Interferon Induced by Endotoxin and Newcastle Disease Virus in Rabbit Macrophage and Kidney Cell Cultures (37050)

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Published literature indicates that interferon induction by viruses and endotoxin may be substantially different. Postic *et al.* (1) found that cortisol inhibited the production of interferon in rabbits injected with endotoxin, but the drug had no effects on animals injected with Newcastle disease virus (NDV). These findings were confirmed by Coraggio *et al.* (2). In contrast, actinomycin D (Act D) had no effect on the appearance of endotoxin-induced interferon (EII) but did suppress synthesis of NDV-induced interferon (VII) in the rabbit (3). Also, hypothermia enhanced EII production in rabbits but VII was unaffected (4). EII and VII of rabbit origin are also known to differ in their molecular weight (5, 6) and resistance to heat (5, 7) and acid pH (8).

Smith and Wagner (9) reported that the synthesis of interferon in rabbit peritoneal macrophages (RM) and rabbit kidney cells (RK) exposed to NDV was inhibited by the addition of 10 μg/ml Act D prior to or shortly after infection. They also reported that synthesis of an interferon in uninfected rabbit macrophages, likely to have been EII, was also sensitive to the addition of 10 μg/ml Act D or 50 μg/ml of puromycin. This communication reports extensions of their research and attempts to correlate the *in vitro* and *in vivo* data concerning the synthesis of rabbit VII and EII. It is based on work performed by the late Thomas J. Smith at the Walter Reed Army Institute of Research.

Methods and Materials. Rabbit kidney (RK) cells were prepared from minced kidney tissues of 3 kg rabbits, as described earlier (9). Rabbit macrophages (RM) were obtained by inducing sterile peritonitis in 3–4 kg rabbits with 50 ml shellfish glycogen (1.0 mg/ml) (Mann Research Laboratories, New York, NY), harvesting 72 hr later. The harvested cells were about 90% macrophages. RM cultures were usually seeded with 2–8 × 10^7 macrophages in 5 ml RM growth medium. The details of the procedure were previously reported (9).

Newcastle disease virus (NDV, strain CG) was serially propagated in the allantoic cavity of 10 day old embryonated chicken eggs. After 2 days at 37°, allantoic fluid was harvested, clarified at 2000 rpm for 10 min at 4°, ampuled, and frozen at —70°. This material averaged $10^{7.5}$ to $10^{9.0}$ embryonated egg LD_{50} ($EELD_{50}$)/0.2 ml. Vesicular stomatitis virus (VSV, strain Indiana CF-7) stocks were prepared in 32 oz prescription bottle cultures of L cells, harvested at 22 hr postinfection (pi). Fluids were clarified at 1500 rpm for 10 min at 4°, ampuled, and frozen at —70°. This material contained about 5 × 10^5 PFU/0.2 ml, assayed on L cells, and about 1.5 × 10^5 PFU/0.2 ml, assayed on RK cells.

Endotoxin-induced interferon (EII) was produced by adding lipopolysaccharide extracted from *Escherichia coli* strains 0127:B8 and 026:B6 (Difco Laboratories, Detroit, MI) to a final concentration of 100 μg/ml at the time the macrophages were seeded. Culture fluids were harvested at various times, depending on the experiment. Stocks of EII were prepared by harvesting fluids from endotoxin-treated RM cultures after 24 hr incubation at 25°. The fluids were clarified by

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[1] This manuscript was compiled by ASL and JAA from data obtained by the late Thomas J. Smith. Address reprint requests to Dr. Anthony S. Lubiniecki, at the above Pittsburgh address.

TABLE I. Effect of Temperature on Production of Interferon in Rabbit Macrophage and Rabbit Kidney Cultures Infected with NDV.[a]

Temp (°)	IF titer (PR$_{50}$/2 ml) in RM cultures	IF titer (PR$_{50}$/2 ml) in RK cultures
25	1280	640
30	1280	1280
33	1280	1280
37	1280	640

[a] RM and RK cultures were infected with NDV at an input multiplicity of 100 EELD$_{50}$. After 1 hr adsorption at 37°, cultures were placed at the indicated temperature for 24 hr. Fluids were harvested and titered in RK flasks by VSV plaque reduction as described in Materials and Methods.

low speed centrifugation, ampuled, and stored at —60°.

