supplemented with 5% FBS.

(3) OVIN is a metastatic rat ovary tumor epithelial cell line, (Turner and Wyche, 1980). This cell line was cultured in Coon's modified F-12 medium supplemented with 5% FBS.

(4) Clone 2-6 is a rat hepatoma cell line and was provided to us by Dr. Nelson Fausto (Pathology Dept., Brown University, Providence, RI). This cell line was cultured in DME/ F-10 (1:1) medium

supplemented with 10% FBS, 10 ng/ml EGF, 10 ug/ml insulin, and 10 nM hydrocortisone.

(5) MCF-7 is a human breast adenocarcinoma cell line. It was provided by Dr. Mark Lippman (Gorgetown University, Gorgetown, MA). This cell line was cultured in MEM medium supplemented with 10% FBS.

(6) A431 is a epithelial cell line derived from human epidermoid carcinoma. It was provided by Dr. Peter Shank (Dept. of Biology, Brown University, Providence, R.I.). This cell line was maintained in DME with 4.5 g/L glucose supplemented with 10% FBS.

(7) G401 is a Wilms' tumor epithelia cell line. It was purchased from American Type Culture Collection. This cell line was maintained in McCoy's 5a medium supplemented with 10% FBS.

(8) DLD-1 clone A is derived from a human colon tumor cell line DLD-1, It was provided to us by Dr. Devasis Chatterjee (Dept. of Biology, Brown University, Providence, R.I.). This cell line was cultured in RPMI 1640 medium supplemented with 10% FBS.

(9) HL-60 is an established human promyelocytic leukemia cell line (Collin et al., 1977). It was purchased from American Type Culture Collection. This cell line was cultured in RPMI 1640 medium supplemented with 20% FBS.

(10) Y79 is a human retinoblastoma cell line. It was provided to us by Dr. Robert Weinberg (Whitehead Biomedical Research Institute, MIT, Boston, MA). This cell line was cultured in RPMI 1640 medium supplemented with 20% FBS.

(11) NIH3T3 is a normal mouse embryonic fibroblast cell line. It was provided to us by Dr. Peter Shank (Dept. of Biology, Brown University,

Providence, R.I.). This cell line was cultured in DME medium with 4.5 g/L glucose supplemented with 10% FBS.

(12) ARK is a normal rat kidney fibroblast cell line. It was originally provided to us by Dr. Harold Moses (Vanderbilt University, Nashville, TN). This cell line was cultured in McCoy's 5a medium supplemented with 10% FBS.

(13) WI38 is a normal human embryonic lung fibroblast cell line. It was provided to us by Dr. Peter Shank (Dept. of Biology, Brown University, Providence, R.I.). This cell line was cultured in DME medium with 4.5 g/L glucose supplemented with 10% FBS. Culture of this cells below passage 15 were used for this study.

(14) GMO 3652A is a normal skin dermal fibroblast cell line derived from a male adult. It was originally obtained from the Human Genetic Mutant Cell Repository, and was provided to us by Dr. Cheryl Conover (Mayo Clinic, Minneapolis, MS). This cell line was cultured in DME medium with 4.5 g/L glucose supplemented with 10% FBS and 10 ng/ml EGF. Cells below passage 20 were used for this study.

(15) Endothelial cells: Primary cultures of Bovine aortic endothelial (BAE) cells and clonally isolated BAE cells were provided by Dr Sharon Round and Mrs. Lilli Hsieh (VA Hospital, Providence, R.I.) and Dr, Robert Auerbach (Dept. of Zoology, Wisconsin University, Madison, WS). The endothelial origin of these cells was confirmed by immunohistological analysis for endothelial specific markers. These cells were cultured in DME medium with 4.5 g/L glucose supplemented with 20% FBS. Cells below passage 20 were used for this study.

(16) L6 is a rat skeletal muscle myoblast cell line. It was provided to us by Dr. Andrew Mendelsohn (Dept. of Molecular Biology, Massachusetts General Hospital, Boston, MS). This cell line was cultured in DME medium supplemented with 10% FBS. Cells between passage 6 and 12 were used for this study.

Note: The media for above cell lines were supplemented with 100 U/ml of penicillin and 50 U/ml of streptomycin. All the stock cultures of above cell line were cultured in T75 tissue culture flasks (Falcon product), in a humidified tissue culture incubator with 5% CO_2, 95% air at 37°C.

Abbreviations

Acidic fibroblast growth factor: aFGF
Adenosine deaminase: ADA
adenosine 3',5'-monophosphate: cAMP
8-Br-adenosine 3',5'-monophosphate: 8-Br-cAMP
8-Cl-adenosine 3',5'-monophosphate: 8-Cl-cAMP
all trans-retinoic acid: tRA
Basic fibroblast growth factor: bFGF
Dimethylformamide: DMS
Dipyridamole: DPD
Dulbecco's modified Eagle's medium: DME
Epidermal growth factor: EGF
fetal bovine serum: FBS
Forskolin: FK
insulin-like growth factor I: IGF-I

insulin-like growth factor II: IGF-II
3-isobutyl-1-methylxanthine: IBMX
N,N'-methylene-bis-acrylamide: bis
Minimum essential medium: MEM
M^6-monobutyryl adenosine 3',5'-monophosphate: MBt-cAMP
N^6,O^2-dibutyryl adenosine 3',5'-monophosphate: DBt-cAMP.
N,N,N'',N'-Tetramethyl-ethylenediamine: TEMED
Phenylmethylsufunyl fluoride: PMSF
Phosphodiesterase: PDE
Platelet-derived growth factor: PDGF
Sodium dodecyl sulfate: SDS
Transforming growth factor alpha: TGFα

Methods

Proliferation Assay: Approximately $3X10^3$ cells in 2 ml of the medium containing 5% calf serum were plated into 35 mm dishes. Alternatively $1X10^3$ cells in 1 ml of medium were plated into each well of 24 well plates. The cells were incubated with 5% carbon dioxide: 95% air and 100% humidity at 37°C for 24 hours. The medium was then removed, and replaced with fresh medium containing 10% fetal bovine serum (FBS) with or without various additions as indicated in the text or legends. The HL-60 and Y9 cells were seeded in the medium containing 10% FBS and treated with various agents at the time of seeding. Cells were cultured for another 3 days (for HL-60 and Y79 it was 4 days). At the end of this assay, cells were trypsinized and the total number of cells per dish was determined.

Alternatively, cell proliferation was determined by counting total numbers of cells per dish daily until two days after the cells reached their maximum density in a monolayer. These data were used for the proliferation of the cell.

Cell Proliferation in Serum-free Media: After an initial 24 hours of pre-incubation as outlined above, the medium for various cell lines was replaced with the appropriate serum-free medium plus or minus various additions. The cells were cultured in this medium for 6 days. The serum-free medium for DLD/1-A, and HL-60 cell lines was basal RPMI 1640 medium supplemented with 10 ug/ml insulin, 10 ug/ml transferrin and 5 ng/ml selenium. For A431 and G401 cell lines, the DME and McCoy's 5a basal media respectively containing 10 ug/ml of insulin, 10 ug/ml of transferrin and 5 ng/ml of selenium. For ARK cell line basal McCoy's 5a medium containing 10 ug/ml insulin, 10 ug/ml transferrin, 5 ng/ml of bFGF, and 5 ng/ml of EGF; For NIH3T3 cells basal DME medium supplemented with 2 ng/ml PDGF, 10 ng/ml EGF, 10 ug/ml insulin, and 10 ug/ml transferrin. For BRL-3A and OVIN cell lines, basal Coons' Modified F-12 medium supplemented with 10 ug/ml insulin, 10 ug/ml transferrin, and 10 ng/ml EGF. The 18-54,SF cell line grew in basal Coon's Modified F-12 medium without any supplements.

Assessment of Chemical Toxicity: Potential cytotoxicity of a chemical in the cell was assessed by its effect on the cell viability. Briefly, after the cells were treated with a chemical, 25 ul of phosphate buffered saline containing 0.4% trypan blue was added to the dishes, and the cells were incubated in this medium for 5 minutes at room-temperature. The percentage of unstained cells among at least 300 cells under a microscope was randomly determined and used as a measurement for cell viability.

***In Vitro* Assay for The Production of proliferation inhibitor(s) from 8-Cl-cAMP:** Approximately 100 ul of the 8-Cl-cAMP stock (100 uM in basal serum-free medium) was mixed with 100 ul of fetal bovine serum (FBS), or 100 ul of heat inactivated (100°C, 5 minutes) fetal bovine serum (HIFBS). The mixture was incubated at 37°C for various times and the incubation was terminated by heating the mixture at 100°C for 3 minutes. This pre-incubated mixture was then mixed with the appropriate serum-free medium in a final volume of 1 ml and added to cells in 24 well tissue culture plates prepared for a growth assay. The cells were cultured in this medium for 3 days, and at the end of this period, the total number of cells per well was determined. Each point was performed in triplicate, and each experiment was repeated two times.

Determination of Intracellular cAMP Level: Cells were quickly detached from the substratum into single cell suspension in 10 ml of PBS containing 1 mM EDTA. One ml of this suspension was used for the determination of cell density, and the cells in the rest of the suspension was pelleted by centrifugation at 10,000 rpm in a bench-top clinical centrifuge (Fisher Centrific Centrifuge) for 1 minute at room-temperature. The cells were resuspended in 50 mM sodium acetate buffer (pH 5.2) at a final density of 10^6 cells per 100 ul of the buffer. This suspension was immediately heated at 100°C for 5 minutes. The lysate was centrifuged at 12,500 g in a microfuge for 5 minutes. The supernatant was transferred into another tube, stored at 4°C and used for cAMP assay. A radioimmunoassay (RIA) originally developed by Biomedical Technology Institute Inc. (BTI Inc., Bedford, MA) was used for the determination of cAMP levels in the extract.

Isolation of HCW-2 as A Variant of HL-60 Cell Line: Approximately $5X10^7$ HL-60 cells in 10 ml of RPMI 1640 medium supplemented with 20% FBS and 100 uM of 8-Cl-cAMP were seeded into 100 mm tissue culture dishes and cultured for 10 days. The medium was gently removed by aspiration and 10 ml of fresh medium containing 10% FBS and 10 uM of 8-Cl-cAMP was added to the dishes. The cells were incubated an additional 2 weeks. The medium was then removed, and dishes were supplemented with fresh medium containing 10% FBS and 10 uM 8-Cl-cAMP. The cells were incubated in medium without 8-Cl-cAMP for another 2 weeks. The dishes were examined under a light microscope, and the location of visible colonies loosely attached to the substratum was marked. One colony subsequently named as HCW-2 was identified, isolated, and transferred into a 35 mm tissue culture dish containing 2 ml of medium and 10% FBS. After these cell grew up and formed a monolayer, they were harvested and re-seeded into a 250 ml tissue culture flask containing 30 ml of medium supplemented with 10% FBS and 10 uM 8-Cl-cAMP. These cells were continuously cultured in 8-Cl-cAMP-containing medium for a period of 2 months with a medium change every week. After this period, they were cultured and subcultured in 8-Cl-cAMP-free medium in a ratio of 1:5, and were used for further studies.

Quantitative Analysis of Differentiation of HL-60 and HCW-2 Cells by NBT Staining Technique: The technique developed by Collins et al. (Collins et al., 1978) was adapted for the determination of terminal myelocytic differentiation of HL-60 and HCW-2 cells. Briefly, after cells were treated with chemicals, approximately $2X10^5$ cells in 200 ul of serum-free RPMI medium were transferred to a test tube. The culture was mixed with a equal volume of PBS containing 0.2% of nitro blue tetrazolium (NBT) and 200 ng/ml of tetradecanoylphorbol acetate (TPA). The mixture was incubated at 37°C for 30 minutes. An aliquot of this mixture was applied to a slide, and the differentiated cells were visualized by their blue-black color under a microscope. The degree of terminally differentiated cells was estimated by the percentage of stained cells among a total of approximately 300 randomly examined cells.

Assay for Clonogenicity of HL-60 and HCW-2 Cells: A well developed soft agarose assay ((Hamburger and Salmon, 1977; Salmon et al., 1978; Human Tumor Cell Cloning Bibliography, 1984) was adapted in this study for the determination of the clonogenicity of the cells. Briefly, approximately $1X10^3$ single cells were suspended in 0.5 ml of 0.30% soft-agarose (Bacto agarose) medium at 37°C. This suspension was layered on top of a 0.5% agarose base layer (0.5 ml) in a 35 mm dish. Both layers of the agar medium were prepared with RPMI 1640 medium containing 20% FBS. After the seeding of cells, dishes were kept at room-temperature until the top layer of agarose medium became semisolid and then they were incubated in carbon dioxide incubator at 37°C for 2 weeks. At the end of this incubation, colonies were visualized under a light microscope and total number of colonies in 10 randomly examined fields of every dish was determined. Each determination was done in triplicate, and the experiments were repeated three times.

Assays for The Tumorigenicity of HL-60 and HCW-2 Cells: In this assay, two week-old male Swiss nude mice were used for assaying tumorigenicity of HL-60 and HCW-2 cells. The mice were weighed and separated into groups. Each group of mice was placed into a single cage. Approximately 2X10^6 cells were suspended in 200 ul phosphate buffered saline (PBS) and injected subcutaneously at the back of each mice. For the controls, 200 ul of PBS was injected into control mice. In order to assess the tumorigenicity of chemical pre-treatment of HL-60 and HCW-2 cells, stock cultures of HL-60 and HCW-2 cells in tissue culture flasks were treated with 10 uM of 8-Cl-cAMP, 2 uM of retinoic acid for 3 days, or with 0.8% of DMF for 8 days with a medium change on the 3rd and 6th day. The treated cells were harvested, pelleted and washed extensively with 20 ml of PBS 5 times. During the washing, cell-suspensions were vigorously pipeted. The cell suspensions were centrifuged at low speed (4,000 rpm) for 2 minutes in a bench-top clinical centrifuge (Fisher Centrific Centrifuge) after each washing. After the final washing, the viability of resuspended cells was determined to be above 90%. The cells were resuspended in PBS at a density of 2X10^6 single cells per 200 ul. The mice were kept in a nude mouse facility for 2 months at Biomedical Center of Brown University. At termination, the mice were sacrificed. Tumors were removed from the mice, weighed, and frozen at -85°C in RPMI 1640 medium supplemented with 20% glycerine for further analysis. The bodies of dead mice were properly disposed of according to the rules of the Animal Care Facility of Brown University.

Effect of 8-Cl-cAMP on The Phosphorylation of Proteins in ARK and BAE Cells: Confluent monolayers of ARK or BAE cells in a 24 well tissue culture plate were washed with serum-free medium and then incubated in 1 ml of phosphate-free and serum-free medium for 3 hours. The medium was removed and the cells were re-fed with phosphate-free medium containing 10 uCi of [^{32}p] phosphoric acid. In order to see the effect of 8-Cl-cAMP and bFGF on the phosphorylation of proteins in the cells, 10 uM 8-Cl-cAMP, 10 ng/ml bFGF or 10 uM 8-Cl-cAMP and 10 ng/ml of bFGF were added to the wells. The cells were incubated in this medium for 24 hours. The medium was removed, and the cells were washed 3 times with 1 ml of serum-free medium. The cells were lysed in 200 ul of lysis solution (10 mM Tris-HCl, pH 6.8; 200 uM PMSF; 100 uM sodium orthovanadate; 200 uM IBMX; 50 uM sodium pyrophosphate; and 1% Triton X-100). The lysate was centrifuged at 12,500 g for 15 minutes, and the supernatant was used as the whole cell extract. The protein concentration of the extract was determined with a assay kit for the determination of microquantity of proteins (Micro-Bradford assay kit, BioRad Inc.). Approximately 10 ug of proteins were analyzed by electrophoresis in a 10% polyacrylamide gel containing 1% SDS prepared by a standard technique (Laemmli, 1970). After electrophoresis, the gel was quickly fixed and stained in 250 ml of solution containing 50% TCA and 0.01% brilliant blue for 5 minutes at room-temperature. The gel was destained in 500 ml of solution containing 10% acetic acid for 30 minutes at room-temperature. The gel was then soaked in a solution containing 10% methanol and 20% glycerine overnight at room-temperature. The gel was dried onto a piece of whatman No.4 paper under a vacuum and exposed to a X-ray film at -80°C overnight. The autoradiogram was developed thereafter.

Preparation of Cell Extracts for The Determination of Adenosine Deaminase (ADA): Monolayers of cells in 100 mm tissue culture dishes were washed once with 10 ml of serum-free medium, and incubated in 10 ml of medium containing 10% FBS or 10% FBS plus other agents for 48 hours or longer. The medium was removed, and the cells were quickly washed with 10 ml of cold (4°C) PBS three times. The cells were scraped off the substratum and harvested with PBS solution (5 dishes/20 ml of PBS). The cells were pelleted by centrifugation at 4°C. The cells were re-suspended in 0.5 ml of 10 mM sodium and potassium phosphate buffer (pH 7.0) containing 200 uM phenylmethylsufonyl fluoride (PMSF), and immediately frozen in liquid nitrogen. The cells were quickly thawed at 37°C, and the freezing and thawing cycle was repeated twice. The lysate was centrifuged at 12,500 g in a microfuge for 15 minutes at 4°C. The supernatant was dialyzed against 4,000 ml of the above phosphate buffer for 10 hours at 4°C in order to remove adenosine from it. After the dialysis, the protein concentration of the extract was determined using a microassay kit for proteins determination (BioRad product) and then used immediately for an assay.

Estimation of Adenosine Deaminase: This assay was a modified method originally developed by Martinek (Martinek, 1963). Briefly, 100 ul of 10 mM adenosine in 10 mM sodium and potassium phosphate buffer (pH 7.0) containing 200 uM PMSF was mixed with 500 ug proteins from the cell extract or various known amount of ADA standard (Sigma product) in tubes, and the volume of the mixture in each tube was adjusted to 250 ul with the phosphate buffer. The tubes were stoppered, and incubated at 37°C for 3 hours. The reaction was terminated by injecting 0.5 ml of phenol color reagent solution (3.6% liquified phenol, 0.02% sodium nitroferricyanide) and 0.5 ml of alkali-hypochlorite reagent solution (0.15 N NaOH, 0.2% sodium hypochlorite) to the tubes using a syringe. The tubes were incubated at 37°C for 20 minutes for color development. For a control, as soon as the cell extracts were mixed with the substrata solution, the phenol color and alkali-hypochlorite reagents were added to the tubes and the tubes were incubated at 37°C for 3 hours. Spectrophotometer reading was made at 640 nm using a Varian DMS 80 spectrophotometer. A standard curve of known amount of ADA activity and the release of ammonia from adenosine was constructed based on the readings and the amount of ADA in the cell extract was deduced from this curve. Each point was performed in triplicate and the experiment was repeated three times.

Measurement of The Uptake of Adenosine in ARK and BAE Cells: Confluent monolyers of ARK and BAE cells in 24 well plate were washed with 1 ml of serum-free medium, and incubated in 0.5 ml of proper medium containing 10% FBS or 10% FBS and various concentrations of 8-Cl-cAMP. The cells were incubated for 48 hours in this medium. Additional 0.5 ml of medium containing 10% FBS and 5 uCi of [^{3}H] adenosine (200 nM) was added to the wells and the cells were incubated in this medium for additional 20 minutes. The medium was removed and the cells were quickly washed with 1 ml of serum-free medium three times. The cells were lysed in 0.5 ml of 1 N NaOH solution overnight at 37°C in a humidified incubator. 0.25 ml of the lysate was transferred to a scintillation vial containing 5 ml of Hydrocount Cocktail LSC solution (J.T. Baker Chemicals). Radioactivity was determined in a scintillation counter (Packard Minaxi beta, Tri-Carb 4000 series). To see the effect of bFGF on this process, the cells were pre-incubated in 0.9 ml of medium containing 10% FBS and various concentrations of bFGF for 20 minutes in an tissue culture incubator. Then 0.1 ml of medium containing 10% FBS and 5 uCi of [^{3}H] adenosine (200 nM) was added to the wells and the cells were incubated at 37°C for another 20 minutes. The cells were washed, lysed and the cell-associated radioactivity was determined in a same way as described above. In this study, the final concentrations of 8-Cl-cAMP and bFGF in the form of presentation was expressed based on 1 ml as the final volume of the incubation medium. Each point was performed in triplicate and each experiment was repeated three times.

Measurement of Adenosine Efflux in ARK and BAE Cells: Confluent monolayers of the cells in 24 well plates were incubated in 1 ml of medium containing 10% FBS and 400 nM of [^{3}H] adenosine at 37°C for 20 minutes in the CO_2 incubator. The medium was then removed, and the cells were quickly washed with 1 ml of serum-free medium 3 times. The cells were re-incubated with 1 ml of medium containing 10% FBS and various concentrations of bFGF at 37°C for 30 minutes in the incubator. The medium was removed and saved. The cells were quickly washed with 1 ml of serum-free medium 3 times. The washing was combined with the previously removed medium. The total radioactivity in this combined medium was determined. The cells were lysed in 0.5 ml of 1 N NaOH solution, and the the total radioactivity in it was determined in a scintillation counter. The effect of bFGF on the efflux of adenosine in the cells was expressed by both the radioactivity in the medium and the cell-associated radioactivity after the treatment.

Adenosine Metabolism: Monolayers of various cell lines in 24 well tissue culture plates were incubated with 0.5 ml of medium containing 10% FBS or 10% FBS and 20 uM 8-Cl-cAMP for 48 hours. Additional 0.5 ml of medium containing 10% FBS and 5 uCi of [^{3}H] adenosine (200 nM) was added to the wells and the cells were incubated in this medium for another 24 hours. The medium was removed, and the cells were quickly washed with 1 ml of serum-free medium three times. The cells were lysed in 0.5 ml of lysis buffer (10 mM Tris, pH 7.5; 150 mM NaCl; 5 mM magnesium chloride, and 0.65% NP-40), The lysate was transferred to a microcentrifuge tube and centrifuged at 12,500 g for 5 minutes at 4°C in order to pellet the nuclei. The supernatant (cytosolic fraction) was transferred to another tube and 2 ml of ice-cold 10% trichloroacetic acid (TCA) solution was added to the tube and the content was well mixed. The mixture was incubated on ice for 30 minutes. The acid insoluble materials was separated from acid-soluble materials by centrifugation. The insoluble material was taken as cytosolic nuclei acids. To the tube containing the pellet of nuclei, 1 ml of 10% TCA was added. The tubes were incubated on ice for 30 minutes. The acid insoluble materials were pelleted by centrifugation. The supernatant was combined with previous acid soluble materials of cytosolic fraction and was used as total cellular acid-soluble nucleotides pool. The acid-insoluble materials from the nuclei was taken as nuclear nucleic acid. The acid-insoluble materials were dissolved in 1 ml of 1 N NaOH overnight at room-temperature and the radioactivity in it was determined. The radioactivity of a aliquot of the acid-soluble materials was also determined. Each treatment was performed in triplicate and each experiment was repeated three times.

