Inhibition of Vesicular Stomatitis Virus Replication in Dexamethasone-Treated L929 Cells (43024)

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Abstract. **We previously demonstrated that dexamethasone treatment of L929 cells inhibited plaque formation by vesicular stomatitis virus (VSV), encephalomyocarditis virus, or vaccinia virus. We now have characterized the antiviral effects of glucocorticoids in L929 cells. Dexamethasone did not directly inactivate VSV nor did steroid treatment of L929 cells affect virion adsorption or penetration. The VSV yield in L929 cells treated with dexamethasone for a period of only 4 or 8 hr was decreased by 50% when cells were infected the day following steroid treatment. Treating L929 cells with dexamethasone for a longer period resulted in greater inhibitions of virus synthesis. Interferon activity (<5 units/ml) was not detected in L929 cell culture fluids and cell sonicates from steroid-treated cells and the addition of antiserum to murine α/β-interferon had no effect on the ability of dexamethasone to inhibit VSV replication. Dexamethasone treatment of L929 cells did not induce the production of double-stranded RNA-dependent protein kinase but did result in a slight elevation of 2-5A oligoadenylate synthetase activity, two enzymatic activities associated with the antiviral state induced by interferon. However, the elevated 2-5A synthetase activity was not associated with an inhibition of VSV RNA accumulation in dexamethasone-treated L929 cells. By contrast, the synthesis of all five VSV proteins was reduced by 50–75% in dexamethasone-treated L929 cells as early as 4 hr after infection. Thus, the dexamethasone-mediated inhibition of VSV replication in L929 cells is associated with decreased production of VSV structural proteins.** [P.S.E.B.M. 1990, Vol 193]

Glucocorticoids have been shown to influence virus replication which appears dependent upon the host cell employed. For example, while glucocorticoid treatment stimulates the production of resident retrovirus (1) or increases the production of retrovirus that is induced by iododeoxyuridine (2, 3), it blocks production of avian sarcoma virus in transformed cells (4). Furthermore, glucocorticoids have been reported to increase herpes simplex virus production in 3T3 cells but not in Swiss mouse embryo fibroblasts (5), to have no effect on herpes simplex virus production in Nc1Ac110 (murine) cells (6), and to inhibit herpes simplex virus production in HEp-2 cells (7). This diversity of reported effects on virus production is likely due to the variety of steroidal effects on cell metabolism that affect the physiologic state of the cell (8).

A previous report from our laboratory documented the glucocorticoid-mediated establishment of an antiviral state that diminished plaque formation by unrelated RNA (vesicular stomatitis virus [VSV], encephalomyocarditis virus) and DNA (vaccinia) viruses in L929 cells, but not in rabbit skin or rat glioma cells. This antiviral activity coincided with other glucocorticoid-induced biochemical activities in L929 cells including inhibition of cell growth and induction of glutamine synthetase (9). The present report expands these initial observations and demonstrates that exposure of L929 cells to dexamethasone for as little as 4 hr established an antiviral state that persisted for 1 to 2 days in the absence of steroid. Additional studies indicated that the dexamethasone-mediated antiviral state was not asso-

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ciated with the synthesis of interferon or with the induction of two enzymatic and biologic activities associated with the antiviral activity of interferon. Dexamethasone-treated L929 cells synthesize VSV proteins at significantly reduced rates throughout the cycle of replication, indicating that the glucocorticoid-mediated antiviral state results in a decreased production of structural components of the virus.

Materials and Methods

Cells and Medium. The L929 cells were grown in Eagle's minimum essential medium in Earle's balanced salt solution and supplemented with 5% heat-inactivated (56°C, 30 min) newborn bovine serum, 20 m*M* Hepes buffer (pH 7.4), and antibiotics (penicillin, 50 units/ml; streptomycin, 50 μg/ml; and neomycin sulfate, 50 μg/ml). Cells received medium containing dexamethasone (10 μ*M*) or 0.1% ethanol (vehicle control) as indicated in the individual experiments.

