GLUCOCORTICOSTEROIDS AND RNA TUMOR VIRUSES

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Abstract

Glucocorticosteroids at physiological concentrations stimulated type-C virus production 2 to 50-fold from mouse fibroblasts induced by 5-iodo-2′-deoxyuridine. They also stimulated virus production from chronically infected mouse myeloid leukemia cell line. Some potential practial applications of these findings are discussed.

In 1971, D. R. Lowy, et al., reported that murine leukemia virus can be induced from previously non-viral producing mouse cells using 5-iodo-2′-deoxyuridine (IUdR) (1). This phenomenon of virus induction from previously non-producing cells is now well established in several systems. The accumulated data from the induction experiments provide strong evidence that the genetic information for virus production is present in every cell of an inducible clone. We have previously suggested that the expression of the provirus genome (to form viral RNA) probably utilizes normal cell mechanisms for transcription and translation of mRNA. Compatible with this proposal are the following recent observations: (A) RNA tumor viruses contain a sequence of poly (A) like mRNA (2); (B) Cordycepin (3′-deoxyadenosine) an inhibitor of poly (A) synthesis (3) which preferentially affects the maturation of mRNA, inhibits virus induction by IdU (4); and (C) cells infected with leukemia virus do not have new RNA polymerase (5).

The glucocorticoids are a group of agents which have been shown to affect host transcriptional and translational mechanisms. It was of interest to establish the role of these and other hormones in virus induction by IdU from "non-producer", and their effect on virus producing cells.

The cells used in this study were BALB/C-3T3 fibroblasts and the same clone transformed by Kirsten murine sarcoma virus (K-MSV) (designated BALB/K-3T3) (both "non-producers"). A mouse myeloid leukemia cell line was chosen as a "producer" (designated M_1, courtesy of Dr. Y. Ichikawa, Kyoto, Japan). Induced cells were treated with IdU for 24 hours and in most of the experiments the hormones were present throughout the whole course of the experiment.

The Effect of Dexamethasone on Virus Production Induced by IdU

The production of K-MSV (after IdU) was measured by assaying reverse transcriptase in particulate fractions released into the media as previously described (4). The effect of dexamethasone on induction is shown in Figure 1. Basically the kinetics of virus induction are the same in the control group (IdU alone) and in those treated with hormone and IdU. Maximum augmentation in this particular experiment is in the second day after induction where relative virus production was stimulated about 50-fold and at the peak (day 6) about 25-fold. The concentration of the hormone was 10^{-6} M. As shown in Table 1, the optimal concentrations for stimulation are between 10^{-5} and 10^{-6} M.

At this point, it was necessary to show that the increase in the reverse transcriptase activity truly reflects an increase in virus production. One approach was to measure the preference of the enzyme for $(dT)_{12\text{-}18}\cdot(rA)n$ over $(dT)_{12\text{-}18}\cdot(dA)n$ as template-primer, a characteristic useful in distinguishing viral from normal cellular polymerase (6, 7, 8, 9). The enzyme derived from the IdU treated cultures showed a substantial preference for $(dT)_{12\text{-}18}\cdot(rA)n$ as template-primer over $(dT)_{12\text{-}18}\cdot(dA)$. This fact together with the fact that the enzyme activity is found in the medium and after high speed centrifugation in the particulate pellet and not found in the control, implies that we are almost certainly dealing with enzyme from viral origin.

A second and more definitive approach was to measure viral biological activity by determining the number of focus forming units (f. f. u.). The results of such an experiment are summarized in Table 2. Both the polymerase activity and the number of f. f. u. increased (5 and 50-fold, respectively). The later assay is a much more sensitive one as it measures single units and provides direct proof that dexamethasone did indeed stimulate virus production.

Table 1. Stimulation of the IdU Induced Virus Production From BALB/K-3T3 Cells By Various Concentrations of Dexamethasone and Prednisolone

Hormone Concentration (M)	Relative Virus Production* pmole/ml	
	Dexamethasone	Prednisolone
0	3.2	3.2
10^{-8}	7.7	N.T.
10^{-7}	22.7	7.6
10^{-6}	56.0	40.4
10^{-5}	29.0	47.8
10^{-4}	N.T.	32.3

* Virus production was measured by RNA directed DNA polymerase activity present in the virus particles. The data were obtained from the peak activity of each culture as shown in Figure 1 (the second day, and third day after induction for prednisolone and dexamethasone, respectively). The data are expressed in picomoles per milliliter of unconcentrated medium. The value of the uninduced control (0.05 pmole/ml) was subtracted from all values. 40 μg/ml of IdU was used in each experiment.

