PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 160, 389–395 (1979)

Enhancement of Adenovirus Transformation by Pretreatment of Rat Embryo Cells with Estrogenic and Androgenic Hormones (40456)

EUSTACE A. VANDERPOOL, P. ROANE, AND W. TURNER

Department of Microbiology, College of Medicine, Howard University, Washington, D.C. 20059

The growth promoting properties of the gonadal hormones and their enhancing effect on macromolecular synthesis in target tissue is well established. Precisely how these hormones act on normal target cells remains undetermined. However, data from the bulk of experimentation, particularly with estrogen, suggest that the hormone enters the cell by simple diffusion (1) where it binds with low molecular weight proteins or receptor molecules present in the cell cytoplasm in relatively low concentration (2). The steroid–receptor complex with increased affinity for chromosomal sites, is translocated to the nucleus, where by an undetermined mechanism, gene expression is modulated (3, 4).

Sex steroids have been reported to inhibit viral infection *in vivo* (5) and either to enhance (6) or inhibit (7) viral oncogenesis in different animal species. Reports on the action of these compounds on viral infection or viral induced transformation *in vitro* are still contradictory. Thus, recent *in vitro* studies by Fong and Ledinko (8) have shown that small amounts of estrogen produced significant enhancement of adenovirus type 12 (Ad-12) transformation of hamster embryo cells. By contrast estrogenic or androgenic hormones were reported to inhibit Ad-12 transformation of hamster embryo cells (9) and to have little or no effect on the transformation of human foreskin cells by feline sarcoma virus (10).

In view of the increased understanding of the mode of action of steroid hormones on target cells and the contradictory observations described above it was of interest to study the influence of selected steroid compounds on *in vitro* viral induced transformation employing the Ad-12 rat embryo transformation system. Thus, the present communication describes, (a) enhancement of Ad-12 transformation of rat embryonic cells (REC) by 17-β-estradiol, estrone, and testosterone, (b) hormonal enhancement of viral adsorption, (c) enhancement of Ad-12 virus transformation in male and female REC and (d) enhanced tumor induction by transformed cells derived from hormone treated virus infected cells.

Materials and methods. Virus. Adenovirus type-12 (Huie Strain) was obtained from American Type Culture Collection, Rockville, MD. The virus was passed three times in human embryonic kidney (HEK) cultures. This virus pool had a mean titer of 10^8 50% tissue culture infectious doses ($TCID_{50}$) per ml and was free of Adeno-associated viruses (AAV) types I, II and III.

Cell cultures. Primary REC were prepared by trypsinization of eviscerated and decapitated embryos from near term Fisher rats. The embryos were separated into male and female groups by genital inspection. From these embryos roller tube cultures were seeded with 2×10^5 cells/ml of either male or female or mixed trypsinized cells resuspended in Eagle's Basal medium (Microbiological Associates, Bethesda, MD) supplemented with 10% fetal bovine serum, 2 m*M* glutamine, 100 units of penicillin and 100 μg/ml of streptomycin. The cultures were maintained at 37°C under 5% CO_2 in air. They were refed every third day and used when confluent, usually 5 days after seeding. HEK cell cultures were obtained from Microbiological Associates, Bethesda, Maryland. These cultures were maintained in Eagles minimum essential medium (EMEM, Microbiological Associates, Bethesda, MD) supplemented with 10% bovine serum, 2 m*M* glutamine, 100 units of penicillin and 100 μg/ml of streptomycin.

Hormones. Stock solutions of reagent grade 17-β-estradiol, estrone, testostrone and cholesterol (Sigma Chemicals) were prepared in 95% ETOH and diluted in EMEM without calcium plus 1% tween 80 and stored at 4°. The final concentration of each hormone that was used in the transformation assays was

0037-9727/79/040389-07$01.00/0

selected from toxicity testing in which monolayers of normal REC cultures were exposed to solutions of varying concentrations (1, 2, 5, 10, 20, 25, 50 and 100 μg/ml) of each hormone tested.

