Killing of Fibroblasts by Dexamethasone or Dibutyryl Adenosine 3′,5′-Monophosphate Is Not a Valid Test for Cystic Fibrosis

Abstract. *Assays based on the counting of total cells and of colony-forming cells were used to demonstrate that neither dexamethasone nor dibutyryl adenosine 3′,5′-monophosphate (cyclic AMP) kills human fibroblasts under a variety of conditions. These results contradict those of previous studies showing that dexamethasone and dibutyryl cyclic AMP kill a higher percentage of fibroblasts from normal humans than from individuals with cystic fibrosis.*

Epstein, Breslow, and their co-workers have reported (*1–4*) that both dexamethasone and dibutyryl adenosine 3′,5′-monophosphate (cyclic AMP) kill cultured fibroblasts derived from skin of normal individuals at concentrations (10^{-10} to $10^{-7}M$) at which they have little effect on the viability of fibroblasts from individuals with cystic fibrosis (CF). This selective effect of dexamethasone has been proposed as the basis of a diagnostic test for the disease in fetuses and in individuals heterozygous for the trait (*2*). In view of the potential clinical significance of such a test, we initiated studies that were designed to confirm these observations. Our results do not agree with the findings reported in (*1–4*). In spite of care to simulate their experimental conditions, we found no significant killing of normal human fibroblasts by dexamethasone or dibutyryl cyclic AMP at the concentrations that they used.

Skin fibroblast strains N381, N57, and N834 were derived from female donors 6, 21, and 28 years of age, respectively, at the Hospital for Sick Children in Toronto. The donors were unrelated and had no history of CF. The methods of culture have been described (*5*). Cells were thawed (except for strain N834, which was not frozen) and passaged twice before use. Cells tested in Eagle's MEM-B [minimal essential medium supplemented with the seven nonessential amino acids (Gibco), glucose (0.9 g/liter), 10 percent fetal bovine serum, penicillin, and streptomycin (*1*)] were subcultured twice in the new medium before being used.

The skin fibroblast strains with the prefix GM were obtained at third through ninth passages from the Human Genetic Mutant Cell Repository at the Institute for Medical Research, Camden, New Jersey. All strains were unrelated except GM1651 and GM1652, which came from sisters. Stock cultures of these cells were frozen over liquid nitrogen after two to seven passages of 1:4 dilutions in Eagle's MEM supplemented with 1.0 m*M* pyruvate, 0.2 m*M* asparagine, 0.2 m*M* serine (*6*), and 10 to 20 percent fetal calf serum (*7*).

In a preliminary study, in which a cloning assay (*1*) was used, cell strains GM323 and GM316 showed, respectively, 104 and 123 percent survival after exposure to $10^{-7}M$ dexamethasone at densities of 100 to 300 cells per 100-mm dish. In contrast, Epstein *et al.* (*1*) reported 15 to 24 percent survival for seven normal fibroblast strains exposed to $10^{-7}M$ dexamethasone, and 10 percent survival for two normal strains exposed to $10^{-6}M$ dexamethasone.

We therefore attempted to find a combination of cell culture techniques that would allow demonstration of significant killing of human skin fibroblasts by dexamethasone. We investigated the uniqueness of serum by using lots from Microbiological Associates (*1*) and Gibco. We tested the effect of antibiotics used by Epstein *et al.* (*1*). We compared the effects of dexamethasone (*1*) to those of dexamethasone phosphate (*2, 3*). We also used different protocols and solvents for dissolving the chemicals and adding them to the cells. In no instance did the average of three dishes show more than 25 percent killing by $10^{-6}M$ dexamethasone or dexamethasone phosphate for any of the four strains tested. Table 1 shows all of the results of a survey of 11 normal cell strains tested as described (*1*) except for the difference in culturing technique discussed below.