N. T. = not tested

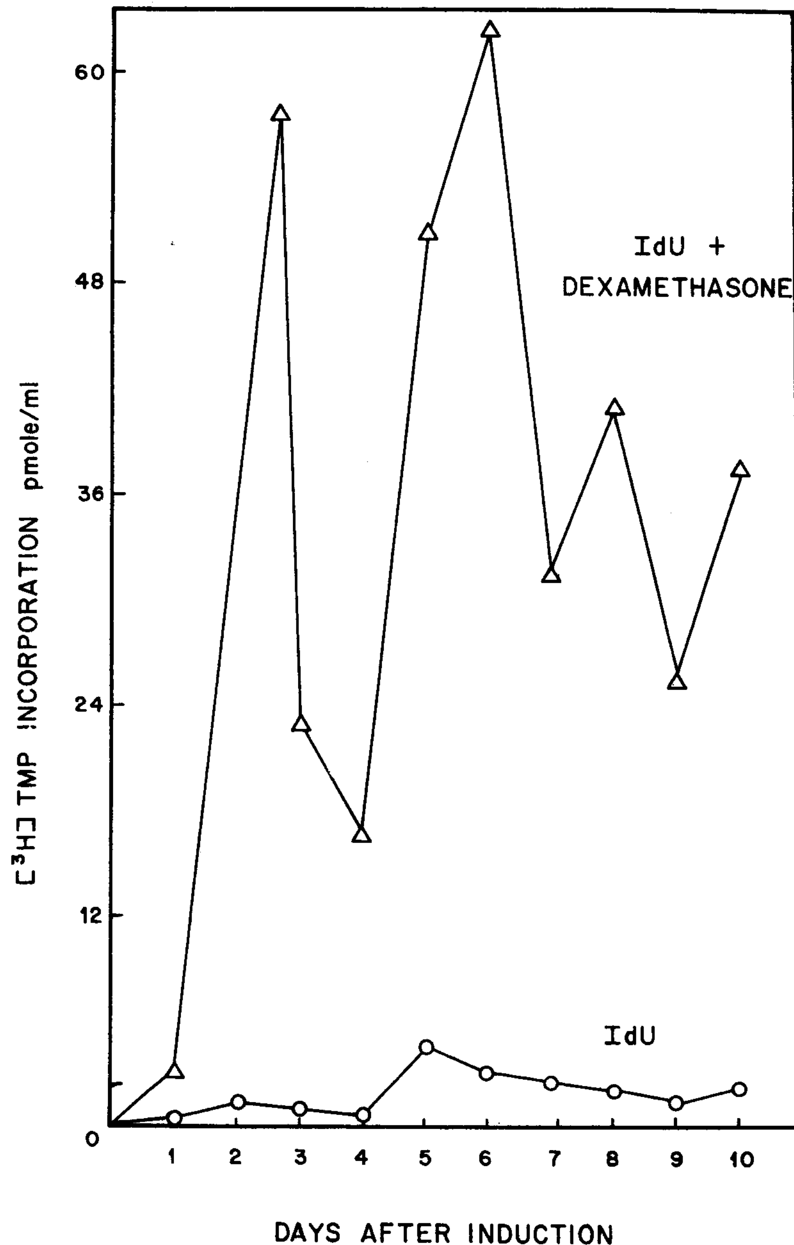

Fig. 1. Kinetics of induction of virus production by IdU and its stimulation by dexamethasone in BALB/K-3T3 cells. 10^6 cells were plated in 100 mm petri dishes. Dexamethasone, when present, was added immediately after plating the cells, and thereafter, daily throughout the course of induction. IdU was added one day after plating the cells for 24 hours. Days after induction started with the addition of IdU, the harvested medium was concentrated 30 fold by ultracentrifugation at 82,000 g for 60 minutes at 4°C. The pelleted particles were resuspended in buffer containing 10 mM Tris HCl, pH 7.9, 20 mM KCl, 1 mM EDTA, 1 mM Dithiothreitol (DDT), and 50 % glycerol. The polymerase activity was assayed in a 50 µl reaction mixture containing 40 mM Tris HCl, pH 7.9, 60 mM KCl, 1 mM DDT, 1 mM Mn Acetate, 0.2 mM EDTA, 20 mM NaF, 0.1 % Triton X-100, 10 µg/ml $(dT)_{12\text{-}18}$. (rA)n (Collaborative Research), 5.4 µM H^3-TTP (Schwarz-Mann Biochemicals, specific activity 13,000 c.p.m./pmole) and 20 µl of virus solution. (dT)n was synthesized at 30°C for 45 minutes and the precipitable radioactivity was then determined. Since the yield of viral enzyme after centrifugation was estimated to be about 20 %, the enzyme activity was corrected accordingly.
Symbols: o—o, Control; △---△, IdU + Dexamethasone