Analysis of cAMP-binding Proteins: After the treatment of the cell when indicated in the text, the cells from 5 dishes (100 mm dishes) were scraped off the substratum and harvested with 10 ml of PBS. The cells were pelleted by centrifugation at 10,000 rpm in a bench-top clinical centrifuge at room-temperature for 1 minute. The pelleted cells were lysed in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.5; 150 mM KCl; 5 mM magnesium chloride; 200 uM PMSF; 0.65% NP-40). The lysate was vortexed for 15 seconds, then centrifuged at 12,500 g for 2 minutes in a microcentrfige. The supernatant was kept as cytosolic fraction, and the pellet was resuspended in 10 ml of lysis buffer, vortexed, recentrifuged at 10,000 rpm in a clinical centrifuge at 4°C for 5 minutes, and the pellet was kept as nuclear fraction. The cytosolic fraction was further centrifuged at 40,000 g for 30 minutes at 4°C, and the supernatant was used as a final cytosolic extract. The nuclear pellet was resuspended in 200 ul of 2 M KCl, incubated on ice for 60 minutes with vortexing every 10 minutes. The incubated nuclear lysate was centrifuged at 12,500 g in a microfuge for 15 minutes at 4°C. The supernatant was used as a final nuclear extract. Both cytosolic and nuclear extracts were dialyzed in 10 liters of binding buffer (10 mM Tris-HCl, pH 7.5; 20 mM KCl; 5 mM magnesium chloride; 200 uM of PMSF; 100 uM IBMX) for 10 hours at 4°C. Concentrations of proteins in the extracts were determined by a protein assay kit (MIcroBradford assay kit, BioRad Inc.). The samples were stored at -20°C for further assaying.

The cAMP-binding proteins were analyzed by a modified method originally developed by Richards and Rolfes, 1980). Briefly, 10 ug proteins (cytosolic extracts) or 20 ug proteins (nuclear extracts) were mixed with 1 uM of $[^{32}p]$ 8-azido-cAMP in a final volume of 20 ul of the binding buffer. The mixture was incubated in the dark at 4°C for three hours. The mixture was then irradiated with UV light (20 watts at a distance of 20 cm) for 60 seconds. The sample was then mixed with 5 volumes of cold acetone (-20°C), and the proteins were precipitated at -20°C overnight. The proteins were pelleted by centrifugation at 12,500 g in a microfuge for 15 minutes at 4°C. The supernatant was discarded accordingly, and the pellet was dissolved in 20 ul of SDS-sample buffer (10 mM Tris-HCl, pH 6.8; 1% SDS; 10 mM mercaptoethanol; 2 M sucrose; 0.01% violet blue), and heated at 100°C for 5 minutes. The proteins in the samples were resolved by electrophoresis in a 10% polyacrylamide gel prepared by a standard technique (Laemmli, 1970). After the electrophoresis, the gel was quickly fixed and stained in 200 ml of a solution containing 50% TCA and 0.01% commassie blue for 5 minutes at room-temperature and destained in 500 ml of destaining solution (10% acetic acid) at room temperature for 30 minutes. The gel was then soaked in a solution containing 10% methanol and 20% glycerine overnight at room-temperature. The gel was dried onto a piece of Whatman No.4 paper under vacuum and exposed to a X-ray film. The autoradiogram was developed and each cAMP-binding protein was localized according to its characterized molecular weight (Haley, 1975; Skare et al., 1977; Richards and Rolfes, 1980). To quantify the labelled bands of cAMP-binding proteins on an autoradiogram, the autoradiogram was analyzed through scanning with the aid of a computer program, Image 1.33. In this analysis, the background of the autoradiogram was taken as zero unit.

Treatment of Fetal Bovine Serum: Ten ml of fetal bovine serum (Sigma product) was placed into dialysis bags with a molecular cut-off size of 12,000 dalton. The serum was dialyzed against 4,000 volumes of PBS at 4°C for 72 hours with a buffer change every 24 hours. For acid- and alkaline-treatment, serum was dialyzed against 4,000 volumes of PBS containing either 0.1 M of acetic acid or 0.1 M NaOH at room-temperature for 24 hours. Acid- and alkaline-treated serum was then dialyzed against 10,000 volumes of PBS at 4°C for 72 hours with a buffer change every 24 hours. For heat-treatment, 5 ml of aliquot of fetal bovine serum in a caped glass tube was heated at 100°C for 5 minutes with the cap on. The tubes were then cooled down to room temperature and used directly in assays. Acetone-treatment of serum was performed as the following: 10 ml of fetal bovine serum was mixed with 5 volumes of cold acetone (-20°C), and serum proteins were precipitated at -20°C overnight. Proteins were pelleted by centrifugation. Residual acetone in the pellet was removed by flushing the centrifuge tubes with nitrogen gas. Precipitated proteins was dissolved in 10 ml of PBS, extracted at room-temperature for 30 minutes, and the non-soluble materials were removed by centrifugation. In order to remove steroids from serum, fetal bovine serum was mixed with sterilized charcoal (Fisher product) in a ratio of 1 gram charcoal/20 ml of serum in a sterilized tube. This mixture was incubated at room-temperature for 24 hours with constant rotations. The charcoal was removed by filtration. For biological assay, all treated serum preparations were aseptically filtered through a 0.22 um filter before use.

RESULTS

A. EFFECT OF 8-Cl-cAMP ON THE PROLIFERATION OF CULTURED TUMOR AND NORMAL CELL LINES

1. Effect of 8-Cl-cAMP on The Proliferation of Tumor Cell Lines: In this initial study, the effect of 8-Cl-cAMP on the proliferation of the human epidermal carcinoma cell line A431, Wilms' tumor cell line G401, colorectal tumor cell line DLD-1 Clone A, breast carcinoma cell line MCF-7, retinoblastoma cell line Y79, promyelocytic leukemia cell line HL-60, and rat hepatoma cell line Clone 2-6, rat ovary metastatic tumor cell line OVIN, and a IGF-II-secreting rat tumor cell line 18-54,SF was studied using the cell proliferation assay. The data obtained are summarized in Table 1. These results indicate that in the cell proliferation assay, the IC_{50} value (a concentration of 8-Cl-cAMP required for inducing 50% inhibition of cell proliferation) of 8-Cl-cAMP was approximately 1 uM for the DLD-1 Clone A, A431, MCF-7, G401, Y79 and Clone 2-6 cells. A maximum of approximately 75-95% inhibition of the proliferation of most cell line was induced by approximately 5-10 uM of 8-Cl-cAMP. In addition, the IC_{50} value of 8-Cl-cAMP for HL-60 cell was approximately 0.25 uM and approximately 95% of the proliferation of these cells was inhibited by 1 uM 8-Cl-cAMP. However, the data in Table 1 also indicate that the serum-independent proliferation of 18-54,SF cells was not affected at all by 8-Cl-cAMP. This observation indicates that the 18-54,SF cells should provide a useful system for studying the mechanism of tumor cell resistance to 8-Cl-cAMP.

2. Effect of 8-Cl-cAMP on The Proliferation of Normal Cell Lines: A key claim made by others was that 8-Cl-cAMP only inhibited the proliferation of tumor-derived or transformed cell lines (Cho-Chung, 1989; Cho-Chung et al., 1989). Therefore, we first screened several normal cell lines for their resistance to 8-Cl-cAMP using the cell proliferation assay. As shown by the data in figure 1, we found that treatment of several normal cell lines including a rat kidney fibroblast cell line ARK, adult human skin fibroblast cell line GMO 3652A, mouse embryonic fibroblast cell line NIH3T3, human lung embryonic fibroblast cell line WI38, and rat skeletal myoblast cell line L-6, with 10 uM 8-Cl-cAMP resulted in approximately 75-80% inhibition of their proliferation. At this concentration of 8-Cl-cAMP, the proliferation of primary bovine endothelial cells (BAE) was inhibited by approximately 45%. These results obviously contradicted others' conclusion that 8-Cl-cAMP did not inhibit the proliferation of normal cell lines (Cho-Chung et al., 1989; Tagliaferri et al., 1989a).

During this course of our study, we noticed that in others' studies the transformed NIH3T3 and NRK cells were cultured in serum-free medium in the cell proliferation assay (Tagliaferri et al., 1988a; Totora et al., 1989a), whereas in our study, all the cells were cultured in media supplemented with 10% fetal bovine serum (FBS). Therefore, we investigated the possibility that a serum factor(s) might somehow influence the ability of 8-Cl-cAMP to inhibit cell proliferation. The data in figure 2 indicate that 8-Cl-cAMP inhibited the proliferation of ARK, NIH3T3 and WI38 cells in the presence of 10% FBS, and the inhibitory effect of 8-Cl-cAMP was dose-dependent. These data indicate that the IC_{50} of 8-Cl-cAMP for all three cell lines was approximately 1.2 uM, and a maximum of approximately 85% inhibition of the proliferation of these cells was induced by as low as 5 uM 8-Cl-cAMP. The data in figure 3 indicate that similar to its inhibitory effect on the proliferation of the normal cell lines, the inhibitory effect of 8-Cl-cAMP on the proliferation of 18-54,SF, BRL-3A, DLD-1 Clone A, A431, G401, MCF-7, and HL-60 was also serum-dependent, because treatment of these cell lines in serum-free medium with as high as 10 uM 8-Cl-cAMP had no effect on their proliferation. These results suggest that the inhibitory effect of 8-Cl-cAMP on the proliferation of both normal and tumor-derived cell lines was serum-dependent.

B. INITIAL CHARACTERIZATION OF THE ACTIVITY OF THE SERUM FACTOR(S) AT THE BIOCHEMICAL LEVEL

1. The Activity of The Serum Factor(s) Varies Among Various Species: The FBS-dependent inhibitory effect of 8-Cl-cAMP on the proliferation of the normal (figure 2) and tumor cells (figure 3) lead us to investigate if other sera might also promote the inhibitory effect of 8-Cl-cAMP on cell proliferation. In our study, 10% of the serum derived from different species or from bovine at different stages of development were supplemented to the culture medium of 18-54,SF cells and the effect of 8-Cl-cAMP on the proliferation of these cells was determined. The data in Table 2 indicate that 8-Cl-cAMP inhibited the proliferation of 18-54,SF cell line in the presence of 10% of bovine sera with an IC_{50} value of approximately 1-1.2 uM. In the presence of 10% pig, horse, rabbit or rat serum, the IC_{50} value was approximately 0.5 uM; in the presence of 10% sheep and chicken serum, IC_{50} value was approximately 5 uM whereas in the presence of 10% human serum, it was approximately 23 uM. These data indicate that all sera tested could promote the inhibitory effect of 8-Cl-cAMP on the proliferation of cells, but the ability of the serum to promote the inhibitory effect of 8-Cl-cAMP on the cell proliferation was in the order of pig = horse = rabbit = rat > sheep = chicken > human.

2. Some Chemical and Physical Properties of The Serum Factor(s): We used the 18-54,SF cell line cultured in the serum-free medium for our initial characterization of some chemical and physical properties of this serum factor(s) that is required for the inhibitory effect of 8-Cl-cAMP on the proliferation of cells. The data in figure 4 (pg indicate that 8-Cl-cAMP did not inhibit the proliferation of these cells when the medium was supplemented with 10% heat-treated FBS (100°C, 5 minutes), acidified FBS (dialyzed against 0.1 M acetic acid for 12 hours and then neutralized), alkalinized FBS (dialyzed against 0.1 M NaOH for 12 hours and then neutralized) and acetone-treated FBS (serum proteins were first precipitated with 5 volumes of acetone at -20°C, and redissolved in PBS). However, extensive dialysis of (dialyzed against 10,000 volumes of PBS at 4°C for 3 days with a daily change of buffer), and treatment of FBS with activated charcoal did not affect its ability to promote the cell proliferation inhibitory effect of 8-Cl-cAMP. These observations suggest that this factor(s) in the serum is non-steroidal and a heat, acid alkaline and acetone-sensitive macromolecule or a small molecule that is tightly associated with a heat, pH or acetone sensitive macromolecule.

3. Development of An *In Vitro* Assay for Studying The Biochemical Mechanism of Serum Activity: The serum-dependent inhibitory effect of 8-Cl-cAMP on the proliferation of various cell lines suggested several possibilities for the involvement of serum factor(s) in this process: (1) The serum contains novel carrier proteins that bind adenosine nucleotides and to facilitate the uptake of 8-Cl-cAMP into the cells; (2) 8-Cl-cAMP itself does not have any inhibitory effect on the proliferation of cells but is converted into a growth inhibitory metabolite(s), which is responsible for exerting the proliferation inhibitory effect on the cells; and (3) This serum factor(s) may stimulate the cell to express mediators, such as type II PKA, that mediate the inhibitory effect of 8-Cl-cAMP. The finding that the activity of this serum factor(s) was heat-sensitive indicated that this property could be exploited in sorting out these possibilities. If the factor(s) in serum were involved in metabolizing 8-Cl-cAMP, then incubation of 8-Cl-cAMP and serum for sufficient length of time *in vitro* before they are treated with heat should result in the production of inhibitory activity of cell proliferation. If on the other hand, the serum factor(s) functions as a carrier protein or to stimulate the expression of mediators of 8-Cl-cAMP in the cells, then heat-treated pre-incubation mixture of 8-Cl-cAMP with serum should not have any inhibitory effect on cell proliferation. Based on these assumptions, we developed an assay. In the first part of this assay, 100 ul of 8-Cl-cAMP stock (100 uM 8-Cl-cAMP in Coon's modified F-12 medium) was mixed with 100 ul of FBS or heat-inactivated FBS (HIFBS) (100°C, 5 minutes) and this mixture was incubated *in vitro* for various length of time at 37°C. The incubation was terminated by heating the mixture at 100°C for 5 minutes, resulting in complete inactivation of the serum factor(s). In the second part of this assay, the heat-treated pre-incubation mixture was aseptically mixed with 0.80 ml of serum-free medium and this final mixture was used as the culture medium for 18-54,SF cells in the cell proliferation assay. The data in figure 5 indicate that incubation of 8-Cl-cAMP with FBS at 37°C resulted in a time-dependent production of cell proliferation inhibitory activities that was no longer dependent on active serum factor(s). In this assay, a minimum of 5 hours of incubation of 8-Cl-cAMP with FBS resulted in the production of the inhibitor(s) that inhibited approximately 90% proliferation of 18-54,SF cells. In contrast, incubation of the same amount of 8-Cl-cAMP with HIFBS for as long as 24 hours did not produce any cell proliferation inhibitory activity. The data in figure 6 demonstrate that the proliferation of other cell lines including ARK, NIH3T3, WI38, DLD-1A, A431, G401, MCF-7, and HL-60 was also very sensitive to the pre-incubation mixture of 8-Cl-cAMP and FBS. These results indicate that this assay provides a excellent system for the investigation of the mechanism by which serum factor(s) promote the growth inhibitory effect of 8-Cl-cAMP. In addition, it suggests that the serum-dependent inhibitory effect of 8-Cl-cAMP on the proliferation of cells probably is not due to the presence of nucleotide-carrier proteins or factors that stimulate the expression of mediators of 8-Cl-cAMP in the serum, but rather the presence of factor(s) which is able to convert 8-Cl-cAMP into some metabolite(s) that inhibits the cell proliferation.

4. The Serum Effect Is Sensitive to The Inhibitor of Cyclic Nucleotide Phosphodiesterase: Cyclic nucleotide phosphodiesterases (PDE) (Butcher and Sutherland, 1962; Beavo et al, 1982) is one of the immediate enzymes that has been shown to be involved in the metabolism of cAMP. The PDE activity can be blocked by isobutylmethylxanthine (IBMX) (Nemecek et al., 1980). Therefore, a potential inhibitory effect of IBMX on the serum-catalyzed production of the proliferation inhibitor(s) from 8-Cl-cAMP was studied. The data in figure 7 indicated that addition of an aliquot of IBMX (50 uM) to the *in vitro* incubation mixture containing 50 uM 8-Cl-cAMP and 50% FBS blocked the production of inhibitor(s) of cell proliferation. Therefore, the IBMX-inhibited production of the proliferation inhibitory activity from 8-Cl-cAMP is probably due to its inhibitory effect on the PDE activity in the serum.

5. Inhibition of Cell Proliferation by 8-Cl-cAMP Is PDE-dependent: To confirm the hypothesis that PDE in the serum was involved in metabolizing 8-Cl-cAMP into metabolites that inhibit the proliferation of cells, we supplemented 10 mU/ml of a partially purified PDE preparation to the appropriate serum-free culture medium of 18-54,SF, DLD-1 Clone A, G401, A43, HL-60, ARK, NIH3T3 and WI38 cells and the effect of 10 uM 8-Cl-cAMP on the proliferation of these cells in this medium was determined. The data in figure 8 indicate that 8-Cl-cAMP inhibited the proliferation of only those cells in the PDE-containing serum-free medium. These results suggest that PDE activity in the serum is probably responsible for catalyzing the metabolism of 8-Cl-cAMP into metabolite(s) that could inhibit the proliferation of cells. However, this observation did not allow us to conclude that PDE might be the only factor that was responsible for this observed effect, because this preparation also contained phosphotases. It could be argued that after the conversion of 8-Cl-cAMP into 8-Cl-AMP by PDE, phosphotases might further catalyze the metabolism of 8-Cl-AMP.

C. IDENTIFICATION OF THE INHIBITORY METABOLITE(S) OF 8-Cl-cAMP

1. Comparative Effects of The Metabolites of 8-Cl-cAMP on Cell Proliferation: To identify the cell proliferation inhibitory metabolite(s) of 8-Cl-cAMP, the effect of 10 uM of 8-Cl-cAMP and its metabolites (8-Cl-AMP, 8-Cl-adenosine, 8-Cl-adenine, 8-Cl-IMP, 8-Cl-inosine, 8-Cl-hypoxanthine, and 8-Cl-xanthine) on the proliferation of ARK, WI38, BAE, HL-60, 18-54,SF, and DLD-1 Clone A cell lines in the serum-containing (10% FBS) medium was studied. The data in figure 9 indicate that the proliferation of all 6 cell lines was inhibited equally by 8-Cl-cAMP, 8-Cl-AMP and 8-Cl-adenosine and the other metabolites had no effect on the proliferation of these cells. These data indicate that 8-Cl-AMP and 8-Cl-adenosine could serve as potential candidates for the proliferation inhibitory metabolite of 8-Cl-cAMP.

2. The Serum-dependent Inhibitory Effect of 8-Cl-cAMP on The Proliferation of Cells Is Sensitive to Adenosine Deaminase (ADA): The above results suggested that if 8-Cl-adenosine was responsible for exerting the inhibitory effect on cell proliferation, then addition of purified ADA to the cells should completely abolish the serum-dependent inhibitory effect of 8-Cl-cAMP on cells. If 8-Cl-AMP was responsible, then the serum-dependent inhibitory effect of 8-Cl-cAMP on cell proliferation should not be affected by ADA. However, if both 8-Cl-AMP and 8-Cl-adenosine additively inhibit the cell proliferation, then exogenous ADA should partially antagonize the serum-dependent inhibitory effect of 8-Cl-cAMP on cell proliferation. To resolve this issue, the effect of 10 uM 8-Cl-cAMP, 8-Cl-AMP or 8-Cl-adenosine on the proliferation of ARK, NIH3T3, WI38, BRL-3A, DLD-1 Clone A, 18-54,SF, A431, G401, HL-60 and BAE cell lines in the medium containing 10% FBS plus or minus 1 unit/ml of ADA was determined. The data in figure 10 indicate that addition of this amount of ADA to the medium completely blocked the serum-dependent inhibitory effect of 8-Cl-cAMP on the proliferation of all the cell lines.

The enzymatic specificity of the ADA towards 8-Cl-adenosine was also investigated. In the experiment, an aliquot of 8-Cl-cAMP, 8-Cl-AMP, or 8-Cl-adenosine was mixed with 1 unit of ADA or an aliquot of buffer for 60 minutes at 37°C *in vitro*. The incubation was terminated by heating the mixture at 100°C for 10 minutes. The effect of the heat-treated mixture on the proliferation of 18-54,SF cells in the presence of 10% FBS was determined. The data in figure 11 indicate that after this pre-incubation, only 8-Cl-adenosine lost its inhibitory effect on cell proliferation. Therefore, these results indicate that the effect of ADA was very specific towards 8-Cl-adenosine.

Together, the data in figure 10 and 11 suggest that 8-Cl-adenosine alone was responsible for mediating the observed inhibitory effect of 8-Cl-cAMP on cell proliferation.

D. SERUM-DEPENDENT PROLIFERATION INHIBITORY ACTION OF 8-Cl-cAMP, ADENOSINE RECEPTORS AND ADENOSINE METABOLISM

1. The Serum-dependent Inhibitory Effect of 8-Cl-cAMP on Cell Proliferation Is Blocked by Dipyridamole: If the serum-catalyzed metabolism of 8-Cl-cAMP occured extracellularly in the cell culture medium, then inhibition of the function of the adenosine nucleotide transport in the plasma membrane should antagonize the serum-dependent inhibitory effect of 8-Cl-cAMP on the proliferation of cells because this transport system regulates the entry of adenosine nucleotides into the cell (Paterson et al., 1983; Best et al., 1979; Parks Jr. et al., 1985). Dipyridamole (DPD) has been shown to inhibit the activity of adenosine transporters in various cells (Afonso, 1970; Henderson et al., 1972; Best et al., 1979; Paterson et al., 1983; Sollevi et al., 1982; Park, Jr. et al., 1985). Therefore, the serum-dependent inhibitory effect of 8-Cl-cAMP on the proliferation of various cell line was studied in the absence and presence of 5 uM dipyridamole. The data in Figure 12 indicate that addition of 5 uM dipyridamole to the medium had no effect on cell proliferation.

