PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **174**, 217–223 (1983)

Glucocorticoids Enhance Viral Transformation of Mammalian Cells (41728)

JOHNG S. RHIM

Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Abstract. The effect of glucocorticoids on the replication of murine sarcoma virus (MSV) in mammalian cells were examined. Glucocorticoids, hydrocortisone (1 to 10 μg/ml) and dexamethasone (5 μg/ml), enhance transformation induced by the Kirsten strain of MSV (Ki-MSV) in normal rat kidney and human cells 10- to 30-fold. The enhancing effect was much more pronounced in normal human colonic mucosal epithelial-like cells. On the other hand, the hormones estradiol, testosterone, and progesterone had no effect (5 μg/ml). Individual foci appeared earlier and were larger in hydrocortisone-treated cells compared with untreated cells. This enhancing effect is further evidenced by the increased virus yield and murine leukemia virus complement-fixing antigen production in the test system. However, such enhancement of hydrocortisone on the Ki-MSV-induced transformation was not observed in mouse embryo cells as previously reported.

Hormones can influence carcinogenesis *in vitro* as well as in experimental animals (1). Glucocorticoid hormones enhanced the production of polyoma virus grown in mouse cells (2) and the production of endogenous type C virus induced by 5-iodo-2′-deoxyuridine from cultured mouse fibroblasts (3). Dexamethasone was found to stimulate the production of murine mammary tumor virus in several cell lines containing relatively low levels of virus (4). Hirschman and Funke (5) reported that the glucocorticoid hormones hydrocortisone and dexamethasone did not promote the production of murine sarcoma virus (MSV) in mouse embryo cells. However, the effects of these hormones on the replication of MSV in other mammalian cells have not been examined. In contrast to the reported lack of effect of steroid hormones on replication of MSV in mouse cells, we found that the glucocorticoid hormones hydrocortisone and dexamethasone enhance transformation induced by MSV in NRK and human cells *in vitro*. Indeed, hydrocortisone enhances the MSV-induced transformation in NRK and human cells 10- to 30-fold.

Materials and Methods. All of the steroid hormones used in these studies were purchased from Sigma Chemical Company, St. Louis, Missouri. Stock solutions of steroid hormones (100×) were prepared in ethanol.

Human skin fibroblast cell strain (PC-109, KD and PF) used in this experiment were found to be highly susceptible to transformation by Ki-MSV (6). The epithelial line from normal human colon mucosa, established recently by Shannon-Danes and Sutanto (7) was obtained from the American Type Culture Collection. The characteristic of this line has been described in detail (7). Continuous cell line of NRK cells was also used.

The Kirsten strain of MSV (Ki-MSV) stock used supernatant fluids from a Ki-MSV-transformed NRK cell line, No. 58967 (8). A type C baboon endogenous virus (BaEV) pseudotype of Ki-MSV [Ki-MSV (BaEV)] was produced in human nonproducer cells (9) by super infection with BaEV. Focus formation by Ki-MSV was assayed on diethylaminoethyl-dextran (DEAE-D)-pretreated cultures (25 μg/ml for 30 min), inoculated with 2×10^5 cells per petri dish for 24 hr before infection. The Ki-MSV was titrated in NRK and human cell by a focus assay. The growth and maintenance medium consisted of Eagle's minimum essential medium (EMEM) with 10% fetal bovine serum (FBS) plus antibiotics. Cells were assayed for complement-fixing (CF) antigen reactive with murine leukemia virus (MuLV) group-specific antiserum. CF tests were done by the microtiter technique (9). Titers were recorded as reciprocals of the highest dilution giving 3+ to 4+ fixation of 1.8 units of complement.

Results. The effect of hydrocortisone on the focus formation of Ki-MSV was determined