Table 2. Relationship Between RNA Directed DNA Polymerase Activity and Viral Transforming Activity*

Dexamethasone	RNA Directed DNA Polymerase Activity** (pmole/ml)	Viral Transforming Activity*** (f. f. u./ml)
None	10.7	0.4 x 10^3
10^{-6} M	49.1	20 x 10^3
Fold Stimulation By Hormone	4.6	50

* The cultured cells (BALB/K-3T3 cells) in the absence and in the presence of hormone were induced with IdU and the media were collected daily and assayed for RNA-directed DNA polymerase and focus forming ability in normal rat kidney cells (NRK). The values presented in the Table were obtained from results 3 days after IdU, the time of maximum virus induction. The polymerase assays were the same as those described in the legend to Figure 1.

** The focus forming assay was assayed as follows: 0.2 ml of virus containing medium was added to DEAE-Dextran pretreated normal rat kidney cells (NRK) in 60 mm plates. After one hour absorption, all plates were washed with 2 ml phosphate-buffered saline and fed with 3 ml DMEM supplemented with 5 % fetal calf serum. Media were changed on days 2 and 4, and the foci were stained with giemsa and counted on day 6.

*** Specific Viral Activity is defined as the amount of RNA-directed DNA polymerase activity (pmole/45 min./ml) per f. f. u.

\# Fold stimulation is defined as follows:

$$\frac{\text{Activity in the presence of hormone}}{\text{Activity in the absence of hormone}}$$

Virus Induction and Cell Growth

Cultures treated with IdU grow slower and reach a lower cell density saturation (4). We, therefore, measured the effect of dexamethasone on cell growth to exclude the possibility that the hormone effect was only to enhance cell growth and as a consequence, more virus production. Cell number was determined daily during the course of induction. The results of this experiment are shown in Figure 2. The upper panel shows the profile of the accumulated cell number during the induction course and the lower panel shows an estimate of virus production by polymerase assay. The hormone had no effect on cell number in the IdU treated or untreated cultures indicating that cell number is not a determining factor for the hormone effect (cells treated with hormone alone did not release any detectable virus).

Optimal Time for Stimulation

It has been reported that IdU incorporation into DNA is essential for virus induction by this agent (1), and only 1 % of the cells (in one study) in the IdU treated culture are actually induced to release virus. Since the hormones used in this study were present throughout the whole course of induction, it is possible that the action of the hormone is a preinduction event, i. e., the steroids might increase the number of cells susceptible to virus induction. We have two pieces of evidence that indicate that