However, in the presence of 5 uM of it, treatment of 18-54,SF, BAE, ARK, WI38, NIH3T3, DLD-1 Clone A, G401, A431, HL-60 and BRL-3A cells with 10 uM 8-Cl-cAMP no longer exhibited inhibition of cell proliferation. These results suggest that uptake of the metabolites of 8-Cl-cAMP into the cytoplasm of the cells was via the adenosine nucleotide transport system and the serum-dependent inhibitory effect of the metabolite of 8-Cl-cAMP, 8-Cl-adenosine, is mediated by intracellular factor(s).

2. 8-Cl-cAMP and cAMP Production: Adenosine and its analogs are known to interact with adenosine receptors and affect the adenylate cyclase activity (Sattin and Rall, 1970; Van Calker et al., 1979; Harris et al., 1979; Londos and Wolff, 1977; Fain, 1973; Londos et al., 1980). To see if the serum-dependent inhibitory effect of 8-Cl-cAMP on the proliferation of cells could be related to such a function of adenosine receptors, various cell lines were treated with 10 uM 8-Cl-cAMP in the presence of 10% FBS for 24 hours prior to the analysis of their intracellular cAMP level. The data in figure 13 indicated that such a treatment of the cells had no effect on their intracellular levels of cAMP. Therefore, it suggested that the serum-dependent growth inhibitory effect of 8-Cl-cAMP may not be mediated by adenosine receptors whose function was shown to modulate the adenylate cyclase in the cells.

3. Adenosine Does Not Alter The Serum-dependent Inhibitory Effect of 8-Cl-cAMP on Cell Proliferation: If the inhibitory effect of 8-Cl-adenosine on cell proliferation was because it interfered with normal functions of adenosine, then it suggests that addition of high concentrations of adenosine to the culture medium of cells might antagonize the serum-dependent inhibitory effect of 8-Cl-cAMP on the proliferation of cells. However, as shown by the data in figure 14, addition of 1 mM adenosine alone to the cells had no significant effect on the proliferation of various cell lines, except it slightly inhibited the proliferation of HL-60 and BAE cells, and it did not affect the ability of 1 uM 8-Cl-cAMP to inhibit the proliferation of any of those cell lines. Under this condition, the concentration of adenosine was 1000 time as higher as that of 8-Cl-cAMP. Because at this concentration it did not alter the serum-dependent inhibitory effect of 8-Cl-cAMP to cell proliferation, it suggests that the mechanism of the serum-dependent inhibition of the cell proliferation by 8-Cl-cAMP might not involve interference of functions of adenosine in the cell.

4. 8-Cl-cAMP and The Metabolism of Adenosine: One other possible mechanism of the serum-dependent inhibition of cell proliferation by 8-Cl-cAMP may involve 8-Cl-adenosine nucleotide-induced inhibition of cellular adenosine metabolism. To investigate this possibility, monolayers of BRL-3A, ARK, NIH3T3, and BAE cell lines were incubated in appropriate medium containing 10% FBS and treated with 10 uM of 8-Cl-cAMP for 48 hours. Then an aliquot of [^{3}H]adenosine was added to the medium, and the cells were continuously incubated in this medium for another 24 hours. The incorporation of labelled adenosine into nuclear nuclei acid, cytosolic nuclei acid and total acid-soluble pools of nucleotides was then analyzed. The data in Table 3 indicate that compared to control cells, the treatment of these cell lines with 8-Cl-cAMP had no effect on the incorporation of labelled adenosine into the nuclear and cytosolic cellular pools. The amount of radioactivity in total acid-soluble materials was not noticeably changed with this treatment. Because the potential total amount of 8-Cl-adenosine nucleotides in the cells was 100 times higher than that of labelled adenosine, therefore these data suggest that treatment of these cells with 8-Cl-cAMP for 72 hours in the presence of 10% FBS had no inhibitory effect on the metabolism of adenosine.

E. ENDOTHELIAL CELLS AS A MODEL SYSTEM FOR STUDYING THE MECHANISM OF SERUM-DEPENDENT INHIBITION OF CELL PROLIFERATION BY 8-Cl-cAMP

1. Reversibility of Serum-dependent Inhibition of Cell Proliferation by 8-Cl-cAMP: To see if 8-Cl-cAMP-induced inhibition of cell proliferation was reversible, 18-54,SF, ARK, NIH3T3, DLD-1 Clone A, A431, and OVIN cell lines were initially incubated in appropriate medium containing 10% FBS and 10 uM 8-Cl-cAMP for 5 days. Then half of the cell culture was shifted into 8-Cl-cAMP-free medium and the other half re-fed with fresh 8-Cl-cAMP containing medium. The cells were continuously incubated for an additional 5 days. During this period, the proliferation of the cells was determined daily. The data in figure 15 indicate that the proliferation of these cell lines, except OVIN, was completely inhibited after the cells were treated with 10 uM 8-Cl-cAMP for 48 hours. The proliferation of OVIN cells was only completely inhibited after 4 days of 8-Cl-cAMP-treatment. However, after removal of 8-

Cl-cAMP from their medium, the 8-Cl-cAMP-induced inhibitory effect on the proliferation of all these cell lines was reversed with a lag period of approximately 24 to 48 hours. These results indicate that there is probably no cytotoxicity associated with the serum-dependent inhibitory effect of 8-Cl-cAMP on cell proliferation.

2. Basic Fibroblast Growth Factor FGF Stimulates The Proliferation of Endothelial Cells in The Presence of Serum and 8-Cl-cAMP: Because the serum-dependent inhibition of cell proliferation did not appear to be due to a cytotoxic effect (figure 15), therefore, we explored the possibility that some growth factor might be able to antagonize this inhibitory effect. In our initial study, the effect of epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor I and II (IGF-I, and IGF-II), basic fibroblast growth factors (bFGF), and insulin (Ins) on the proliferation of ARK, BAE, 18-54,SF, L-60 and HL-60 cells cultured in the medium containing 10% FBS and 10 uM 8-Cl-cAMP was determined. The data in figure 16 indicate that in the presence of 10% FBS and 10 uM 8-Cl-cAMP, none of these growth factors could stimulate any observable degree of proliferation of these cell lines except that addition of 10 ng/ml bFGF to the medium of BAE cells stimulated approximately 120% increase in their proliferation. These results indicate that bFGF can selectively antagonize the serum-dependent inhibitory effect of 8-Cl-cAMP on the proliferation of BAE cells.

The data in figure 17 indicate that bFGF was able to stimulate the proliferation of independently isolated strains of endothelial cells such as, BA14B, BA3A, PA5B and BAE 1665 in the presence of 100 uM

8-Cl-cAMP and 10% FBS. This suggests that the mitogenic function of bFGF in endothelial cells might be generally resistant to the serum-dependent inhibitory effect of 8-Cl-cAMP.

The data in figure 18 indicate that although treatment of BA14B, BA3A, PA5B and BAE 1665 cells with 100 uM 8-Cl-cAMP, 8-Cl-AMP, or 8-Cl-adenosine inhibited the proliferation of these cells by approximately 50%, addition of 10 ng/ml of bFGF antagonized the inhibitory effect of all three analogs on the proliferation of these cells. The data in figure 19 indicate that, in the cell proliferation assay, after 5 days of treatment of BA14B cells with 100 uM 8-Cl-cAMP, the cell proliferation was completely inhibited and cells became quiescent. However, even after a 10-days treatment of these cells with 8-Cl-cAMP, addition of 10 ng/ml of bFGF to the 8-Cl-cAMP-containing culture stimulated these cells to re-enter the proliferating state after approximately 48 hours of a lag period. These results, therefore, indicate that studies on the mechanism of how bFGF stimulates the proliferation of the endothelial cells in the presence of serum and 8-Cl-cAMP should provide specific information on the mechanism of the inhibition of the cell proliferation by 8-Cl-cAMP.

3. cAMP Antagonizes The Serum-dependent Inhibition of The Proliferation of ARK and BA14B Cells by 8-Cl-cAMP: Because metabolism of 8-Cl-cAMP into 8-Cl-adenosine is required for the inhibition of cell proliferation by 8-Cl-cAMP, it would be expected to see that addition of high doses of cAMP to the culture medium might antagonize the serum-dependent inhibitory effect of 8-Cl-cAMP on cell proliferation. To confirm this, we cultured ARK and BA14B cells in the medium containing 10% FBS plus or minus 2 mM cAMP, or 50 uM forskolin (FK). As a control, 10 nM PDBu was also supplemented to the medium. The effect of 10 uM 8-Cl-cAMP on the proliferation of these cells was determined in the cell proliferation assay. The data in figure 20 indicate that both cAMP and FK were able to partially antagonize the serum-dependent inhibitory effect of 8-Cl-cAMP on the proliferation of both cell lines. In contrast, PDBu had no effect, suggesting activation of cellular protein kinase C had no effect on this process.

4. Basic FGF Does Not Affect The Cellular Production of cAMP in ARK and BA14B cells: To see if bFGF might selectively stimulate the production of cAMP in BA14B cells and therefore antagonize the serum-dependent inhibition of their proliferation by 8-Cl-cAMP, the effect of bFGF on the production of cAMP in ARK and BA14B cells was determined. As a control, ARK and BA14B cells were treated with 50 uM FK for the same period of time. The data in figure 21 indicate that treatment of ARK and BA14B cells with FK increased their intracellular Levels of cAMP by approximately 1.5 and 2 fold respectively. However, treatment of ARK and BA14B cells with 10 ng/ml of bFGF for as long as 24 hours had no effect on the production of cAMP in both cell lines. Therefore, it suggests that the ability of bFGF to antagonize the inhibition of BA14B cells by 8-Cl-cAMP is probably not mediated by cAMP.

5. Basic FGF Does Not Affect The Production of Adenosine Deaminase in ARK and BA14B Cells: A second possibility would be that bFGF stimulated intracellular accumulation of ADA in the cells, resulting in intracellular conversion of 8-Cl-adenosine into non-inhibitory molecules, such as 8-Cl-inosine. Therefore, we investigated this possibility. The data in figure 22 indicate that when compared to the control, treatment of these cells with bFGF, at concentrations as high as 100 ng/ml, for 24 hours had no effect on the levels of cell-associated ADA activity in both cell lines. This data indicate that the mechanism of bFGF stimulation of BAE cell proliferation in the presence of 8-Cl-cAMP and serum may not involve changes in cellular ADA activity.

6. Basic FGF and The Activity of Adenosine Nucleotide Transport in ARK and BA14B Cells: In order to see if bFGF would somehow differentially affect the process of uptake of adenosine in ARK or BA14B cells, the effect of bFGF on the activity of adenosine nucleotide transport in ARK and BAE cells was studied. Cells were treated with 10 ng/ml of bFGF for 20 minutes prior to the addition of [^{3}H]adenosine to their culture medium. The data in figure 23 indicate that in BA14B but not in ARK cells, bFGF reduced the uptake of adenosine in a dose-dependent manner. Addition of 10 ng/ml of bFGF maximally inhibited approximately 30% uptake of adenosine in BAE cells. These observations suggest that bFGF may play a regulatory role in adenosine uptake in endothelial cells. Therefore, bFGF-induced inhibition of the adenosine transporter activity in BAE cells may be partially responsible for bFGF to antagonize the inhibitory effect of 8-Cl-adenosine on the cell proliferation.

In a second assay, the ARK and BAE cells were pre-loaded with [^{3}H]adenosine and then treated with bFGF to see if bFGF might cause changes in the efflux of adenosine. The data in figure 24A and 24B indicate that such a treatment of cells with bFGF had no effect on the amount of labelled adenosine associated with ARK and BAE cells and in their culture mediums respectively. This indicates that bFGF does not induce efflux of adenosine or possibly 8-Cl-adenosine in these cells.

7. Basic FGF Selectively Antagonizes The Inhibitory Effect of 8-Cl-cAMP on The Phosphorylation of Proteins in BA14B Cells: Since the transduction of the mitogenic signals of bFGF has been found to involve phosphorylation of cellular proteins (Coughlin et al., 1988; Nilsen-Hamilton and Hamilton, 1979), it would be interesting to see if the ability of bFGF to induce protein phosphorylation in ARK and BA14B cells might be differentially regulated by 8-Cl-cAMP-treatment. In the experiment, cells were incubated in phosphate-free medium supplemented with 10 % FBS and [^{32}p]phosphoric acid, and treated with 10 uM 8-Cl-cAMP, 10 ng/ml bFGF, or 10 uM 8-Cl-cAMP and 10 ng/ml bFGF for 24 hours prior to the analysis of phosphorylated proteins. The data in figure 25 represents a typical autoradiogram obtained from such an experiment. It indicates that serum-stimulated ARK and BA14B cells possessed many phosphorylated proteins and the addition of 10 uM 8-Cl-cAMP caused approximately a 75% decrease in the phosphorylation of almost all the proteins seen. Basic FGF on the other hand, enhanced the phosphorylation of the same proteins in both cell lines in the presence of 10% FBS. Basic FGF was unable to antagonize the inhibitory effect of 8-Cl-cAMP-treatment on the phosphorylation of the proteins in ARK cells. However, it completely restored the level of the phosphorylation of every protein to the control (10% FBS). These data indicate that the ability of bFGF to selectively antagonize inhibitory effect of 8-Cl-cAMP on the phosphorylation of proteins in BA14B cells was very well correlated to its' ability to selectively stimulate the proliferation of BA14B cells in the presence of 8-Cl-cAMP and serum. Therefore, it suggests that the inhibitory effect of 8-Cl-cAMP on the phosphorylation of cellular proteins might be part of the mechanism for the serum-dependent inhibitory effect of it on cell proliferation.

8. 8-Cl-cAMP and Subcellular Distribution of RI and RII of PKA in ARK and BA14B Cells: We investigated potential alterations on the expression of nuclear RI and RII of PKA in ARK and BAE cell lines in response to treatment with 5, 10, and 20 uM of 8-Cl-cAMP for 48 hours. The data in figure 26 indicate that when incubated in serum-containing (10% FBS) medium, BA14B cells possessed nuclear RIβ and RIIβ, whereas ARK cells possess RIβ and RIIα. These data also indicate that treatment of BA14B cells with 8-Cl-cAMP for 48 hours did not have a dramatic effect on the level of nuclear RIIβ, but increased the level of RIβ in a dose-dependent fashion. At a concentration of 20 uM 8-Cl-cAMP, the nuclear RIβ content in BA14B cells increased by approximately 3 fold, resulting in a significant reduction in nuclear RIIβ/RIβ ratio in these cells. The data in figure 26 also indicate that treatment of ARK cells with 10 uM of 8-Cl-cAMP for 48 hours caused simultaneous increases in the level of nuclear RIIα and RIβ, resulting in no net change in the nuclear RIIα/RIβ ratio. In addition, this data indicate that 8-Cl-cAMP-treatment did not induce the expression of any demonstrative amount of RIIb in the nucleus of ARK cells.

The data in figure 27A indicate that (1) BA14B cells possessed high levels of cytosolic RIβ and RIIβ; (2) Treatment of BA14B cells with 10-20 uM 8-Cl-cAMP for 48 hours induced approximately up to a 68 and 75% decrease in the cytosolic content of RIβ and RIIβ in the cells respectively; and (3) there were additional cAMP-binding proteins with molecular weight ranging from 39 Kd (p^{39}) to 35 Kd (p^{35}) which appeared in the cytoplasm of BA14B cells. The data in figure 27B indicate that (1) ARK cells possessed very high levels of cytosolic RIβ and RIIα; (2) 8-Cl-cAMP-treatment had no effect on cytosolic levels of RIIα in ARK cells; and (3) treatment of ARK cells with 10 uM 8-Cl-cAMP for 48 hours resulted in a dose-dependent increase in cytosolic content of RIβ.

9. Comparative Effects of Basic FGF and 8-Cl-cAMP on Subcellular Distribution of RI and RII of PKA in ARK and BA14B Cells: If high levels of nuclear RII (particularly RIIβ) are indeed involved in 8-Cl-cAMP-induced inhibition of the growth of various cell lines (Katsaros et al., 1987; Cho-Chung et al., 1989; Tagliaferri et al.,1988a; also see review by Cho-Chung, 1989), then bFGF might alter the levels of nuclear RIIb in BAE cells since it selectively antagonized 8-Cl-cAMP-induced inhibitory effect on the growth of BA14B cells. To investigate this possibility, a comparative study on the effect of bFGF on the levels of nuclear RI and RII between ARK and BA14B cells were studied. The cells were incubated in a medium containing 10% FBS and 10 uM 8-Cl-cAMP, and were treated with 5, 10, and 20 ng/ml bFGF for 48 hours prior to the analysis of their nuclear RI and RII. The data in figure 28 indicate that such a treatment of ARK cells with bFGF caused a dose-dependent decrease in both their nuclear RIIα and RIβ levels, and at a concentration of 10 ng/ml, bFGF induced approximately a 35 and 52% reduction in the contents of nuclear RIIα and RIβ, respectively, in ARK cells. The data in figure 28 also indicate that a similar treatment of BA14B cells with bFGF caused no significant changes in the content of nuclear RIIβ in the cells. However, at concentrations as low as 5 ng/ml, bFGF caused an approximate 65% reduction in the content of nuclear RIβ in BA14B cells, resulting in a higher nuclear RIIβ/RIβ ratio.

The data in figure 29 indicate that when incubated in a medium containing 10% FBS and 10 uM of 8-Cl-cAMP, (1) ARK cells contained very high levels of RIIα and RIβ in their cytoplasm; and (2) treatment of ARK cells with bFGF had no effect on the level of RIIα in the cells. The data in figure 29 also indicate that under similar culture conditions to that employed with ARK cells, (1) BA14B cells had high levels of both RIIβ and RIβ in their cytoplasm; (2) bFGF at concentrations of 10-20 ng/ml caused increases in cytosolic levels of RIIβ and RIβ in BAE cells.

F. ISOLATION OF HCW-2 CELLS AS ANOTHER UNIQUE SYSTEM FOR STUDYING THE MECHANISM OF THE SERUM-DEPENDENT INHIBITION OF CELL PROLIFERATION BY 8-Cl-cAMP

1. 8-Cl-cAMP Selectively Induces Cytotoxic Effect On HL-60 Cells: During the course of our study, we noticed that treatment of HL-60 cells with 8-Cl-cAMP also induced cell death, suggesting that 8-Cl-cAMP was cytotoxic to these cells. The data in figure 30A indicate when cultured in the medium containing 10% FBS, 8-Cl-cAMP-induced cytotoxic effect on the HL-60 cells in a dose-dependent fashion with a IC_{50} value of approximately 6 uM. However, this effect of 8-Cl-cAMP was sensitive to addition of 1 U/ml of ADA. This suggesting that 8-Cl-adenosine was responsible for causing the observed cytotoxic effect. In comparison, the data in figure 30B show that the IC_{50} value of 8-Cl-cAMP for inhibiting the proliferation of the HL-60 cells was only 0.25 uM and this effect was also sensitive to ADA. The data in figure 31 indicate that when cultured in serum-free medium, only 8-Cl-adenosine induced death of the HL-60 cells, whereas in the presence of 10% FBS, 8-Cl-cAMP, 8-Cl-AMP and 8-Cl-adenosine were cytotoxic to the same cells. Therefore, these results suggest that 8-Cl-adenosine has inhibitory effects on both the proliferation and viability of the HL-60 cells.

2. Isolation of HCW-2 Cells As A Variant of HL-60 Cell Line: We decided to use 8-Cl-cAMP as a positive selection agent to select variant that might survive the cytotoxic effect of 8-Cl-cAMP on HL-60 cells. HCW-2 cells were originally isolated in a single colony developed from $5X10^7$ HL-60 cells that had been extensively treated with 100 uM 8-Cl-cAMP for 2 weeks. As shown by the data in figure 32, treatment of HL-60 cells with 10 uM of 8-Cl-cAMP, 8-Cl-AMP or 8-Cl-adenosine for 5 days induced approximately 80% death of HL-60 cells, whereas treatment of HCW-2 cells with the same amount of 8-Cl-cAMP for the same length of time had no effect on their viability. However, as shown by the data in figure 33, the proliferation of HCW-2 remained to be sensitive to 8-Cl-cAMP-treatment. The IC_{50} value of 8-Cl-cAMP for inhibiting the proliferation of HCW-2 cells in the presence of 20% FBS was approximately 0.5 uM. In addition, the data in figure 33 also show that the inhibitory effect of 8-Cl-cAMP on the proliferation of HCW-2 cells was very sensitive to the presence of ADA, because addition of 1 unit/ml of exogenous ADA abolished the inhibitory effect of 8-Cl-cAMP on the proliferation of HCW-2 cells. These results indicate that HCW-2 cells should provide a system for studying the mechanism of the non-cytotoxic effect of 8-Cl-cAMP on the proliferation of cells.

3. Subcellular Distribution of RI and RII of PKA in HL-60 and HCW-2 Cells: It has been reported that treatment of HL-60 cells with synthetic anti-sense oligonucleotides against the mRNA of human RIIβ reduced the expression of RIIβ proteins in, and antagonized the inhibitory effect of 8-Cl-cAMP on the proliferation of these cells (Tortora et al., 1990). Thus, it appeared that RIIβ might play an essential role in mediating the inhibitory effect of 8-Cl-cAMP on the proliferation of the HL-60 cells. The sensitivity of the proliferation of both HL-60 and HCW-2 cells to 8-Cl-cAMP-treatment suggested that these cells may have very high levels of nuclear RIIβ. The data in figure 34 indicate that (1) the nuclear fraction of HL-60 cells contained high levels of both RIIα and RIIβ, but an extremely low level of RI; (2) treatment of HL-60 cells with 10 uM 8-Cl-cAMP for 72 hours induced an approximately 58% increase in their nuclear RIIα, but no correspondent effect could be seen on nuclear RIIβ; (3) treatment of HL-60 cells with 10 uM 8-Cl-cAMP for 24 hours abolished the expression of RI in their nuclear fraction and this effect lasted for 48 hours. However, after 72 hours treatment, cytosolic levels of RI came back to that of control, and even higher after 96 hours; and (4) there was no demonstrative presence of nuclear RII or RI in HCW-2 cells. Treatment of the cells with 10 uM 8-Cl-cAMP for up to 2 months had no effect on the expression of nuclear RII or RI in HCW-2 cells.

