Differential Inhibitory Effects of Interferon on Deoxythymidine Kinase Induction of Vaccinia-Infected Cell Cultures (32646)

S. BARBAN AND S. BARON

U. S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Biology of Viruses, Bethesda, Maryland 20014

Although the mechanism of action of interferon has not been elucidateed, accumulating evidence suggests that it exerts its antiviral effects on some early event in virus replication. Evidence that interferon inhibits the induction of deoxythymidine kinase in vaccinia infected chick embryo cell cultures has been reported (1,2). The purpose of this paper was to extend these observations and examine various parameters of the inhibitory effect of interferon on deoxythymidine kinase induction. Interferon was observed to inhibit enzyme induction by vaccinia virus in chick embryo cell cultures under conditions of inhibition of viral DNA synthesis suggesting that interferon acts on viral synthesis before replication of viral DNA. No inhibition of deoxythymidine kinase induction was found in mouse embryo cultures treated with mouse interferon under similar experimental conditions.

Materials and Methods. Cells. Primary chick embryo cultures were prepared by trypsinization of 9- to 10-day-old embryos and plating in Eagle's medium (3) with 10% (v/v) calf serum. Primary mouse embryo cell cultures were obtained in a similar manner from near term mouse embryos. Cells were grown to confluent sheets in 60 mm plastic petri dishes in a 5% (v/v) CO_2 incubator at 37°C.

Two strains of vaccinia virus, one obtained from Dr. Aaron Shatkin and the other from Dr. Saul Kit. Vaccinia virus infectivity was determined as plaque forming units in HeLa cell cultures. Before assay, all samples were frozen and thawed and sonicated to release vaccinia virus from cells and aggregates.

To achieve the high virus to cell ratio (12:1 for chick embryo and 6:1 for mouse embryo), required for a single cycle of infection and for maximum induction of enzyme, it was necessary to infect each plate with $10^{7.6}$ pfu or $10^{7.3}$ pfu, respectively) of vaccinia virus. After 2 hours at 37° the plates were washed 4 times to remove unadsorbed virus. This washing procedure was usually sufficient to decrease residual input virus to a titer of $10^{5.9}$/ml. Thus, the full extent of viral inhibitory effect of interferon and inhibitors of DNA could not be determined. Therefore, virus titers in the table are noted as less than (<) the residual input virus titer whenever virus yield was inhibited to the level of residual virus. Control experiments, using as many as 13 washings over a 30-min period to remove more of the input virus, demonstrated that the doses of interferon and DNA inhibitors which were employed inhibited vaccinia virus growth at least 30-fold.

Interferon. Mouse interferon was prepared as pools of serum from mice injected intravenously with Newcastle disease virus (4). Chicken interferon was prepared from allantoic fluid of NWS influenza infected embryonated eggs (5). The titer of interferon was expressed as the reciprocal of the highest dilution of the interferon preparation which inhibited 50% of vesicular stomatitis virus plaques in homologous cell cultures.

Preparation of enzyme extracts and assay. After appropriate periods of incubation, the supernatant fluid was removed, the cells washed once in phosphate buffered saline (pH 7.0) and harvested by scraping with a rubber policeman into a Tris buffer (0.01 *M*, pH 8.0) containing 0.15 *M* KCl and 0.003 *M* dithiothreitol reducing agent. After centrifugation, the cells were resuspended in 3 vol. of the same buffer solution and subjected to sonic vibrations in a 10 KC Raytheon sonic oscillator for 5 min. The sonically disrupted cell suspensions were then centrifuged for 15 min at 18,000 *g* and the supernatant fraction was employed immediately for enzyme assay. Thymidine kinase assay was essentially the same as described by Kit *et al.* (6).

Each assay was performed in duplicate. Enzyme activity was found to be proportional to the protein content of the cell extracts.

Protein assay. The protein content of the cell extracts was assayed by the method of Lowry *et al.* (7) using bovine serum albumin as a standard.

Results. Kinetics of deoxythymidine kinase induction in vaccinia-infected chick embryo and mouse embryo cell cultures. In the uninfected confluent cell cultures the activity of deoxythymidine kinase was initially low and declined during the course of the experiment. In the vaccinia virus-infected chick embryo cultures, enzyme activity began to increase about 6 hours postinfection and continued to increase rapidly, reaching maximum activity about 24 hours postinfection and then declined. The specific enzyme activity of infected mouse embryo cultures rose after 3.5 hours and reached maximum activity after 5–7 hours and then declined. Employing a different vaccinia virus strain (obtained from Dr. Saul Kit), a maximum enzyme activity was observed 18 hours postinfection.