In addition to fibroblast strains obtained from the Institute for Medical Research (Table 1), three other normal cell strains (N57, N381, and N834) were tested independently in similar experiments in Toronto in order to determine the effects on their cloning efficiency of dexamethasone and dexamethasone phosphate (10^{-10} to $10^{-5}M$) in both Eagle's MEM-B and α medium (*5*) in which fetal calf serum obtained from Flow Laboratories was used. Inhibition of cloning efficiency never exceeded 26 percent, with an average of 8 percent at $10^{-7}M$ and 16 percent at $10^{-5}M$. The average survival of two cell strains from Toronto and ten strains from the Institute for Medical Research, grown in Eagle's MEM-B and exposed to dexamethasone or dexamethasone phosphate (10^{-7} to $10^{-5}M$), was 105 ± 4 percent (± standard error; 29 determinations). Similar results were obtained with eight skin fibroblast strains from subjects with CF.

In a negative report of this type it is important to establish the availability and efficacy of the drugs used. The presence of dexamethasone phosphate at the

Table 1. Effect of dexamethasone (dex) on the cloning efficiency of normal human fibroblasts. For this type of experiment, strains from the Institute for Medical Research were thawed and passaged once or twice at 2500 cells per square centimeter before being cultured at densities of 2 to 10 cells per square centimeter (here, 300 cells per 100-mm dish) in 10 ml of Eagle's MEM-B without antibiotics (inocula of 1000 cells per dish sometimes resulted in dishes too crowded with clones to permit accurate counting). After 24 hours we added 1 ml of Eagle's MEM-B containing 1 percent ethanol with or without the test agent at a concentration ten times greater than the desired final concentration (here, $10^{-5}M$ dexamethasone phosphate diluted in medium from $10^{-3}M$ in ethanol). Twenty-four hours later the cells were washed with one or two 10-ml rinses of Hanks salts or Eagle's MEM (here, Eagle's MEM) and fed with 10 ml of Eagle's MEM-B. The medium was replaced after 7 to 10 days (here, 10) and the cells were fixed after 13 to 15 days (here, 15) with 70 percent ethanol. The cells were stained for about 1 minute with a filtered solution of Giemsa (0.2 g) in methanol (100 ml) and for 30 seconds after removal of the excess stain with water. Cloning efficiency was defined as the number of colonies with about 30 or more cells (about five doublings) divided by the calculated number of cells originally cultured. Survival was defined as cloning efficiency after exposure to the test agent divided by cloning efficiency without exposure.

Strain	Sex	Donor age (years)	Clones per dish		Survival (%)
			With dex	Without dex	
GM323	M	11	43, 48, 65	73, 60	78
GM1582	F	11	29, 23, 29	23, 23, 20	123
GM497	M	4	3, 2, 0	2, 3, 0	
GM316	M	12	40, 36, 38	47, 46, 44	83
GM409	M	7	1, 7, 3	5, 0, 1	
GM500	M	10	14, 15, 14	11, 13	120
GM1651	F	11	18, 15, 15	16, 17, 10	112
GM1652	F	13	27, 36, 42	14, 29	162
GM495	M	29	33, 34, 36	33, 32, 32	106
GM2037	M	13	37, 39, 28	25, 22, 31	134
GM498	M	3	86, 68	83, 72, 80	98
Mean ± standard error					113 ± 9

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correct concentration ($10^{-4}M$) was ascertained spectrophotometrically (wavelength, 239 nm; absorption coefficient, 14,000 in ethanol) when dissolved in ethanol, Hepes-buffered Eagle's MEM, and Hepes-buffered Eagle's MEM plus 1 percent fetal calf serum (*8*). Dexamethasone phosphate was biologically potent, as indicated by its effect on S-49 lymphoma cells (*9, 10*); significant inhibition of the increase in cell number was observed after 32 hours and complete cessation of growth occurred after 3 days in the presence of the chemical at $10^{-6}M$.

The validity of the fibroblast cloning system as a measure of cell killing was demonstrated by measuring the effects of ouabain (*11, 12*). Cells from strains GM498 and GM323 were cultured at a density of 300 cells per dish and exposed to 10^{-10}, 10^{-9}, 10^{-8}, and $10^{-7}M$ ouabain and $10^{-6}M$ dexamethasone phosphate. Concentrations of ouabain $10^{-8}M$ and lower had little or no effect, but $10^{-7}M$ ouabain lowered cloning efficiency to less than 2 percent of control; $10^{-6}M$ dexamethasone phosphate caused no more than 20 percent inhibition of cloning efficiency.