0037-9727/83 $1.50

as follows. One-day-old NRK monolayers (pretreated with DEAE-D 25 μg/ml for 30 min) were fed with EMEM + 10% FBS with and without various concentrations of the steroid and were infected with 0.2 ml of increasing dilutions of Ki-MSV. The cultures were incubated at 37°C in 5% CO_2, refed once with the same medium, and transformed foci counted on the fifth day after infection. The results (Table I, Experiment 1) show that hydrocortisone significantly enhanced focus formation. The maximum enhancing effect, a 30-fold increase in focus formation, was seen at a hydrocortisone concentration of 5 μg, as compared with untreated cultures. A hydrocortisone concentration as low as 1 μg/ml also had a significant effect. At concentrations of 10 μg/ml or higher a toxic effect became evident. In the hydrocortisone-exposed NRK cells, Ki-MSV induced large and well delineated foci (Fig. 1A) which could be counted on the fourth day without a microscope. On the seventh or eighth day round cells predominated in the foci and plaque-like areas developed as transformed foci became dense and the cells sloughed off into the medium (Figs. 1D–F). In contrast, in unexposed control cultures the foci composed mainly of fusiform cells were smaller and showed minimal cell piling (Figs. 1B, C). To determine whether the effect of hydrocortisone was due to its glucocorticoid activity an experiment comparing various steroid hormones, all at 5 μg/ml, was performed (Table I, Experiment 2). The data illustrate that dexamethasone, a synthetic glucocorticoid, stimulated focus formation as well as hydrocortisone. On the other hand the hormones estradiol and progesterone, which have antiglucocorticoid activity (10) as well as testosterone had no effect at the 5-μg/ml dose tested.

The effect of hydrocortisone and DEAE-D on production of Ki-MSV in NRK cells was also studied. The DEAE-D-pretreated and untreated 1-day-old NRK cells (2×10^5 per plate) were infected with a multiplicity of infection of 0.01 and fed with EMEM + 10% FBS with and without hydrocortisone (5 μg). The cultures were examined for foci after seven days of incubation and the infected cells were harvested to assay for the growth of virus. The fluids were assayed for infectivity and the cell pack (10×) preparations for the CF test were made by a previously described method (9). The results confirm the enhancing effect of hydrocortisone as evidenced by the yields shown in Table II of Ki-MSV-infected NRK cells. In the presence of a 5-μg/ml dose of hydrocortisone the yield of sarcoma virus was enhanced 20-fold. Enhancement of CF antigen activity was also demonstrated in the infected cultures. In addition, hydrocortisone at a concentration of 5 μg/ml significantly stimulated Ki-MSV production in NRK cells by the po-

TABLE I. EFFECT OF STEROID HORMONES ON REPLICATION OF Ki-MSV IN NRK CELLS[a]

Expt No.	Hormones	Concentration (μg)	Virus titer (FFU[b]/ml)	Enhancement (fold)
1	Virus control	—	1.0×10^5	—
	Hydrocortisone	1	1.8×10^6	18
		2	2.3×10^6	23
		5	3.2×10^6	32
		10	9.1×10^5	9
2	Virus control	5	1.2×10^5	—
	Hydrocortisone	5	3.6×10^6	30
	Dexamethasone	5	3.2×10^6	26.7
	Progesterone	5	1.5×10^5	1.3
	Testosterone	5	2.1×10^5	1.7
	17-β-estradiol	5	1.0×10^5	0.8

[a] One-day-old NRK cells (2×10^5) pretreated with DEAE-D (25 μg/ml for 30 min) were inoculated with Ki-MSV and fed with EMEM + 10% FBS containing various concentrations of the steroid hormones. The infected cultures were incubated at 37°C in 5% CO_2, refed once with the same medium, and foci were counted on the sixth day after infection.

[b] Focus-forming units.

TABLE II. EFFECT OF HYDROCORTISONE (5 μg) AND DIETHYLAMINOETHYL-DEXTRAN (DEAE-D) ON GROWTH OF Ki-MSV IN NRK CELLS[a]

Experiment	Virus titer (FFU[b]/ml)	CF titer[c] vs MuLV	Enhancement (fold)
Virus control	1.0×10^4	1:4	—
+ hydrocortisone	2.0×10^5	1:8	20.0
+ DEAE-D	5.2×10^4	1:8	5.2
+DEAE-D-hydrocortisone	2.5×10^5	1:16	25.0

[a] One-day-old NRK cells (2×10^5) pretreated and untreated DEAE-D (25 μg/ml for 30 min) were inoculated with Ki-MSV at a multiplicity of infection of 0.01 and were fed with EMEM + 10% FBS with and without hydrocortisone (5 μg). The cultures were examined for foci for 7 days of incubation, and the infected cells were harvested. The fluids were assayed for infectivity, and the cell pack (10×) preparations were examined for the presence of MuLV CF antigens.

[b] Focus-forming units.