The data in figure 35 indicate that (1) HL-60 cells had moderate levels of cytosolic RI, but extremely low levels of RII; (2) treatment of the cells with 10 uM 8-Cl-cAMP for 24 hours rapidly reduced a 7 fold increase in the cytosolic level of RIIβ and decreased the level of RI to a level that was almost undetectable. But after 48 hours treatment of these cells with 8-Cl-cAMP, the cytosolic level of RIIb declined to the original control level, and RI level was restored to a level that was twice as high it was before the treatment; The data also indicated that (1) there were very high levels of cytosolic RIIb and the amount of RIα was approximately 1/10 of that of RIIβ in HCW-2 cells; (2) treatment of these cells with 10 uM of 8-Cl-cAMP for up to 2 months had no effect on the levels of RIIβ, but after 48 hours increased that of RIα by approximately 10 folds. This high level of RIα in these cells remained through out the entire treatment. These data indicate that nuclear localization of RII or RI may not be an absolute requirement for mediating the serum-dependent inhibitory effect of 8-Cl-cAMP in these cells. However, the presence of high levels of cytosolic RIIβ in HCW-2 cells and a transient increase in cytosolic RIIβ in HL-60 cells induced by 8-Cl-cAMP-treatment may be potential involved in the mechnism.

G. STUDIES ON THE ABILITY OF 8-Cl-cAMP TO INHIBIT THE PROLIFERATION AND INDUCE DIFFERENTIATION OF HL-60 CELLS

1. 8-Cl-cAMP and Differentiation of HL-60 and HCW-2 Cells: The ability of 8-Cl-cAMP to induce maturation of HL-60 cells was compared to that of all trans-retinoic acid (tRA) and dimethylformamide (DMF), the two well known agents that induce terminal differentiation of HL-60 cells (Collins et al., 1978, 1980; Lazenby et al., 1987). In addition, the effect of these chemicals on potential differentiation of HCW-2 cells was also studied. The cells were treated with 10 uM 8-Cl-cAMP, or 2 uM of tRA for 3 days, or with 0.8% of DMF for 8 days. Terminal differentiation of the cells was determined by the ability for terminally differentiated cells to convert nitro blue tetrazolium (NBT) into blue-black colored deposit of formazan inside the cells (Collins et al., 1978, 1980; Bewburger et al., 1979). The data in Figure 36 indicate that: (1) treatment of HL-60 cells with 2 uM of tRA for 3 days and 0.8% DMF for 8 days induced approximately 92 and 76%, respectively, of the cells to become terminally differentiated. HCW-2 cells were seen to be none of the terminally differentiated using the same treatment; and (2) 8-Cl-cAMP treatment of both HL-60 and HCW-2 cells did not induce terminal differentiation of these cells. This inability of 8-Cl-cAMP to induce terminal differentiation of HL-60 cells indicate that 8-Cl-cAMP is not a maturation agent for HL-60 cells.

2. Long-term Effects of 8-Cl-cAMP, tRA and DMF on The Proliferation of HL-60 and HCW-2 Cells: Potential long-term effect of the treatment of HL-60 and HCW-2 cells with 8-Cl-cAMP (72 hours), tRA (72 hours) or DMF (8 days) on the proliferation of these cells was studied in the cell proliferation assay. The data in figure 37 indicate that the pre-treatment of HCW-2 cells with 10 uM 8-Cl-cAMP, or 2 uM tRA for 72 hours or with 0.8% DMF for 8 days did not cause the loss of their ability to proliferate. Similarly, 8-Cl-cAMP-treated HL-60 cells proliferated normally. However, both tRA and DMF-treated HL-60 cells could no longer proliferate. These results might be explained by the ability of tRA and DMF to induce the HL-60 cells to differentiate into non-proliferative mature cells whereas 8-Cl-cAMP could not.

3. Comparative Effects of 8-Cl-cAMP, tRA and DMF on The Clonogenicity of HL-60 and HCW-2 Cells: Clonogenicity assay has been widely used as a short-term *in vitro* assay for assessing the ability of cancerous cells to form tumors in vivo (Hamburger and Salmon, 1977; Salmon et al., 1978; Van Hoff et al., 1983; Human Tumor Cell Cloning Bibliography), and the ability of a chemical to induce terminal differentiation of cancer cells has been correlated to its ability to inhibit the clonogenicity of cancer cells (Von Hoff et al., 1983; Salmon et al., 1978; Human Tumor Cell Cloning Bibliography, 1984). Therefore, potential effect of 8-Cl-cAMP on the clonogenicity of HL-60 was compared with that of tRA and DMF. In addition, the clonogenicity of HCW-2 cells was also determined. The data in figure 38 indicate that the clonogenicity of HCW-2 cells was approximately 4 times as higher as that of HL-60 cells. In addition, pre-treatment of HL-60 cells with 10 uM 8-Cl-cAMP or 2 uM tRA for 3 days, or with 0.8% DMF for 8 days abolished the clonogenicity of these cells. In contrast, the same treatment of HCW-2 cells had no significant inhibitory effect on the clonogenicity of these cells. These results indicate that the clonogenicity of HCW-2 cells is resistant to these agents.

4. Comparative Effects of 8-Cl-cAMP, tRA, and DMF on The Tumorigenicity of HL-60 and HCW-2 Cells: A better way for determining anti-cancer effect of a chemical is to study the effect of this chemical on the tumorigenicity of the cells *in vivo* To see how effective 8-Cl-cAMP, tRA or DMF might be in suppressing the tumorigenicity of HL-60 and HCW-2 cells, we treated these cells with 10 uM 8-Cl-cAMP, or 2 uM tRA for 3 days, or with 0.8% of DMF for 8 days *in vitro* before they were injected into nude mice subcutaneously. The data in Table 4 indicate that: (1) after 2 months, all 5 mice developed tumors from HL-60 cells with a average weight of 18.01 grams per tumor; (2) five out of 10 mice developed tumors from HCW-2 cells with an average weight of 11.2 grams; (3) One out of 5 mice developed a tumor (weighing 5.8 grams) from 8-Cl-cAMP-treated HL-60 cells (10 uM 8-Cl-cAMP and 72 hours), and the *in vitro* treatment of HL-60 cells with 2 uM tRA for 3 days, or with 0.8% DMF for 8 days completely abolished their tumorigenicity; (4) treatment of HCW-2 cells with 10 uM 8-Cl-cAMP for 72 hours *in vitro* abolished the tumorigenicity of the cells; and (6) treatment of HCW-2 cells with 2 uM tRA for 72 hours or with 0.8% DMF for 8 days *in vitro* had no effect on their tumorigenicity, because 3 out of 5 mice developed tumors (weighing approximately 11.43 grams) from tRA treated cells, and 2 out of 5 mice developed tumors (weighing approximately 12.30 grams) from DMF-treated cells. These data indicate that HL-60 cells were much more tumorigenic than HCW-2 cells, and the potential for these cells to form tumors *in vivo* was very sensitive to the treatment of the cells with 8-Cl-cAMP. The differential effect of in vitro tRA- and DMF-treatment on the potential of HL-60 and HCW-2 cells to form tumors *in vivo* suggested that, in comparison to HL-60, the tumorigenicity of HCW-2 cells was no longer dependent on processes that could be affected by both t-RA and DMF.

8-Cl-adenosine has been shown to be responsible for mediating the inhibitory effect of 8-Cl-cAMP on the proliferation of cells in general. However, the data in the Table 4 indicate that treatment of both HL-60 and HCW-2 cells with 10 uM 8-Cl-adenosine alone *in vitro* for 72 hours did not have any inhibitory effect on these cells ability to form tumors *in vivo*, and the tumors formed from 8-Cl-adenosine-treated HL-60 cells had approximately the same size as that from untreated cells. In contrast, 8-Cl-cAMP-treated HL-60 and HCW-2 cells no longer formed any tumors *in vivo*. These results suggested that 8-Cl-cAMP-induced inhibition of the tumorigenicity of HL-60 and HCW-2 cells was not mediated by 8-Cl-adenosine, suggesting that the anti-tumor effect of 8-Cl-cAMP may involve additional metabolites.

DISCUSSION

Experimental data presented in this report indicate that the observed inhibitory effect of 8-Cl-cAMP on the proliferation of various cell lines is not a direct effect of 8-Cl-cAMP *per se*, but rather more direct effect of 8-Cl-adenosine which is a metabolic product of 8-Cl-cAMP through serum-catalyzed reactions. A similar conclusion also has been independently made in a recent study in which it was demonstrated that incubation of 100 uM of 8-Cl-cAMP with Chinese hamster ovary (CHO) cells in the presence of 10% FBS for 3 days resulted in approximately 70% conversion of 8-Cl-cAMP into 8-Cl-adenosine in the culture medium, and 8-Cl-adenosine was observed to inhibit the proliferation of CHO and Molt-4 lymphoblast cells (Van Lookeren Campagne et al., 1991).

The inhibitory effect of 8-Cl-adenosine on cell proliferation has been shown in another report (Tagliaferri et al., 1988a). However, the authors of that report dismissed the possibility that 8-Cl-cAMP could be metabolized into 8-Cl-adenosine which in turn may be responsible for exerting inhibitory effect on the proliferation of the cell. The reason for those authors to dismiss 8-Cl-adenosine as a potential inhibitor of cell proliferation was because using HPLC analysis, no formation of 8-Cl-adenosine in the growth medium of and extracts from the cell was detected (Tagliaferri et al., 1988a). Based on this study, it was proposed that 8-Cl-cAMP, but not its metabolites that induced inhibition of the proliferation of tumor cells (Tagliaferri et al., 1988a; Cho-Chung et al., 1989; Cho-Chung, 1989). However, it now appears, as pointed out by Van Lookeren Campagne et al., (Van Lookeren Campagne et al., 1991), that the failure to detect the formation of 8-Cl-adenosine from 8-Cl-cAMP in that reported study was probably due to the design of the analytical method in which a buffer with a pH value of 8.3 was used for anion-exchange HPLC analysis of the metabolites of 8-Cl-cAMP (Tagliaferri et al., 1988a), because at pH 8.3 8-Cl-adenosine should be positively charged. Therefore, it was not retained on an anion exchange column and was probably obtained in subsequent eluted fractions (Tagliaferri et al., 1988a).

One of the key claims that was described to 8-Cl-cAMP was that it did not inhibit the proliferation of normal cell lines, tumor-derived or transformed cells were selectively inhibited (Cho-Chung, 1989; Cho-Chung et al, 1989). However, this conclusion was made prematurally. As shown clearly by our study (figure 2 and 3), treatment of both normal and tumor cells in serum-free medium with 8-Cl-cAMP did not cause any observable degree of cell proliferation inhibition, because 8-Cl-cAMP by itself is not inhibitory, and it is 8-Cl-adenosine, a serum-catalyzed metabolite of 8-Cl-cAMP, that was responsible for inhibiting the proliferation of both normal and tumor cells (figure 10).

The inability of 8-Cl-cAMP to inhibit the proliferation of various cell lines in serum-free medium suggests that these cells might possess very low levels of extracellular PDE activity. Thus when cells are incubated in media without serum, 8-Cl-cAMP would not be metabolized into 8-Cl-adenosine, and therefore did not inhibit the proliferation of those cell lines. However, it is interesting to note that the proliferation of v-Ki-*ras* and Ha-murine sarcoma virus-transformed fibroblast cells cultured in serum-free media was greatly inhibited by 8-Cl-cAMP (Tagliaferri et al., 1988b; Tortora et al., 1989a). This observation suggests that in those transformed fibroblast cells, there may be high levels of PDE and other enzymes that are required for metabolizing of 8-Cl-cAMP into 8-Cl-adenosine. Rous sarcoma virus-tramsformed chicken embryonic fibroblast cells have been shown to have substantially reduced production of cAMP (Otten et al., 1972; Anderson et al., 1973a, 1973b; Gidwitz et al., 1980), but such reduction in cAMP production was found to be due to a deficiency in the activity of adenylate cyclase (Anderson et al., 1973a, 1973b; Gidwitz et al., 1980). It would be interesting to see if 8-Cl-cAMP-induced inhibition of the growth of transformed fibroblast cells may also be due to higher than normal levels of PDE activity being expressed in those cells.

In reviewing the literature, several reports were found to have documented findings that metabolites of other cAMP analogs inhibited the proliferation of various cell lines. For instance, it was reported that: (1) DBt-cAMP was deacylated into MBt-cAMP inside the cells (Kaukel and Hilz, 1972a, 1972b; O'Neil et al., 1975; Friedman et al., 1976). Since butyrate was a very strong inhibitor of cell proliferation (D'Anna et al., 1980), the possibility that butyrate actively inhibited the growth of various cell lines could not be ignored; (2) butyrated adenosine molecules were produced in cells treated with DBt-cAMP and were suggested to be responsible for the observed inhibitory effect of DBt-cAMP on the cell proliferation (Schroder and Plagemann, 1971; Granner et al;, 1975; O'Neill et al., 1975; Bevers et al., 1976; Hargrove and Granner, 1980), In addition, cAMP was found to stimulate, whereas DBt-cAMP to inhibit DNA synthesis in HeLa cells (Kaukel et al., 1972); (3) 8-Br-cAMP was found to be metabolized into 8-Br-AMP (Niles et al., 1979) and a 8-Br-cAMP resistant cell line was also shown to be resistant to 8-Br-AMP treatment (Martin and Ronning, 1981); and (4) Ara-cAMP was found to be metabolized into araAMP in L-cells and demonstrated to be responsible for inhibiting the growth of those cells (LePage and Hersh, 1972).

Besides the effect of cAMP analogues on cell proliferation, there have been numerous studies to suggest that the observed inhibitory effect of various cAMP analogues on other cellular functions might also be an "analogue" effect rather than a true "cAMP" effect. In studies in which effects of cAMP and DBt-cAMP on various cellular activities, such as metabolism of lipid and transport and oxidation of glucose in isolated fat cells (Solomon et al, 1970) and glycogen synthesis in HeLa cells (Hilz and Tarnowski, 1970), it was found that cAMP stimulated these activities in the cells, whereas DBt-cAMP inhibited all activities in the same cells.

These observations suggests that the use of synthetic analogues of cAMP including 8-Cl-cAMP in studies on the regulatory role of cAMP in cell proliferation can lead to an incorrect conclusion evoke regulatory mechanisms of cell proliferation that were misleading. Thus, it appears that in order to evaluate how specifically a cAMP analogue-induced cellular function could be related to the function of cAMP, It may be helpful to compare the effect of a cAMP analogue on a particular biological activity with that of a hormone that is known to specifically stimulate the production of cAMP in those cells.

In the past, a variety of adenosine analogues were found to inhibit the proliferation of different cell lines because these analogues were either incorporated into DNA or RNA and caused abnormal functions of either DNA or RNA or protein synthesis (Biesele et al., 1951; 1954; Tatibana and Yoshikawa, 1962; Brockman and Anderson, 1963; Klenow, 1963; Acs et al., 1964; Brink and LePage, 1964; Ellis and LePage, 1965; Shigeura and Grodon, 1965; Shigeura et al., 1965, 1966a,1966b; Heildberger,1967; Divekar et al.,1972). Alternatively, certain adenosine analog-derived nucleotides could become inhibitors for various enzymes, such as ribonucleotides polymerase thereby inhibiting the synthesis of RNA (Shigeura and Gordon, 1962), therefore, inhibit basic cellular metabolism and resulted in inhibition of the cell proliferation. This type of inhibition is certainly due to the cytotoxicity of these adenosine analogues (Brockman and Anderson, 1963).

Several studies on the subject of adenosine analogue-induced cytotoxicity in cells appeared to indicate that an initial conversion of adenosine analogues into nucleotide analogues was necessary for their inhibitory effect on the proliferation of cells, because it was found that mutant cells whose proliferation was resistant to the cytotoxic effect of various adenosine analogues generally did not have active adenosine kinase or purine ribonucleoside kinase (Brockman, 1961; Brockman et al.,1961; Ellis and Lepage,1963; Lepage and Junga, 1963; Bennett, Jr. et al., 1966, 1968; Caldwell et al., 1967; Divekar et al., 1972). Although 8-halogen-substituted adenosine analogues were demonstrated to be good substrata for adenosine kinase, and were found to be converted into various 8-halogen-substituted nucleotides (Miller et al., 1979), our study does not seem to suggest that the serum-dependent inhibitory effect of 8-Cl-cAMP on the proliferation of cells was due to a cytotoxic effect of 8-Cl-adenosine, because: (1) treatment of most cell lines except HL-60 with 10 uM of 8-Cl-cAMP for a long period of time (5 days) in the presence of 10% FBS did not cause any inhibitory effect on the viability of the cells (figure 15); (2) the serum-dependent inhibitory effect of 8-Cl-cAMP on the proliferation and viability of HL-60 cells appeared to be mediated by different mechanisms, the concentrations of 8-Cl-cAMP required to affect the viability of HL-60 cells was much higher (IC_{50} = 6 uM) than that required for affecting the cell proliferation (IC_{50} = 0.25 uM) (figure 30); (3) at a concentration of 1.5 uM, 8-Cl-cAMP completely inhibited the growth of HCW-2 cells. However, treatment of the same cells with concentrations of 8-Cl-cAMP as high as 10 uM for as long as 5 days did not cause any cytotoxic effect on these cells (figure 32); and (4) bFGF specifically reversed the 8-Cl-cAMP-induced inhibitory effect on BAE cells (figure 19). The fact that bFGF reversed 8-Cl-adenosine-induced inhibition of the proliferation of BAE cells (figure 20) indicated that bFGF did not act in generic manner in antagonizing the inhibitory effects of adenosine analogues. In addition to the above findings, another experiment in this study demonstrated that 10 uM 8-Cl-cAMP had no effect on subsequent incorporation of tritium labelled adenosine into macromolecules and general acid soluble nucleotides pools (Table 3). Therefore, it indicated that the mechanism for the serum-dependent inhibitory effect of 8-Cl-cAMP on cell proliferation did not involve a general inhibition of 8-Cl-adenosine on normal metabolism of adenosine in the cells as most adenosine analogues did (Heidelberger, 1967; Biesele, 1951; Brockman and Anderson, 1963).

The possibility that 8-Cl-adenosine might interact with adenosine receptors does not appear to be likely, because (1) based on extensive studies, it was concluded that C-8 substituted adenosine analogues generally lacked binding activity to adenosine receptors and lacked adenosine-like biological activities (Agarwal, 1987); and (2) treatment of different cell lines with 8-Cl-cAMP in the presence of serum did not seem to have any regulatory effect on their intracellular levels of cAMP (figure 13), whereas adenosine receptors are known to regulate intracellular levels of cAMP in various cells (Sattin and Rall, 1970; Fain, 1973; Harris et al., 1976; Londos et al., 1977; Van Calker et al., 1979; Londos et al., 1980; Wolff et al., 1981); and (3) treatment of cells with excess amount of adenosine did not have any effect on their proliferation nor did it have any effect on modifying the inhibitory effect of 8-Cl-cAMP on the proliferation of cells (figure 14). Therefore, it suggests that the mechanism by which 8-Cl-cAMP induced the inhibition of the cell proliferation might be independent from adenosine receptors.

The fact that dipyridamole, a specific inhibitor of adenosine nucleotide transport system (Afonso, 1970; Best et al.,1979; Sollevi et al.,1982; Paterson et al.,1983; Parks et al.,1985) blocked the serum-dependent inhibitory effect of 8-Cl-cAMP on various cell lines (figure 12) suggests that a intracellular mediator is required for mediating the inhibitory effect of 8-Cl-adenosine in cells. There has been studies on intracellular adenosine receptors, but the biological functions of these receptors, particularly to their role in the regulatory processes of the proliferation of cells, are still unknown. However, It was reported that S-adenosylhomocysteine (SAH) hydrolase could serve as a specific intracellular adenosine receptors in lymphocytes (Hershfield and Kredich, 1978). It was also reported that accumulation of adenosine in lymphocytes could inhibit the hydrolysis of SAH, leading to a accumulation of it in the cells (Kredich and Martin, 1977). SAH has been shown to serve as inhibitors for the methylation process that required S-adenosine-L-methionine (SAM) (Hurwitz et al., 1964; Zappia et al., 1969; Pegg, 1971; Hildeschein et al., 1972; Kerr, 1972; Glick et al., 1975), and because SAM was demonstrated to serve as a methyl donor for the methylation of DNA (Brown and Attardi, 1965; Vanyushi et al., 1970), mRNA (Desrosiers et al., 1974; Perry and Kelley, 1974), rRNA (Greenberg and Penman, 1966; Maden and Salim, 1974), tRNA (Kerr and Borek, 1972), and proteins (Paik and Kim, 1975). In addition, 2-F-adenosine has been shown to be metabolized into 2-F-S-adenosylhomocysteine and 2-F-S-adenosylmethionine inside cells (Zimmerman et al., 1979, 1980). Therefore, it would be interesting to investigate whether 8-Cl-adenosine alone could either directly, or it could be metabolized into S-8-Cl-adenosylhomocysteine first and then causes inhibitory effect on the methylation of cell proliferation-associated macromolecules, resulting in inactivation of these molecules and inhibition of the proliferation of cells.

The proliferation of different cell lines was believed to be regulated by different growth factors and hormones present in serum (Hayashi and Sato, 1976; Barnes and Sato, 1980; Barnes et al., 1987). We have found that bFGF specifically and completely antagonized this inhibitory effect on the proliferation of endothelial cells (figure 16). The ability of bFGF to stimulate the proliferation of BAE cells in the presence of excess amount of 8-Cl-cAMP, 8-Cl-AMP and 8-Cl-adenosine (figure 18) also indicated that no matter what the mechanism may be for the serum-dependent proliferation inhibitory effect of 8-Cl-cAMP on the cells, it certainly does not involve the inhibition of cellular activities that could be too basic for the proliferation of the cells. Otherwise we should have not seen the stimulatory effect of bFGF on the proliferation of the endothelial cells when it was greatly inhibited by 8-Cl-cAMP.

The proliferation of endothelial cells is a very important part of angiogenesis and as well normal development. It is also interesting to study angiogenesis, since this process is very critical in the pathological development of solid tumors in the body (Tannock, 1968; Folkman, 1971; Folkman and Cotran, 1976; Miller, 1980; Kreisle and Ershler, 1988; Folkman, 1989; Folkman et al., 1989;). In this process, bFGF has been demonstrated to be an primary angiogenesis factor *in vivo* (Thomas et al., 1985; Esch et al., 1985; Lobb et al., 1985), and *in vitro* (Folkman, 1984; Folkman and Klagsbrun, 1987; D'Amore and Braunhut, 1988). Therefore, a good understanding of some of the more unique aspects of endothelial cell-associated mitogenic function of bFGF should provide information on the development of chemotherapeutic strategies that is specifically targeted to bFGF and and angiogenesis in tumor development. The ability of bFGF, a mitogen for both fibroblast and endothelial cells (Gospodrowicz, 1985), to selectively stimulate the proliferation of endothelial cells in the presence of serum and 8-Cl-cAMP (figure 16) indicate that there must be some unique BAE cell-associated aspects of the mitogenic action of bFGF.

