THE INFLUENCE OF CORTISONE ON EXPERIMENTAL VIRAL INFECTION

VI. Inhibition by Hydrocortisone of Interferon Synthesis in the Chick Embryo*

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The several and sometimes paradoxical effects of cortisone and its analogues on the course of a variety of infections may be duplicated in the relatively simple chick embryo–influenza virus model in which the interrelationships of a single obligate intracellular parasite and the cells of a single target organ (the allantoic entoderm) may be examined. Even in this simple system the influence of cortisone may be favorable (1, 2) or detrimental (3, 4) to the host depending on experimental conditions. The observation that the administration of cortisone to chick embryos (3) or in tissue culture (5) increases final yields of influenza B virus although it reduces early viral synthesis (2) is explicable as a result of inhibition of viral autointerference (4). A preliminary report has indicated that the interferon synthesis that accompanies influenza virus infection of the chick embryo is inhibited by cortisone and by a substituted C-19-nor-steroid that is also active in inhibiting viral interference (6). This report had suggested also that cortisone inhibited the action of preformed interferon as well as its synthesis.

The present paper presents further evidence for the effect of cortisone (hydrocortisone) on interferon formation in the chick embryo and on interference *in vitro* and reexamines the question of whether or not interferon action is also moderated.

Materials and Methods

Viruses.—The Lee strain of influenza B virus and the PR8 strain of influenza A virus used in earlier studies (2) were employed in the present experiments as aliquots from two pools of

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seed virus with the following characteristics: *Lee:* $EID_{50}10^9$/ml; HA (hemagglutinin concentration) $10^{2.8}$/ml. *PR8*: EID_{50} $10^{9.3}$/ml; HA $10^{3.7}$/ml. Both viruses were diluted to contain 10^2 EID_{50}/0.1 ml as inocula for interferon production. The Lee strain was diluted to contain $10^{6.5}$ EID_{50}/0.1 ml when used as a challenge in the in vitro interferon assay.

Chick Embryos.—Ten-day-old white Leghorn embryos obtained from Shamrock Farms, 380 Church Lane, North Brunswick, New Jersey, were used for the production of interferon. Chorioallantoic membrane (CAM) cultures for interferon assay were prepared from groups of 4 embryos 11 to 13 days of age.

Hydrocortisone.—A salt of high aqueous solubility, (28 mg/ml) hydrocortisone diethylaminoacetate hydrochloride, that had been used in previous studies (2, 4, 6) was used in the present experiments after sterilization as an aqueous solution (1 mg/ml) at 15 lb. pressure for 15 min.

Interferon.—Partially purified interferon was prepared as described previously (6).

Interferon Assays.—Dialyzed preparations of allantoic fluid were assayed for interferon activity in shake cultures of chorioallantoic membranes suspended in Earle's solution with 2% chick serum and incubated at 36°C. After 18 to 24 hr exposure to the test materials, membranes were washed, then exposed to $10^{6.5}$ EID_{50} of Lee virus in a volume of 1.0 ml. The inoculum was removed following 2 to 3 hr of adsorption. Interferon content (activity) is expressed as the difference (decrease) in the 48 hr Lee virus hemagglutinin yield ($\log_2$) between control and experimental groups.

Interferon was also assayed in chick embryo fibroblast cultures by a plaque reduction method using the NWS strain of influenza virus or VSV (vesicular stomatitis virus) as challenges. For these experiments monolayers of cells were prepared by addition of 9×10^6 cells derived from minced, trypsinized skin and muscle from 10-day-old chick embryos to each 60 mm plastic Petri dish. The growth medium consisted of Earle's balanced salt solution, 0.5% lactalbumin hydrolysate, and 5% chicken serum. Full monolayers were attained after 48 hr incubation at 35°C. The fluid maintenance medium was 199 with 0.5% chicken serum.

