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Effects of Steroid Hormones on Replication of Murine Sarcoma Virus in Mouse Embryo Cultures (38900)

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(Introduced by Hans Popper)

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Recent reports have indicated an enhancing effect of steroid hormones on endogenous production of oncornaviruses by cells in culture. Paran *et al.* (1) demonstrated that adrenal corticosteroids enhanced production of type C virus induced by 5-iodo-2′-deoxyuridine (IdU) in cultured mouse fibroblasts. Viral production was stimulated 5- to 25-fold by glucogenic adrenal corticosteroids after induction by IdU of both fibroblasts, previously infected with the murine Kirsten sarcoma virus but not producing virus, and uninfected 3T3 cells. Dexamethasone was found to stimulate the production of murine mammary tumor virus (MMTV) in several cell lines containing relatively low levels of virus (2). Large quantities of oncornavirus-like particles were produced when the Chinese hamster ovary cell line (CHO-K1) was treated with the combination of testosterone propionate and dibutyryl cyclic AMP (3).

The present studies were undertaken to examine the effects of steroid hormones on the production of murine sarcoma virus in mouse embryo fibroblast (MEF) cultures after exogenous infection with virus. Morhenn *et al.* (4) had shown that physiologic concentrations of glucocorticoids promoted large increases in the production of polyoma virus both in primary MEF and mouse 3T3 cells after exogenous infection with virus. In contrast to the reported stimulation of endogenous virus production by steroid hormones we found no effects on production of oncornavirus by steroid hormones after exogenous infection.

Another observation resulting from our studies was the inhibition by testosterone of focus formation by murine sarcoma virus in MEF cells. It further was shown that the inhibition of focus formation by testosterone was abetted by cyclic AMP. Although focus formation was inhibited by testosterone, there was no interference with the replication of the sarcoma virion.

Materials and Methods. The steroid hormones used in these studies, 17-β-estradiol, progesterone, cortisol, dexamethasone, testosterone, and testosterone propionate, and the cyclic nucleotides, cyclic AMP, cyclic GMP, dibutyryl cyclic AMP, and dibutyryl cyclic GMP were purchased from Sigma Chemicals, St. Louis, MO. Diethylaminoethyl-dextran (DEAE-D), molecular weight 2×10^6, was purchased from Pharmacia Inc., Piscataway, NJ. Minimal essential medium with Earle's salt (MEM), fetal calf serum (FCS), agamma fetal calf serum, phosphate-buffered saline (PBS), penicillin G–streptomycin (PSN), and glutamine were purchased from Grand Island Biologicals (GIBCO), Grand Island, NY. Concentrated stock solutions of steroid hormones (100×) were prepared in ethanol and were added to the various media to the desired concentration.

The sarcoma–leukemia (Moloney) virus complex studied in these experiments (lots 36 and 42) (5), obtained from Dr. Robert Holdenreid of the National Cancer Institute, was prepared from mouse rhabdomyosarcomas by the method given by Moloney (6). The techniques for the preparation of primary and secondary NIH Swiss MEF cultures, the passage of virus in MEF cultures, and titration of sarcoma virus by focus-forming assay have been previously described (7, 8). The titration pattern of the sarcoma virus moiety of the complex (MuSV(M)) was determined by methods previously reported (7, 9). Generally, 4×10^5 secondary MEF cells in 60-mm plastic Falcon plates were infected with 0.2 cc of increasing dilutions of virus. After allowing 60 min for viral adsorption at 37°, the plates were fed with 5 cc of media, and foci were

TABLE I. EFFECT OF STEROID HORMONES ON REPLICATION OF MUSV(M) IN MOUSE EMBRYO CULTURES.

Hormone	Concentration (μM)	Virus Titer (FFU/ml $\times 10^{-3}$)
Virus control	—	4.51
17-β-Estradiol	0.5	5
Protesterone	2	5
	0.5	4.82
Cortisol	10	3.99
	2	3.98
	0.5	4.4
Dexamethasone	25	3.9
	10	4.33
	2	4.62
	0.2	4.42
Testosterone	17	1.8
	9	3.97

counted after 6-day incubation at 37° in 7% CO_2. In experiments in which DEAE-D was used, the cultures were first treated with DEAE-D at a concentration of 25 μg/ml for 45 min and then infected with virus (5). Methods for harvesting cell cultures, enumerating cell counts and the percentage of viable cells using trypan blue have been described previously (7). The titration patterns of MuSV(M) stocks used were that of competent virus showing "one-hit" kinetics (5, 7, 9).