Effect of interferon on deoxythymidine kinase induction in chick embryo cell cultures. Initial experiments confirmed the original observations of Ohno and Mozima (1) that pretreatment of the cells with interferon inhibited the induction of deoxythymidine kinase in vaccinia-infected cells. The representative experiments in Table I show that chick interferon-inhibited deoxythymidine kinase induction by 40–60% and also inhibited multiplication of vaccinia virus by at least 67%.

Effect of dose and pretreatment of interferon. In order to characterize more fully that the deoxythymidine kinase inhibitor was interferon (in our crude preparations), certain physiochemical and biological properties were examined. As can be seen (Table I), the inhibiting effect of interferon on deoxythymidine kinase activity of the vaccinia-infected cell cultures was dose dependent and was correlated with its antiviral effect. In addition, following treatment of the interferon preparation with recrystallized pancreatic trypsin, no inhibition of deoxythymidine kinase induction was observed.

The results obtained on heat treatment of the interferon preparations indicated relatively high heat stability of the inhibitor up to 80°. The inhibitor was also stable to pH 2 and could not be washed off cells. The inhibition of deoxythymidine kinase by interferon demonstrated species specificity (Table I, gp. 12). The results as shown in Table I indicate that

TABLE I. Effect of Dose and Pretreatment of Interferon on Its Inhibition of Deoxythymidine Kinase in Vaccinia-Infected Chick Embryo Cell Cultures.

Group	Treatment[a]	Deoxythymidine kinase[f]	Virus titer $\log_{10}$/ml
1	Uninfected	0.05	—
2	Infected	10.40	7.1
3	Infected + control fluid[b]	8.90	7.1
4	Infected + interferon (30 units)	4.04	<6.3
5	Infected + interferon (3 units)	6.79	6.7
6	Infected + interferon (0.3 units)	8.90	6.8
7	Infected + interferon (trypsin)[c]	9.18	6.7
8	Infected + interferon (trypsin inhibitor)[d]	5.16	<6.3
9	Infected + interferon (70°)[e]	4.20	<6.3
10	Infected + interferon (80°)	4.40	6.6
11	Infected + interferon (90°)	7.54	6.6
12	Infected + mouse interferon	8.92	7.1

[a] Confluent cell cultures were preincubated for 18 hours with the interferon preparations. Cells were harvested 18 hours postinfection.

[b] Control cultures received normal allantoic fluid.

[c] Interferon, 3 units, pretreated with 0.2 μg/ml pancreatic trypsin 60 min at 37°C then stopped with an equal concentration of soybean trypsin inhibitor.

[d] Interferon, 3 units, treated with soybean trypsin inhibitor.

[e] Temperatures at which 3 units of interferon held for 1 hour prior to experiment.

[f] The values represent millimicromoles of deoxythymidine monophosphate formed per milligram protein in 15 min and are the averages of two to three experiments.

TABLE II. Effect of Interferon and Bromodeoxyuridine on Deoxythymidine Kinase Activity of Vaccinia-Infected Chick Embryo Cell Cultures.

Group	Treatment[a]	Deoxythymidine kinase	Virus titer $\log_{10}$/ml
1	Uninfected	.09	—
2	Infected	12.90	6.7
3	Infected + BUDR	5.79	<5.9
4	Infected + interferon	1.08	<5.9
5	Infected + interferon + BUDR	1.75	<5.9

[a] Similar conditions as in Table I; BUDR at 25 μg/ml was added ½ hour prior to virus inoculation and present in medium during entire incubation period (24 hours); virus multiplicity = 5 ID_{50}; chick interferon, 50 units/ml.

TABLE III. Effect of Interferon and Cytosine Arabinoside on Deoxythymidine Kinase Activity of Vaccinia-Infected Chick Embryo Cells.

Group	Treatment[a]	Deoxythymidine kinase	Virus titer $\log_{10}$/ml
1	Uninfected	0.03	—
2	Uninfected + NALF + CA	0.15	—
3	Uninfected + interferon	0.11	—
4	Uninfected + interferon + CA	0.26	—
5	Infected	9.67	7.6
6	Infected + interferon	5.13	<7.1
7	Infected + NALF + CA	15.83	<7.1
8	Infected + interferon + CA	7.77	<7.1

[a] Confluent cell cultures were preincubated 18 hours with chick interferon (33 units/culture). Control cultures received normal allantoic fluid (NALF). Cytosine arabinoside (CA) 250 μg/ml was added 1 hour prior to virus inoculation and was present throughout the incubation period (20 hours).

the inhibitor of vaccinia virus has the same properties as that of the inhibitor of induction of deoxythymidine kinase and coincide with those reported for chicken interferon (8).