Virus Yield Studies. L929 cell cultures in Leighton tubes (Bellco, Vineland, NJ) received medium containing 10 μ*M* dexamethasone for 4, 8, or 12 hr. At these times the medium was removed, the cell cultures were washed three times with saline (0.15 *M* NaCl), and medium containing 0.1% ethanol was added for the remainder of a 24-hr period. Control cell cultures received medium supplemented with 0.1% ethanol throughout the 24-hr incubation. At 24 hr, the cells were infected with VSV at a multiplicity of infection of 10 plaque-forming units/cell (moi = 10 PFU/cell). The cell cultures were frozen at 12 hr after infection, thawed, sonicated (20 kilocycles/sec for 15 sec at 4°C), and the total virus content was determined by plaque assay (10).

Virus Adsorption and Penetration. The effect of dexamethasone treatment on virus attachment and penetration of L929 cells was determined as described previously (11). Briefly, L929 cell cultures were incubated with 10 μ*M* dexamethasone or 0.1% ethanol for 24 hr at 37°C. The cells were then incubated with ^{35}S-labeled VSV that was prepared by infection of BHK-21 cells incubated with methionine-free medium supplemented with actinomycin D (5 μg/ml) and [^{35}S] methionine (50 μCi/ml) for 24 hr. The virus was purified by pelleting and two cycles of rate-zonal centrifugation as described (11). A 400-μl volume containing a moi = 5 PFU/cell (10^7 PFU) and 104,000 cpm was added to each cell culture. At the times indicated, the cells were extensively washed and the penetrated VSV, measured as trypsin-resistant radioactivity, was determined.

Interferon and Anti-Interferon. Mouse interferon (MuIFN-β) was purchased from Lee Biomolecular Inc., San Diego, CA. Mouse α/β-interferon antiserum was the kind gift of Dr. Maureen Myers, NIH, Bethesda, MD.

2-5A Synthetase Activity. L929 cell monolayers (75 cm^2) were treated with ethanol, dexamethasone, epicortisol, or MuIFN-β as indicated in the experiments. At the times indicated, the cell cultures were washed three times with a solution containing 35 m*M* Hepes (pH 7.5), 140 m*M* NaCl, and 3 m*M* $MgCl_2$ at 0°C. The cells were lysed in 1 ml containing 20 m*M* Hepes (pH 7.5), 5 m*M* $MgCl_2$, 120 m*M* KCl, 1 m*M* dithiothreitol, 10% (v/v) glycerol, and 0.5% (v/v) NP-40. The cell lysate was collected and centrifuged at 4°C in an Eppendorf microfuge (12,600*g*) for 15 min to remove the nuclei. Cytosolic fractions were assayed for protein concentration according to the method of Lowry *et al.* (12), with bovine serum albumin as the standard.

A volume of cell cytosol containing 1 mg of protein was incubated in a column containing 100 μl of packed polyriboinosinic-polyribocytidylic acid-Agarose (P. L. Biochemicals, Milwaukee, WI) for 30 min at room temperature. Columns were washed with 1 ml of buffer A (50 m*M* KCl, 3 m*M* magnesium acetate, 20 m*M* Hepes [pH 7.5], and 20% glycerol) to remove nonadsorbed material. One-hundred twenty-five microliters of the reaction mixture which contained 90 m*M* KCl, 11 m*M* magnesium acetate, 20 m*M* Hepes (pH 7.5), 5 m*M* [^{3}H]ATP (10 Ci/mmol), and 20% glycerol were added to the washed column. The agarose columns were incubated for 16 hr at 30°C and then eluted with 250 μl of buffer A. The eluates were added to DEAE-Sephadex columns which were washed with 10 ml containing 25 m*M* KCl, 3 m*M* magnesium acetate, and 20 m*M* Hepes (pH 7.5). Synthesized 2-5A (ppp(A′p)nA, n ≥ 2) was eluted with 300 m*M* KCl, 3 m*M* magnesium acetate, and 20 m*M* Hepes (pH 7.5). 2-5A Synthetase activity was expressed as nmoles of ATP converted to 2-5A/mg protein/hr.