[c] CF titers = reciprocal of dilution giving 3+ to 4+ fixation of 1.8 units of complement.

lycation DEAE-D. The enhancing effect of DEAE-D on MSV replication in rat cells has previously been reported (11). Thus, DEAE-D and hydrocortisone appear to exercise an added effect on Ki-MSV production in NRK cells.

The effect of hydrocortisone on the focus formation of Ki-MSV in human skin fibroblasts and NIH 3T3 cells was next examined. One-day-old human skin fibroblasts and NIH 3T3 mouse embryo cells were fed with EMEM + 10% FBS with and without hydrocortisone (5 μg) and were infected with Ki-MSV (BaEV) and Ki-MSV, respectively. The infected cultures were examined for focus formation at 5 days for NIH 3T3 cells and at 10 days for human skin fibroblasts. The results (Table III) show that a 5-μg/ml dose of hydrocortisone significantly enhanced focus formation in human skin fibroblasts but did not enhance focus formation in NIH 3T3 cells. The enhancing effect, a 12- to 14-fold increase in focus formation was seen in three strains of human skin fibroblasts tested. In the hydrocortisone-exposed human skin fibroblast Ki-MSV (BaEV) produced large and well defined foci (Fig. 1G) which could be counted on the seventh day after infection. In contrast, in unexposed human skin fibroblasts, small foci, composed mainly of fusiform cells, were barely visible (Fig. 1H) and could not be counted until 14 days after infection. In the hydrocortisone exposed NIH 3TC cells Ki-MSV induced large and well delineated foci which could be counted on the fifth day after infection.

The effect of hydrocortisone on the focus formation of Ki-MSV (BaEV) in human colonic mucosal cells (HCMC) was also examined. Transformed foci, consisting of mainly round to polygonal cells (Figs. 2A and C) appeared in cultures within 1 week of infection. Without hydrocortisone, no foci or very few foci were produced. The same virus preparations, used here at 1.0 ml per culture, contained between 2.0×10^5 and 2.0×10^6 focus-forming units/ml when assayed on NRK cells

TABLE III. EFFECT OF HYDROCORTISONE (5 μg) ON REPLICATION OF Ki-MSV IN HUMAN SKIN FIBROBLASTS AND NIH 3T3 MOUSE EMBRYO CELLS[a]

Cells	Hydrocortisone	Virus titer (FFU[b]/ml)	Enhancement (fold)
Human skin[c]			
PF	−	1.8×10^4	—
	+	2.5×10^5	13.9
KD	−	8.0×10^3	—
	+	1.0×10^5	12.5
PC-109	−	3.0×10^4	—
	+	3.2×10^5	14.0
NIH 3T3	−	2.0×10^5	—
	+	2.0×10^5	1

[a] One-day-old human skin fibroblasts and NIH 3T3 cells pretreated with DEAE-D (25 μg/ml for 30 min) were inoculated with Ki-MSV and fed with EMEM + 10% FBS with and without hydrocortisone (5 μg). The infected cultures were examined for foci for 5 to 7 days of incubation.

[b] Focus-forming units.

[c] A type C baboon endogenous virus (BaEV) pseudotype of Ki-MSV was used.