Transformation assays. To examine the effect of steroid hormones on Ad-12 virus transformation standard *in vitro* transformation procedures, previously described (11) were modified to include selected concentrations of β-estradiol, estrone testosterone or cholesterol. Briefly, 27 replicate REC cultures in roller tubes were exposed for 24 hr to 1 ml of 5 μg/ml of hormone solution prepared in low calcium medium. An equal number of treated and untreated cell cultures were included as non-infected and virus infected controls respectively. The cultures were washed two times and subsequently inoculated with Ad-12 virus at input multiplicities of 30–50.

The cultures were maintained at 37° and refed every 3 to 4 days with EMEM containing 0.1 m*M* $CaCl_2$ and supplement with 5% dialized calf serum, 2% fetal bovine serum, 2 m*M* glutamine, 0.1 m*M* nonessential amino acids, penicillin and streptomycin in concentrations of 100 units and 100 μg/ml respectively. All cultures were scored for transformed foci over a 70-day period.

Viral adsorption studies. Replicate REC cultures pretreated for 24 hr with 5 μg/ml of β-estradiol or 5 μg/ml of testosterone and untreated control cultures were inoculated with Ad-12 containing 10^8 TCID 50/tube. The cultures were placed in a rotating roller tube drum at 37°. During the period of adsorption three tubes from each group of cell cultures were removed periodically over a duration of 4 hr. The fluids were pooled and assayed in HEK cell cultures to determine the amount of unadsorbed virus.

Oncogenicity studies. Ad-12 transformed cell lines were established by seeding 4 tissue culture flasks with several transformed foci either from hormone-treated Ad-12 infected cell cultures or from untreated Ad-12 infected cell controls. These cells were maintained on EMEM containing 0.1 m*M* $CaCl_2$ supplemented with 5% dialized calf serum, 2% fetal bovine serum, 2 m*M* glutamine and antibiotics.

In order to determine the oncogenic potential of these transformed cells an inoculum containing 10^6 cells was injected subcutaneously into weanling Fisher rats and weanling Syrian hamsters. An equal number of animals were inoculated similarly with 10^6 REC and 10^6 hormone-treated REC for controls. The animals were observed and palpated routinely for tumor growth.

Results. Hormonal effect on viral transformation in REC cultures. Replicate REC cultures treated with 5 μg/ml of selected steroid compounds and untreated controls were routinely scored for changes in viral transformation post Ad-12 infection. Enhanced transformation was based on an increase in the average number of morphologically transformed foci in hormone-treated cultures over the number observed in untreated control cells. It is clear from the graphs in Fig. 1 that two- to eight-fold increases in the average number of identifiable foci occurred in Ad-12 infected REC cultures pretreated with 17-β-estradiol, estrone or testosterone as compared with cholesterol-treated and untreated virus infected controls. It is also evident by the slope of the curves that transformed foci occurred earlier in hormone-treated cultures than in untreated controls. Transformation did not occur in hormone-treated noninfected cell cultures.

Hormonal effect on viral transformation in male and female REC cultures. In these stud-

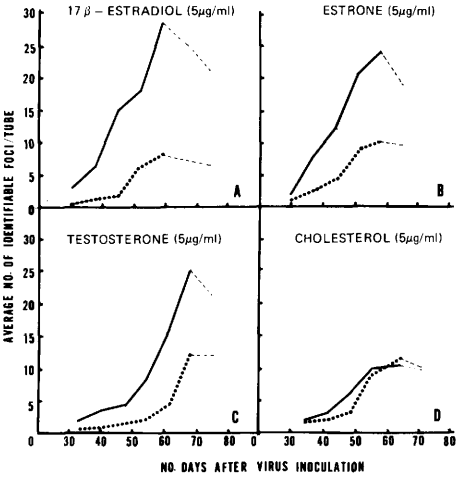

FIG. 1. Effect of selected steroid hormones (5 μg/ml) on Ad-12 transformation in REC cultures as monitored by mean foci count. (——) Hormone-treated; (●) untreated; (– – –) coalescence of foci.