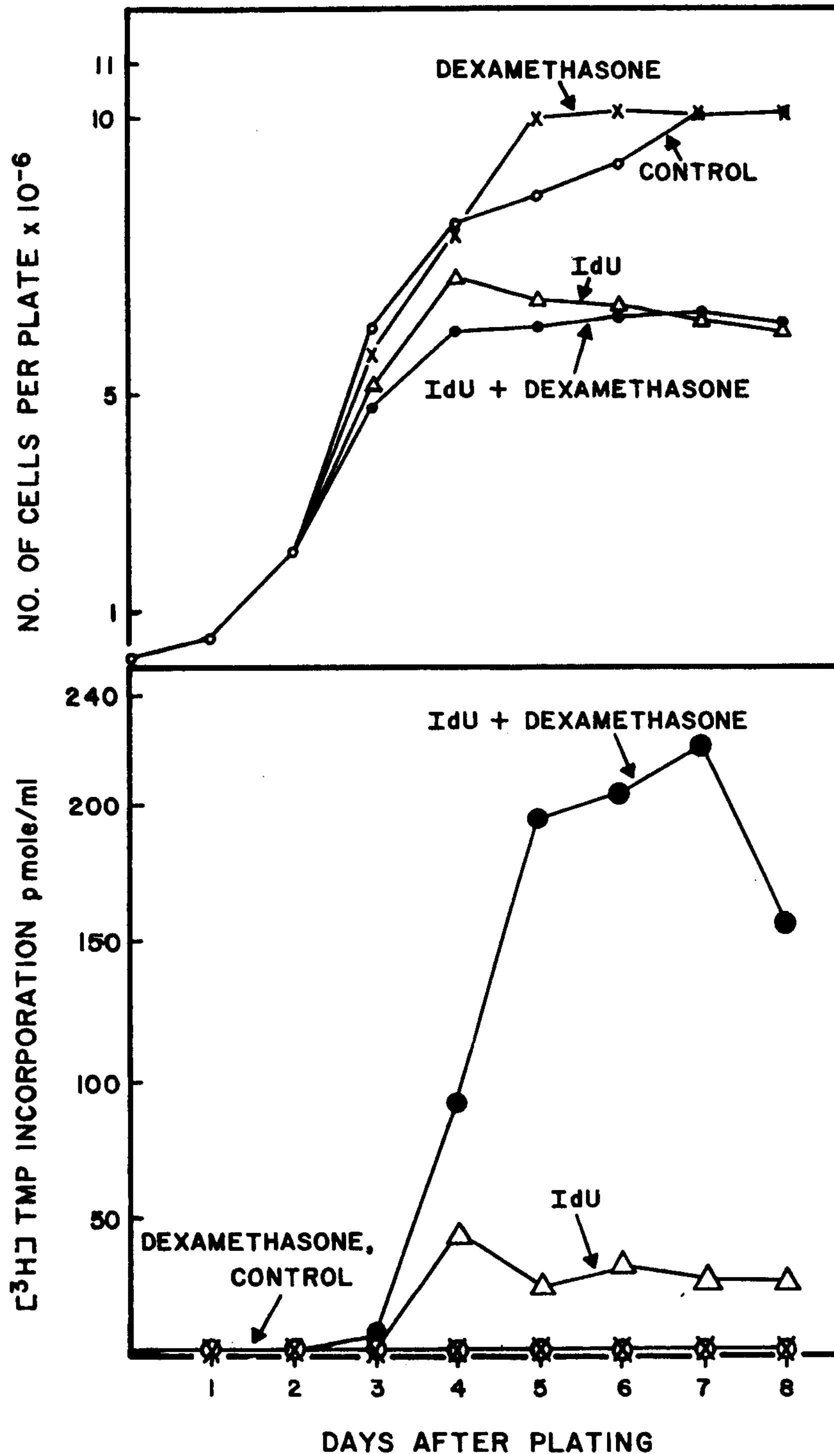

Fig. 2: Effect of dexamethasone on cell number and virus production induced by IdU from K-3T3 cells.
Upper Panel: The accumulated cell number during the course of induction of viruses by IdU in cells.
Symbols: o---o, Control; △---△; ●---●, IdU + Dexamethasone; x---x, Dexamethasone alone.
Lower Panel: The rate of induction of virus production assayed by RNA directed DNA polymerase activity. The enzyme assays were performed as described in the legend to Figure 1.

this is not the case, The first, which we won't go into detail about, is the absence of a significant increase in the number of infectious centers (10). The second derives from results obtained from a time course experiment. In these experiments, dexamethasone was added at various time intervals during the course of virus production. The results are shown in Table 3.

Maximum stimulation of virus production occurs when dexamethasone is present between 24 to 48 hours after IdU. Since IdU was present only in the first 24 hours, stimulation by dexamethasone must occur in the absence of IdU. The results show also that pretreatment of the cells with dexamethasone and its presence at periods other than that between 24 to 48 hours are not very effective in promoting virus induction.

Dexamethasone Increases the Detectability of Induced Viruses

The optimal IdU concentration for viral induction is between 40 and 200 μg/ml, and concentrations below 10 μg/ml apparently are not very effective. Table 4 summarizes the results of an experiment in which we used as low as 4 μg/ml of IdU. As indicated above, very little or no virus induction can be detected at this concentration of IdU. However, when dexamethasone was added virus was readily detected. Further, when 10 μg/ml of IdU and hormone are utilized, virus induction was greater than with 40 or 200 μg/ml of IdU alone.