Measurement of Protein Kinase Activity. L929 cell monolayers were untreated, IFN-treated (100 units/ml), treated with ethanol (0.1%), or treated with 10 μ*M* dexamethasone. After 24 hr at 37°C, S10 cell extracts were prepared as described previously (13). Fifteen-microliter samples of the cell extracts were incubated with 1 μCi of [γ-^{32}P]ATP (sp act 4000 Ci/mmol; ICN, Irvine, CA) and 12.5 nmol of unlabeled ATP in the presence or absence of 0.4 μg/ml polyriboinosinic-polyribocytidylic (poly I:C) acid. After 5 min at 30°C, each reaction received 50 μg of calf thymus histone IIA (Sigma Chemical Co., St. Louis, MO) to act as exogenous substrate for the double-stranded RNA-activated protein kinase (14) and incubation was continued for an additional 20 min. The reaction was terminated by the addition of 2X Laemmli sample buffer. The products were examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (15) and autoradiography.

VSV RNA Synthesis. L929 cell cultures were incubated with medium or medium containing 0.1% ethanol, 10 μ*M* dexamethasone, or 100 units/ml interferon for 24 hr at 37°C. The cell cultures were infected with VSV (moi = 100 PFU/cell) in the presence of 5

μg/ml actinomycin D and 200 μg/ml cycloheximide. At 1 hr, the inoculum was removed and medium supplemented with the drugs and 10 μCi/ml [^{3}H]uridine (sp act 35 Ci/mmol; ICN) was added. At the times indicated, the cells were lysed by the addition of 1% SDS and the trichloroacetic acid-precipitable radioactivity was determined.

Dexamethasone Effect on VSV Protein Synthesis. L929 cell cultures were incubated with medium alone or medium supplemented with 0.1% ethanol, 10 μ*M* dexamethasone, or 100 units/ml interferon for 24 hr at 37°C. The cell cultures were then infected with VSV (moi = 10 PFU/cell). At 2, 4, 6, and 8 hr postinfection, cell cultures from each test group received medium containing 2 μCi/ml [^{35}S]methionine (sp act 800 Ci/mmol; New England Nuclear, Boston, MA) for 30 min at 37°C. The cultures were washed and the cells lysed in 1% SDS, heat denatured following the addition of buffer. A volume equivalent to 5×10^4 cells was loaded in each lane and subjected to SDS-polyacrylamide gel electrophoresis (13) and autoradiography. Bands representing the VSV proteins were quantitated using an LKB XL laser densitometer.

Results

The time course for the establishment of the antiviral state in L929 cells treated with dexamethasone was examined. Cells received medium containing steroid for 4, 8, or 12 hr followed by medium without steroid for the remainder of a 24-hr period. At this time the cells were infected with VSV and the virus yield was determined 12 hr after infection. Cells exposed to steroid for 4 or 8 hr produced 50% less VSV. Virus yields were inhibited by 75% in cells treated with steroid through 24 hr (Table I). These data indicated that the antiviral state, once established, persisted in the absence of steroid for an additional 24–32 hr.

The ability of VSV to attach to and penetrate dexamethasone-treated L929 cells was assessed. Dexamethasone and vehicle control-treated cells were exposed to radiolabeled VSV and monitored for intracellular radioactivity. The data indicated VSV attached to and penetrated control and dexamethasone-treated L929 cells equally well (Table II). Additional studies showed that dexamethasone and other glucocorticoids do not inactivate VSV directly (data not shown), collectively indicating that dexamethasone exerts effects on the intracellular phase of the VSV replication cycle.

Since previous studies have shown that dexamethasone and interferon trigger some of the same biochemical pathways (16), we tested whether dexamethasone treatment of L929 cells induced an antiviral state by inducing enzymatic activities induced by interferon and/or by inducing the production of interferon itself. Assays of the culture fluids alone or in combination with sonicated cells failed to reveal the presence of any interferon activity (<5 units/ml; data not shown). However, if steroid-induced synthesis of low levels of interferon was responsible for the observed inhibition of VSV, then the addition of anti-interferon serum should block the dexamethasone-mediated inhibition of VSV replication. The presence of 1500 units/ml anti-α/β-interferon did not preclude the dexamethasone-mediated inhibition of VSV, although it neutralized 20 units/ml exogenously added interferon (Table III).