We also measured the effects of dexamethasone on cell growth by determining cell number with a particle counter. If a high percentage of cells was killed by dexamethasone, a difference in cell number compared to controls should be evident within a few cell doubling times. In four experiments of this type, we used five normal cell strains in Dulbecco's MEM and Eagle's MEM-B at high and low cell densities. Exposure to $10^{-7}M$ dexamethasone for 24 hours resulted in no inhibition of growth after 10 days of culture. Exposure to $10^{-6}M$ dexamethasone phosphate produced 19 to 25 percent inhibition of growth for strain GM498 and 7 percent inhibition for strain GM316 after 6 days of culture. Ouabain, however, caused 80 to 90 percent inhibition of growth at $10^{-7}M$, with greater inhibition at $10^{-6}M$ and no significant inhibition at $10^{-8}M$.

Still another experiment was designed to monitor cell number and cloning efficiency simultaneously for two strains at 500 cells per 100-mm dish. Strain GM498 (carried 14 days before cloning, rather than 6 days as in Table 1) exhibited a cloning efficiency of 5.3 percent (average of four to six dishes); cell number was 156,000 cells (average of duplicates). Strain GM499 showed a cloning efficiency of 1.8 percent and averaged 57,000 cells. Exposure of cells to 10^{-7} and $10^{-6}M$ dexamethasone phosphate or 10^{-9} and $10^{-8}M$ ouabain did not decrease these values significantly, but $10^{-7}M$ ouabain decreased both cloning efficiency and cell number by 95 percent.

Table 2. Effect of dibutyryl cyclic AMP on the survival of normal human fibroblasts. In experiment 1, strain N834 was used and 212 cells were cultured per dish; in experiment 2, strain N381 was used at 221 cells per dish. Cells in the fifth through tenth generations were passaged from a late exponential phase and cultured in 100-mm dishes in 9 ml of Eagle's MEM-B supplemented with 10 percent fetal calf serum. After 24 hours, 1 ml of dibutyryl cyclic AMP (Sigma) at a concentration ten times greater than the desired final concentration was added in complete medium. The medium and drugs were removed after 24 hours and the cells were washed twice with Earle's balanced salt solution and fed again with 10 ml of medium. The cells were fed 7 days after the start of the experiment and stained and counted 7 days later.

Concentration of dibutyryl cyclic AMP (M)	Clones per dish	Average cloning efficiency (%)	Survival (%)
	Experiment 1		
0	65, 56, 50, 51	26	
10^{-8}	47, 59, 71, 54	27	104
10^{-6}	49, 86, 61	31	119
10^{-4}	49, 50	24	92
	Experiment 2		
0	16, 15, 19, 18	7.7	
10^{-8}	20, 31, 17	10.2	132
10^{-6}	20, 12	7.2	94
10^{-4}	18, 13, 11, 16	6.6	86

Thus the results of the five experiments involving cell counts support the conclusion, based on the cloning experiments, that concentrations of dexamethasone between 10^{-7} and $10^{-5}M$ do not kill more than a small percentage (certainly less than 25 percent) of cells from all tested strains of normal human skin fibroblasts. Epstein *et al.* (*1*) observed a 75 to 90 percent killing of normal skin fibroblasts at 10^{-7} to $10^{-5}M$ dexamethasone. We attempted to uncover causes for the disparity by considering the manner in which dexamethasone was applied, the availability and potency of the dexamethasone phosphate used, the serum source, and the presence or absence of antibiotics. None of these factors appear to be involved (*13, 14*).

We are aware of at least one possible explanation for the differences between our results and those of Epstein and his co-workers. Their procedure (*1-4, 12*) has been to add enough cells to each experimental dish to produce 75 to 100 colonies. Therefore, more cells were cultured on experimental dishes than on control dishes. The results of an experiment relating size of the inoculum to apparent cloning efficiency suggest that even without dexamethasone, the cloning efficiency decreases at higher cell densities. The cloning efficiency of GM498 cells decreased progressively from 22.9 to 12.6 percent as the size of the inoculum was increased from 100 to 1000 cells. The cloning efficiency of GM323 cells likewise decreased from 31.7 to 16.6 percent (*15*). Possibly the large clones covered up smaller clones that would have been observed at lower clone densities; especially susceptible to such obscuration would be the small clones exhibiting a dispersed morphology (*16*). Another factor leading to fewer detectable clones after introduction of a large inoculum would be the faster depletion of growth factors in the medium by the larger numbers of cells.