NDV-induced interferon (VII) was prepared similarly in either RK or RM cultures by infecting with 5–10 EELD$_{50}$/cell. After 24 hr at 37°, fluids were harvested, clarified by low speed centrifugation, and extensively dialyzed for several days at pH 2.0 to inactivate the virus. The VII-containing fluids were then dialyzed against pH 8.0 Hanks' balanced salt solution (HBSS) to return the pH to physiological levels. The interferon was then ampuled and stored at —70°.

Interferons were assayed on RK monolayers using VSV as a challenge virus, as described earlier (9). One unit of interferon is the reciprocal of the highest dilution which inhibited the development of at least 50% of the VSV plaques at 48 hr pi (PR$_{50}$). Two-fold dilutions were employed for all interferon titrations.

Cortisone acetate (Cortone) and actinomycin D (Act D) were both obtained from Merck, Sharpe, and Dohme, West Point, PA.

Results. Cultures of RM and RK cells were infected with NDV and incubated for 24 hr at various temperatures after the adsorption period. Table I shows that VII production was identical at 25, 30, 33, and 37° over the 24 hr experimental period in both cell types. The experiment was repeated several times with similar results. Differences in VII production between RM and RK cultures or among various temperatures were random and seldom greater than 2-fold, which is nonsignificant in the interferon assay employed for this study.

The ability of endotoxin to induce interferon was also examined in RM and RK cultures. After 1 hr at 37°, endotoxin-treated and mock-treated cultures were incubated at 25 or 37°. At 4, 8, and 24 hr, samples were taken and assayed for interferon. The results of this experiment (Table II) indicate that (a) endotoxin did not stimulate interferon production in RK cells, (b) production of EII in RM cells at 37° was virtually complete by 8 hr, whereas at 25°, much of the EII was produced after 8 hr, and (c) more EII was produced at 25° in RM cultures than 37°. The enhancement of total EII yield at 25°, although small and of marginal significance in any one experiment, was seen in all of eight separate experiments and must be considered real.

Table II also shows that small amounts of interferon-like activity were also present in RM cultures receiving no endotoxin. In 8 experiments, the titer of the "spontaneous

TABLE II. Effect of Temperature on Interferon Production in Rabbit Macrophage and Rabbit Kidney Cultures Exposed to Endotoxin or Medium.[a]

Endotoxin (μg/ml)	Temp (°)	IF titer (PR$_{50}$/2 ml) in					
		RM cultures at			RK cultures at		
		(hr): 4	8	24	4	8	24
0	25	<10	20	40	<10	<10	<10
0	37	20	10	10	<10	<10	<10
200	25	80	80	320	<10	<10	<10
200	37	80	160	10	<10	<10	<10

[a] RM and RK cultures were exposed to 200 μg/ml endotoxin or plain medium for 1 hr at 37°. Cultures were washed, refed, and placed at the indicated temperature. At times indicated, fluids were harvested and titrated as before. No interferon was detectable at 1 hr after the addition of endotoxin in any culture.

TABLE III. Effect of Act D and Cortisone on Interferon Production in Rabbit Macrophage Cultures Infected with NDV.[a]

Act D (μg/ml)	Cortisone (μg/ml)	IF titer (PR_{50}/2 ml)
0	0	160
0.5	0	80
1.0	0	<10
2.5	0	<10
5.0	0	<10
10.0	0	<10
0	25	160
0	50	80
0	100	160
0	200	160

[a] RM cultures were infected with NDV in the presence of the drugs. After 1 hr at 37°, the cultures were washed with HBSS and refed with medium. Cortisone-treated cultures were again treated with cortisone at the same concentration present during virus adsorption. After 8 hr at 37°, fluids were harvested and titrated as before.

interferon" from untreated macrophages ranged from 4- to 16-fold lower than that of EII from endotoxin-treated RM cultures, averaging 8-fold lower. "Spontaneous interferon" has been previously reported in this system (9), and remains of unknown origin. It is felt that the low titer of this spontaneous material does not seriously effect the interpretation of experiments concerning EII production.