Effect of bromodeoxyuridine and cytosine arabinoside on deoxythymidine kinase inhibition by interferon. It has been shown that induction of deoxythymidine kinase synthesis by vaccinia virus does not require DNA synthesis (9). To determine whether interferon could act to inhibit an event which occurs prior to replication of viral DNA, experiments were carried out to determine the effect of interferon on deoxythymidine kinase induction in infected cells treated with inhibitors of DNA synthesis. The results of experiments are presented in Tables II and III. Bromodeoxyuridine in these experiments (Table II) partially repressed deoxythymidine kinase induction, suggesting that the enzyme was saturated with BUDR and thus had a reduced capacity to phosphorylate the deoxythymidine. However, interferon pretreatment resulted in a marked inhibition of the induction of deoxythymidine kinase activity in the presence or absence of the inhibitor BUDR. It may be noted that viral replication was inhibited both by bromodeoxyuridine and interferon.

As summarized in Table III, high concentrations of cytosine arabinoside 250 μg/ml as compared to bromodeoxyuridine had no inhibiting effect on deoxythymidine kinase induction. In fact, it appeared that it had a stimulating effect on the enzyme activity in both uninfected and infected cell cultures. As measured by the incorporation of labeled deoxythymidine into acid insoluble material 6-hours postinfection, treatment with cytosine arabinoside inhibited DNA synthesis by greater than 95%, but had little effect on protein synthesis. As can be seen, interferon inhibited deoxythymidine kinase activity by 50% in the presence of doses of cytosine arabinoside which strongly inhibit viral and cellular DNA synthesis. These findings suggest that the antiviral activity of interferon may be directed against an event which occurs before replication of viral DNA.

Lack of effect of interferon on inhibition of deoxythymidine kinase induction in vaccinia infected mouse embryo. To determine whether the experimental results obtained with vaccinia virus-infected chick embryo occurred in another cell culture system, experiments were performed using mouse embryo cell cultures. Levels of deoxythymidine kinase induced in these cell cultures were approximately 50% lower than those achieved in the vaccinia

TABLE IV. Lack of Inhibition of Deoxythymidine Kinase Activity by Interferon in Vaccinia-Infected Mouse Embryo Cell Cultures.

Group	Treatment	Deoxythymidine kinase		Virus titer $\log_{10}$/ml
		5 hours P. I.	18 P. I.	
1	Uninfected	.05	.06	
2	Infected + normal mouse serum	0.36	1.84	6.6
3	+ interferon no. 38	0.44	1.78	<6.0
4	+ interferon no. 44	0.47	1.80	<6.0
5	+ chick interferon	0.48	1.85	6.6

[a] Similar conditions as in Table I. Two different batches of mouse serum employed: no. 38, titer 50 units/ml; no. 44, titer 63 units/ml.

virus-infected chick embryo cell system. Paradoxically, various attempts to demonstrate the inhibiting effect of mouse interferon on deoxythymidine kinase iduction were unsuccessful. Results of a representative experiment are present in Table IV. Employing different batches of mouse prepared interferon, and two different vaccinia strains, mouse interferon did not inhibit deoxythymidine kinase induction. To confirm that the inhibitor of vaccinia virus in mouse serum was actually interferon it was determined that the inhibitor was stable to pH 2, could not be washed off cells, was inactivated by trypsin, was species specific in its action, and was active against an unrelated virus (vesicular stomatitis virus).

Discussion. Infection of cells with vaccinia virus leads to enhanced incorporation of deoxythymidine into DNA via an increase in the activities of deoxythymidine kinase and DNA polymerase (9, 10). There is strong evidence that deoxythymidine kinase is synthesized *de novo* upon virus infection (11). The studies described here, together with the previous findings show that interferon treatment of chick embryo cells significantly reduced the induction of deoxythymidine kinase associated with infection by vaccinia virus. The present paper further characterized the inhibitor of the enzyme induction in vaccinia-infected cells as interferon by using the following criteria:

(a) Strict species specificity of interferon as shown by the failure of the interferons to inhibit deoxythymidine kinase in heterologous cell culture systems.

(b) Loss of activity of the interferon after treatment with trypsin.

(c) Relative heat stability of the chick interferon to 80°.

(d) Stability at pH 2.

(e) Inability to separate the inhibitor from cells by washing.

Dose response study indicated that the degree of inhibition of enzyme induction was related to the amount of interferon added and was correlated with its antiviral effects.

The data presented on the effect of DNA inhibitors are of interest because they demonstrate that interfron may be directed against an event which occurs before replication of viral DNA. In the presence of both inhibitors, vaccinia virus replication was almost completely inhibited, but not kinase induction. Under these conditions of inhibition of DNA synthesis, interferon was found to significantly inhibit deoxythymidine kinase induction in the chick embryo cell system.