ies it was of interest to determine whether the augmented effect of the selected steroid compounds on Ad-12 transformation is dose-dependent or sex-dependent. Thus, replicate tube cultures were prepared by seeding with 2×10^5 cells from either male or female rat embryos. Prior to Ad-12 infection the cultures were treated for 24 hr with varying concentrations of 17-β-estradiol or testosterone. Table I shows that at day 42, postviral infection the number of transformed foci is increased in both male and female infected cell cultures as the concentration of each hormone is increased from 0.1 μg/ml to 2 μg/ml. It is also apparent that the mean number of transformed foci is decreased as the concentration of each homone increases from 5 μg/ml to 200 μg/ml. The data in Table II show that, generally, the average number of transformed foci in cell cultures treated with 2 μg/ml of either hormone was significantly higher than the untreated controls ($p < 0.001$). It is apparent that based on the difference in the magnitude of enhancement, neither of the steroid compounds are selective in their action, although both types of cell culture appeared to exhibit a greater sensitivity to testosterone. It is also clear that based on the mean values of transformed foci in virus infected control cultures, female REC appeared

TABLE I. HORMONAL EFFECT ON Ad-12 TRANSFORMATION IN MALE AND FEMALE RAT EMBRYO CELLS. DOSE RESPONSE RELATIONSHIP.

Hormones (μg/ml)	Mean No.[a] of foci/male REC culture			Mean No.[a] of foci/female REC culture		
	Days post viral infection			Days post viral infection		
	35	42	50[b]	35	42	50[b]
17-β-Estradiol						
0.1	3 ± 0.8[c]	10 ± 1.5	12 ± 1.8	11 ± 1.6	19 ± 2.3	14 ± 1.8
1.0	6 ± 1.6	12 ± 1.9	10 ± 1.6	14 ± 1.9	24 ± 2.6	11 ± 1.9
2.0	12 ± 1.9	21 ± 2.7	18 ± 2.7	14 ± 2.1	34 ± 3.3	22 ± 2.5
5.0	9 ± 2.1	17 ± 2.5	9 ± 1.8	12 ± 1.9	25 ± 2.9	19 ± 2.1
10.0	5 ± 1.3	11 ± 2.1	14 ± 2.1	8 ± 1.4	20 ± 2.5	17 ± 1.9
20.0	3 ± 0.9	4 ± 0.8	7 ± 1.3	6 ± 1.3	10 ± 1.9	12 ± 1.3
Untreated virus						
Infected control	4 ± 1.3	12 ± 1.6	14 ± 2.3	10 ± 1.7	20 ± 2.6	16 ± 2.2
Testosterone						
0.1	7 ± 1.5	11 ± 1.5	14 ± 2.3	7 ± 1.4	16 ± 2.1	12 ± 1.6
1.0	7 ± 1.4	12 ± 1.8	15 ± 2.2	6 ± 1.2	18 ± 2.7	13 ± 1.8
2.0	14 ± 2.0	27 ± 2.3	24 ± 2.5	19 ± 2.3	34 ± 3.2	17 ± 2.1
5.0	17 ± 2.6	25 ± 2.9	23 ± 2.4	19 ± 2.2	28 ± 3.1	18 ± 2.4
10.0	16 ± 2.9	21 ± 2.6	23 ± 2.5	19 ± 1.9	28 ± 3.3	21 ± 2.3
20.0	11 ± 1.8	16 ± 2.3	19 ± 2.1	11 ± 1.6	23 ± 2.5	21 ± 2.4
Untreated virus						
Infected control	6 ± 1.5	12 ± 1.9	14 ± 2.0	8 ± 1.2	18 ± 2.3	15 ± 2.1

[a] Mean number of transformed foci in nine replicate tube cultures per hormone concentration.
[b] Foci have loss individual identity due to coalescence.
[c] Standard deviation of the Mean.

TABLE II. STATISTICAL ANALYSES OF HORMONAL INFLUENCE ON VIRAL TRANSFORMATION IN MALE AND FEMALE REC CULTURES. (Student t test).

Cell culture	Average No. of transformed foci/tube[a]			p-Value	% Enhancement
	Virus alone	β-estradiol[b] + Virus	Testosterone[b] + Virus		
Male REC	12	21		<0.01	75
"	12		27	<0.001	125
Female REC	20	34		<0.001	70
"	18		34	<0.001	90

[a] Mean number of transformed foci in nine replicate tube cultures.
[b] 2 μg/ml.

to be more responsive to Ad-12 induced transformation.