In experiments designed to study the effects of the various hormones on focus formation, secondary MEF cells in the logarithmic phase of growth (8) were first infected with 0.2 cc of increasing dilutions of MuSV(M). After allowing 60 min for viral adsorption, the plates were fed with 5 cc of MEM containing 10% FCS and various concentrations of hormones. Infected cultures not containing steroid hormones but with the same concentration of ethanol were included in each study as additional controls. The cultures were incubated at 37° in 7% CO_2 and foci counted on the sixth day. The effects of the hormones on production of MuSV(M) in MEF cells were studied by methods previously described (7, 8). MEF cells (4×10^5) per plate were infected at a multiplicity of infection (m.o.i.) of 0.01 and, after viral adsorption, were fed

TABLE II. EFFECT OF CORTISOL (1 μM) AND DIETHYL AMINOETHYL-DEXTRAN ON GROWTH OF MUSV(M) IN MOUSE CULTURES.

Experiment	Virus titer (FFU/ml)
Virus control	2.17×10^3
+ cortisol	2×10^3
+ DEAE-D	4.57×10^4
+ DEAE-D + cortisol	4.39×10^4

with 5 cc of media containing the various hormones. The cultures were examined for foci after 5 days of incubation and the cells harvested in the feeding media by scraping the cells free with a rubber policeman. The cell suspensions were frozen and thawed and titrated for MuSV(M) by the focus assay. Cultures also were harvested for enumeration of viable cell counts. Focus assays in the present studies showed standard deviations of $\pm 15\%$ and cell counts showed standard deviations of $\pm 12\%$.

Results. Neither 17-β-estradiol, progesterone, nor the glucogenic adrenal corticosteroids, cortisol and dexamethasone, had a significant effect on the replication of MuSV(M) in mouse embryo cultures as measured by the focus-forming assay (Table I). Cortisol, at a concentration of 1 μM, had no effect on the stimulation of MuSV(M) replication in MEF cells by the polycation DEAE-D (5, 10, 11) (Table II).

However, testosterone, at a concentration of 17 μM, showed significant inhibition of focus formation by MuSV(M) in secondary MEF cells (Table I). The inhibitory effect of testosterone on focus formation by MuSV(M) was enhanced by the addition of cyclic AMP at a concentration of $10^{-4} M$ (Fig. 1). None of the cyclic nucleotides—cyclic AMP, dibutyryl cyclic AMP, cyclic GMP, and dibutyryl cyclic GMP—by themselves had a significant effect either in the number of foci produced or in the titration pattern of MuSV(M) (Table III).

Titration of MuSV(M) in the presence of increasing concentrations of testosterone propionate in combination with various concentrations of dibytyryl cyclic AMP and dibutyryl cyclic GMP (Fig. 2) showed: (1) an increasing inhibition of focus formation with increasing concentrations of testoster-

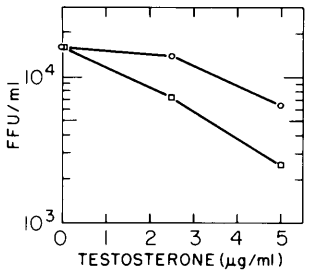

FIG. 1. Effect of testosterone on focus formation by MuSV(M): O——O, testosterone; □——□, testosterone plus cyclic AMP ($\times 10^{-4}$ *M*). Secondary MEF cultures containing 4×10^5 cells/plate were infected with various dilutions of MuSV(M) stock. After allowing 60 min for viral adsorption at 37°, the plates were fed with 5 cc MEM containing 10% FCS and the various indicated concentrations of testosterone and cyclic AMP. The cells were incubated for 6 days at 37° in 7% CO_2 and examined for the presence of foci.

TABLE III. EFFECT OF CYCLIC NUCLEOTIDES ON REPLICATION OF MUSV(M) IN MOUSE EMBRYO CULTURES.