In this study, we have shown that bFGF partially inhibited the uptake of adenosine in endothelial cells, whereas it had no such effect on ARK cells (figure 23). Since it has been well established that cellular uptake of various nucleotides including many adenosine analogues was mediated by specific cell membrane-associated transporters, such as adenosine transporters (Paterson et al., 1983; Pearson et al., 1978; Parks, Jr. et al.,1985). Therefore such a selective inhibitory effect of bFGF on the uptake of adenosine in endothelial cells may be partially responsible for bFGF-stimulated proliferation of endothelial cells but not ARK cells in the presence of excess amount of 8-Cl-cAMP and serum. This suggests that in endothelial cells besides its' mitogenic function, bFGF may also interact with adenosine transport and modulate its' activity.

Phosphorylation of proteins in various cell lines is very important in keeping the functions of not only growth-associated proteins (Maller, 1990; Nurse, 1990), but also the functions of proteins involved in basic metabolism (Ingebritsen and Cohen, 1983; Cohen and Cohen, 1989; Cohen, 1989). The differential effect of bFGF on the phosphorylation of proteins in ARK and BA14B cells in the presence of serum and 8-Cl-cAMP (figure 25) was an important bit of data indicating that the effect of bFGF on fibroblast and endothelial cells were differentially regulated at the biochemical levels. The fact that bFGF could selectively maintain general phosphorylation of proteins in endothelial but not in fibroblast cells incubated in medium containing 10% FBS and 10 uM 8-Cl-cAMP (figure 25) probably suggests that such an inhibitory effect of 8-Cl-cAMP on serum-stimulated general phosphorylation of proteins in both ARK and BAE cells may very well be part of the mechanism for serum-dependent inhibitory effect of 8-Cl-cAMP on the proliferation of cells.

In some studies it was reported that treatment of different cell lines with 8-Cl-cAMP for 72 hours induced increases in the level of nuclear RIIβ and a concomitant decrease in the level of nuclear RI. Therefore, nuclear RIIβ was proposed to be responsible for mediating the growth inhibitory effect of 8-Cl-cAMP (katsaros et al., 1987; Tagliaferri et al., 1988a; Cho-Chung et al., 1989; also see review by Cho-Chung, 1989). However, we have observed that (1) ARK cells do not possess RIIβ and a 48 hours treatment of the cells with 8-Cl-cAMP did not induce the expression of RIIβ in these cells (figure 26). (2) Although BAE cells contained RIIβ and RI in their nucleus, treatment of these cells with 8-Cl-cAMP for 48 hours did not have any effect on the level of RIIβ but, nonetheless, increased the level of RIβ in the nucleus (figure 27). (3) bFGF antagonized the serum-dependent inhibitory effect of 8-Cl-cAMP on the proliferation of BA14B cells (figure 17), but did not have any effect on the level of nuclear RIIβ in these cells (figure 27). (4) Although 8-Cl-cAMP greatly inhibited the proliferation of HCW-2 cells (with an IC_{50} = 0.5 uM) (figure 33), these cells did not have any demonstrative nuclear

RII or RI proteins (Figure 34). Thus, it appears that the presence of RIIβ in the nucleus of cells could not be an absolute requirement for mediating the inhibitory effect of 8-Cl-cAMP on cell proliferation. However, It could be argued that the observed increases in nuclear RIIb was a result but not a cause of 8-Cl-cAMP-induced inhibition of the cell proliferation.

It was reported that a treatment of various leukemia cell lines with 8-Cl-cAMP not only inhibited their growth but also induced differentiation of these cells (Tortora et al., 1988, 1989b, 1990). Because it has been believed that mature blood cells, such as erythrocytes, granulocytes, and macrophages, were the results of terminal differentiation of proliferative precursor cells (progenitor cells) and the development of various types of leukemia is due to a genetic blockage that prevents the progenitor cells from becoming committed to differentiate into mature cells that are no longer proliferative. Hence, those cells remain at a state with highly proliferative activity (Gallo, 1975; Greaves, 1982; Sachs, 1985; Sartorelli, 1983; Bloch, 1984; Sachs, 1987; Potter, 1988). Therefore, it was proposed that activation of type II PKA by 8-Cl-cAMP was also responsible for inducing cell differentiation and 8-Cl-cAMP might potentially be a good anti-leukemia agent (Tortora et al., 1988, 1989b, 1990). However, in this study when compared to the effect of tRA and DMF, two well characterized agents that induce terminal differentiation of HL-60 cells (Friend et al., 1971; Collins et al., 1978, 1980; Breitman et al., 1980; Reuben et al., 1980; Takenaga et al., 1980; Lazenby et al., 1987), treatment of HL-60 cells with 10 uM of 8-Cl-cAMP for 3 days did not induce any degree of terminal differentiation of these cells (figure 36). The high degree of tRA- and DMF-induced terminal differentiation of HL-60 cells in this study (figure 36) served as good controls for the evaluation of the capability of 8-Cl-cAMP to do so. This inability for 8-Cl-cAMP to induce terminal differentiation of HL-60 cells was in contradiction from the conclusion made by others in their study in which it was stated that treatment of HL-60 cells with 10 uM 8-Cl-cAMP for 72 hours induced significant degree of the differentiation of these cells (Tortora et al., 1990). It seems that this contradiction could be explained as a result of the use of different techniques between these studies. In those reported studies, 8-Cl-cAMP-induced differentiation of HL-60 cells was determined by morphological changes, such as ruffled cell surface, increased expression of monocyte-specific surface antigen and a decrease in markers of immature progenitor cells, and decreased ratio of cytoplasm to nucleus, that were associated with 8-Cl-cAMP treatment (Tortora et al., 1988, 1990). In this study, terminal differentiated HL-60 cells was determined by NBT-staining which has been prove to be a better technique for determining chemically-induced and terminally differentiated HL-60 cells (Collins et al., 1978, 1980; Bewburger et al., 1979). It could be argued that those early reported studies (Tortora et al., 1988, 1989b, 1990) on 8-Cl-cAMP-induced differentiation of HL-60 cells was a reflection of initial steps during a progressive process of a terminal differentiation of these cells, and unlike tRA and DMF, 8-Cl-cAMP-treatment did not force the cells to become committed for terminal differentiation. Although, 8-Cl-cAMP-treatment did not induce terminal differentiation of HL-60 cells, it effectively inhibited the clonogenicity (figure 38) as well as tumorigenicity of these cells (Table 4). One of the possibilities that may explain why 8-Cl-cAMP-treatment reduced tumorigenicity of HL-60 cells is that because treatment of HL-60 cells with 8-Cl-cAMP *in vitro* might initiate the process of differentiation of these cells, and in the *in vivo* environment, these cells continued their differentiation and become non-proliferative and therefore non-tumorigenic.

HCW-2 cells was initially isolated from the population of HL-60 cells after an extensive treatment of those cells with 8-Cl-cAMP, and then subsequently cultured in 8-Cl-cAMP-free medium. Based on our observation that some cells in the HCW-2 population were tumorigenic and a short-term treatment of these cells with 8-Cl-cAMP inhibited the tumorigenicity of these cells (Table 4), it suggests that the inhibitory effect of 8-Cl-cAMP-treatment on the tumorigenicity of these cells might be reversible, because If the inhibition was irreversible, then there should have been no tumors formed from HCW-2 cells. This observation points out a potential down side in the potential use of 8-Cl-cAMP as an anti-promyelocytic leukemia agent. However, until more in vivo experiments are performed, it remains to be seen of how effective it could be used as a anti-leukemia agent.

Our initial studies on the serum-dependent inhibitory effect of 8-Cl-cAMP on cell proliferation has led us to the conclusion that 8-Cl-adenosine derived from 8-Cl-cAMP was primarily responsible for exerting the inhibitory effect on various cell lines including HL-60 and HCW-2. However, the observed differential effect of the treatment of HL-60 and HCW-2 cells with 8-Cl-cAMP and 8-Cl-adenosine (Table 4) on their tumorigenicity certainly indicated that 8-Cl-cAMP-treatment of the cells was far better than 8-Cl-adenosine-treatment. The fact that the tumorigenicity of 8-Cl-adenosine-treated HL-60 and HCW-2 cells remained almost the same as that of untreated cells (Table 4) indicates that treatment of the cells with 8-Cl-adenosine alone had no inhibitory effect at all on their tumorigenic potential. Therefore, although 8-Cl-adenosine, as a metabolite of 8-Cl-cAMP, was responsible for exerting the serum-dependent inhibitory effect on the growth of the cells, the inhibition of tumorigenicity of both HL-60 and HCW-2 cells by 8-Cl-cAMP-treatment might involve other metabolites.

In summary, this study has provided new information on the mechanism of how 8-Cl-cAMP inhibits the proliferation of various cells. Our data have indicated that the previously proposed mechanism for 8-Cl-cAMP-induced inhibition of cell proliferation was flawed and requires modification. In addition, this study has also suggested to us that in the search for biological functions of potential signal molecules, great caution must be taken in interpreting experimental results when modified synthetic signal molecules are used.

TABLE 1. 8-Cl-cAMP inhibits the proliferation of tumor cell lines

Cell Line	IC_{50}	I_{max}	C_{max}
18-54,SF	-	0	-
OVIN	-	45%	12 uM
BRL-3A	-	55%	10 uM
G401	1 uM	90%	5 uM
A431	1 uM	80%	10 uM
MCF-7	1 uM	85%	10 uM
DLD 1 Clone A	0.8 uM	92%	10 uM
HL-60	0.25 uM	96%	1 uM
Y79	0.82 uM	85%	5 uM

Cells (except 18-54,SF cells were cultured in serum-free medium) were cultured in medium containing 10%FBS, and treated with various concentrations of 8-Cl-cAMP for 3 days in the cell proliferation assay. In the case of HL-60 and Y-79 cells, the cells were cultured in medium containing 20% FBS and treated with 8-Cl-cAMP at the time of seeding (see Methods section). The degree of cell proliferation at the end of the assay was determined by the total number of cells per dish. Abbreviations: IC_{50}, the concentration of 8-Cl-cAMp that was required to inhibit 50% cell proliferation. I_{max}, the maximum inhibition of cell proliferation by 8-Cl-cAMP. C_{max}, the minimum concentration of 8-Cl-cAMP required for inducing maximum degree of cell proliferation.

Table 2. Inhibitory effect of 8-Cl-cAMP on the proliferation of the 18-54,SF cells cultured in medium containing different sera

Serum Species and developmental stage	IC_{50} of 8-Cl-cAMP
Bovine	
Fetal	1.0 uM
Neonatal	1.8 uM
Newborn (wet cord)	1.2 uM
Newborn (5 day old)	0.8 uM
Newborn (16 week old)	0.8 uM
Calf (10 month old)	0.8 uM
Adult	1.8 uM
Horse	
Fetal	0.4 uM
Adult	0.5 uM
Human	23 uM
Chicken	5.0 uM
Rabbit	0.6 uM
Sheep	4.5 uM
Pig	0.4 uM
Rat	0.4 uM

18-54,SF cells were cultured in medium containing 10% of the above serum, and the effect of 8-Cl-cAMP at various concentrations on the proliferation of cells was determined in the cell proliferation assay (see Methods section). IC50, the concentration of 8-Cl-cAMP that inhibited 50% cell proliferation.

Table 3. Effect of 8-Cl-cAMP treatment on the metabolism of [^{3}H]adenosine in different cell lines

	Incorporation of [^{3}H]adenosine into cellular nucleotide pool					
	Control cells			8-Cl-cAMP-treated cells		
	Acid-insoluble molecules			Acid-insoluble molecules		
Cell Line	nuclear	cytosolic	ASM	nuclear	cytosolic	ASM
BRL-3A	1652	22450	33246	1726	25680	34562
ARK	2451	12344	62726	2102	10442	63214
NIH3T3	5763	34559	30210	6012	33018	31243
BAE	12010	56782	43107	9102	57894	41251

Monolayers of the listed cells in 24-well plates were incubated in 0.5 ml of medium containing 10% FBS plus 20 uM 8-Cl-cAMP for 48 hours. Then an equal volume of the medium containing 10% FBS and 200 nM [^{3}H]adenosine was added to each well and the cells were incubated in this medium for another 24 hours. After this incubation, cells were fractionized into nuclear and cytosolic fractions. The acid-soluble and acid-insoluble materiales were obtained (see Methods section). The radioactivity in each fraction was determined and used to represent the incorporation of [^{3}H]adenosine into various pools of nucleotides in these cells. The acid-soluble material from both the nuclear and cytosolic fractions were combined to represent the total acid-soluble molecules in the cell. Abbreviation: ASM, acid-soluble molecules.

Table 4. Effect of short-term *in vitro* treatment of HL-60 and HCW-2 cells with 8-Cl-cAMP, 8-Cl-adenosine, all trans retinoic acid, and dimethylformamide on their tumorigenicity *in vivo*

Cells and treatment	No. of mice with tumor	Tumor weight
HL-60 cells		
Control	5/5	18.08 grams
2 uM tRA, 72 hrs	0/5	
0.8% DMF, 8 days	0/5	
10 uM 8-Cl-cAMP, 72 hrs	1/5	5.80 grams
10 uM 8-Cl-Ado., 72 hrs	4/5	11.60 grams
HCW-2		
Control	5/10	10.60 grams
2 uM tRA, 72 hrs	3/5	11.43 grams
0.8% DMF, 8 days	2/5	8.30 grams
10 uM 8-Cl-cAMP, 72 hrs	1/10	0.80 grams
10 uM 8-Cl-Ado., 72 hrs	1/5	7.30 grams

HL-60 and HCW-2 cells were treated with the listed agents for the indicated time period in vitro. After the treatment, approximately 2X106 viable cells were injected s.c. in the back of Swiss nude mice. Tumor development was determined two months after the transplantation of the cells. Abbreviation: tRA, all trans retinoic acid; DMF, dimethylformamide; 8-Cl-Ado., 8-Cl-adenosine.

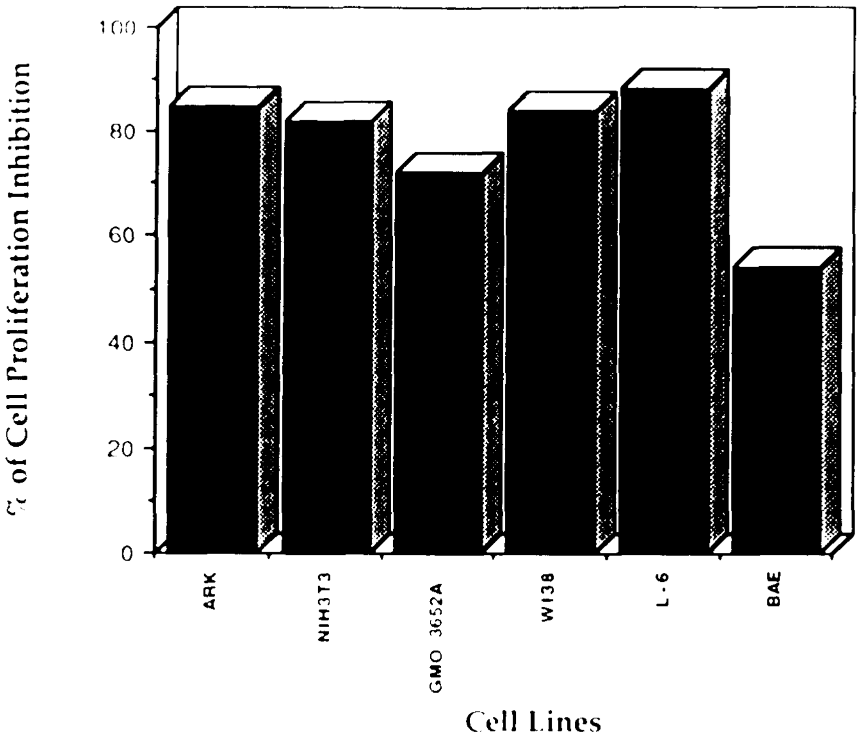

FIGURE 1. INHIBITORY EFFECT OF 8-Cl-cAMP ON THE PROLIFERATION OF NORMAL CELLS: The effect of 10 uM 8-Cl-cAMP on the proliferation of ARK, NIH3T3, WI38, L-6 and bovine aorta endothelial (BAE) cells was determined in the cell proliferation assay (see Methods section). The proliferation of the cell in the absence of 8-Cl-cAMP is used as the 100% index. The data presented here are the average of three independent experiments. In each experiment every point was determined in triplicate.

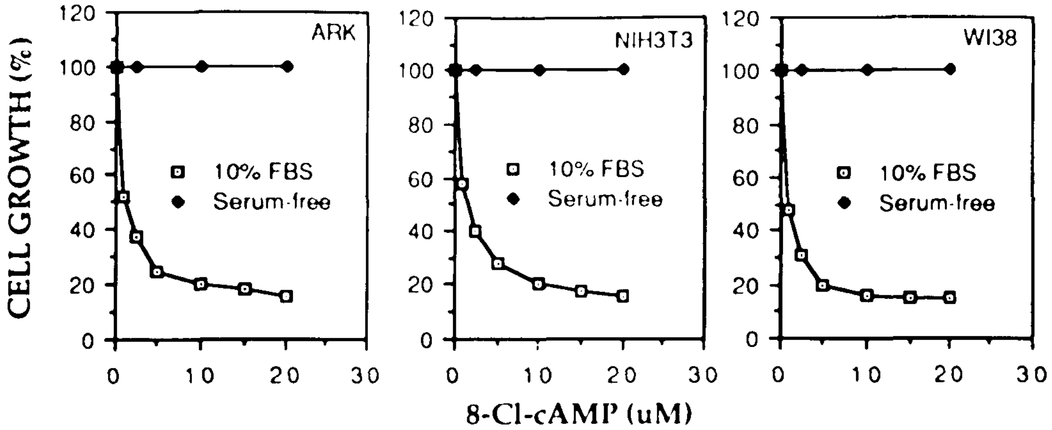

FIGURE 2. THE SERUM-DEPENDENT INHIBITORY EFFECT OF 8-Cl-cAMP ON NORMAL FIBROBLAST CELLS: The effect of 8-Cl-cAMP on the proliferation of ARK, NIH3T3 and WI38 cells was determined in the cell proliferation assay in which the cells were cultured in either serum-free or serum-containing (10% FBS) medium. The proliferation of the cell in the absence of 8-Cl-cAMP is taken as the 100% index. The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

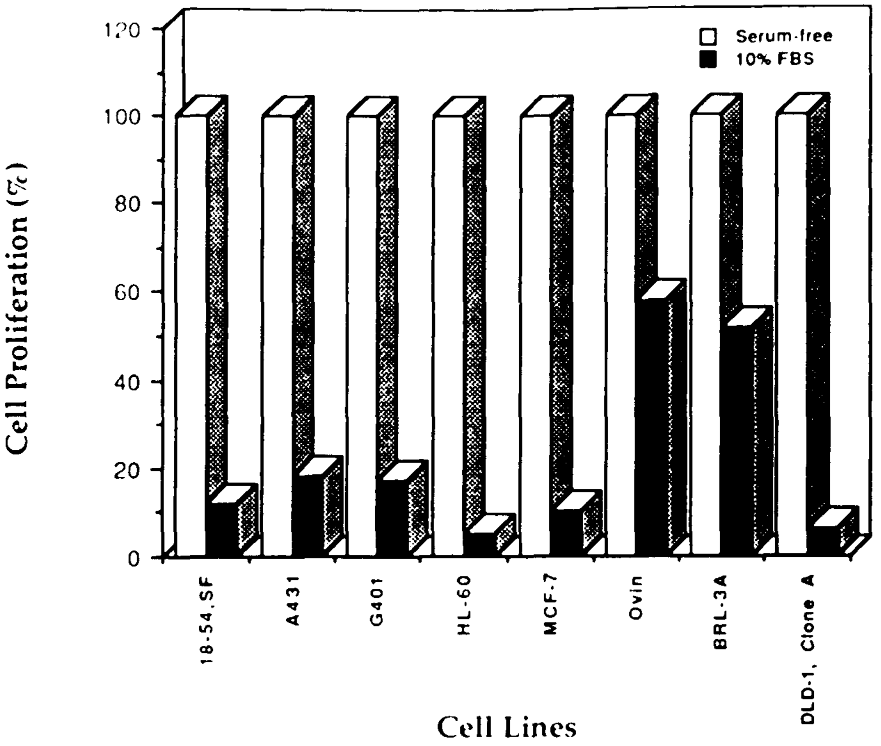

FIGURE 3. THE SERUM-DEPENDENT INHIBITORY EFFECT OF 8-Cl-cAMP ON TUMOR CELLS: The effect of 8-Cl-cAMP on the proliferation of the listed tumor cell lines was determined in the cell proliferation assay in which the cells were cultured in either serum-free or serum-containing (10% FBS) medium. The proliferation of the cell in the absence of 8-Cl-cAMP is taken as the 100% index. The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

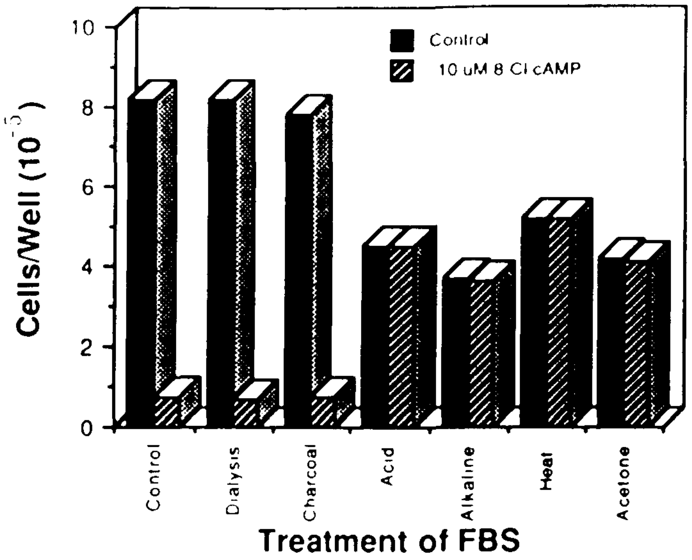

FIGURE 4. COMPARATIVE EFFECTS OF 8-Cl-cAMP ON THE PROLIFERATION OF 18-54,SF CELLS IN THE PRESENCE OF DIFFERENT PREPARATIONS OF FETAL BOVINE SERUM: The effect of 10 uM 8-Cl-cAMP on the proliferation of 18-54,SF cells cultured in the medium containing 10% of the indicated preparations of fetal bovine serum was determined in the cell proliferation assay. The data presented here are the average of three independent experiments. Each point was determined in triplicate in every experiment.