Hormonal effect on viral adsorption. REC cultures treated for 24 hr with 5 μg/ml of 17-β-estradiol or testosterone and untreated controls were washed and exposed to Ad-12 for 4 hr. At hourly intervals, after infection the inoculum in each of three treated and untreated cultures was removed for assay. The results from this experiment (Fig. 2) show that higher concentrations of virus are removed from the inocula more rapidly in hormone treated cultures than untreated controls. Accordingly only one log of virus appears to be adsorbed by untreated cultures, whereas two to three logs of virus seem to be taken up at an accelerated rate by testosterone and β-estradiol-treated cultures respectively.

Oncogenicity of transformed cells. Cell cultures derived from transformed foci of hormone-treated virus infected cells and from nontreated infected controls were subcultured for over 100 generations. At this passage level both groups of transformed cells had an average generation time of 24 hr. This is consistent with a previous report of the growth characteristics of Ad-12 transformed cells (11). In order to determine the oncogenic potential of these transformed cells weanling rats and hamsters were inoculated with 0.5 ml volumes of 10^6 cells. The data illustrated in Table III show that 17-β-estradiol-treated Ad-12 transformed cells induced tumors in nine of eleven rats and three of five hamsters. It is also apparent that generally, tumors appeared 5–10 days earlier in animals implanted with viral transformed cells derived from 17-β-estradiol treated REC cultures.

TABLE III. ONCOGENICITY OF Ad-12 TRANSFORMED CELLS DERIVED FROM HORMONE-TREATED VIRUS INFECTED REC CULTURES.

Cell line	Cellular inoculum	No. Tumors developed per no. of animals Inoculated		No. of days after inoculation
		Rats	Hamsters	
REC	10^6/0.5	0/11	0/5	60
REC + β-estradiol	10^6/0.5	0/11	0/5	60
REC + Testosterone	10^6/0.5	0/11	0/5	60
REC + Ad-12	10^6/0.5	1/11	0/5	35
REC + β-estradiol + Ad-12	10^6/0.5	9/11	3/5	25
REC + Testosterone + Ad-12	10^6/0.5	1/11	N.D.[a]	30

[a] Not done.

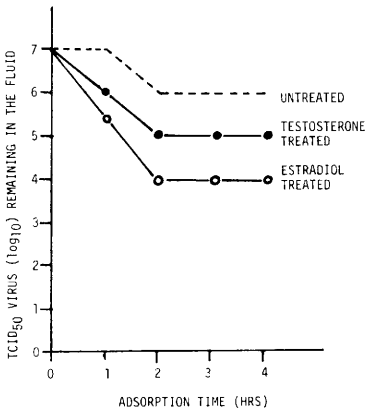

FIG. 2. Enhanced Ad-12 adsorption to REC pretreated for 24 hr with 5 μg/ml of β-estradiol (○) or testosterone (●). (– – –) untreated control.

Effect of hormones on viral induced cytopathology. One interesting feature observed in the cell cultures of this study was the expression of viral CPE which is known to occur in rat embryo cells inoculated with high input multiplicities of Ad-12 virus. While characteristic viral CPE (i.e. rounding up of cells) is indicated in hormone free infected cells similar alterations are markedly diminished in cell cultures pretreated with low doses (1–5 μg/ml) of estrogenic or androgenic hormones (Fig. 3). Additionally, characteristic transformed foci, previously described as morphologically altered areas consisting of a core of multilayers of small tightly packed epithelioid cells (11), is apparent in hormone-free virus infected cells. However, in hormone-treated virus infected cells, transformed foci appear as clusters of small epithelioid cells in monolayer arrangement.

Discussion. Based on the data generated by these studies we conclude that sex steroids play an important and perhaps a determinant role in viral induced transformation *in vitro*. Accordingly, REC cultures treated with low doses of estrogenic and androgenic hormones and subsequently infected with Ad-12 exhibited significantly higher numbers of transformed foci than untreated infected cells.