Nucleotide	Concentration (*M*)	Virus titer (FFU/ml $\times 10^{-4}$)
Virus control	—	1.85
Cyclic AMP	10^{-4}	2
	10^{-5}	1.9
Dibutyryl cyclic AMP	10^{-3}	1.79
	10^{-4}	1.86
	10^{-5}	1.91
Cyclic GMP	10^{-4}	1.85
	10^{-5}	1.9
Dibutyryl cyclic GMP	10^{-3}	2.01
	10^{-4}	1.81
	10^{-5}	1.93

one propionate. No foci were observed at a concentration of testosterone propionate of 10 μg/ml; (2) dibutyryl cyclic AMP enhanced the inhibition of focus formation by testosterone propionate. No foci were formed at a concentration of $10^{-4}M$ of dibutyryl cyclic AMP in combination with 5 μg/ml of testosterone propionate, or when testosterone propionate at a lower concentration of 2.5 μg/ml was combined with $10^{-3}M$ dibutyryl cyclic AMP; and (3) the addition of dibutyryl cyclic GMP did not significantly alter the effect of testosterone propionate (Fig. 2). In these experiments there were no differences in the rates of cell growth nor the final viable cell counts when compared to appropriate control cultures. Dibutyryl cyclic AMP was more effective than cyclic AMP in enhancing the inhibition of focus formation by testosterone and testosterone propionate. Adenosine-5′-monophosphate had no effect in these studies.

Experiments were carried out to determine whether the inhibition of focus formation by testosterone propionate alone, and in combination with cyclic AMP, represented interference only with focus formation or with viral replication. MEF cultures were infected with high concentrations of MuSV(M) in the presence of the various hormone concentrations (Table IV). Plates not containing hormones showed a confluence of foci (Table IV). No foci were observed in plates containing testosterone propionate at concentrations of 10 and 5 μg/ml. Cultures containing the combination of testosterone propionate (2.5 μg/ml) and dibutyryl cyclic AMP ($10^{-4}M$) rarely contained foci: no foci were observed in cultures containing testosterone propionate (2.5 μg/ml) in combination with dibutyryl cyclic AMP at a concentration of $10^{-3}M$ (Table IV). After the fifth day of growth the cultures were harvested and titered for the presence of MuSV(M). Cell counts were similar for all the cultures (Table IV). There were no sig-

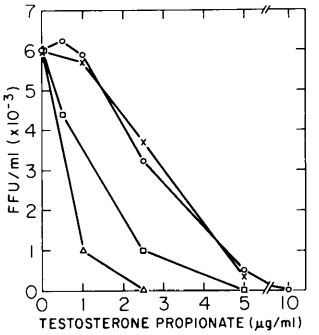

FIG. 2. Effects of testosterone propionate in combination with cyclic AMP and cyclic GMP on focus formation by MuSV(M): O——O, testosterone propionate; □——□, plus dibutyryl cyclic AMP ($10^{-4}M$); △——△, plus dibutyryl cyclic AMP ($10^{-3}M$); ×——×, plus dibutyryl cyclic GMP ($10^{-4}M$). For experimental details see text and legend to Fig. 1.

TABLE IV. TITRATIONS TO DETECT REPLICATION OF MuSV(M) IN MEF CULTURES SHOWING INHIBITION OF FOCUS FORMATION.

Experiment	Foci in original culture	Cell count of original culture at harvest (cells/plate × 10^{-6})	Titration of culture for MuSV(M) (FFU/ml × 10^{-3})
Virus control	Confluent	9.3	6.03
Testosterone propionate (10 μg/ml)	None	8.7	4.93
Testosterone propionate (5 μg/ml)	None	9.5	7.05
Testosterone propionate (2.5 μg/ml) + dibutyryl cyclic AMP ($10^{-4}M$)	Rare	9.5	5.46
Testosterone propionate (2.5 μg/ml) + dibutyryl cyclic AMP ($10^{-3}M$)	None	10.3	1.01

nificant differences in the titers of MuSV(M) found in control cultures and cultures containing testosterone propionate alone or combined with dibutyryl cyclic AMP at a concentration of $10^{-4}M$ (Table IV). These results indicated that there was normal replication of MuSV(M) although focus formation was inhibited. The original cultures also were examined for the presence of leukemia virus by the XC cell assay (12). Again there were no significant differences in the titers of MuLV(M) under the various experimental conditions given in Table IV. However, there seemed to be some inhibition also of viral growth when testosterone propionate (2.5 μg/ml) was combined with dibutyryl cyclic AMP at the high concentration of $10^{-3}M$.

Discussion. Previous reports of stimulatory effects of glucogenic adrenal corticosteroids on production of oncornaviruses in cell cultures dealt with systems examining endogenous virus production. We found no effects by adrenal corticosteroids on exogenous infection of mouse embryo cells with MuSV(M).