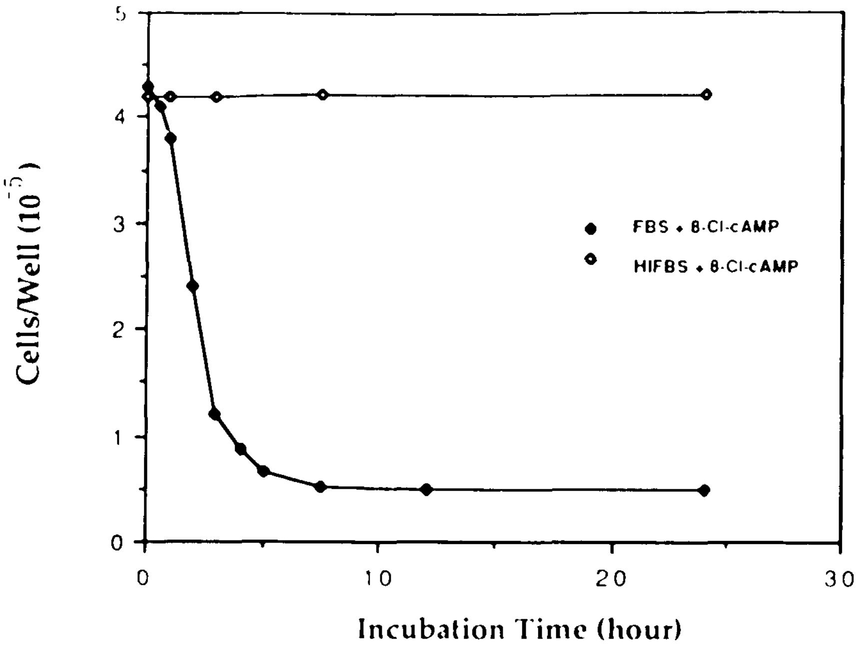

FIGURE 5. TIME-DEPENDENT METABOLISM OF 8-Cl-cAMP INTO PROLIFERATION INHIBITORY ACTIVITY *In Vitro*: One hundred ul of 8-Cl-cAMP stock solution in F-12 medium (100 uM) was mixed with 100 ul FBS or heat-inactivated FBS (HIFBS) and incubated at 37°C for the indicated length of time. The incubation was terminated by heating the mixture at 100°C for 5 minutes. The mixture was aseptically mixed with 0.8 ml of F-12 medium and this final mixture was used as the culture medium for 18-54,SF cells pre-plated in 24-well plates for the cell proliferation assay (see Methods section). The data presented here are the average of three independent experiments. Each point was determined in triplicate in every experiment.

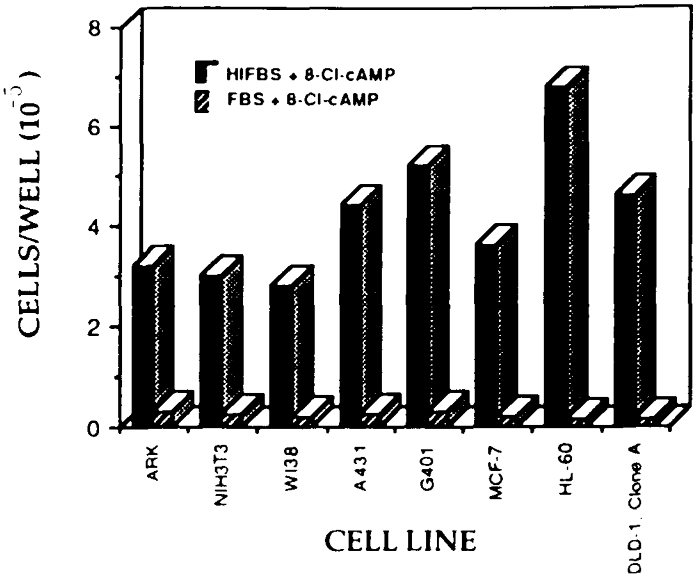

FIGURE 6. CELL PROLIFERATION IS SENSITIVE TO THE PRE-INCUBATION OF 8-Cl-cAMP WITH FBS *In Vitro*: One hundred ul of 8-Cl-cAMP stock solution in F-12 medium (100 uM) was mixed with 100 ul FBS or heat-inactivated FBS (HIFBS) and incubated at 37°C for 5 hours prior to the heat-treatment. The heated mixture was combined with 0.8 ml of serum-free medium and used as the culture medium for the indicated cells. The proliferation of these cell lines was determined in the cell proliferation assay.(see Methods section). The data presented here are the average of three independent experiments. Each point was determined in triplicate in every experiment.

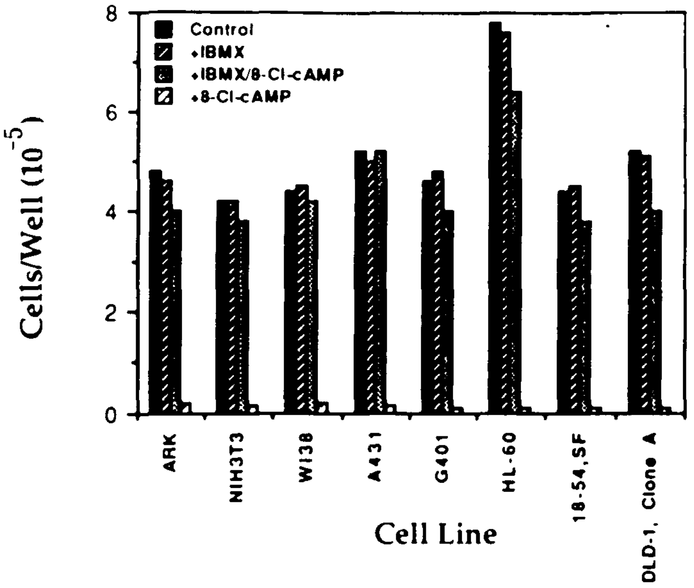

FIGURE 7. IBMX ANTAGONIZES THE SERUM-CATALYZED PRODUCTION OF PROLIFERATION INHIBITORY ACTIVITY FROM 8-Cl-cAMP *In Vitro*: Approximately100 ul of FBS was mixed with 100 ul F-12 medium (control), or with 100 ul of 8-Cl-cAMP in F-12 medium (100 uM), or with 100 ul of IBMX in F-12 medium (200 uM) or with 100 ul of solution containing 100 uM and 200 uM IBMX. The mixtures were incubated at 37°C for 5 hours prior to the heat treatment (100°C, 5 minutes). The heated mixture was then combined with 0.8 ml of F-12 medium and used as the culture medium for the indicated cell lines pre-plated in 24-well plates for the cell proliferation assay. (see Methods section). The data presented here are the average of three independent Each point was determined in triplicate in every experiment.

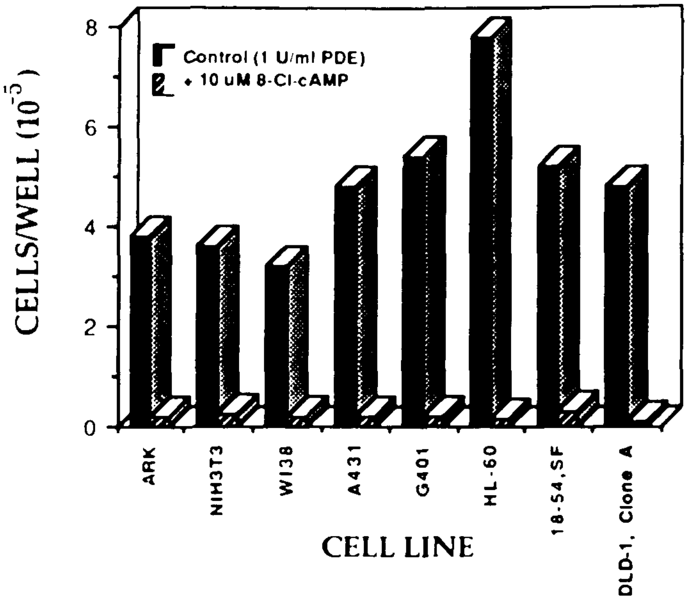

FIGURE 8. THE INHIBITORY EFFECT OF 8-Cl-cAMP ON CELL PROLIFERATION IS DEPENDENT ON THE CYCLIC NUCLEOTIDE PHOSPHODIESTERASE (PDE): In the cell proliferation assay, the indicated cells were cultured in serum-free medium plus or minus 10 mU/ml of PDE preparation, and the effect of 10 uM 8-Cl-cAMP on the proliferation of the cell was determined (see Methods section). The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

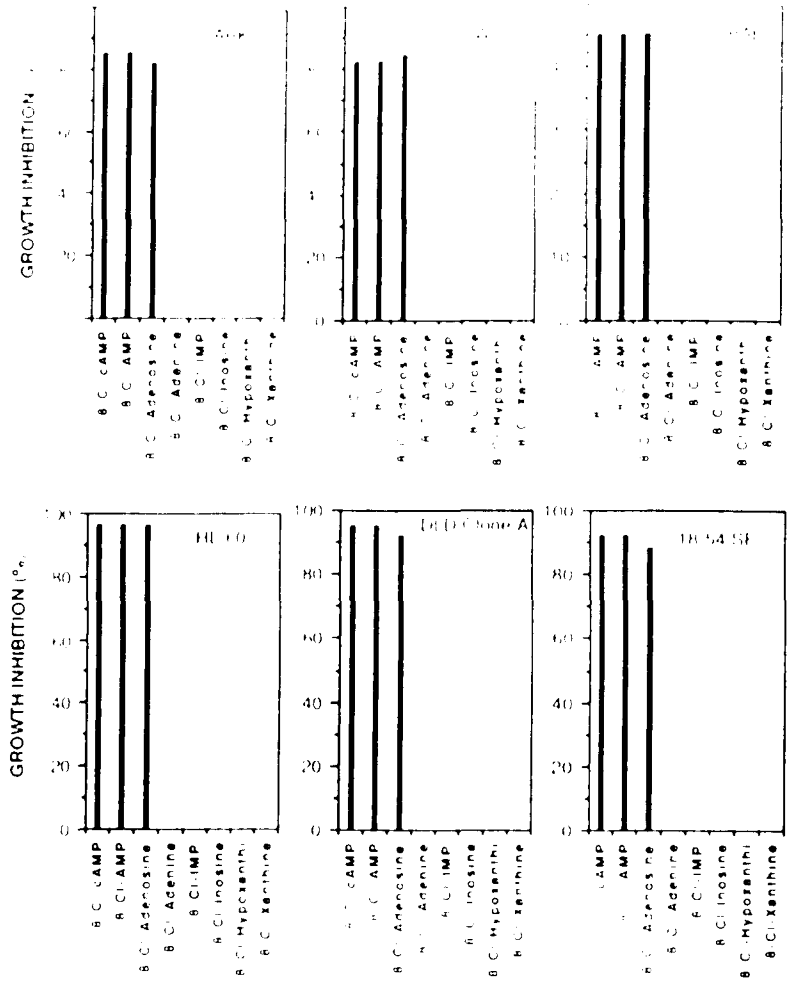

FIGURE 9. COMPARATIVE EFFECTS OF THE METABOLITES OF 8-Cl-cAMP ON CELL PROLIFERATION: In the cell proliferation assay, the indicated cell lines were cultured in the medium containing 10% FBS and the effect of 10 uM of the listed metabolites of 8-Cl-cAMP on the proliferation of the cell was determined (see Methods section). 8-Cl-Hypoxanthi. represents 8-Cl-Hypoxanthine.

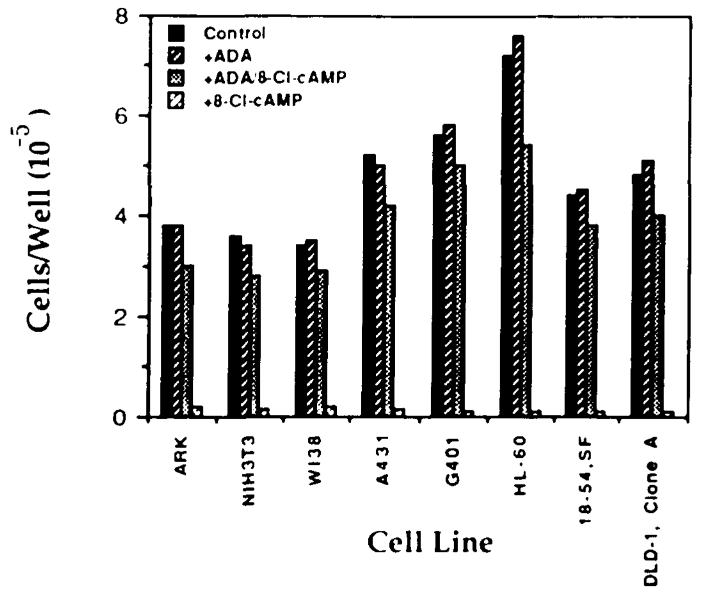

FIGURE 10. THE SERUM-DEPENDENT INHIBITORY EFFECT OF 8-Cl-cAMP ON CELL PROLIFERATION IS SENSITIVE TO ADENOSINE DEAMINASE (ADA): In the cell proliferation assay, the listed cells were cultured in the medium containing 10% FBS plus or minus 1 U/ml ADA, and the effect of 10 uM 8-Cl-cAMP on the cell proliferation was determined. The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

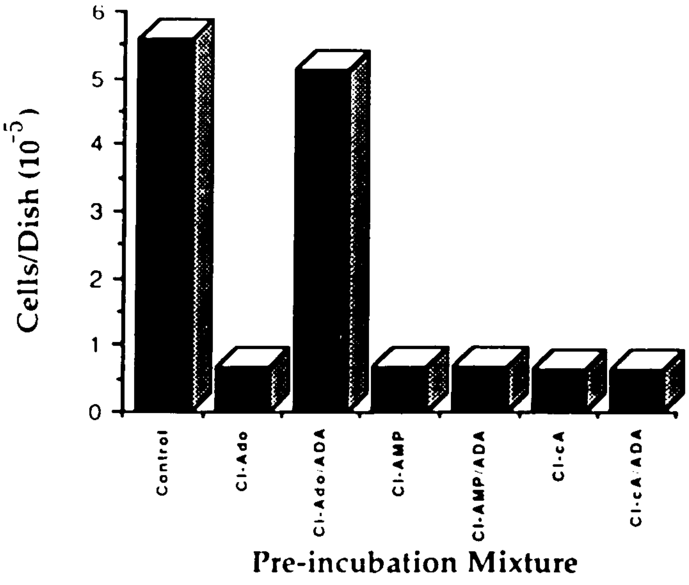

FIGURE 11. THE ACTIVITY OF ADA IS SPECIFIC TOWARDS 8-Cl-cAMP: Approximately 50 ul of 8-Cl-cAMP (200 uM), 8-Cl-AMP (200 uM), or 8-Cl-adenosine (200 uM) in Coon's modified F-12 medium was mixed with 1 unit of purified ADA (in 50 ul of Coon's modified F-12 medium). The mixture was incubated at 37°C for 60 minutes prior to the heat treatment (100°C,10 minutes). The mixture was then combined with 0.7 ml of fresh medium and 100 ul FBS. The effect of this final mixture on the proliferation of 18-54,SF cells was determined in the cell proliferation assay (see Methods Section). The data presented here are the average of five independent experiments. In each experiment, every point was determined in triplicate.

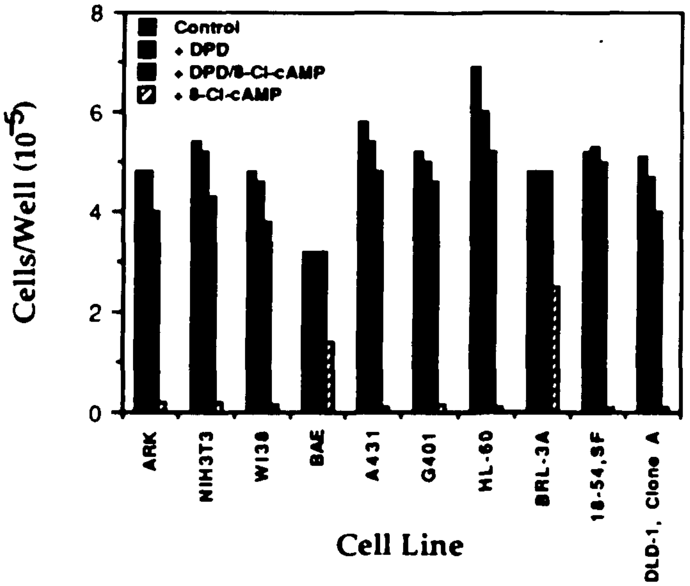

FIGURE 12. DIPYRIDAMOLE (DPD) BLOCKS THE SERUM-DEPENDENT INHIBITORY EFFECT OF 8-Cl-cAMP ON CELL PROLIFERATION: In the cell proliferation assay, the listed cell lines were cultured in the medium containing 10% FBS plus or minus 5 uM DPD, and the effect of 10 uM 8-Cl-cAMP on cell proliferation was determined (see Methods section). The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

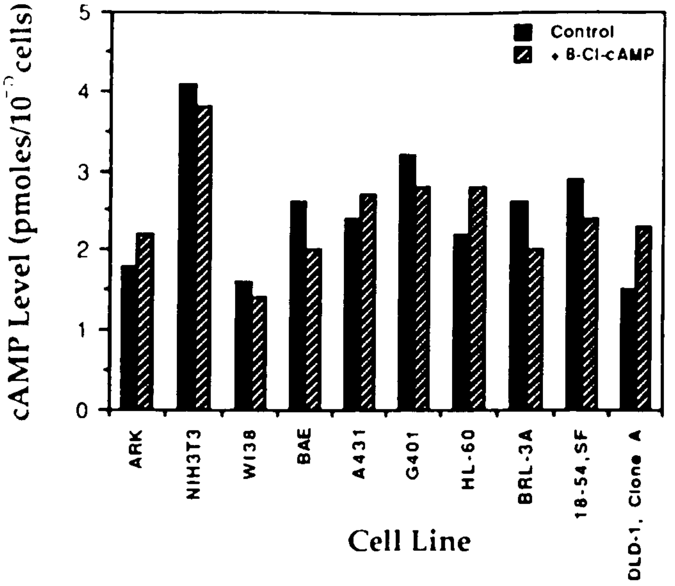

FIGURE 13. 8-Cl-cAMP HAS NO EFFECT ON THE INTRACELLULAR LEVELS OF cAMP IN VARIOUS CELL TYPES: Confluent monolayers of the listed cell lines were cultured in the medium containing 10% FBS and treated with 10 uM 8-Cl-cAMP for 24 hours prior to the analysis of intracellular levels of cAMP (see Methods section). The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

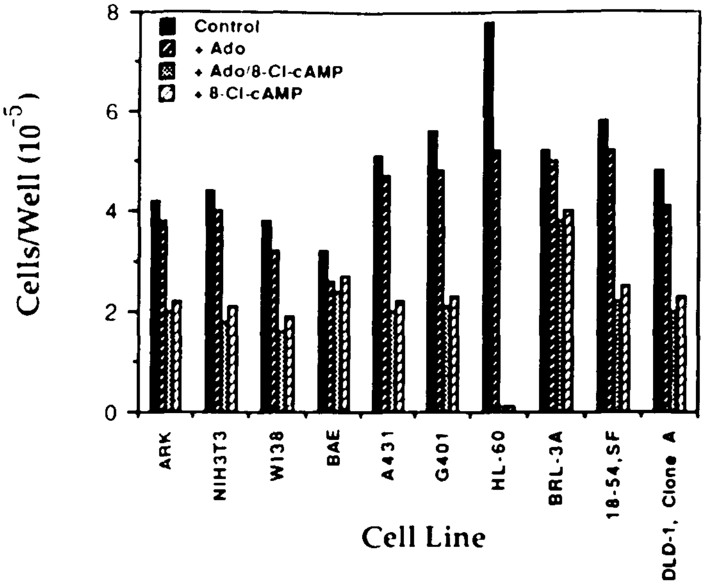

FIGURE 14. ADENOSINE DOES NO MODIFY THE SERUM-DEPENDENT INHIBITORY EFFECT OF 8-Cl-cAMP ON CELL PROLIFERATION: In the cell proliferation assay, the listed cell lines were cultured in the medium containing 10% FBS plus or minus 1 mM adenosine (Ado), and the effect of 1 uM 8-Cl-cAMP on the cell proliferation was determined. The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

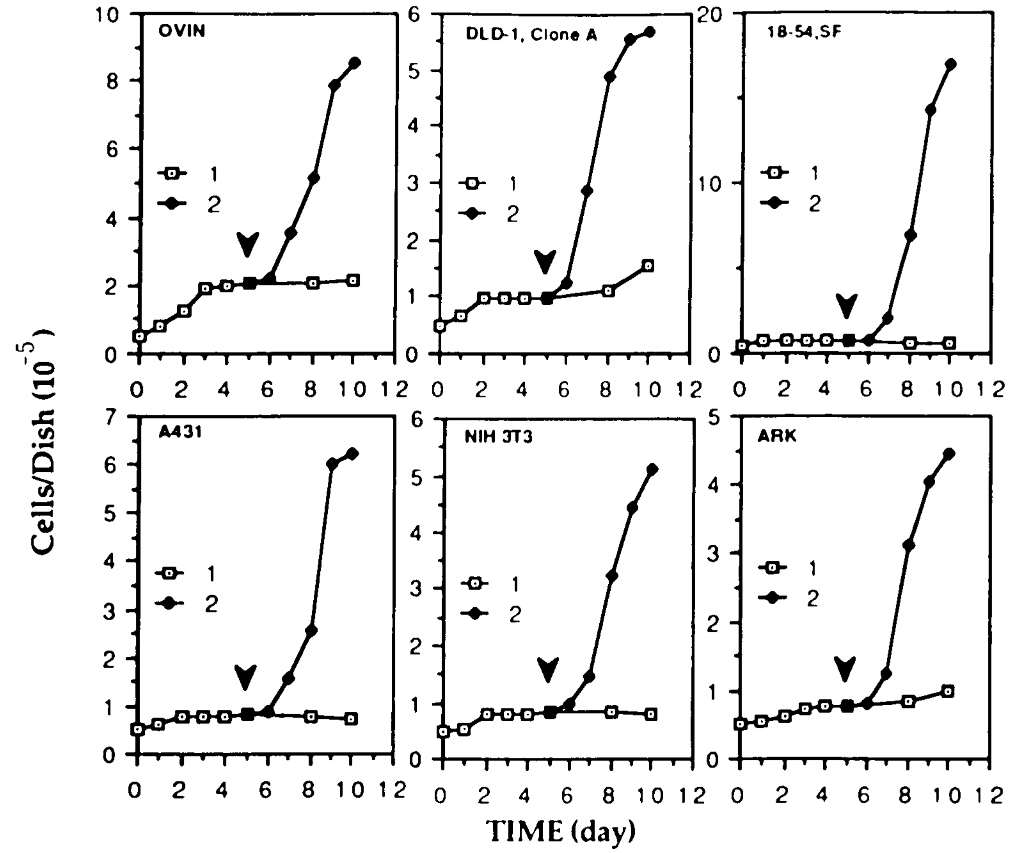

FIGURE 15. THE SERUM-DEPENDENT INHIBITORY EFFECT OF 8-Cl-cAMP ON CELL PROLIFERATION IS REVERSIBLE: In the cell proliferation assay, the listed cell lines were initially cultured in the medium containing 10% FBS and 10 uM 8-Cl-cAMP. Then the medium of half of the cell culture was replaced with 8-Cl-cAMP-free medium, and the other half in fresh 8-Cl-cAMP-containing medium. The cells were cultured for additional days as indicated in the figure. The arrow indicated the time when the medium change occured. Curve 1 represents the kinetics of the cell proliferation in 8-Cl-cAMP-containing medium, and curve 2 represents the kinetics of the proliferation of the cells after the withdral of 8-Cl-cAMP. The data presented here are the average of three independent experiments. In each experiment, every point was determined in duplicate.