Studies have linked the antiviral activity of interferon to the induction of two double-stranded RNA-dependent enzymes, a protein kinase and 2-5 oligomeric A synthetase (17-22). The possibility that dexamethasone treatment of L929 cells resulted in induction of these enzymes was investigated. Although the induction of both enzymatic activities was apparent in interferon-treated L929 cells, protein kinase activity was not induced (Fig. 1) and 2-5 oligomeric A synthetase was only slightly elevated in dexamethasone-treated L929 cells (Table IV).

VSV replication is dependent upon the synthesis of RNA and structural proteins. Since the VSV genome has negative polarity, an essential initial step in the synthesis phase of the replication cycle is the primary transcription of the infecting genome. This was examined in L929 cells treated with dexamethasone. The data indicated that primary VSV RNA transcription was inhibited in interferon-treated L929 cells but was not inhibited in dexamethasone-treated cells (Fig. 2). The synthesis of VSV-directed structural proteins was

Table I. Inhibition of VSV Yield in Dexamethasone-Treated L929 Cells[a]

Length of steroid treatment (hr)	Virus yield from (PFU/cell) experiment					Mean (SEM)	% Control
	1	2	3	4	5		
0(control)	310	294	215	175	383	275 (37)	—
4	214	82	63	97	126	116 (26)	42[b]
8	190	104	20	158	211	137 (34)	50[b]
12	118	59	23	78	194	94 (25)	34[b]
24	114	63	29	41	94	68 (16)	25[b]

[a] L929 cell cultures received medium containing 10 μ*M* dexamethasone only for the time period indicated followed by medium containing 0.1% ethanol (vehicle) for the remainder of a 24-hr period. Controls received 0.1% ethanol for 24 hr. The cells were then infected with VSV (moi = 10) and the total virus content was determined at 12 hr after infection.
[b] $P < 0.05$; two-tailed Student's *t* test.

Table II. VSV Attaches to and Penetrates Dexamethasone-Treated L929 Cells

Time of incubation (min)	Cell treatment[a]	
	Vehicle control (cpm)	Dexamethasone (cpm)
0	83	76
30	3222	3505
60	6396	6140
90	7828	8367

[a] L929 cell cultures were incubated with 10 μM dexamethasone or 0.1% ethanol (vehicle control) for 24 hr at 37°C. The cells were washed, exposed to ^{35}S-labeled VSV, and the trypsin-resistant acid-precipitable radioactivity (penetrated virus) was determined as described (11). Values listed are means of duplicate samples which varied ±5% from the mean.

Table III. Dexamethasone-Mediated Antiviral State is Resistant to Treatment with Anti-Interferon Serum

Treatment	Concentration	PFU/cell	% Respective control[a,b]
Medium control		1150	100a
Vehicle control (EtOH)	0.1%	860	100b
Dexamethasone	10 μM	240	28b
Interferon	20 units/ml	13	1a
Anti-interferon	1500 units/ml	1040	90a
Interferon and anti-interferon	20 units/ml 1500 units/ml	1160	101a
Dexamethasone and anti-interferon	10 μM 1500 units/ml	260	30b

[a,b] L929 cell cultures were treated for 24 hr with supplements as indicated, then infected with VSV (moi = 10). The total virus content in replicate pooled samples was determined at 24 hr after infection and expressed as a percentage of the VSV yield in cells incubated with medium alone (a) or medium supplemented with 0.1% ethanol (b).