The Effect of Dexamethasone on Chronically Infected Cells

In order to determine the effect of the hormone on chronically infected cells which are low producers, a mouse myeloid leukemia cell line (M_1) was treated with dexamethasone at a concentration of 10^{-6} M. Table 5 summarizes the results of these experiments. It was shown that after 6 days in culture, the treated cells produced five

Table 3. Dexamethasone Enhances Type C Virus Production After Removal of IdU

Presence of Dexamethasone*	Relative Virus Production #
(hours)	(pmole/ml)
none	119.2
−24 to 0	161.5
0 to 24	446.2
24 to 48	692.3
48 to 72	126.9

* The time of addition of IdU is taken as 0 time.

The procedure for virus induction from BALB/K-3T3 cells is the same as that described in the legend of Fig. 1 except that dexamethasone was added at the time indicated. IdU was present only in the first 24 hours of induction.

Table 4. Stimulation of IdU Virus Production from BALB/K-3T3 Cells by Dexamethasone

IdU μg/ml	Relative Virus Production* pmole/ml	
	– Dexamethasone	+ Dexamethasone
4	1.8	3.6
10	3.1	23
20	5.5	47
40	10.9	60
200	12.5	75

* Relative virus production is expressed as pmole/ml of RNA directed DNA polymerase activity.

Table 5. Stimulation of Virus Production from Mouse Myeloid Leukemia Cell Line (M_1) by Dexamethasone

Days in Culture	Relative Virus Production pmole/ml/10^6 Cells*	
	– Dexamethasone	+ Dexamethasone (10^{-6}M)
1	33	39
2	26	86
4	29	96
6	46	230

* Relative virus production is expressed as pmole/ml of RNA directed DNA polymerase activity per 10^6 cells.

times more virus than the non-treated cells. It is worthwhile to mention here that the hormones do not augment virus production from cells that already produce large amounts of virus.

Effect of Other Steroid Hormones on Virus Induction

All the natural and synthetic glucogenic hormones we tested augmented the induction of virus as seen in Table 6. The magnitude of stimulation with the BALB/K-3T3 cells range from 2-fold (cortisone) to 25-fold (dexamethasone) and with the BALB/3T3 cells from 1.8-fold (cortisone) to 7.5-fold (dexamethasone). Apparently this stimulatory activity is specific for the glucocorticosteroids.

Insulin was shown to enhance a post-transcriptional synthesis of TAT in rat hepatoma cells (11). To test whether insulin could also control virus production stimulated by dexamethasone the effect of insulin alone and together with dexamethasone was measured. As shown in Table 6, insulin does not enhance IdU induced virus production or the stimulation by dexamethasone. On the contrary, a small degree of inhibition was observed in both cases.

Table 6. Effect of Various Steroid Hormones and Insulin in Induction of Type C Virus by IdU from BALB/K-3T3 and BALB/3T3 Cells

Hormones*	Relative Virus Production** pmole/ml	
	.BALB/K-3T3	BALB/3T3
None	3.7	4.4
Dexamethasone	92.5	33.5
Prednisolone	81.0	18.5
Fludrocortisone	76.5	17.5
Hydrocortisone	61.0	14.8
Corticosterone	41.2	10.4
Cortisone	7.2	7.6
Androstenedione	5.9	2.5
Testosterone	4.4	2.5
Progesterone	3.4	4.7
B-estradiol	2.4	1.2
Insulin	1.8	–
Dexamethasone + Insulin	50.0	–

* The concentration of all steriod hormones is 10^{-6}M. The concentration of insulin is 5 to 10 μg/ml.

** Relative virus production is expressed as pmole/ml of RNA direct DNA polymerase activity. For details of these data, see the footnote to Table 1.

Discussion

This phenomenon of stimulation of RNA tumor virus production by steroid which was shown to be unique to a glucocorticosteroids (12) might have several applications among which include: 1) increase in the level of detectability of virus production which might be useful in attempting to induce viruses from human tumor cells. Advancement of this application may be important in the understanding of the etiology of human oncogenesis and as a diagnostic tool; 2) greater production of type-C virus from mammalian virus producing cell lines; and 3) in the light of the recent biochemical evidence for footprints of viral information in human leukemic cells such as virus-like reverse transcriptase (13, 14, 15), antigenic cross reactivity between this enzyme and primate viral enzyme (16), and the relatedness of nucleic acid sequences between human leukemic cells and murine type-C viruses (14) and particularly to primate (ape and monkey) type-C sarcoma viruses (17) it may be appropriate to consider our results with corticosteroid prior to their use in diseases where their value is in question such as myeloblastic leukemia (18).

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