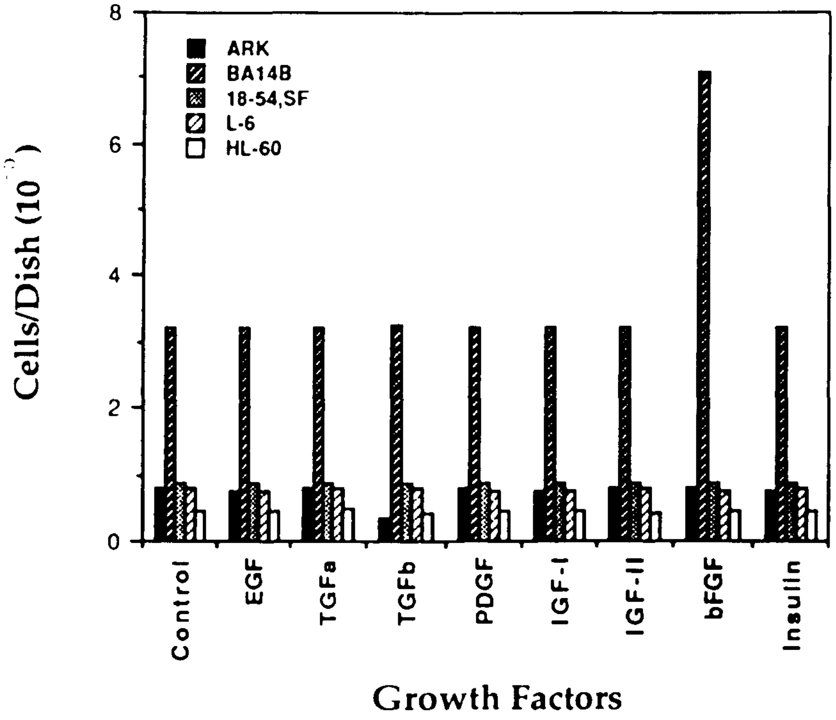

FIGURE 16. BASIC FGF (bFGF) SELECTIVELY ANTAGONIZES THE SERUM-DEPENDENT INHIBITION OF ENDOTHELIAL CELL PROLIFERATION BY 8-Cl-cAMP: In the cell proliferation assay, all cell lines were cultured in the medium containing 10% FBS and 10 uM 8-Cl-cAMP and treated with various growth factors. Concentrations of growth factors: 10ng/ml of EGF, TGFa, PDGF and bFGF; 1 ng/ml TGFb; 50 ng/ml IGF-I, IGF-II and insulin. The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

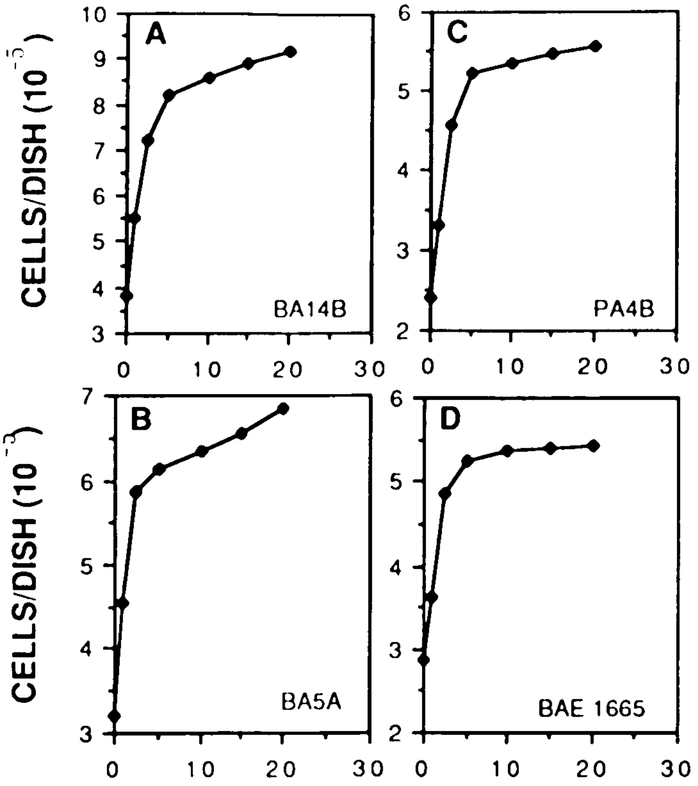

FIGURE 17. BASIC FGF (bFGF) STIMULATES THE PROLIFERATION OF DIFFERENT STRAINS OF ENDOTHELIAL CELLS IN THE PRESENCE OF 8-Cl-cAMP: In the cell proliferation assay, the endothelial cells were cultured in the medium containing 10% FBS and 100 uM 8-Cl-cAMP and the effect of various concentrations of bFGF on the cell proliferation was determined. The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

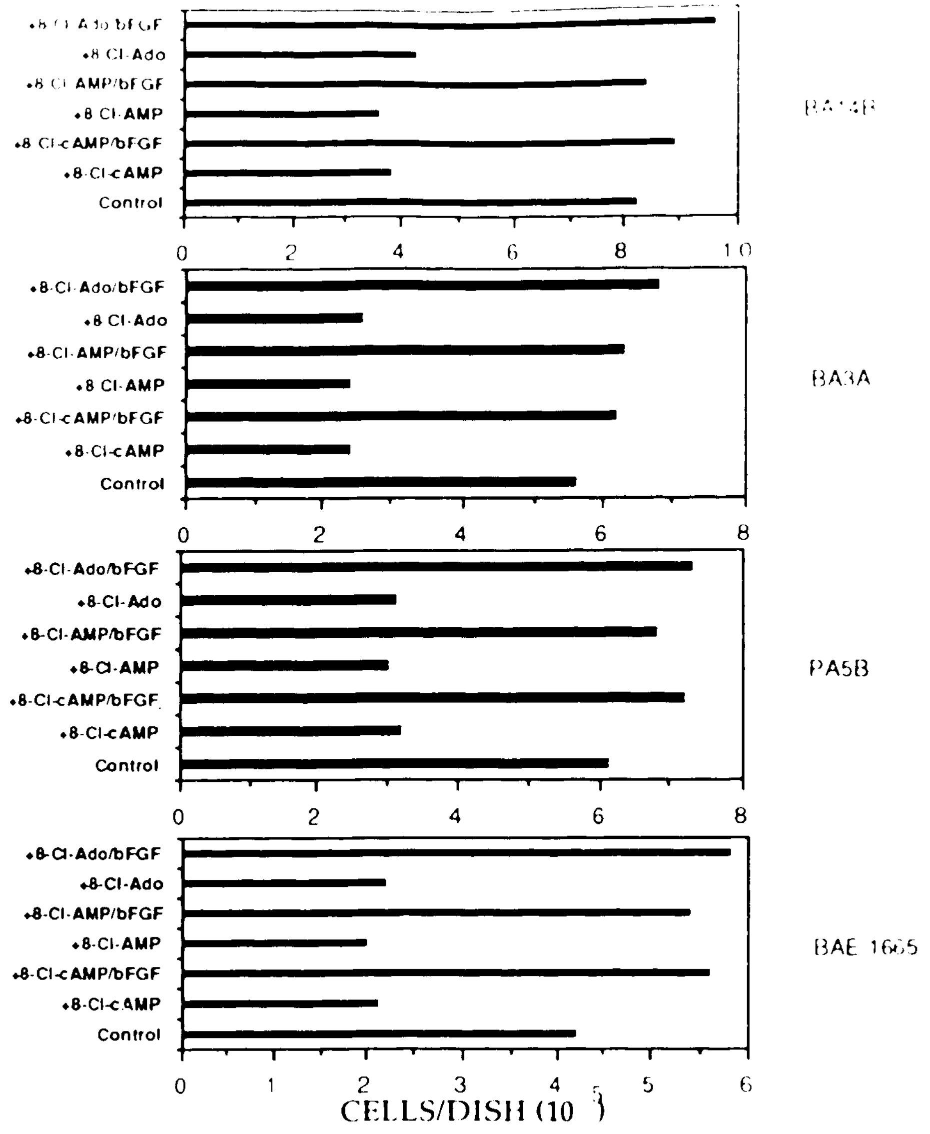

FIGURE 18. BASIC FGF ANTAGONIZES THE INHIBITORY EFFECT OF 8-Cl-CAMP, 8-Cl-AMP, AND 8-Cl-ADENOSINE ON THE PROLIFERATION OF ENDOTHELIAL CELLS: In the cell proliferation assay, cells were cultured in the medium containing 10% FBS or 10% FBS plus100 uM of 8-Cl-cAMP, 8-Cl-AMP or 8-Cl-adenosine and the effect of 10 ng/ml bFGF on cell proliferation in the presence of these analogs was determined. The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

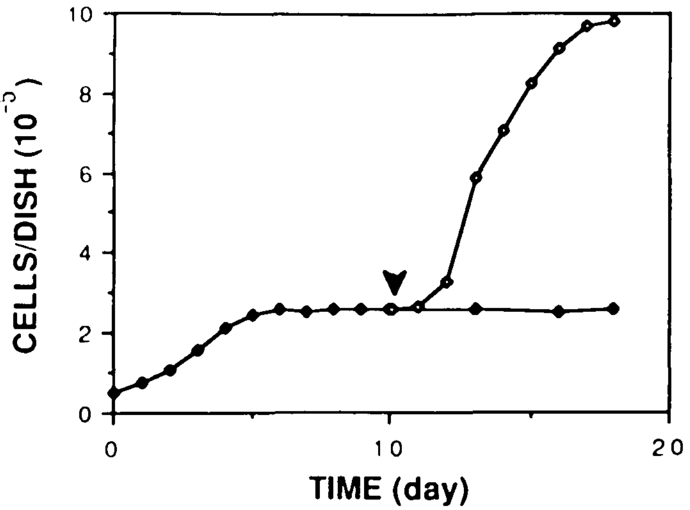

FIGURE 19. BASIC FGF STIMULATES THE PROLIFERATION OF 8-Cl-cAMP-INDUCED QUIESENT ENDOTHELIAL CELLS: In the cell proliferation assay, BA14B cells were initially cultured in the medium containing 10% FBS and 100 uM 8-Cl-cAMP. Then half of the cell culture were shifted into fresh 8-Cl-cAMP-containing medium (solid circle), the other half to the same medium plus 10 ng/ml of bFGF (open circle). The cells were cultured for additional days indicated in the figure. The arrow indicates the time when the medium was changed. The data presented here are obtained from a representative experiment. Each point was determined in triplicate.

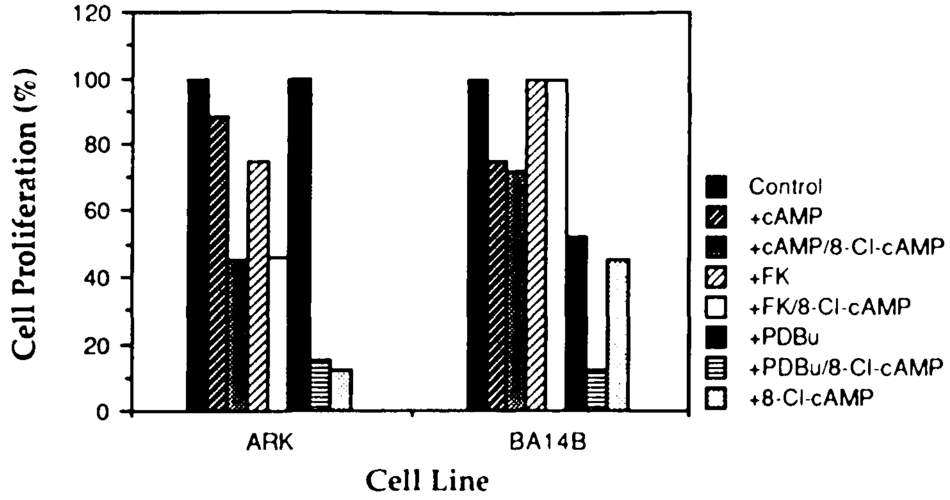

FIGURE 20. Cyclic AMP AND FORSKOLIN (FK) PARTIALLY ANTAGONIZES THE SERUM-DEPENDENT INHIBITORY EFFECT OF 8-Cl-cAMP ON CELL PROLIFERATION: ARK and BA14B cells were cultured in the medium containing 10% FBS plus or minus 2 mM cAMP, 50 uM FK or 10 nM PDBu, and the effect of 10 uM 8-Cl-cAMP on the proliferation of these cells was determined in the cell proliferation assay (see Methods section) The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

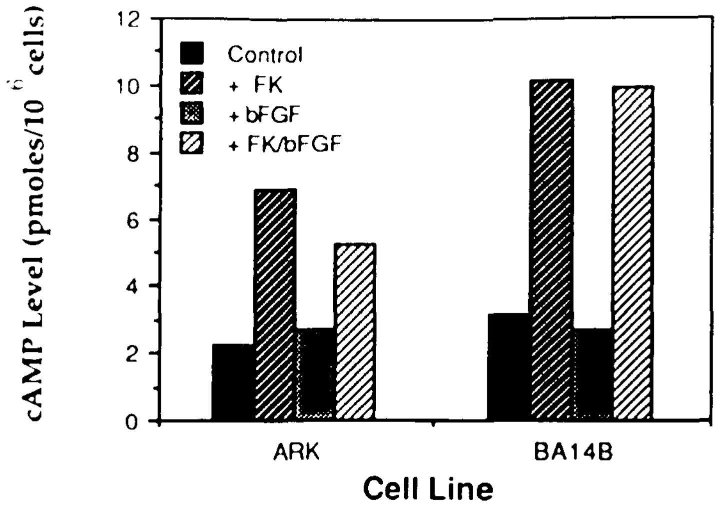

FIGURE 21. BASIC FGF HAS NO EFFECT ON INTRACELLULAR LEVELS OF cAMP ON ARK AND BA14B CELLS: Confluent monolayers of ARK and BA14B cells were cultured in the medium containing 10% FBS and treated with 10 ng/ml bFGF, 50 uM FK or 10 ng/ml bFGF plus 50 uM FK for 24 hours prior to the analysis of intracellular levels of cAMP (see Methods section) The data presented here are the average of five independent experiments. In each experiment, every point was determined in triplicate.

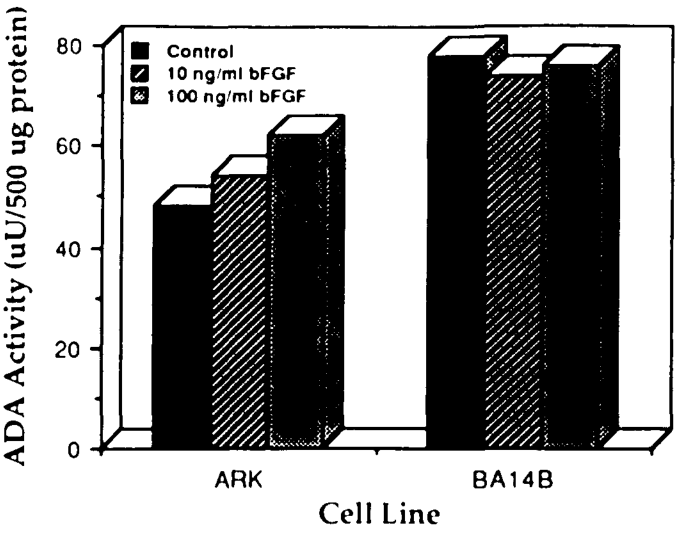

FIGURE 22. BASIC FGF HAS NO EFFECT ON INTRACELLULAR LEVELS OF ADENOSINE DEAMINASE (ADA) IN ARK AND BA14B CELLS: Confluent monolayers of ARK and BA14B cells were cultured in the medium containing 10% FBS and treated with 10 and 100 ng/ml bFGF for 24 hours prior to the analysis of cellular levels of ADA activity (see Methods section) The data presented here are the average of five independent experiments. In each experiment, every point was determined in triplicate.

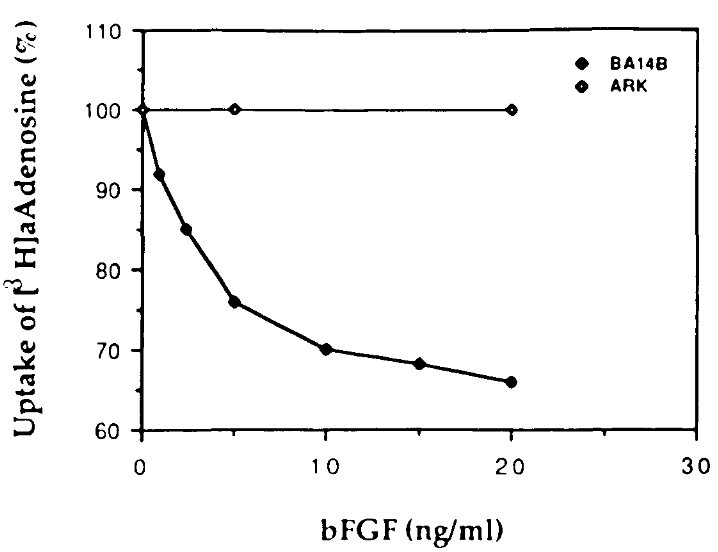

FIGURE 23. BASIC FGF PARTIALLY INHIBITES THE ACTVITY OF THE ADENOSINE NUCLEOTIDE TRANSPORT SYSTEM IN BA14B CELLS: Confluent monolayers of BAE and ARK cells were cultured in the medium containing 10%FBS and various concentrations of bFGF for 20 minutes prior to the addition of [^{3}H]adenosine in the assay for cellular uptake adenosine as described in the methods section. The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

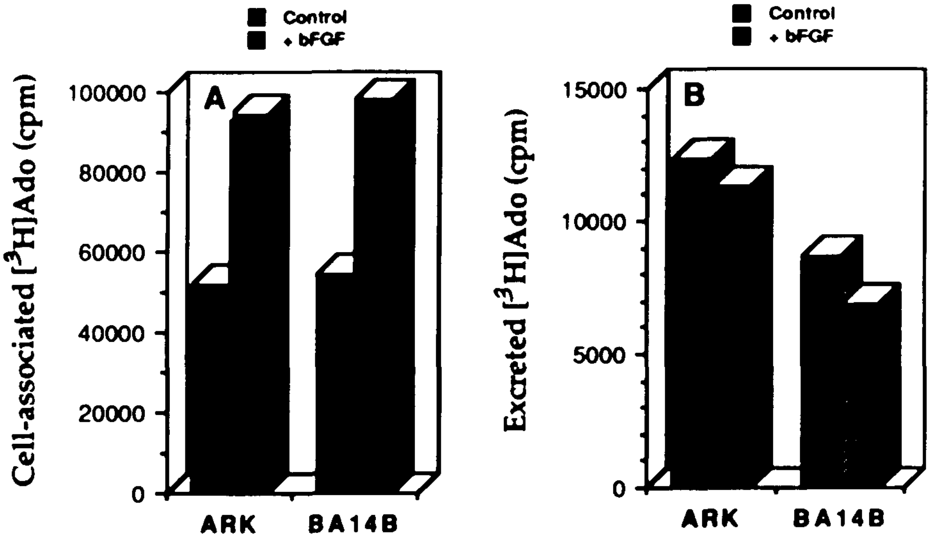

FIGURE 24. BASIC FGF HAS NO REGULATORY EFFECT ON THE EFFLUX OF ADENOSINE IN ARK AND BA14B CELLS: The [3H]adenosine-loaded BA14B and ARK cells were incubated in the medium containing 10%FBS plus or minus 10 ng/ml of bFGF for 30 minutes. After the incubation, the radioactivity remained in the cell (A) and released into the medium (B) were determined and used to represent cell-associated and excreted adenosine nucleotides (see Methods section). The data presented here are the average of five independent experiments. In each experiment, every point was determined in triplicate.

FIGURE 25. BASIC FGF SELECTIVELY ANTAGONIZES 8-Cl-cAMP-INDUCED INHIBITION OF THE PHOSPHORYLATION OF PROTEINS IN BA14B CELLS: Both BAE (lane 1-5) and ARK (lane 6-10) cells were cultured in phosphate-free medium supplemented with 10 uCi [^{32}P]phosphate and 10% FBS for 24 hours. Alternatively, cells were treated with 10 ng/ml bFGF (lane 2 and 7), or 10 uM 8-Cl-cAMP (lane 3 and 8), or 10 ng/ml bFGF plus 10 uM 8-Cl-cAMP (lane 4 and 9) for 24 hours. In addition, cells were first treated with 10 uM 8-Cl-cAMP for 24 hours and then treated with 10 ng/ml bFGF for additional 30 minutes. Approximately 10 ug proteins from the whole cell extract were analyzed in a 10% SDS-gel (see <u>Methods</u> section). This is a representative autoradiogram from a typical experiment. This experiment was repeated three times.