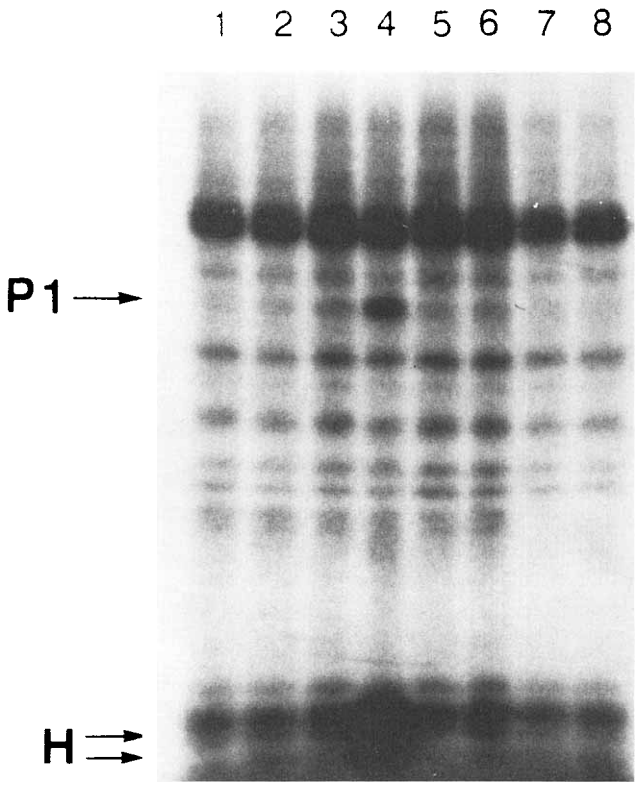

Figure 1. Failure of dexamethasone to induce double-stranded RNA-dependent protein kinase. L929 cell monolayers were untreated (1,2), IFN-treated (3,4), ethanol-treated (5,6) or dexamethasone-treated (7,8) as described in Materials and Methods. Cell extracts were prepared and assayed for the IFN-associated protein kinase in the absence (1,3,5,7) or presence (2,4,6,8) of poly I:C. The relative migration position of the M_r 68,000 P1 protein (13) and the histone proteins are indicated.

also monitored in dexamethasone-treated cells. The data indicate that the synthetic rate of all five structural virion proteins is clearly diminished in dexamethasone-treated L929 cells relative to cells that were mock infected or treated with ethanol (vehicle control; Fig. 3). Quantitation of the autoradiogram by densitometry indicated that relative to ethanol-treated cells, the rate of synthesis of VSV proteins was diminished by 50% or more at 4 hr after infection. The rate of glycoprotein G synthesis was most sensitive to the dexamethasone treatment of the L929 cells, being reduced by 75% through 8 hr after infection (Table IV). The reduction of VSV yield (12 hr after infection) in concordantly infected cells was 85% (not shown). Although virus production is decreased, dexamethasone-treatment did not affect the VSV-mediated inhibition of cell protein synthesis (Fig. 3).

Discussion

Since glucocorticoids stimulate specific biologic activities which can vary with the cell, they can differentially affect the physiologic state of different cells. Alternatively, the physiologic state of the cells may also affect their response to glucocorticoids (8, 23). It is likely that this accounts for the seemingly contradictory observations reported in previous studies of the effects of glucocorticoids on virus replication. Our previous report documents three coincident biologic activities in glucocorticoid-treated L929 cells: growth inhibition of cells, induction of glutamine synthetase, and inhibition of virus plaque formation (9). Data in the present report indicate that the anti-VSV activity of dexamethasone is not due to steroid-mediated inactivation of the virion. Dexamethasone exerts its effects during intracellular events in the VSV replication cycle as it does not interfere with VSV attachment and penetration.

It is noteworthy that a 4-hr exposure to steroid renders L929 cells resistant to VSV replication. The steroid can be removed, and the virus replication remains inhibited even though the cells have been maintained for as long as 32–44 hr (through the end of a 12- or 24-hr virus replication cycle) in the absence of dexamethasone. Extending the length of infection from 12 (Table I) to 24 hr (Table III) results in higher VSV yields per cell but the ratio of VSV produced relative to control remains constant, indicating that dexamethasone treatment inhibits the production of progeny

Table IV. Effect of 10 μM Dexamethasone on the Induction of 2-5 Oligo A Synthetase in L929 Cell Cultures