Table III shows that VII production in NDV-infected RM cultures was insensitive to cortisone up to a concentration of 200 μg/ml. In contrast, VII production was eliminated by as little as 1.0 μg/ml Act D. When these experiments were repeated using endotoxin as the interferon inducer (Table IV), an opposite pattern was observed. As little as 25 μg/ml of cortisone completely abolished EII production while Act D did not completely stop EII production until a concentration of 5.0 μg/ml was employed. A concentration of 1.0 μg/ml had no effect on EII production; lower concentrations of Act D may have actually increased EII production slightly. This slight accentuation at very low Act D concentration was observed in each of three separate experiments in endotoxin-treated RM cultures. While this fourfold difference would not normally be considered important, the repeatability of the actinomycin-mediated enhancement argues in favor of its significance. These experiments indicated the existence of fundamental differences in the synthesis, control, or release of VII and EII in rabbit macrophage cultures.

To examine the possibility of differences in the action of EII and VII, RK cultures were treated with EII or VII and infected with VSV. The effect of temperature and Act D on the ability of interferon to suppress VSV replication was tested. Table V shows that pretreatment with either VII or EII was effective in reducing VSV yields at 30, 33, and 37°; however, interferon treatment after infection was of little value in suppressing VSV replication. Table VI shows that the antiviral state induced by EII and VII was completely inhibited by concomitant treatment with 1.0 μg/ml Act D. However, 0.1 μg/ml of Act D prevented the development of EII antiviral activity, but possibly permitted partial antiviral activity (5-fold reduction of VSV titer) in the VII-treated cultures. These two experiments failed to detect major differences between the modes of action of EII and VII, although minor one(s) may well exist.

TABLE IV. Effect of Act D and Cortisone of Interferon Production in Rabbit Macrophage Culture Exposed to Endotoxin.[a]

Act D (μg/ml)	Cortisone (μg/ml)	IF titer (PR_{50}/2 ml)
0	0	80
0.1	0	320
0.5	0	160
1.0	0	80
2.5	0	10
5.0	0	<10
0	25	<10
0	50	<10
0	100	<10
0	200	<10

[a] RM cultures were exposed to 200 μg/ml endotoxin and drugs for 1 hr at 37°. Cultures were washed with HBSS, refed with medium, and supplemented with the same concentration of cortisone as present during adsorption. After 24 hr at 25°, culture fluids were harvested and assayed for interferon.

TABLE V. Effect of Temperature on the Ability of EII and VII to Reduce VSV Replication in Rabbit Kidney Culture.[a]

Time of IF treatment (hr pi)	Temp (°)	VSV yield (PFU/0.5 ml) in cultures treated with EII	VII	no IF
−4	30	2.3×10^3	2.0×10^3	1.8×10^4
−4	33	2.4×10^3	2.6×10^3	2.4×10^5
−4	37	7.8×10^2	6.3×10^2	3.4×10^5
+1	37	1.5×10^5	1.6×10^5	3.6×10^5

[a] RK cultures were treated with 100 PR_{50} of VII or EII or medium for 1 or 4 hr at the temperatures indicated above. They were infected with VSV at an input multiplicity of 0.1 PFU/cell at 0 hr. After 8 hr at the temperature indicated, fluids were harvested and titrated in RK cultures.

Discussion. The results presented here have demonstrated four major differences in the induction process of interferons induced by endotoxin and NDV in rabbit macrophage and rabbit kidney cells. First, EII was only produced by RM cultures, while VII was inducible in both RM and RK cultures. This is in agreement with the results of Smith and Wagner (9) and a host of other workers.