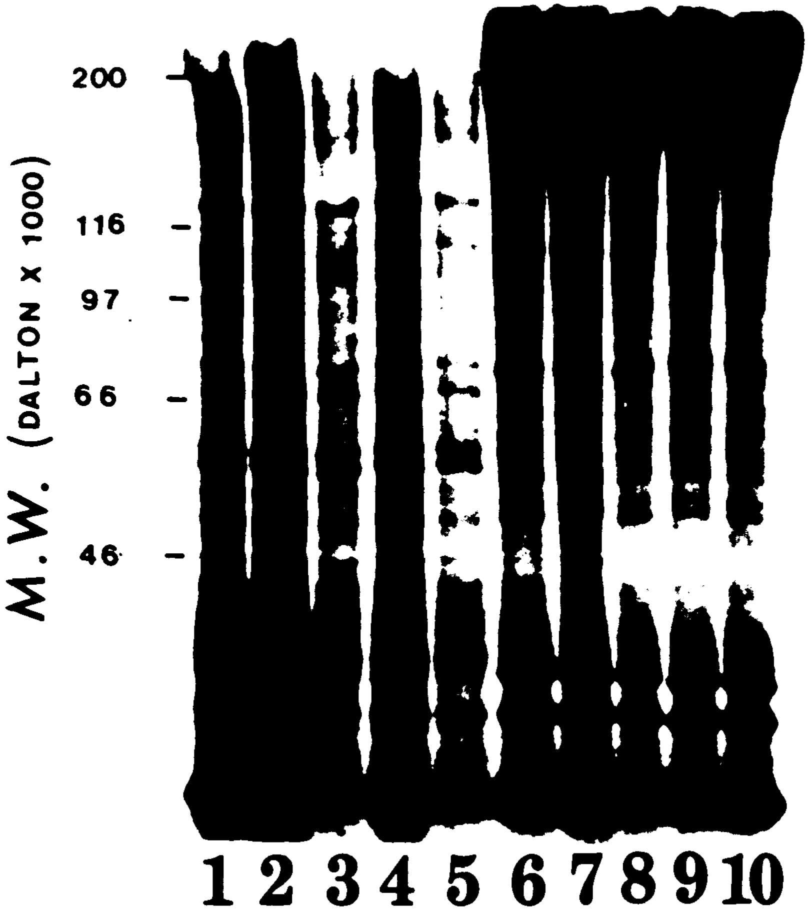

FIGURE 26. EFFECT OF 8-Cl-cAMP-TREATMENT ON THE NUCLEAR CONTENT OF RI AND RII OF PKA IN BA14B AND ARK CELLS: Both ARK (lane 1-4) and BA14 (lane 5-8) cells were cultured in the medium containing 10% FBS and treated with 5 uM (lane 2 and 6), 10 uM (lane 3 and 7), or 20 uM 8-Cl-cAMP (lane 4 and 8) for 24 hours before the analysis of cAMP-binding proteins. Approximately 20 ug proteins were used for the photoaffinity labelling and SDS-PAGE analysis using [^{32}p]8-azido-cAMP as the binding ligand for cAMP-binding proteins, and the RI and RII were identified according to Richards and Rolfes (Richards and Rolfes, 1980).

FIGURE 27. EFFECT OF 8-Cl-cAMP-TREATMENT ON THE CONTENT OF CYTOSOLIC RI AND RII IN BAE AND ARK CELLS: Both BAE (A) and ARK (B) cells were cultured in the medium containing 10% FBS and treated with 5 uM (lane 2 and 6), 10 uM (lane 3 and 7), or 20 uM 8-Cl-cAMP (lane 4 and 8) for 24 hours before the analysis of cAMP-binding proteins. Approximately 10 ug proteins were used for the photoaffinity labelling and SDS-PAGE analysis using [^{32}p]8-azido-cAMP as the binding ligand for cAMP-binding proteins, and the RI and RII were identified according to Richards and Rolfes (Richards and Rolfes, 1980).

FIGURE 28. EFFECT OF bFGF ON THE NUCLEAR CONTENT OF RI AND RII OF PKA IN 8-Cl-cAMP-TREAT BA14B AND ARK CELLS: Both ARK (lane 1-4) and BA14B (lane 5-8) cells were cultured in the medium containing 10% FBS and 10 uM 8-Cl-cAMP and treated with 5 ng/ml (lane 2 and 6), 10 ng/ml (lane 3 and 7), or 20 ng/ml of bFGF (lane 4 and 8) for 48 hours before the analysis of cAMP-binding proteins. Approximately 20 ug proteins were used for the photoaffinity labelling and SDS-PAGE analysis using [^{32}p]8-azido-cAMP as the binding ligand for cAMP-binding proteins, and the RI and RII were identified according to Richards and Rolfes (Richards and Rolfes, 1980).

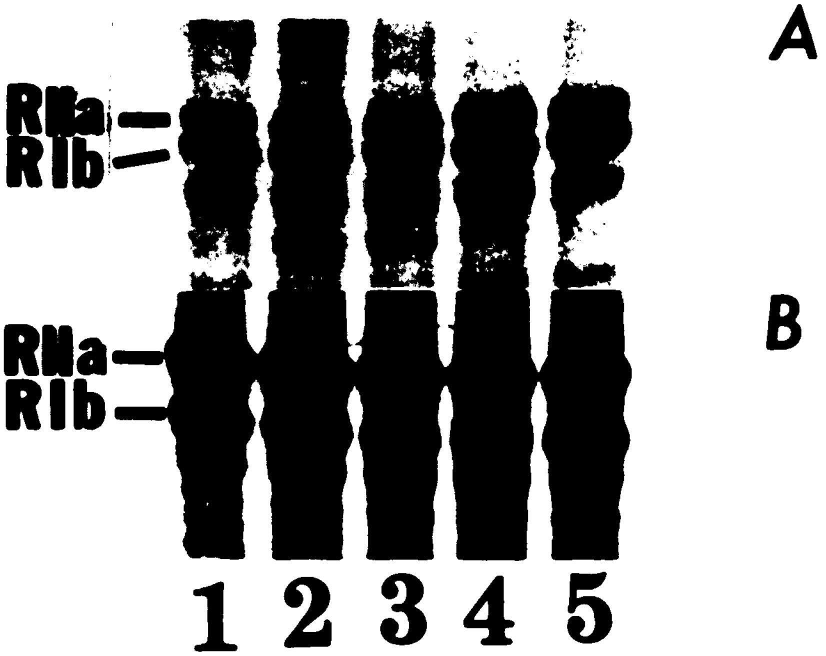

FIGURE 29. EFFECT OF bFGF ON THE CYTOSOLIC CONTENT OF RI AND RII OF PKA IN 8-Cl-cAMP-TREAT BA14B AND ARK CELLS: Both BA14B (A) and ARK (B) cells were cultured in the medium containing 10% FBS and 10 uM 8-Cl-cAMP and treated with 1 ng/ml (lane 2), 5 ng/ml (lane 3), 10 ng/ml of bFGF (lane 4) and 20 ng/ml bFGF (lane 5) for 48 hours before the analysis of cAMP-binding proteins. Approximately 10 ug proteins were used for the photoaffinity labelling and SDS-PAGE analysis using [^{32}p]8-azido-cAMP as the binding ligand for cAMP-binding proteins, and the RI and RII were identified according to Richards and Rolfes (Richards and Rolfes, 1980).

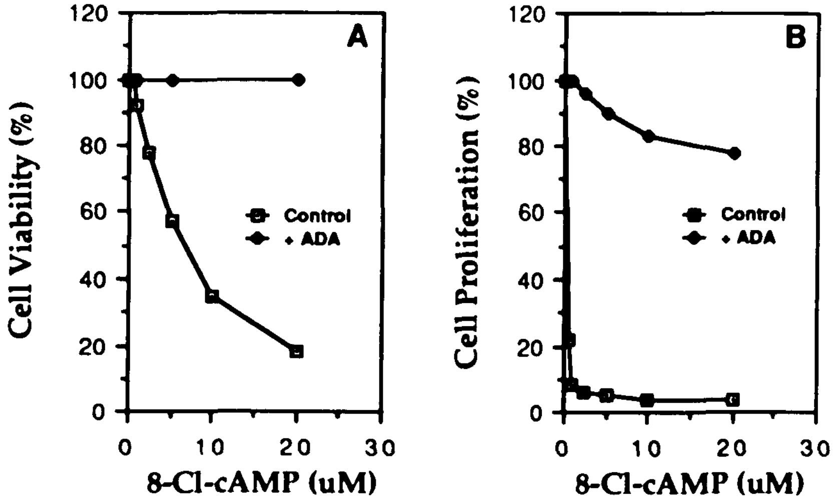

FIGURE 30. 8-Cl-cAMP-INDUCED INHIBITION OF THE PROLIFERATION AND VIABILITY OF HL-60 CELLS IS SENSITIVE TO ADENOSINE DEAMINASE (ADA): HL-60 cells were cultured in the medium containing 20% FBS plus or minus 1 unit/ml of ADA, and treated with 8-Cl-cAMP for 5 days. The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

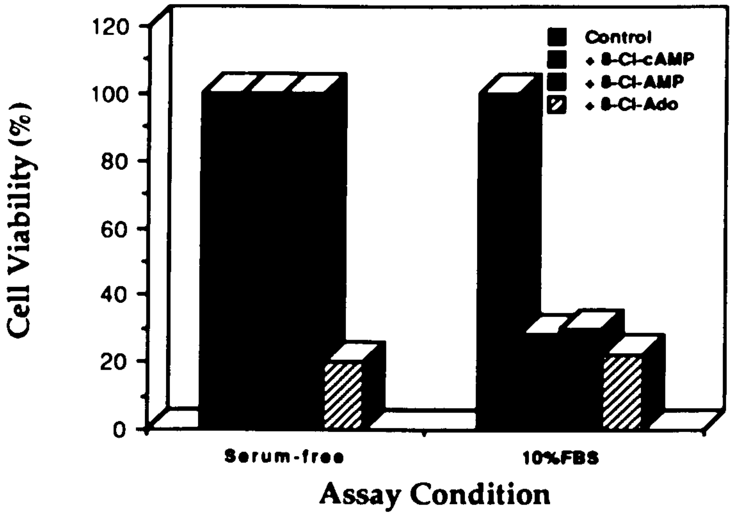

FIGURE 31. THE INHIBITORY EFFECT OF 8-Cl-cAMP AND 8-Cl-AMP ON THE VIABILITY OF HL-60 CELLS IS SERUM-DEPENDENT: The HL-60 cells were cultured in the serum-free or serum-containing (20% FBS) medium and treated with 10 uM of 8-Cl-cAMP, 8-Cl-AMP or 8-Cl-adenosine for 5 days prior to the determination of cell viability.(see Methods section). The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

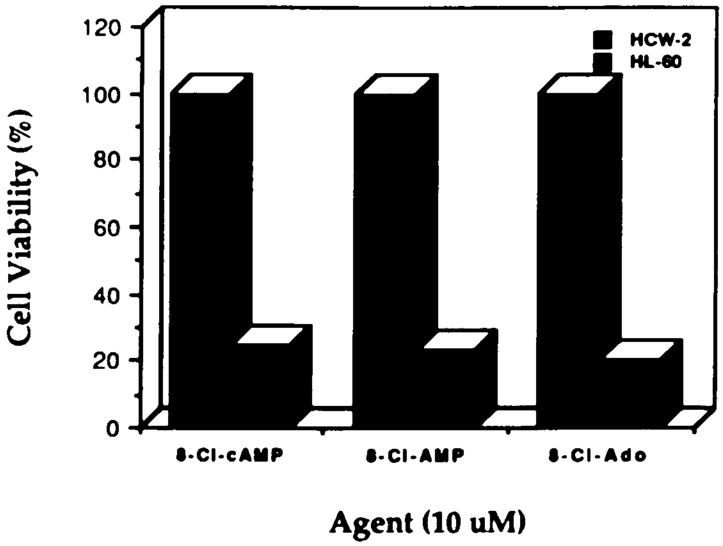

FIGURE 32. COMPARATIVE EFFECTS OF 8-Cl-cAMP, 8-Cl-AMP AND 8-Cl-ADENOSINE ON THE VIABILITY OF HL-60 AND HCW-2 CELLS: The HL-60 and HCW-2 cells were cultured in the medium containing 20% FBS and treated with 10 uM of 8-Cl-cAMP, 8-Cl-AMP or 8-Cl-adenosine for 5 days prior to the determination of cell viability.(see Methods section). The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

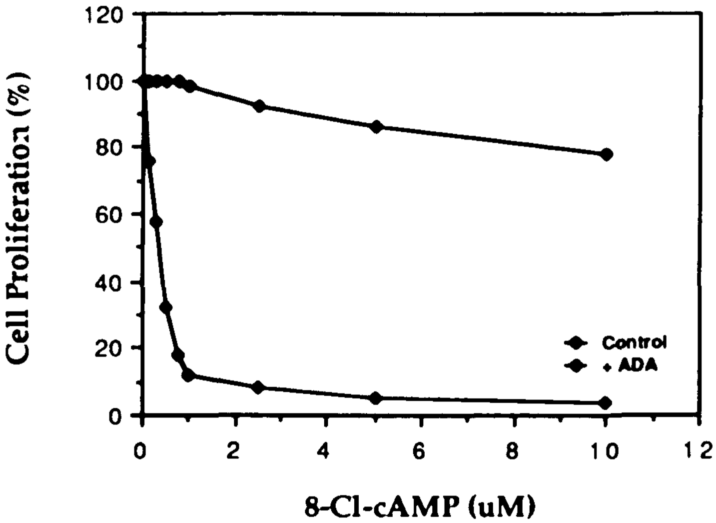

FIGURE 33. 8-Cl-cAMP-INDUCED INHIBITION OF THE PROLIFERATION OF HCW-2 CELLS IS SENSITIVE TO ADENOSINE DEAMINASE (ADA): HCW-2 cells were cultured in the medium containing 20% FBS or 20% FBS plus 1 unit/ml of ADA, and the effect of various concentrations of 8-Cl-cAMP on the cell proliferation was determined in the cell proliferation assay (see <u>Methods</u> section). The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

FIGURE 34. HCW-2 CELLS DO NOT POSSESS NUCLEAR RI AND RII OF PKA: The HL-60 (lane 1-5) and HCW-2 (lane 6-12) cells were cultured in the medium containing 20% FBS (lane 1 and 6), and treated with 10 uM 8-Cl-cAMP for 24 (lane 2 and 7), 48 (lane 3 and 8), 72 (lane 4 and 9) and 96 hours (lane 5 and 10). In addition, HCW-2 cells were treated with 8-Cl-cAMP for up to 1 (lane 11) and 2 months (lane 12) prior to the preparation of nuclear extract. Approximately 20 ug proteins from the extract was used for the analysis of cAMP-binding proteins in the photoaffinity labelling and SDS-PAGE analysis using [^{32}p]8-azido-cAMP as the binding ligand (Richards and Rolfes, 1980).

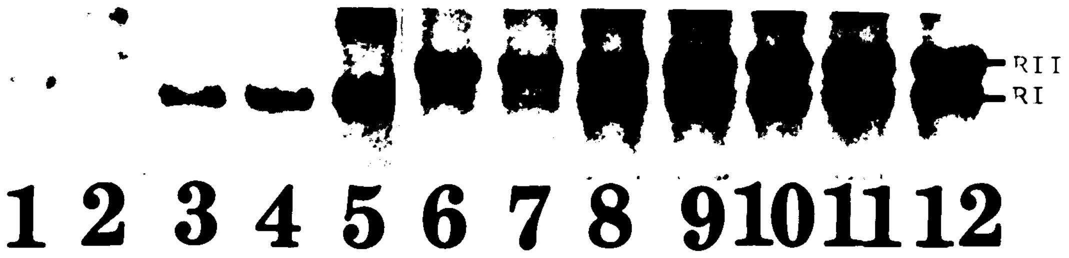

FIGURE 35. EFFECT OF 8-Cl-cAMP-TREATMENT ON THE LEVELS OF CYTOSOLIC RI AND RII OF PKA IN HL-60 AND HCW-2 CELLS: The HL-60 (lane 1-5) and HCW-2 (lane 6-12) cells were cultured in the medium containing 20% FBS (lane 1 and 6), and treated with 10 uM 8-Cl-cAMP for 24 (lane 2 and 7), 48 (lane 3 and 8), 72 (lane 4 and 9) and 96 hours (lane 5 and 10). In addition, HCW-2 cells were treated with 8-Cl-cAMP for up to 1 (lane 11) and 2 months (lane 12) prior to the preparation of nuclear extract. Approximately 10 ug cytosolic proteins were used for the analysis of cAMP-binding proteins in the photoaffinity labelling and SDS-PAGE analysis using [^{32}p]8-azido-cAMP as the binding ligand (Richards and Rolfes, 1980).

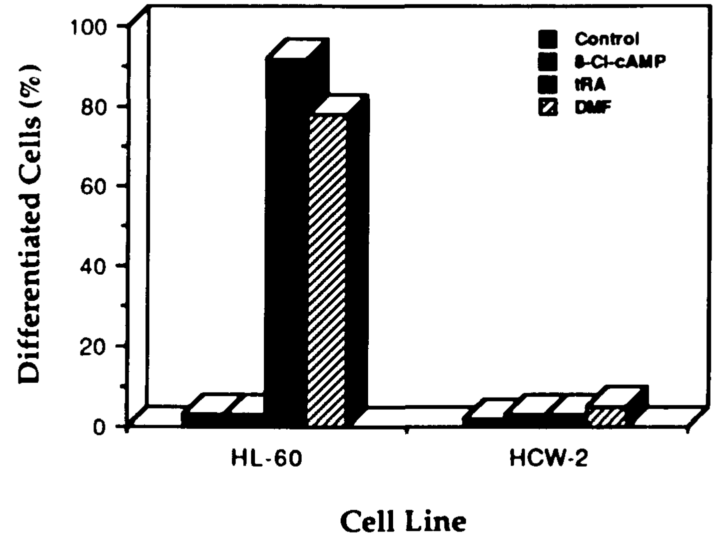

FIGURE 36. COMPARATIVE EFFECTS OF 8-Cl-cAMP, tRA, AND DMF ON THE DIFFERENTIATION OF HL-60 AND HCW-2 CELLS: The HL-60 and HCW-2 cells were incubated in the medium containing 20% FBS and treated with 10 uM 8-Cl-cAMP or 2 uM tRA for 3 days or with 0.8% DMF for 6 days. After this treatment, cells were stained by NBT-staining technique and the percentage of terminally differentiated cell was determined (see Methods section). The data presented here are the average of three independent experiments.

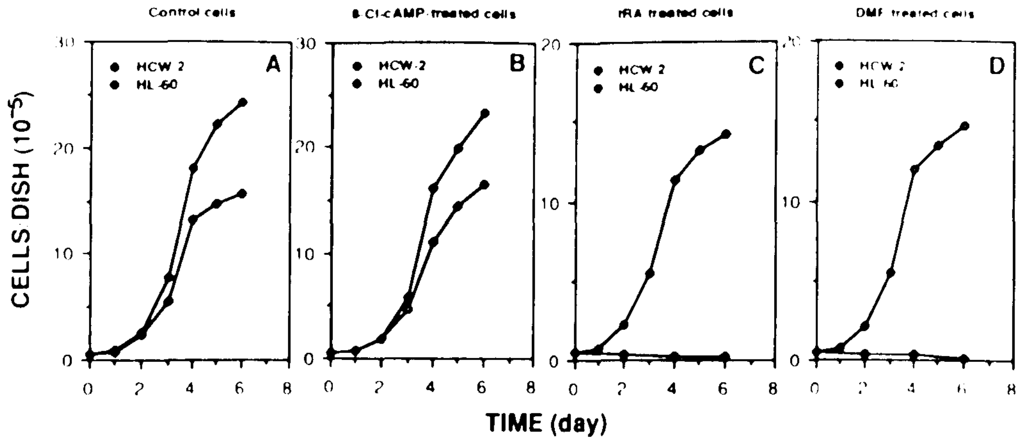

FIGURE 37. LONG-TERM EFFECT OF 8-Cl-cAMP-, tRA- AND DMF-TREATMENT ON THE PROLIFERATION OF HL-60 AND HCW-2 CELLS: Stock cultures of the HL-60 and and HCW-2 cells were incubated in the medium containing 20% FBS and treated with 10 uM 8-Cl-cAMP, or 2 uM tRA for 72 hours, or with 0.8% DMF for 8 days. After the treatment, the cells were washed and the proliferation of these cells was determined in the absence of those agents (see Methods Section). The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

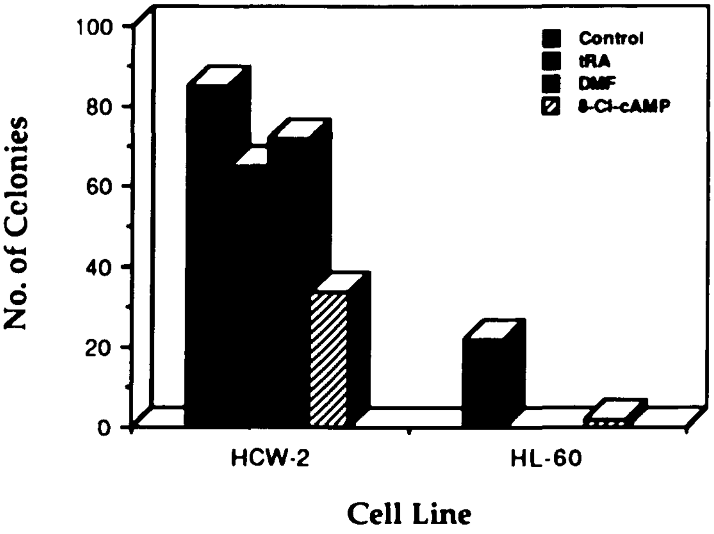

FIGURE 38. COMPARATIVE EFFECTS OF 8-Cl-cAMP, tRA, AND DMF ON THE CLONOGENICITY OF HL-60 AND HCW-2 CELLS: Stock cultures of HL-60 and HCW-2 cells were incubated in the medium containing 20% FBS and treated with 10 uM 8-Cl-cAMP or 2 uM tRA for 3 days or with 0.8% DMF for 8 days. The cells were washed and seeded into soft-agarose medium supplemented with 20% FBS and incubated for two weeks.Total number of colonies per ten microscopic field was independently determined by two persons. The clonogenicity of the cells is represented by the average number of colonies. The data presented here are the average of three independent experiments. In each experiment, every point was determined in triplicate.

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