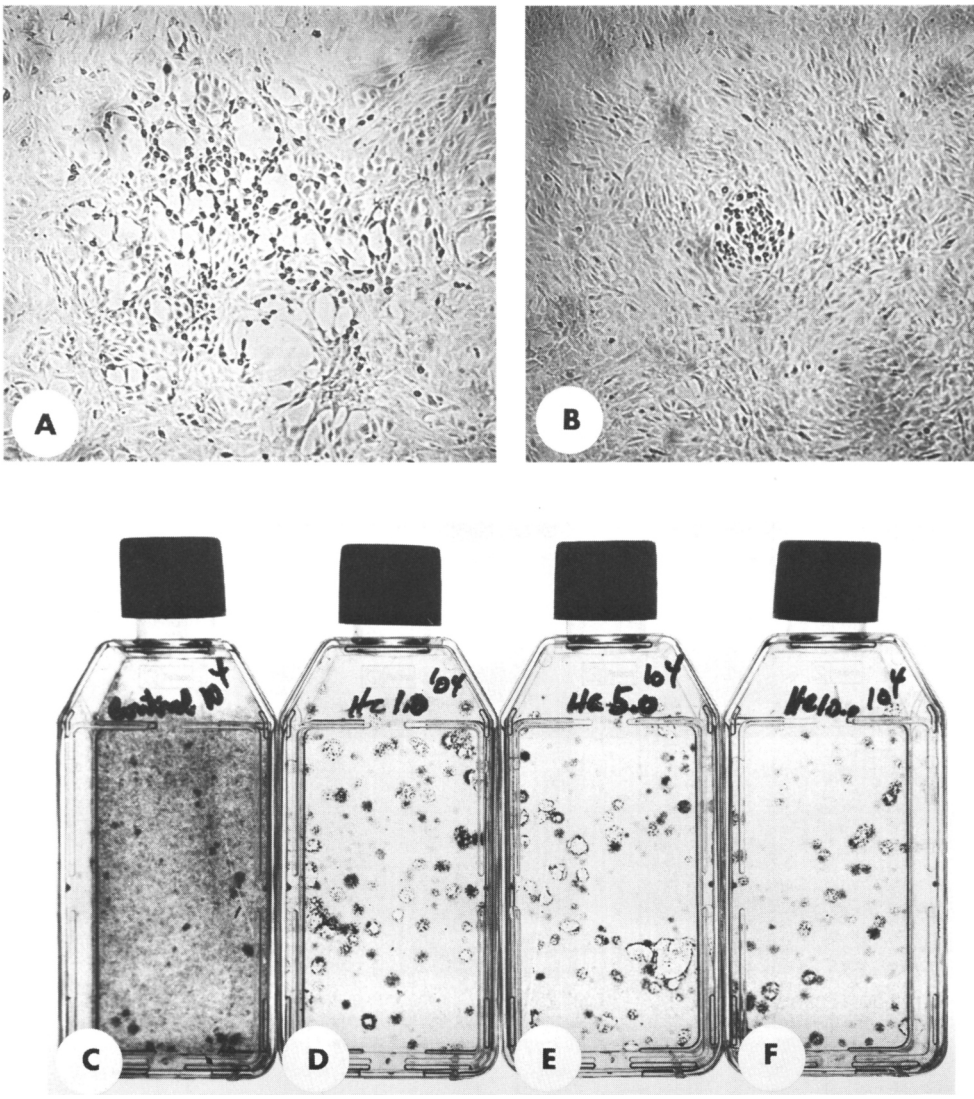


FIG. 1. Transformed foci induced by Ki-MSV in NRK cells. Cells were stained with Giemsa 7 days after infection with Ki-MSV (C–F). (A) A larger focus induced by Ki-MSV in NRK cells in the hydrocortisone medium on Day 4 after infection unstained, ×30. (B) A small focus induced by Ki-MSV in NRK cells in the medium without hydrocortisone on Day 4 after infection, ×30. (C) A few foci induced by Ki-MSV in NRK cells in the medium without hydrocortisone. (D–F) Numerous foci induced by Ki-MSV in NRK cells in the medium containing hydrocortisones. Note plaque-like areas develop as transformed foci become dense and the cells slough off into medium. (G) Several large foci induced by Ki-MSV (BaEV) in human skin fibroblasts in the hydrocortisone (5 μg/ml) medium on Day 7 after infection, unstained, ×30. (H) A small focus induced by Ki-MSV (BaEV) in human skin fibroblasts in the medium without hydrocortisone on Day 7 after infection, unstained, ×30.

(Table IV, Experiments 1 and 2). Unlike Ki-MSV (BaEV)-induced foci in human skin fibroblasts (6), foci of HCMC cultures reverted to normal morphology within 3 weeks (Figs. 2B and D). The HCMC cells grew in clusters of small "sheets." When confluent, the cells had a polygonal shape with many cytoplasmic connections (Fig. 2D) rather than the spindle-shape of the fibroblast.