It might be argued that in our experiments adrenal corticosteroids enhanced replication of defective virus (7, 9) that would not be measured by the focus-forming assay; however, this is highly unlikely. Indeed, Paran *et al.* (1) found that dexamethasone treatment after induction by IdU enhanced virus production as measured both by reverse transcriptase activity and the focus-forming assay. In fact, there was a concordance of the amount of stimulation of viral production as measured by the two assays. Thus, the stimulatory effect of glucogenic corticosteroids on induction of endogenous oncornavirus by IdU was not observed with exogenous infection by MuSV(M). Noteworthy, Paran *et al.* (1) found that dexamethasone itself without prior induction by IdU had no effect on endogenous virus production. However, dexamethasone itself was found to stimulate type B particle production without prior treatment of cells with halogenated pyrimidines (2).

Tihon and Green (3) found that the combination of testosterone propionate ($1.5 \times 10^{-5}M$) and dibutyryl cyclic AMP ($10^{-3}M$) led to the production of oncornavirus-like particles in cultured CHO-Kl cells as measured by the reverse transcriptase reaction and increased cell agglutination. The mechanism of the hormone activity was unknown (3). We found no increase in production of MuSV(M) in secondary MEF cells by the combination of testosterone propionate and dibutyryl cyclic AMP.

Although there were no effects of steroid hormones on production of MuSV(M) in MEF cells after exogenous infection with virus, we did find that testosterone interfered with focus formation by MuSV(M). The inhibition of focus formation by testosterone was enhanced by cyclic AMP. These observations are in keeping with the effects of the combination of dibutyryl

cyclic AMP and testosterone propionate on the morphology of the Chinese hamster ovary cell line, CHO-Kl, reported by Hsie and Puck (13). The CHO-Kl cell is a proline-requiring mutant containing only 20 chromosomes developed by Kao and Puck (14). The cell cultures are compact, multilayered and randomly oriented. Hsie and Puck (13) found that concentrations of dibutyryl cyclic AMP $\geq 10^{-4}M$ caused the compact, well-separated, and poorly oriented CHO-Kl cells to elongate to the spindle shape characteristic of the mammalian fibroblast. The action of dibutyryl cyclic AMP ($10^{-4}M$) was greatly enhanced by testosterone propionate ($1.5 \times 10^{-6}M$), the combination producing rapid conversion of the CHO-Kl cell line into cells with fibroblast-like appearance. In contrast to our findings in MEF cells, testosterone propionate by itself had no effect on CHO-Kl cells. These effects of dibutyryl cyclic AMP, alone and in combination with testosterone propionate, were prevented by colcemid and vinblastine indicating that microtubular protein assembly may be involved (13). The morphological conversion of the CHO-Kl cells was attended by the acquisition of strict contact inhibition of growth, the induction of collagen synthesis, and a decrease in the ability of the cells to be agglutinated and rounded up by plant agglutinins and specific cell antibodies (15). Hsie *et al.* (15) further showed that estradiol, hydrocortisone, insulin, glucagon, and epinephrine were inactive. However, prostaglandins E_1, E_2, and, to some extent, $F_{2\alpha}$ were synergistic with dibutyryl cyclic AMP in the conversion of CHO-Kl cells to a fibroblast-like appearance (15).

Johnson *et al.* (16) showed that dibutyryl cyclic AMP alone or in combination with theophylline restored the normal morphology of L-929 and Rous sarcoma virus-transformed hamster cells. Protein synthesis but not RNA synthesis was required for the response. Again, these authors hypothesized that microtubular proteins may be involved in this response. Sheppard (17) reported that dibutyryl cyclic AMP in combination with theophylline had a very marked effect in bringing saturation densities of cells transformed with polyoma virus back toward normal. Indeed, it was shown that polyoma virus-transformed cells had decreased concentrations of cyclic AMP (18). Johnson and Pastan (19) reported that dibutyryl cyclic AMP with and without theophylline restored the morphology of many transformed cell lines toward normal. Otten *et al.* (20) observed that dibutyryl cyclic AMP in combination with theophylline prevented morphologic transformation on downshift of temperature by a temperature-sensitive mutant of Rous sarcoma virus.