Second, RM cultures produced more EII at 25° than at 37°, but produced it earlier at 37° than at 25°. In contrast, VII was produced in both RM and RK cultures equally well at 25, 30, 33, and 37°. This finding parallels the results of Postic *et al.* (4), who found that the optimum temperatures for EII production in rabbits was lower than that of VII. Also, EII production in mouse peritoneal cell cultures shows the same optimum at cooler temperatures (25–26°) (10), and slower rates of production at cooler temperatures (11). Also, Ho, Ke and Armstrong (12), found that tissue slices from the thymus, spleen, lungs, kidneys, and omentum of endotoxin-treated rabbits produced more interferon at 23 than 37°.

Third, induction of EII in rabbit macrophage cultures was completely inhibited by cortisone, while NDV-induced VII production was unaffected by the drug. This observation also correlates well with the *in vivo* rabbit data reported by Postic *et al.* (1). Fourth, EII production was found to be more resistant to Act D than VII, much as was found with intact rabbit (3). Initially, Smith and Wagner (9) reported that Act D completely suppressed production of both EII and VII, but 10.0 μg/ml was the only dose level examined. Tables III and IV clearly show that VII production was abolished completely by 1.0 μg/ml Act D, while EII was unaffected.

Low levels of Act D (0.1 and 0.5 μg/ml) seemed to accentuate the production of EII in RM cultures as previously shown in spleen and lung tissue slices of endotoxin-treated rabbits (12). Since cycloheximide is known to enhance EII production in mouse peritoneal leukocyte cultures (11), both classes of drugs (antitranscriptional and antitranslational) are capable of accentuating interferon yields, presumably by blocking a regulatory mechanism which normally terminates interferon synthesis after a certain time (13, 14). The present work and that of Lackovic, Borecky and Youngner (11) indicate that induction (and hyperinduction) of EII in peritoneal cells may possess a control mechanism similar to those regulating interferons induced by other

TABLE VI. Effect of Act D on the Ability of EII and VII to Reduce VSV Replication in Rabbit Kidney Cells at 37°.[a]

Act D (μ/ml)	at hr	IF	at hr	VSV yield (PFU/0.5 ml)
—	—	—	—	1.6×10^5
—	—	EII	−2	8.3×10^3
—	—	VII	−2	4.2×10^3
0.1	−2	—	—	3.3×10^5
0.1	−4	EII	−2	3.5×10^5
0.1	−4	VII	−2	6.4×10^4
1.0	−2	—	—	2.2×10^5
1.0	−2	EII	−2	1.7×10^5
1.0	−2	VII	−2	1.5×10^5

[a] Cultures were treated with Act D and/or 100 PR_{50} interferon at time indicated. All cultures were infected at 0 hr, harvested at 8 hr, and titrated in RK cultures, as in Table V.

substances in other tissues and cell cultures. Ho, Ke and Armstrong (12) also postulated that NDV, poly I:poly C, and endotoxin-induced interferons all possess a similar type of control mechanism in rabbit tissue slices. As recently discussed by Lackovic *et al.* (10), this control mechanism may also account for the increased production of EII at 25–26°, compared with 37°.

The inhibitory effect of cortisone on the appearance of EII in rabbit serum (1) and RM culture fluids (and its complete lack of effect on VII in both systems) is quite puzzling. Preliminary investigation has shown that the cortisone must be present continuously to suppress EII production. Removal of the drug after 1 hr at 37° results in a partial (25–50%) EII response. Preliminary investigation has also shown that cortisone at a concentration of 1000 μg/ml (40 times the concentration known to inhibit EII production) had no effect on the 8 hr yield of VSV from RM cultures. This rules out any gross toxic effects on the macrophages, as well as major disruption of the proteosynthetic processes of these cells. Postic *et al.* (1) found that rabbits treated with endotoxin alone or endotoxin and cortisone (resulting in a failure of EII to appear in the serum) were hyporeactive or tolerant to endotoxin on the following day, just as if the EII had been produced in cortisone-treated animals in response to the first injection of endotoxin. In view of these findings, and the known ability of corticosteroids to alter membrane permeability (15, 16) and stabilize lysosomes (17), it could be hypothesized that EII is synthesized normally in the presence of cortisone, but may not be released. It is, of course, also possible that EII and VII are synthesized in different cell populations in rabbit macrophage cultures.