The addition of hydrocortisone to cultures

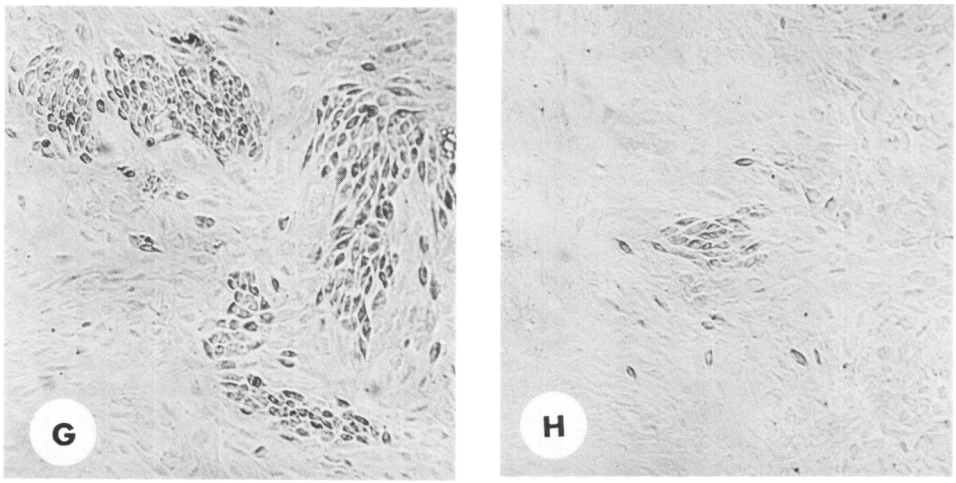


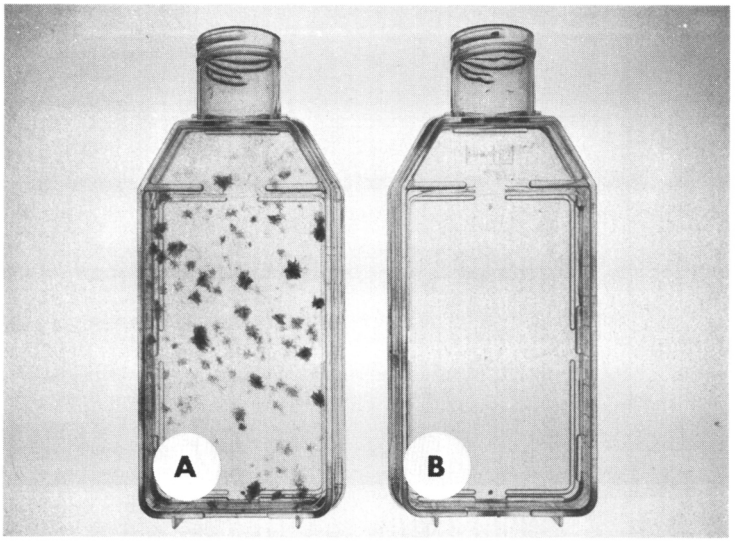
FIG. 1—*Continued.*

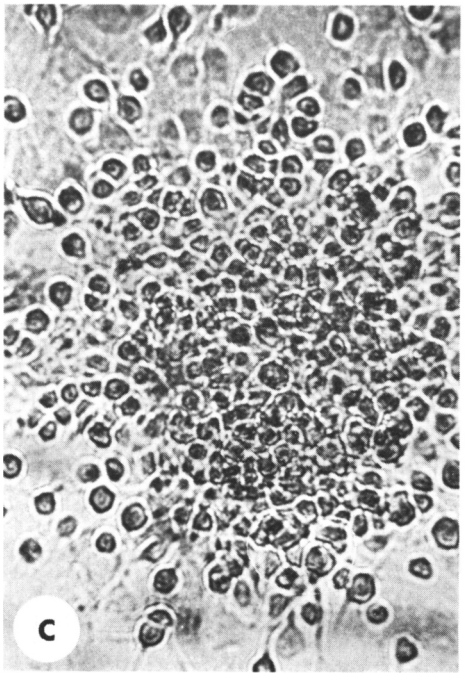
at the time of viral infection produced a dramatic increase in the incidence of transformed foci (Table IV). The maximum enhancing effect, an 80-fold increase in focus formation, was seen at a hydrocortisone concentration of 5 μg as compared with untreated cultures. A hydrocortisone concentration as low as 1 μg/ml also had a significant effect, a 30-fold increase in focus formation (Table IV, Experiment 3). No toxic effect was observed in the HCMC cultures exposed to a 10-μg/ml dose of hydrocortisone. In the hydrocortisone-exposed HCMC cells, Ki-MSV (BaEV) induced well-delineated foci (Fig. 2C) which could be counted on the 10th day with a microscope. Foci gradually increased in size and showed a pronounced proliferative effect with multilayered cell growth. By 20 days, the foci seen stained darkly with Giemsa could be counted (Fig. 2A).