	Treatment	Concentration	Length of treatment (hr)	2-5A synthetase activity[a] (experimental/control)
Experiment 1	Vehicle control	0.1% EtOH	24	1.0 ± 0.1
	Epicortisol	10 μM		0.8 ± 0.2
	MuIFN-β	100 units/ml		5.3 ± 0.1
	Dexamethasone	10 μM		1.4 ± 0.1
Experiment 2	Vehicle control	0.1% EtOH	24	1.0 ± 0.1
	Epicortisol	10 μM		1.0 ± 0.1
	MuIFN	100 units/ml		4.7 ± 0.3
	Dexamethasone	10 μM		2.2 ± 0.1
	Vehicle control	0.1% EtOH	48	1.0 ± 0.4
	Epicortisol	10 μM		0.6 ± 0.1
	MuIFN	100 units/ml		4.7 ± 0.1
	Dexamethasone	10 μM		2.1 ± 0.1

[a] L929 cell monolayers were treated as shown and at the times indicated replicate samples were processed and assayed for 2-5A synthetase activity expressed as nmoles of ATP converted into ppp(A2′p)nA, ($n \geq 2$)/mg protein/hr, as described in Materials and Methods. The 2-5A synthetase activities for the three control preparations listed were 4.4 (Experiment 1), 0.9, and 1.0 nmol (Experiment 2, 24 and 48 hr, respectively).

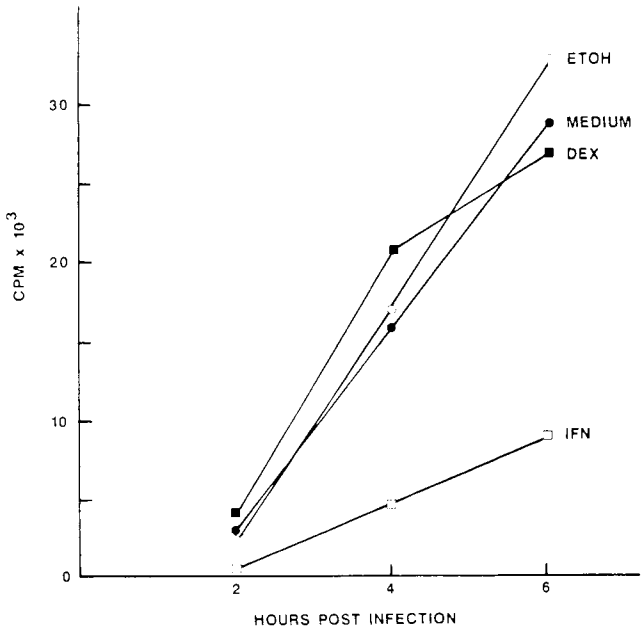

Figure 2. Primary VSV transcription in dexamethasone-treated L929 cells. L929 cell cultures received medium (MEDIUM), or medium supplemented with 100 units/ml interferon (IFN), 0.1% ethanol (ETOH), or 10 μM dexamethasone (DEX) for 24 hr at 37°C. Each cell culture was then infected with VSV (moi = 100) in the presence of 5 μg/ml actinomycin D and 200 μg/ml cycloheximide, and then received medium containing the drugs and 10 μCi/ml [^{3}H]uridine at 1 hr after infection. Cell cultures were harvested at the times indicated and the acid-precipitable radioactivity was quantitated as described in Materials and Methods.