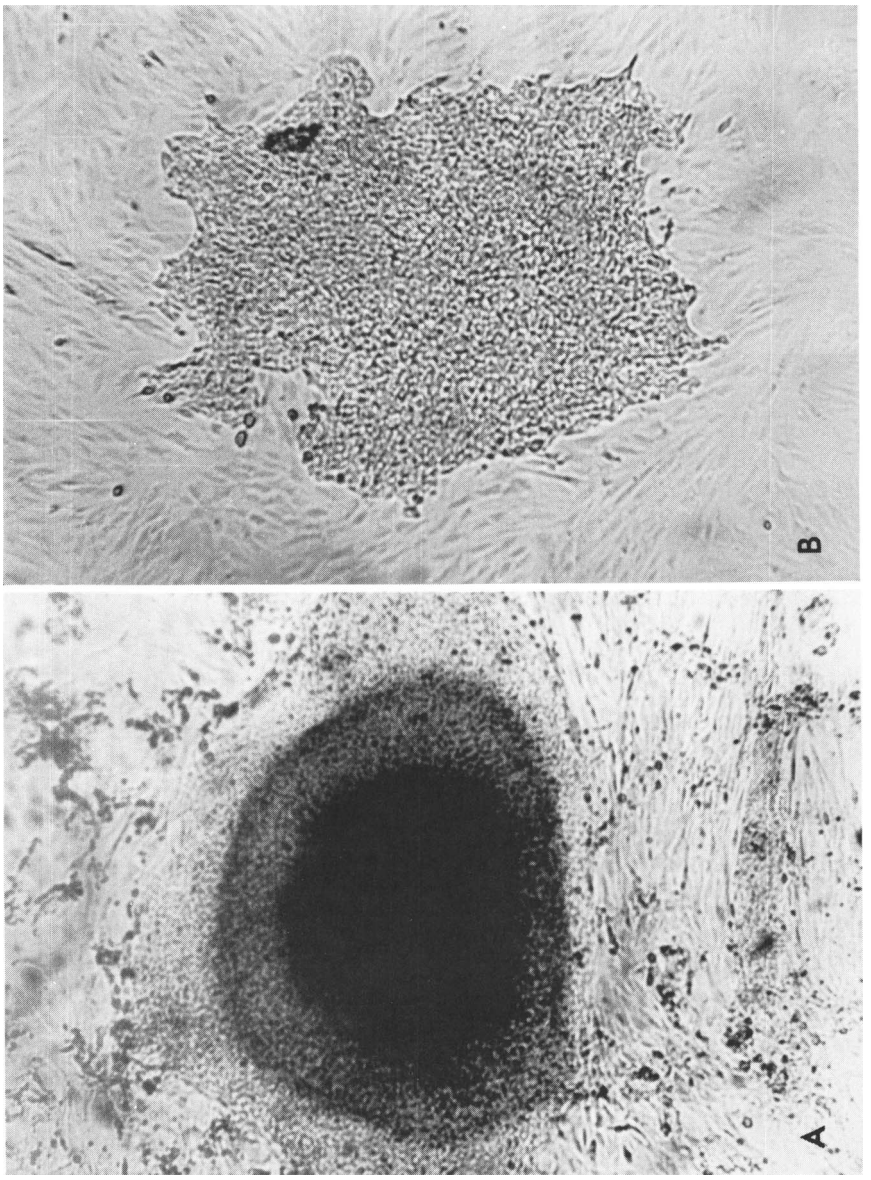

FIG. 3. Morphology of Ad-12 transformed foci. A. Typical transformed focus in untreated infected REC culture with CPE in surrounding fibroblasts. B. Typical transformed focus in hormone treated infected REC culture with absence of CPE in surrounding fibroblasts.

In the present communication primary foci development was observed in non-hormone treated cells 30–35 days post infection (PI) and exhibited a range of 0–3 transformed foci per culture. This is consistent with Ad-12 induced transformed foci development in the REC system as previously reported (11). By contrast, primary foci development in hormone-treated cells appeared as early as 20–25 days PI and exhibited a range of two to five transformed foci per culture. The differences between the total number of transformed foci in hormone-treated and nontreated infected cell cultures is magnified at 40–60 days PI when foci formation appears to be at its maximum and is feasibly quantitative. However since no measures were taken to inhibit metastasis of primary transformed cells, the possibility of satellite foci development cannot be excluded.