Chorioallantoic Membrane (CAM) Extracts.—Extracts of CAM for assay of interferon content were prepared after dissection of entire membranes from embryos, rinsing them twice in phosphate-buffered saline (pH 7.2) and then once in Earle's medium with 2% chicken serum. The CAM extracts suspended in groups of six in 3 ml of medium were minced, then ground in glass tubes with Teflon grinders. The preparations were clarified by centrifugation for 10 min at room temperature, at 8,200 *g*. The supernatants were brought up to a final volume of 7 ml of medium per 6 CAM, this material being termed the CAM extract. Equilization of the volume of the various extracts was a necessary correction since the edematous reaction of the CAM in cortisone-injected embryos would otherwise tend spuriously to decrease their apparent interferon content. Subsequent preparation of the CAM extracts for interferon assay was the same as that described above for the allantoic fluid pools.

Ultraviolet Inactivation of Virus.—Viral particles were separated from small molecular weight ultraviolet-absorbing materials by dialysis or by ultracentrifugation and exposed in 15 ml volume, in 100 mm Petri dishes during agitation by a magnetic stirrer, to irradiation with a 7½ watt GE lamp at 7 inches distance.

RESULTS

Kinetic Studies of Interferon Increase—Parallelism of Cell-Associated (CAM) and Free (Allantoic Fluid) Interferon.—Earlier studies had shown that cortisone does not merely delay the release of interferon into the allantoic fluid but rather reduces the amount detectable in both allantoic fluid and chorioallantoic membrane (6). It has also been demonstrated that changes in the dis-

tribution ratios of allantoic fluid and CAM virus are affected by cortisone only as the secondary result of reduction in viral synthesis (7). With studies of the kinetics of interferon formation in prospect, it was essential to determine whether measurement of allantoic fluid interferon would be representative of tissue concentrations at various time intervals in both cortisone-injected and control embryos.

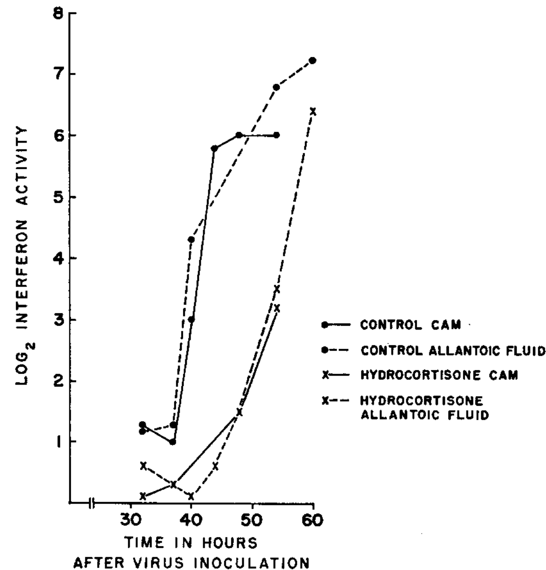

FIG. 1. Parallelism of incremental curves of cell-associated (CAM) and free (allantoic fluid) interferon in control and hydrocortisone-injected chick embryos. Interferon activity is expressed per milliliter of allantoic fluid or CAM extract diluted 1:2.

Ten-day-old chick embryos (in groups of six) were injected in the allantoic sac with 10^3 EID_{50} of Lee virus 2 hr after injection of 0.1 mg of hydrocortisone or distilled water. Allantoic fluid and CAM pools were harvested at twelve intervals between 2 and 60 hr after viral inoculation and fluids and CAM extract were titrated for interferon activity. The combined results of three experiments are shown in Fig. 1. Significant amounts of interferon were not detected in fluids or membranes of either control or steroid-injected eggs at 2, 4, 8, 18, or 24 hr. At 32 hr interferon first appeared in the CAM and allantoic fluids of control eggs and increased rapidly to attain near maximal levels 12 hr later (at 44 hr). Temporary inhibition by hydrocortisone of interferon formation is reflected by measurement of interferon both in CAM extracts and allantoic fluid, and it is also clear that concentrations of interferon in CAM and allantoic fluid increased in parallel without significant (i.e., $> 1 \log_2$) deviation in both control and hydrocortisone-treated groups.