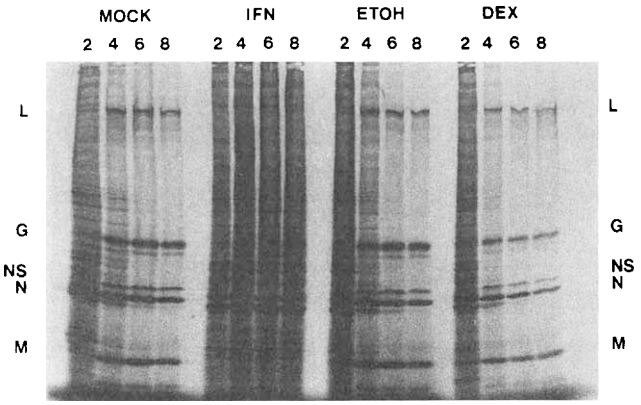

Figure 3. Inhibition of VSV protein synthesis in dexamethasone-treated L929 cells. L929 cell cultures were incubated with medium (MOCK) or medium supplemented with 100 units/ml interferon (IFN), 0.1% ethanol (ETOH), or 10 μM dexamethasone (DEX). At 24 hr the cultures were infected with VSV (moi = 10) and at the times indicated after infection, the cultures received [^{35}S]methionine for 30 min. The cells were lysed, the proteins were separated by SDS-polyacrylamide gel electrophoresis (15), and the gel was subjected to autoradiography.

virus and does not simply delay the cycle of replication. Thus, dexamethasone may function in an inductive manner, resulting in the development of a relatively long-lasting antiviral state that does not require the continued presence of glucocorticoid.

The initial observations of a glucocorticoid-mediated antiviral state that rendered L929 cells resistant to plaque formation by unrelated viruses could be accounted for if the glucocorticoid steroids induced the L929 cells to produce interferon. A previous report suggested that noradrenaline treatment mediated development of an antiviral state in myocardial cells (24). In addition, glucocorticoid treatment of Daudi cells significantly increased the cellular content of 2-5, oligoadenylate synthetase, an enzymatic activity associated with the antiviral activity of interferon, although the viral susceptibility of these glucocorticoid-treated cells was not reported (16). We studied the induction of interferon and interferon-associated enzymatic activities in dexamethasone-treated L929 cells. The data clearly indicate that interferon itself was not produced by dexamethasone-treated cells. In addition, double-

standed RNA-dependent protein kinase activity was unaffected in glucocorticoid-treated L929 cells (Fig. 1). However, 2-5A synthetase activity in dexamethasone-treated L929 cells was increased 2-fold; interferon treatment on the other hand resulted in a 5-fold enzyme induction. If the 2-5A synthetase was activated by binding to double-stranded RNA, this would result in synthesis of 2-5A which subsequently could activate the endogenous endoribonuclease to cleave virion (and cell) plus strand RNA (25, 26). However, the data indicated primary VSV transcription was inhibited only in interferon-treated and not dexamethasone-treated L929 cells. Thus, we conclude that the antiviral state established by dexamethasone is independent of interferon production and the activity of two known enzymes associated with interferon activity.

The data presented (Table V and Fig. 2) indicate the rate of synthesis of all five VSV proteins is inhibited by 50–75% as early as 4 hr after infection while cellular protein synthesis is inhibited by approximately 20% throughout a 24-hr glucocorticoid treatment (data not shown). In addition, there is an approximate parity between the decrements in synthesis of VSV proteins and VSV yields. An inhibition of one or more VSV proteins, e.g., glycoprotein G, could account for the decreased virus yield in L929 cells. Thus, the inhibition of VSV replication in dexamethasone-treated L929 cells is associated with an inhibition in the synthesis of VSV structural proteins. It is interesting to note that the steroid-mediated inhibition of VSV does not preclude the virion-mediated inhibition of cell protein synthesis.

Recent studies indicate that glucocorticoids induce the production of lipocortins, proteins that inhibit the activity of phospholipase A_2 (27, 28). Lipocortin, which is believed to be responsible for many of the anti-inflammatory properties of glucocorticoids, can also regulate the physiologic state and biologic activities in mammalian cells (29, 30). The relationship between dexamethasone-mediated lipocortin induction, phospholipase A_2 activity, and susceptibility of cells to virus infection is at present unknown and is currently under investigation.

Table V. Quantitation of VSV Proteins Synthesized in Dexamethasone-Treated L929 Cells

Time after VSV infection (hr)	VSV Protein synthesis[a] (% control)			
	M	N/NS	G	L
4	28	53	36	41
6	34	35	28	47
8	54	34	26	51

[a] Quantitation of VSV protein synthesis in dexamethasone-treated L929 cells from the autoradiogam in Figure 2. These values are expressed as a percentage of the desitometer tracings for VSV proteins synthesized in ethanol-treated cell cultures.

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