The data illustrated in Fig. 1 primarily support an augmented effect on viral induced transformation by the sex steroid compounds employed at 5 μg/ml quantities in a system comprised of a mixed population of male and female cells. In addition, the data generated by our experiments in male and female REC cultures indicate that enhancement or inhibition of viral induced transformation is dependent upon the concentration of the hormone employed. Focus formation in both male and female cells is significantly stimulated by 17-β-estradiol or testosterone at the restricted concentration level of 2 μg/ml. By contrast, focus formation is inhibited in male and female infected cells treated with 20 μg/ml of 17-β-estradiol. The concentration at which testosterone inhibits focus formation was not achieved but an inhibitory action is suggested by the steady decrease in the number of transformed foci over a range of 5–20 μg/ml (Table I). The reason for the apparent bimodal action of the steroid compounds in these experiments is not clear. It may be that these hormones exert their effect on viral transformation by a dual mechanism at two different concentration levels. This may explain the stimulatory and inhibitory effects observed for this class of hormones when tested in other transformation systems (8, 9).

An interesting feature of the present communication is the apparent influence of 17 beta-estradiol and testosterone on the rate of Ad-12 virus adsorption to REC. It is widely assumed that certain steroids, because of their lipophilic properties, enter cells with little or no obstruction from the cell membrane (1, 12). However this does not rule out the possible existence of surface receptors to these hormones. Specific membrane receptors have been demonstrated for certain peptide hormones (13). Recently, receptor components specific for 17-β-estradiol have been demonstrated on the surface membrane of estrogen responsive cells (14). The increased uptake of Ad-12 virus to REC cultures in these experiments may suggest some interaction of the steroid compounds with cell surface components. However, since biologically important receptors for these hormones are largely intracytoplasmic, caution must be exercised in the interpretation of this data without further defined experiments on the effect of these compounds on the sequential order of viral infection in the present virus-cell system.

The striking increase in the time and frequency of tumor formation observed in animals inoculated with transformed cells derived from 17-β-estradiol-treated virus infected REC is particularly exciting. However explanation of these results awaits further experimentation.

Summary. Pretreatment of REC cultures for 24 hr with low concentrations of estrogenic and androgenic hormones modified the expression of Ad-12 lytic activity and significantly enhanced the frequency of viral transformation. The augmented effect of the steroid compounds does not appear to be sex-dependent since both male and female REC cultures exhibited enhanced transformation. However, stimulation or inhibition of Ad-12 induced transformation in these *in vitro* cell systems appears to be dependent on the concentration of hormones employed. The enhancing effect of the sex steroids appears to extend beyond the *in vitro* transformation state. This is indicated by the markedly increased tumor production in animals implanted with 17-β-estradiol derived transformed cells.

1. Jensen, E. V., and Jacobson, H. I., *in* "Biological Activities of Steroids in Relation to Cancer" (G. Pincus and E. P. Vollmer, eds.), pp. 161. Academic Press, New York (1960).

2. Groski, J., Toft, D., Shyamala, G., Smith, D., and Notides, A., Rec. Progr. Horm. Res. **24,** 45 (1968).
3. Yamamoto, K. R., J. Biol. Chem. **249,** 7068 (1974).
4. Yamamoto, K. R., and Alberts, B., Cell **4,** 301 (1975).
5. Foley, G. E., and Aycock, W. L., Endocrinology **37,** 245 (1945).
6. Dmochowski, L., Adv. Cancer Res. **1,** 104 (1953).
7. Ahlström, G. C., and Johnson, N., Acta Endocrinol. **34,** 437 (1960).
8. Fong, C. K., and Ledinko, N., Cancer Res. **30,** 889 (1970).
9. Milo, G. E., Schaller, J. P., and Yohn, D. S., Cancer Res. **32,** 2338 (1972).
10. Schaller, J. P., Milo, G. E., Blakeslee, J. R., Olsen, R. G., Jr., and Yohn, D. S., Cancer Res. **36,** 1980 (1976).
11. Freeman, A., Black, P., Vanderpool, E. A., Henry, P. H., Austin, J. B., and Huebner, R. J., Proc. Nat. Acad. Sci. **58,** 1205 (1967).
12. Granner, D. K., Chase, L., Aurbach, G. D., and Tomkins, G. M., Science **162,** 1018 (1968).
13. Cuatrecasas, P., Fed. Proc. **32,** 1838 (1973).
14. Pietras, R. J., and Szego, C. M., Nature (London) **265,** 69 (1977).

Received June 20, 1978. P.S.E.B.M. 1979, Vol. 160.