Without hydrocortisone, all foci reverted within 3 weeks and the infected cultures became stationary and morphologically similar to uninfected controls (Figs. 1B and D). The infected cultures without hydrocortisone as well as uninfected cultures grew slowly and could be carried through only a few subcultures. They underwent nonspecific, progressive deterioration. However, transformed foci persisted in cultures with hydrocortisone, and did not revert to normal morphology and in the continued presence of hydrocortisone gave rise to a population of rapidly growing morphologically altered cells. Several morphologically transformed lines were established. At present they have survived more than seven serial passages.

Discussion. The experiments described above demonstrated clearly that the glucocorticoid hormones enhance Ki-MSV transformation of NRK and human cells. Individual foci appeared earlier and were larger in hormone-treated cells compared with untreated cells. This enhancing effect is further evidenced by the increased virus yields and CF antigen production in the test system. Hydrocortisone did not promote the production of Ki-MSV in mouse embryo cells as previously reported (5). However, individual foci in hormone treated cells appeared also earlier and the size of foci were larger than those appearing in untreated cells. Thus, the hydrocortisone treatment offers a simple, rapid, sensitive transformation assay method for MSV in mammalian cells and may be also of practical use in achieving greater production of type C virus from certain retrovirus producing mammalian cell lines.

It has been reported that glucogenic steroids bind to cytoplasmic receptor sites "steroid-binding protein," followed by the appearance of hormone-receptor complexes in the nucleus (12, 13). The latter interaction in turn influences gene expression to favor the accumulation of certain mRNAs (14). Certain steroids such as progesterone act as hormone antag-





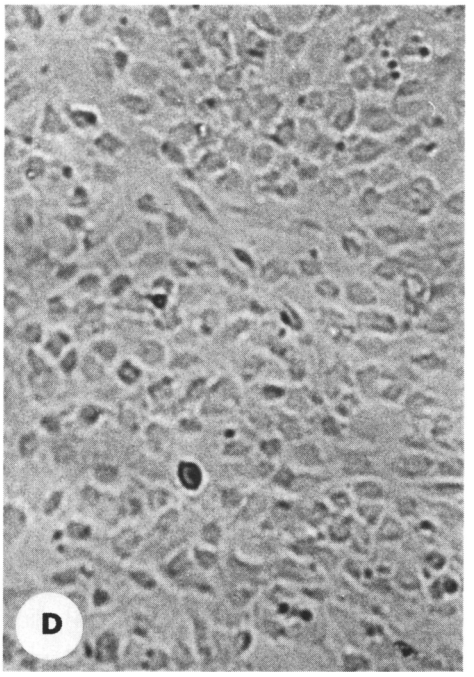


FIG. 2. Transformed foci induced by Ki-MSV (BaEV) in normal human colonic mucosal epithelial-like cells (HCMC). Cells were stained with Giemsa 20 days after infection with Ki-MSV (BaEV). (A) Numerous foci induced by Ki-MSV (BaEV) in HCMC cells in the hydrocortisone medium. ×30. (B) No Ki-MSV (BaEV)-induced foci were seen in HCMC cells without hydrocortisone medium, ×30. (C) A focus induced by Ki-MSV (BaEV) in HCMC cells in the hydrocortisone medium on Day 10 after infection, unstained, ×120. (D) The HCMC cells infected with Ki-MSV (BaEV). No foci were observed.

onists by competing for cytoplasmic receptor sites, thus preventing the changes in the receptor required for nuclear binding. The data on the steroid specificity of the effect reported here together with reported evidence that progesterone has antiglucocorticoid activity (10),

TABLE IV. EFFECT OF HYDROCORTISONE ON THE INCIDENCE OF TRANSFORMED FOCI IN Ki-MSV (BaEV)-INFECTED NORMAL HUMAN COLONIC MUCOSAL EPITHELIAL-LIKE CELLS[a]

Expt No.	Ki-MSV (BaEV) (FFU/ml)	Hydrocortisone (μg/ml)	Foci per culture
1	0	0	0
	0	5	0
	2.0×10^5	0	0
	2.0×10^5	5	85
2	2.0×10^6	0	2
	2.0×10^6	5	162
3	2.0×10^6	0	3
	2.0×10^6	1	90
	2.0×10^6	2	128
	2.0×10^6	5	240
	2.0×10^6	10	160

[a] Foci were counted 12 days after infection when number was maximal.

suggest that the hormonal enhancement of Ki-MSV multiplication like hormonal enzyme induction may involve specific cytoplasmic receptors.