The findings suggest that interferon may act to inhibit vaccinia virus synthesis at a stage preceding replication of viral DNA. Interferon has been reported not to inhibit viral adsorption (8) nor appearance of vaccinia virus messenger RNA (12), then the inhibition of deoxythymidine kinase may occur because a functional polysome-messenger RNA complex needed for its synthesis is not made (13, 14). This interpretation assumes that deoxythymidine kinase induced by vaccinia virus infection is specified by the viral genome since interferon does not inhibit host messenger translation. However, this has not been demonstrated conclusively.

The observed lack of inhibition of mouse interferon on deoxythymidine kinase induction in vaccinia-infected mouse cell cultures was unexpected and is difficult to explain. Kinetic

studies of kinase activity in vaccinia-infected mouse cell cultures indicated that induction occurred earlier in this system as compared to the chick embryo system. It is possible that differences in the kinetics of expression of the viral genome in the two systems studied could account for the differences in the inhibition of deoxythymidine kinase activity. Another possibility is that the mouse antiviral substance which is thought to be induced in interferon treated cells acts somewhat differently from the antiviral substance of chicken cells. Speculatively, the different action could reflect a different spectrum of interferon activity on the various virus messenger RNAs.

Summary. The effect of interferon on the induction of deoxythymidine kinase has been studied in vaccinia-infected chick embryo and mouse cell culture systems. Antecedent treatment of vaccinia-infected chick embryo cells with interferon inhibited deoxythymidine kinase induction. The inhibitor of deoxythymidine kinase was shown to share many properties with interferon. In the presence of concentrations of cytosine arabinoside or bromodeoxyuridine which suppressed vaccinia replication, interferon effectively inhibited deoxythymidine kinase induction, indicating an interferon effect on virus prior to replication of viral DNA. These results are consistent with other studies which indicate that interferon may act to prevent the function of viral mRNA. In contrast, under similar experimental conditions, mouse interferon did not inhibit deoxythymidine kinase induction in the mouse embryo cell culture system although interferon effectively inhibited viral multiplication.

We wish to thank Dr. Hilton Levy for helpful discussion during the course of this work. The excellent technical assistance of Charles E. Buckler and Henry O. Schulze is gratefully acknowledged.

1. Ohno, S. and Nozima, T., Acta Virol. (Prague) **8**, 479 (1964).
2. Gosh, S. N. and Gifford, G. E., Virology **27**, 186 (1965).
3. Eagle, H., Science **130**, 432 (1959).
4. Baron, S. and Buckler, C. E., Science **141**, 1061 (1963).
5. Lampson, G. P., Tytell, A. A., Nemes, M. M., and Hilleman, M. R., Proc. Soc. Exptl. Biol. Med. **118**, 441 (1965).
6. Kit, S., Piekarski, L. J., and Dubbs, D. R., J. Mol. Biol. **6**, 22 (1963).
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1953).
8. Isaacs, A., Advan. Virus Res. **10**, 1 (1963).
9. Hanafusa, T., Biken's J. **4**, 97 (1961).
10. Green, M., Cold Spring Harbor Symp. Quant. Biol **27**, 219 (1962).
11. Kit, S., Dubbs, D. R., and Piekarski, L. J., Biochem. Biophys. Res. Commun. **11**, 176 (1963).
12. Joklik, W. K. and Merigan, T. C., Proc. Natl. Acad. Sci. U. S. **56**, 558 (1966).
13. Marcus, P. I. and Salb, J. M., Virology **30**, 502 (1966).
14. Levy, H. B., Carter, W. A., Buckler, C. E., Snellbaker, R., and Baron, S., Bacteriol. Proc. 119 (1966).

Received July 27, 1967. P.S.E.B.M., 1968, Vol. 127.

Deoxycorticosterone Secretion in Chronic Experimental Heart Failure and during Infusion of Angiotensin II* (32647)

JAMES O. DAVIS, STUART S. HOWARDS, C. I. JOHNSTON,[1] AND FRED S. WRIGHT
(With the surgical assistance of Alfred Casper)
Section on Experimental Cardiovascular Disease, Laboratory of Kidney and Electrolyte Metabolism, National Heart Institute, U. S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, Bethesda, Maryland 20014

Hypersecretion of aldosterone occurs in chronic experimental heart failure (1, 2) and aldosterone, in association with an extra-adrenal sodium-retaining factor (3), leads to marked sodium retention. The quantitative

* Address requests for reprints to Dr. James O. Davis, Department of Physiology, University of Missouri School of Medicine, Columbia, Missouri 65201.
[1] Research Fellow, National Heart Foundation of Australia.