Other reports offer some perspective on the effects of ouabain and dexamethasone on fibroblasts. Concerning the effects of ouabain on growth of human skin fibroblasts, Mankovitz *et al.* (*11*) observed that the effect of ouabain on cell growth was negligible up to $10^{-8}M$ but caused 80 percent inhibition at $10^{-7}M$ for two normal strains. Epstein and Breslow (*12*) measured a progressive increase in inhibition of cell growth by ouabain, from 40 percent at $10^{-10}M$ to 90 percent at $10^{-6}M$. Although it is true that the two research groups measured the ouabain effect with different assays, the rationalization put forth by Epstein and Breslow (*12*) cannot explain the lack of killing at ouabain concentrations of $10^{-8}M$ or less noted by Mankovitz *et al.* (*11*). In both of our laboratories, we confirmed the concentration-effect relationship for ouabain whether we assayed by cell number or by cloning efficiency (*17*). We suggest that the progressive decrease in survival between 10^{-10} and $10^{-6}M$ ouabain measured by Epstein and his co-workers represents the peculiarities of their cloning assay more than the effect of ouabain; indeed, all of their survival curves are similar whether they measured the effect of dexamethasone (*1*), ouabain (*12*), dibutyryl cyclic AMP, isoproterenol, theophylline (*4*), or three sex steroids (*3*). The shape of these dose-response curves cannot be reconciled with the presently understood interactions of these substances with their receptors.

Finally, we tested dibutyryl cyclic AMP, another putative fibroblast killer (*4*), on the cloning efficiency of cell strains N834 and N381 (Table 2). In contrast to the data of Epstein *et al.* (*4*), which showed 30 percent inhibition at $10^{-10}M$ dibutyryl cyclic AMP, isoproterenol, or theophylline increasing to 90 percent inhibition at $10^{-5}M$, we found

very little inhibition (10 to 15 percent) at concentrations of dibutyryl cyclic AMP as high as $10^{-4}M$. Although the cells used in the experiment described in Table 2 were trypsinized from stock cells with 0.25 percent trypsin, another experiment comparing 0.25 percent trypsin in citrate saline with 0.05 percent trypsin in 0.5 mM Na_2EDTA (*4*) also showed no killing by 10^{-8}, 10^{-6}, and $10^{-4}M$ dibutyryl cyclic AMP.

We know of no other evidence showing that human fibroblasts are killed by glucocorticoids. The nonhuman fibroblast cell line most studied in this respect is the mouse L cell. However, whereas steroids inhibit the growth rate of these cells, they do not cause the cytolysis that occurs in some nonfibroblast cell types, such as thymic lymphocytes (*18*).

Other investigators have found some stimulation of fibroblast growth by glucocorticoids (*19, 20*), some have found no effect (*21, 22*), and one group reported some inhibition with $3 \times 10^{-6}M$ cortisol and prednisolone (*23*). Milo *et al.* (*24*) showed that estradiol and cortisol at concentrations as high as $2 \times 10^{-6}M$ did not inhibit cloning of human embryonic lung and newborn foreskin fibroblasts; in fact, glucocorticoids, under certain conditions, are required for best clonal growth (*24, 25*).

An entire field of well-documented research is concerned with the lengthening of the lifetime in vitro of human embryonic lung fibroblasts by $10^{-5}M$ cortisone and cortisol (*26-28*). Although the lifetimes in vitro of skin fibroblasts are not uniformly lengthened by glucocorticoids, no large inhibitions of cell growth are observed (*29*). In particular, strain GM316, which both we and Breslow *et al.* (*2*) have tested, was found to be unaffected (*29*).