In a recent meticulous scanning and transmission electron microscopic study of the effects of dibutyryl cyclic AMP plus synergizing compounds such as testosterone propionate on CHO-Kl cells, Porter *et al.* (21) confirmed the hypothesis that cyclic AMP caused assembly and alignment of the microtubular system within the Chinese hamster ovary cell. To obtain conversion of CHO-Kl cells to the fibroblast-like form, either $2 \times 10^{-4}M$ or $10^{-3}M$ dibutyryl cyclic AMP supplemented with testosterone propionate, 5 μg/ml, were added to the growth media. The treated cells elongated, developed pronounced assymmetry, becoming bipolar, and lost their knobs or blebs. The surfaces of the cells showed an exaggerated development of microvilli and a greater degree of ruffling, especially at the extreme ends of the cells. These morphologic changes were accompanied by an increase in the number of microtubules per unit volume of cytoplasm and a change in the orientation of the microtubules from random arrangement to an orderly alignment in parallel to each other and the long axis of the cell (21). Thus, the enhancing effects of cyclic AMP on inhibition of focus formation by testosterone observed in our experiments are consonant with the reports showing restoration of the morphology of transformed cell lines toward normal by cyclic AMP. However, the inhibitory effects we found with testosterone and testosterone propionate alone remain unexplained.

Of interest is the observation that testosterone propionate did not interfere with viral replication. Thus, these findings may offer a system for the study of the differen-

tial biochemistry of viral replication and focus formation.

Summary. The androgenic steroid hormone, testosterone, inhibited focus formation by the murine Moloney sarcoma virus in mouse embryo cells. The inhibition of focus formation was enhanced by cyclic AMP. Although focus formation was inhibited, there was no inhibition of viral replication. The glucogenic adrenal corticosteroids, cortisol and dexamethasone, and 17-β-estradiol and progesterone did not affect focus formation by MuSV(M).

1. Paran, M., Gallo, R. C., Richardson, L. S., and Wu, A. M., Proc. Nat. Acad. Sci. USA **70,** 2391 (1973).
2. Parks, W. P., Scolnick, E. M., and Kozikoski, E. H., Science **184,** 158 (1974).
3. Tihon, C., and Green, M., Nature New Biol. **244,** 227 (1973).
4. Morhenn, V., Rabinowitz, Z., and Tomkins, G. M., Proc. Nat. Acad. Sci. USA **70,** 1088 (1973).
5. Hirschman, S. Z., and Funke, V., Proc. Soc. Exp. Biol. Med. **148,** 527 (1975).
6. Moloney, J. B., Nat. Cancer Inst. Monogr. **22,** 139 (1966).
7. Hirschman, S. Z., Fischinger, P. J., Zaccari, J. J. and O'Connor, T. E., J. Nat. Cancer Inst. **42,** 399 (1969).
8. Hirschman, S. Z., Fischinger, P. J. and O'Connor, T. E., Int. J. Cancer **4,** 671 (1969).
9. O'Connor, T. E., and Fischinger, P. J., Science **159,** 325 (1968).
10. Duc-Nguyen, H., J. Virol. **2,** 643 (1968).
11. Vogt, P. K., Virology **33,** 175 (1967).
12. Klement, V., Rowe, W. P., Hartley, J. W., and Pugh, W. E., Proc. Nat. Acad. Sci. USA **63,** 753 (1969).
13. Hsie, A. W., and Puck, T. T., Proc. Nat. Acad. Sci. USA **68,** 358 (1971).
14. Kao, F., and Puck, T. T., Proc. Nat. Acad. Sci. USA **60,** 1275 (1968).
15. Hsie, A. W., Jones, C. and Puck, T. T., Proc. Nat. Acad. Sci. USA **68,** 1648 (1971).
16. Johnson, G., Friedman, R. M., and Pastan, I., Proc. Nat. Acad. Sci. USA **68,** 425 (1971).
17. Sheppard, J. R., Proc. Nat. Acad. Sci. USA **68,** 1316 (1971).
18. Sheppard, J. R., Nature New Biol. **236,** 14 (1972).
19. Johnson, G. S., and Pastan, I., J. Nat. Cancer Inst. **48,** 1377 (1972).
20. Otten, J., Bader, J., Johnson, G. S., and Pastan, I., J. Biol. Chem. **247,** 1632 (1972).
21. Porter, K. R., Puck, T. T., Hsie, A. W., and Kelley, D., Cell **2,** 145 (1974).

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