The Influence of Residual ("Carry-over") Hydrocortisone in Allantoic Fluids in the (CAM) Interferon Assay System.—Biologically active hydrocortisone inoculated intraallantoically is almost completely removed by dialysis during the preparation of interferon samples. This was ascertained by comparing the ability of an allantoic fluid pool from hydrocortisone-injected eggs to increase the yield of Lee virus in the in vitro CAM system before and after dialysis. In six experiments in which "interferon" was prepared from hydro-

TABLE I

Influence of Hydrocortisone on Viral Interference in Vitro. Differing Effects with Inactivated Virus and Preformed Interferon

Group	Initial injection	HA $\log_2$ yield after Lee virus challenge*		Increase with hydrocortisone
		Pretreatment‡		
		None	Hydrocortisone	
1	Interferon§	4.3	4.8	0.5
2	Interferon (A)	5.0		
3	UV–PR8‖	3.0	6.3	3.3
4	UV–PR8 (A)	8.0		
5	None	8.0	8.3	0.3

* Challenge with Lee virus $10^{6.5}$ EID_{50} 20 hr after initial injection.

‡ Hydrocortisone 0.1 μg administered 2 hr before initial injection.

§ Spinco supernatant of allantoic fluid from chick embryos infected 72 hr before with PR8 virus; adsorbed with human "O" RBC; residual hemagglutination titer — $<10^{-0.3}$, residual infectivity titer $10^{1.5}$/0.1 ml. (A), same as above, but dialyzed at pH 2.0 to destroy residual virus.

‖ Spinco sediment of above, partially purified by adsorption-elution from human "O" RBC; irradiated with ultraviolet (UV) 20 sec; residual infectivity titer $10^{3.2}$/0.1 ml. (A), same as above, etc.

cortisone-treated but uninfected embryos, the average increase over control values of Lee HA yields in the in vitro assay system was 0.3 $\log_2$. This small but consistently observed effect on the yield of the challenge virus is in accord with the observation of DeMaeyer (8) who noted that 10% of tritiated cortisone was not removed by dialysis. In any case, this increase is below the level of significance in any single experiment and it may be concluded that any observed effects in cortisone-treated embryos are mediated in ovo and not secondarily in the in vitro interferon assay system.

Influence of Hydrocortisone on Viral Interference in Vitro.—The inactivation of influenza viral infectivity by ultraviolet (UV) irradiation or heat produces a preparation of high interfering activity. It has been shown that cortisone may

negate this effect in the chick embryo so that final yields of virus are not reduced by the interfering effects of a high multiplicity of inactivated viral particles (4). Similar reduction in viral yield is observed in CAM tissue cultures inoculated with inactivated influenza viruses (9). That this in vitro effect may also be inhibited with hydrocortisone is demonstrated in Table I.

Inoculation of cultures with partially purified UV-inactivated PR8 (group 3) caused a 32-fold (5 $\log_2$) reduction in the yield of HA following subsequent inoculation of Lee virus. That this effect was dependent upon the presence of intact virus is shown by group 4 in which the inoculum had been subjected to acid dialysis to destroy virus in the preparation. Pretreatment of CAM cultures by introduction of 0.1 μg of hydrocortisone 2 hr before inactivated virus

TABLE II

Effect of Hydrocortisone on the Action of Preformed Interferon in Vitro

Experiment	Control		Interferon*		Hydrocortisone-induced increase	
		Hydrocortisone‡		Hydrocortisone	Control	Interferon
1	6.8§	6.5	3.8	3.8	(0.3)	0.0
2	6.3	7.3	2.0	4.3	1.0	2.3
3	7.3	8.3	4.0	6.5	1.0	2.5
4	7.5	8.8	1.5	4.5	1.3	3.0

* pH_2 dialyzed 72 hr PR8 (1-3), 48 hr Lee (4), 1:5, at −20 hr.

‡ Hydrocortisone, 0.1 μg, at −22 hr.

§ 44 to 48 hr HA $\log_2$, Lee virus $10^{6.5}$ EID_{50} inoculated in 11-day (1, 3), 12-day (4), or 13-day (2) CAM shake cultures.

almost completely inhibited the interfering effect (group 3). When the Spinco supernatant of the partially purified PR8 virus (interferon) was employed, either in untreated (group 1) or acid-dialyzed form (group 2), reduction in Lee virus yield was effected as expected. However, in contrast to its effect with interference induced with inactivated virus, hydrocortisone did not reduce interference from preformed interferon (group 1). (The formation of interferon following inoculation of partially inactivated virus, and its inhibition with hydrocortisone are discussed in a later section.)