Thus, as discussed by Grove *et al.* (*29*), fibroblasts may vary in their response to glucocorticoids depending on the tissue source and the age of the donor. In addition, skin fibroblasts may differ according to the type of skin and the microenvironment of the particular sample. However, killing or large inhibition of growth is not seen even in the continual presence of steroids; thus the observation by Epstein and his co-workers (*1-3*) that exposure to low doses of dexamethasone for 24 hours causes significant killing of human skin fibroblasts is unique. When we measure the effect of dexamethasone, dibutyryl cyclic AMP, and ouabain in straightforward cloning assays performed independently, we observe a killing effect by ouabain only. Thus, there appears to be no basis for the diagnosis of CF in homozygotes or heterozygotes by a technique based on a differential kill by dexamethasone of their fibroblasts compared to normal fibroblasts (*2*).

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7. Media powder, Hanks salts, and trypsin were from Gibco; pyruvate, dexamethasone, and Hepes buffer were from Sigma; asparagine and serine were from Calbiochem; glucose, sodium citrate, and potassium chloride were from Baker; sodium chloride, dimethyl sulfoxide, and Giemsa stain were from Fisher; ouabain was from Lilly; penicillin G was from Parke, Davis; and streptomycin was from Pfizer. The dexamethasone phosphate was a gift from Merck Sharp & Dohme. Fetal bovine serum was from both Microbiological Associates and Gibco.
8. Since medium containing 10 percent serum blocks transmission, we dissolved dexamethasone phosphate to $10^{-3}M$ in medium plus 10 percent fetal calf serum, filtered it to sterilize, and diluted it with Hepes-buffered Eagle's MEM (1:9) to give an absorbance of 2.736 at 239 nm compared to the control (without dexamethasone) absorbance of 1.246. Thus the chemical dissolves easily in medium and passes through a 0.22-μm filter in the presence of 10 percent serum.
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13. Use of a minimum of 60 cells per colony (as done by Epstein and his co-workers) does not unmask any effect of dexamethasone. Besides using this higher minimum in three experiments, we also reinspected the fixed dishes that were used to grow strains GM1582, GM316, and GM498 (Table 1). Values for cloning efficiency decreased 2 to 5 percent, but the survival percentage showed no change from that given in Table 1.
14. Our differences with Epstein and his co-workers do not arise from the use of different cell strains, since we have tested at least nine normal cell strains. For GM316 they observed 51 percent survival at $10^{-10}M$ dexamethasone phosphate, decreasing to 10 percent at $10^{-6}M$; for GM1582 their values were 57 and 22 percent at 10^{-10} and $10^{-6}M$ dexamethasone phosphate, respectively (*2*). Our values for these strains are given in Table 1.
15. The cultures were fed at 8 days and fixed at 13 days; colonies of more than about 60 cells were counted on five dishes for each condition by three observers. Each observer reported a steady decrease in cloning efficiency with increasing size of the inoculum.
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22 August 1979

Metabolism of Theophylline to Caffeine in Human Fetal Liver

Abstract. *Caffeine (1,3,7-trimethylxanthine) is a biotransformation product of theophylline (1,3-dimethylxanthine) in the human fetus. Liver explants, obtained from human fetuses with gestational ages of 12 to 20 weeks, were incubated with theophylline and produced caffeine and, in lesser amounts, 1,3-dimethyluric acid and 3-methylxanthine. These findings suggest that the predominant pathway in theophylline metabolism in the fetus and newborn infant is the methylation reaction producing caffeine. This may contribute to the neonate's exceedingly slower elimination of caffeine relative to theophylline. Caffeine produced from theophylline may add to the pharmacologic effects of theophylline in newborn infants with apnea.*

Theophylline (1,3-dimethylxanthine), an active alkaloid from tea, is a potent central nervous system and cardiovascular stimulant with diuretic and bronchial smooth-muscle relaxant properties. Recently, this drug was shown to be effective in the prevention and treatment of apnea in the premature newborn infant (*1*); indeed, it has gained increasing acceptance as the major pharmacotherapeutic agent for the management of neonatal apnea. Despite its widespread use, however, little is known about the biotransformation of this drug in the newborn infant. In adults, the major metabolic pathway involves demethylation and oxidation reactions producing monomethylxanthines and methyluric

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