The development of the antiviral state appeared to be quite similar in RK cultures treated with EII or VII, except that EII was slightly more sensitive than VII to 0.1 μg/ml Act D. Their antiviral effects were similarly diminished by decreasing temperature and higher levels (1.0 μg/ml) of Act D. Basically, EII and VII seem to confer antiviral resistance upon recipient cells through a similar mechanism, in spite of the differences in their modes of synthesis and physical properties.

Summary. Interferon induction by endotoxin and NDV in rabbit macrophages showed several differences. The former was inhibited by cortisone, and also by Act D at a concentration of 5.0 μg/ml. At lower concentrations, no effect or slight accentuation of EII production occurred. In contrast, 1.0 μg/ml Act D completely inhibited VII synthesis in rabbit macrophages, while cortisone had no effect. Also, production of EII was greater at 25 than 37°, but VII was synthesized equally well at all temperatures from 25 to 37°. These *in vitro* findings correlate well with published findings on the effects of cortisone, temperature, and Act D on EII and VII production in rabbits.

EII and VII were active to similar extents in preventing the replication of VSV in RK cells at 30, 33, and 37°. The action of EII and VII was sensitive to inhibition by Act D at 1.0 μg/ml. EII was perhaps somewhat more sensitive than VII to 0.1 μg/ml Act D, but only a factor of 5. This difference may be significant, but generally no major differences were observed between antiviral states induced by EII and VII.

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1. Postic, B., De Angelis, C., Breinig, M. K., and Ho, M., Proc. Soc. Exp. Biol. Med. **125**, 89 (1967).
2. Coraggio, F., Coto, V., Daleota, C. A., and Georgiades, J., Boll. Ist. Sieroter. Milan. **47**, 308 (1968).
3. Ho, M., and Kono, Y., Proc. Nat. Acad. Sci. U.S.A. **53**, 220 (1965).
4. Postic, B., De Angelis, C., Breinig, M. K., and Ho, M., J. Bacteriol. **91**, 1277 (1966).
5. Smith, T. J., and Wagner, R. R., J. Exp. Med. **125**, 579 (1967).
6. Ke, Y., Ho, M., and Merigan, T. C., Nature (London) **211**, 541 (1966).
7. Ho, M., Science **146**, 1472 (1964).
8. Ke, Y., and Ho, M., J. Virol. **1**, 883 (1967).
9. Smith, T. J., and Wagner, R. R., J. Exp. Med. **125**, 559 (1967).
10. Lackovic, V., Borecky, L., Sikl, D., Master, L., and Bauer, S., Acta Virol. **11**, 500 (1967).
11. Lackovic, V., Borecky, L., and Youngner, J. S., Acta Virol. **16**, 217 (1972).
12. Ho, M., Ke, Y., and Armstrong, J. A., presented Int. Congr. Endotoxin, 2nd, Warrenton, VA, June 2, 1972.

13. Vilcek, J., Rossman, T. G., and Vacarelli, F., Nature (London) **222**, 682 (1969).

14. Ho, M., and Ke, Y., Virology **40**, 693 (1970).

15. Kinkelstein, J. D., and Schnacter, D., Amer. J. Physiol. **203**, 873 (1962).

16. Kimberg, D. V., Baerg, R. D., Gershon, E., and Graudusius, R. T., J. Clin. Invest. **50**, 1309 (1971).

17. Janoff, A., Weissman, G., Zweilach, B., and Thomas, L., J. Exp. Med. **116**, 451 (1962).

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