In other related experiments it has also been shown that with inocula of infective virus in the chick embryo the augmenting effect of hydrocortisone on final yields of virus is unrelated to the interferon content of the viral inocula. These experiments were at variance with those reported earlier (6) in that they failed to demonstrate any significant influence on the viral-inhibiting effect of preformed interferon. Accordingly, this question was investigated further.

Effect of Hydrocortisone on the Action of Preformed Interferon.—In four

separate experiments (Table II) the influence of hydrocortisone in 0.1 μg/ml concentration on the action of preformed interferon was studied. It will be noted that hydrocortisone induced increases of virus above control levels in interferon-treated cultures. However, lesser but consistent increases were also effected by hydrocortisone in the absence of preformed interferon. This observation suggested that cortisone was influencing in part the autointer-

TABLE III

Lack of Effect of Hydrocortisone on Action of Preformed Interferon in Chick Embryo Fibroblast Monolayer Cultures

Experiment	Virus	Log_{10} dilution	Interferon*	Incubation period‡	Plaque count		
					Control	Hydro-cortisone§	Concen-tration
				days			*μg*
1	VSV	4.5	None	5	53, 45, 50	37, 39, 40	1
			1:2	5	0	0	
			1:20	5	7	6	
			1:50	5	20	17	
2	VSV	4.5	None	1	28, 26, 34	30, 29, 21	1
			1:20	1	3, 3, 1	1, 2, 1	
3	VSV	4.5	None	3	24, 24, 22	23, 24, 27	10
			1:20	3	5, 6, 4	1, 2, 5	
4	NWS	6.5	None	5	28, 27, 29	19, 22, 17	1
			1:2	5	11	4	
			1:20	5	20	6	
			1:50	5	23	11	

* Introduced into cultures at −20 hr.

‡ After viral inoculation.

§ Hydrocortisone introduced in fluid medium at −24 hr and also in same concentration in agar overlay after viral inoculation; viral inoculation at time 0.

ference associated with the high multiplicity of infective challenge virus per cell (4-20/1) that is required to produce measurable hemagglutination in the CAM culture system. The slight additional relative increase seen in the presence of interferon remains unexplained, but it was obvious that no unequivocal answer to the question of cortisone effect on interferon action was obtainable in the CAM test system. Therefore, further information on this point was sought in monolayer cell cultures of chick embryo fibroblasts in which inhibition of plaque formation at end point dilutions (low virus/cell multiplicity) of challenge virus might be studied. The results of four experiments are summarized in Table III. In three experiments in which inhibition of plaque for-

mation by VSV was the end-point for interferon assay, preliminary treatment of monolayers with 1 to 10 μg/ml of hydrocortisone (at 20 to 24 hr) did not influence the reduction in plaque count that resulted from the application of interferon in various dilutions. Results were the same, whether plaques were counted at 1, 3, or 5 days after viral inoculation.

In an effort to make the plaque reduction assay more comparable to results obtained in CAM culture, a plaque-producing influenza A virus (NWS) was used as the challenge agent. Again, hydrocortisone in 1.0 μg/ml concentration did not influence even the minimal activity of interferon observed in this experiment (Table III, Experiment 4). Indeed, a trend toward reduction in plaque count was observed with hydrocortisone. It is of interest that NWS was also relatively resistant to interferon inhibition in the CAM assay system.

TABLE IV

Augmentation by Hydrocortisone of Yield of Lee Virus Hemagglutinin and of Interferon Following Inoculation of Partially Inactivated Virus

Group	Inactivated Lee virus*	Hydrocortisone‡	Mean $\log_2$ viral HA	Mean $\log_2$ interferon
1	+	+	10.3	6.2
2	+	0	2.7	0.7
3	0	+	—	0.6
4	0	0	—	0.4

* Virus Δ48 hr at 36°C reduced infectivity titer from $10^{-9.3}$/ml to $<10^{-1}$/ml; inoculum 0.1 ml/egg of 10^{-1} dilution of virus.

‡ Hydrocortisone 0.01 mg/egg 2 hr before viral inoculation. Incubation period, 96 hr.