It is interesting to note that the enhancing effect of hydrocortisone on the Ki-MSV (BaEV)-induced transformation was much more pronounced in normal epithelial-like HCMC cell. In this cell system, transformation seems to be a two-step process. Infection with Ki-MSV (BaEV) is necessary but not usually sufficient for transformation. The effect of the virus thus resembles that of an "initiator" as described for chemical carcinogenesis. Similarly, hydrocortisone appears to act here as a chemical promotor. Such an action would be in keeping with *in vivo* studies suggesting that certain hormones may act as tumor-promoting agents in their ability to enhance the incidence of cancers (15). It has recently been reported that epidermal growth factor, a naturally occurring polypeptide hormone, similarly enhanced Ki-MSV-induced transformation of rat granulosa cells and appeared to act as a tumor promotor in the retrovirus-induced transformation of a mesodermally derived epithelium (16).

1. Furth, J. Hormones as etiological agents in neoplasia. In: Becker FF, ed. Cancer, A Comprehensive Treatise. New York, Plenum, Vol **1**:75, 1975.
2. Morhenn V, Rabinowitz Z, Tomkins GM. Effect of adrenal glucocorticoids on polyoma virus replication. Proc Natl Acad Sci USA **70**:1088, 1973.
3. Paran M, Gallo RC, Richarson LS, Wu AM. Adrenal corticosteroids enhance production of type C virus induced by 5-iodo-2′-deoxyuridine from cultured mouse fibroblasts. Proc Natl Acad Sci USA **70**:2391, 1973.
4. Parks WP, Scolnick EM, Kozikowski EH. Dexamethasone stimulation of murine mammary tumor virus expression: A tissue culture source of virus. Science **184**:158, 1974.
5. Hirschman SZ, Funke V. Effect of steroid hormones on replication of murine sarcoma virus in mouse embryo cultures. Proc Soc Exp Biol Med **149**:790, 1975.
6. Rhim JS, Trimmer R, Huebner RJ, Papas TS, Jay G. Differential susceptibility of human cells to tranformation by murine and avian sarcoma virus. Proc Soc Exp Biol Med **170**:350, 1982.
7. Shannon-Danes B, Sutanto E. Epithelial line from normal human colon mucosa. J Natl Cancer Inst **69**:1271, 1982.
8. Klement V, Freedman HH, McAllister RM, Huebner RJ. Difference in susceptibility of human cells to mouse sarcoma virus. J Natl Cancer Inst **47**:65, 1971.
9. Rhim JS, Cho HY, Huebner RJ. Nonproducer cells of murine sarcoma virus transformed human cells. Int J Cancer **15**:23, 1975.
10. Samuels HH, Tomkins GM. Relation of steroid structure to enzyme induction in hepatoma tissue culture cells. J Mol Biol **52**:57, 1970.
11. Duc-Nguyen H. Enhancing effect of diethylaminoethyl-dextran on the focus-forming titer of a murine sarcoma virus (Harvey strain). J Virol **2**:643, 1968.
12. Rousseau GG, Baxter JD, Tomkins GM. Glucocorticoid receptors: Relations between steroid binding and biological effects. J Mol Biol **67**:99, 1972.
13. Baxter JD, Rousseau GG, Benson MC, Ito J, Tomkins GM. Role of DNA and specific cytoplasmic receptors in glucocorticoid action. Proc Natl Acad Sci USA **69**:1892, 1972.
14. Scott WA, Shields R, Tomkins GM. Mechanism of hormonal induction of thyrosine amino transferase studied by measurement of the concentration of growing enzyme molecules. Proc Natl Acad Sci USA **69**:2937, 1972.
15. Troll W. Blocking tumor promotor by protease inhibitors. In: Magee PN, ed. Fundamentals in Cancer Prevention. Baltimore, Univ Park Press/Tokyo Univ of Tokyo Press, p41, 1976.
16. Harrison J, Auersperg N. Epidermal growth factor enhances viral transformation of granulosa cells. Science **213**:218, 1981.

Received March 24, 1983. P.S.E.B.M. 1983, Vol. 174.