The Augmenting Effect of Hydrocortisone on Interferon Synthesis under Certain Conditions with Inocula Containing Large Amounts of Inactivated Virus.—It has been proposed that in vivo viral synthesis may be regulated to a degree by corticosteroids (2) and that the regulation of infection may depend on a balance of the effects of endogenous corticosteroids on viral and interferon synthesis (6). It is interesting in this connection that experimental conditions may be so adjusted that in the chick embryo the synthesis of interferon may be strikingly *augmented* by the administration of hydrocortisone. Such an experiment is outlined in Table IV. In this experiment a partially inactivated preparation of Lee virus was injected into groups of chick embryos (in groups of six) to which a preceding injection of H_2O or hydrocortisone had been administered. As expected, yields of virus were increased in steroid-treated embryos (group 1), but paradoxically, a 45-fold increase in interferon titer also occurred in comparison with control embryos (group 2) in which viral yields were greatly restricted through autointerference. Significant increases in interferon were not observed in appropriate control allantoic fluids from embryos not inoculated with virus.

This experiment emphasizes, for one thing, the exhibition of extensive viral interference in the absence of demonstrable interferon formation and also the sequential effect in this instance of steroid-mediated viral increase and a resultant increased production of interferon. Cortisone does not influence the kinetics of adsorption and elution of influenza virus (7) except that in high dosage it may delay viral release in vitro (10). The present result is therefore not explicable on the basis of change by hydrocortisone of available viral receptors or facilitation of viral or interferon release, but is most probably explained as a sequel of a restrictive effect on initial synthesis of interferon during the first infectious cycle. This effect would allow for an additional cycle or cycles to occur, with a consequent stimulation of interferon synthesis—as hydrocortisone is not completely inhibitory to interferon synthesis in the usual multicyle infection in which a low virus/cell multiplicity of infective virus is inoculated.

In other experiments with a higher multiplicity of inactivated virus, CAM extracts were examined early after viral inoculation for interferon, and it was found to be present in low concentration 4 hr after infection in eggs not treated with hydrocortisone, at which time it was not detectable in allantoic fluid. At the same time period (4 hr), interferon was not detectable in either CAM or allantoic fluids of hydrocortisone-injected embryos, although it appeared subsequently. These observations are compatible with the explanation proposed of an early inhibitory effect of hydrocortisone on interferon formation, and demonstrate as well the early presence of interferon (probably inhibitory to continued viral multiplication) in embryos injected with virus only.

It is also clear that the interferon detected early in the CAM was not residual preformed interferon from the inoculum, because (*a*) the inoculum had been partially purified to leave principally a viral sediment, and (*b*) interferon was not detectable in the CAM 2 hr after inoculation.

DISCUSSION AND SUMMARY

The initial observations that cortisone may act as an inhibitor of viral interference (11, 4) are now explicable as an inhibitory effect on interferon synthesis. The suggestion that the action of interferon is also inhibited by cortisone or its analogues (6) has not been confirmed in a plaque reduction type of interferon assay system in which autointerference by the challenge inoculum is a lesser problem. In this respect, the present results are in accord with those obtained by DeMaeyer and DeMaeyer (8) with hydrocortisone in a system in which a low multiplicity (0.1) Sindbis virus infection in monolayer culture was employed with cytopathic effect (CPE) as an end-point. It has been shown that hydrocortisone is restrictive to the synthesis of interferon induced by inoculation of either infective or inactivated virus into the chick embryo, and that this inhibitory effect is temporary. However, in another study in the chick embryo, three spaced injections of hydrocortisone (0.25 mg/dose) prevented

the appearance of detectable interferon during the entire 64 hr observation period following inoculation of $10^{3.3}$ EID_{50} of Lee virus (12).

The importance of explicit definition of experimental conditions in assessing hormonal effects on infection is illustrated by the capacity of hydrocortisone either to inhibit or increase interferon synthesis in vitro, depending on the proportion of inactivated and infective virus in the inoculum employed, and the time at which interferon is measured. As suggested previously, it is not unlikely that similar shifts in hormone–virus–interferon balance may operate in vivo to influence the outcome of infection.

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