SOME FACTORS AFFECTING THE PRODUCTION OF INTERFERON

D. C. BURKE AND A. ISAACS

From the National Institute for Medical Research, Mill Hill, London, N.W.7

Received for publication April 24, 1958

PREVIOUS studies on viral interference have identified a product of the interference reaction, called interferon, which itself had the property of inducing interference against a number of viruses (Isaacs and Lindenmann, 1957). Interferon was formed by the incubation of inactivated influenza virus with chick chorio-allantoic membranes. In the present paper we have described some factors affecting the production of interferon, including the use of virus inactivated by different methods, different strains of virus and alternative cell systems. The production of interferon during prolonged incubation of live influenza virus with chorio-allantoic membranes has been investigated also.

MATERIALS AND METHODS

These were generally those of a previous paper (Lindenmann, Burke and Isaacs, 1957).

Virus strains

The virus strains used were the Melbourne (MEL) and PR8 strains of influenza A, the LEE strain of influenza B, the Dutch strain of fowl plague, the Herts strain of Newcastle disease and the haemagglutinating virus of Japan or Sendai virus. Incomplete virus was prepared by the technique of von Magnus (1951) in which three passages of undiluted infective allantoic fluid were made in the allantoic cavity. Third passage virus was found to have a ratio of infectivity/haemagglutinin titre of $< 0{\cdot}1$ per cent of standard virus.

Inactivation of virus

Ultra-violet irradiation of virus, partially purified by adsorption on to chick red cells and elution into saline, was carried out as described previously, the dose being $9{\cdot}5 \times 10^5$ ergs per sq. cm. per min. Virus was inactivated at 56° and partially inactivated virus was prepared by heating a chick red cell eluate at 37° for 6–7 days.

General

Pepsin was a crystalline preparation of porcine origin from Armour and Company (lot 12655). Bottles of fully-grown monkey kidney and human amnion cells were kindly provided by our colleague Dr. F. T. Perkins and HeLa cells by our colleague Dr. H. G. Pereira.

RESULTS

Different methods of virus inactivation

Virus inactivated by heating at 56°.—In earlier work it was found that the production of interferon by heat-inactivated virus was largely complete in 24 hr. (Isaacs and Lindenmann, 1957). More recently it has been found that while interferon production is largely restricted to the first 24 hr., with large doses of heat-inactivated virus some interferon is produced in the second 24-hr. period.

Ultra-violet inactivated virus.—Unlike virus inactivated by heating at 56°, interferon production after a single inoculum of ultra-violet inactivated virus was found to continue into the third day. The yield of interferon was not appreciably raised by using three daily doses of inactivated virus instead of one. The effect of the time of ultra-violet irradiation of the virus on the yield of interferon produced over 3 days has been investigated and the results of a representative experiment are shown in Table I. Virus (1000 agglutinating doses per membrane) was irradiated for different times and the amount of interferon released on 3 successive days was measured. None of the virus specimens was infective after irradiation.

TABLE I.—*Effect of Time of Irradiation of Virus on Interferon Yield*

Time of irradiation of virus	Interferon yield as virus produced after challenge					
	First day		Second day		Third day	
	$-\log_2$ HA titre	Per cent control group	$-\log_2$ HA titre	Per cent control group	$-\log_2$ HA titre	Per cent control group
30 sec.	<1	<0·9	<1	<0·9	2·2	4
1 min.	2·6	5·5	2·2	4	2·6	5·5
2 ,,	4·0	14	2·4	5	3·3	9
Buffer	6·8	100	—	—	—	—

* In this and the following tables $-\log_2$ HA titre represents the mean $\log_2$ of the haemagglutination titre end-point of a group of six pieces of chorio-allantoic membrane treated with the materials shown.

It can be seen that irradiation for the shortest period led to the maximal interferon yield; this is in general agreement with earlier results in chorio-allantoic membranes (Lindenmann, Burke and Isaacs, 1957) and on the chick chorion (Burke and Isaacs, 1958), where slight irradiation damage was found to be necessary for maximal interferon yields. By using a single dose of virus irradiated for 30 seconds it has been possible to economise on virus for the production of interferon.

Virus inactivated by heating at 37°.—Tyrrell and Tamm (1955) have shown that the LEE strain of influenza B after heating for some days at 37° was an efficient interfering agent and it was of interest therefore to see if virus inactivated at 37° produced interferon.

A chick red cell eluate of PR8 virus was left at 37° for 7 days after which its infectivity had dropped to less than $10^{4.3}$ ID_{50}/ml. The virus was then used at a concentration of 1600 agglutinating doses per membrane to prepare interferon. Both single and three daily doses of inactivated virus were tested and the yields of interferon over a period of 3 days were measured. While no haemagglutinin was produced during the 3-day period, interferon was produced as shown in Table II.

It has also been found that virus inactivated at 37° with a high residual titre of infective virus ($10^{6.3}$ ID_{50}/ml.) behaved similarly to the less infective material used in the above experiment, although in this case haemagglutinin was produced.

In this and other experiments it was necessary to measure the amount of interferon in the presence of live virus. This was done by making use of the stability of interferon at pH 2: dialysis at pH 2 at 2° overnight destroyed live

TABLE II.—*Production of Interferon by Virus Inactivated at* 37°

Time	Interferon as shown by virus yield after challenge: One dose of inactivated virus, $-\log_2$ HA titre	One dose of inactivated virus, Per cent control group	Three doses of inactivated virus, $-\log_2$ HA titre	Three doses of inactivated virus, Per cent control group
First 24 hr. . .	3·0	9	3·3	11
Second 24 hr. . .	5·0	35	2·0	4·5
Third 24 hr. . .	4·8	30	5·2	40
Buffer . . .	6·5	100	—	—

virus and the interfering ability of irradiated virus, and left interferon available for testing.

Formaldehyde-treated virus.—Formolised virus is known to be a poor interfering agent (Henle, 1950 ; Isaacs and Edney, 1950) and it was of interest to see whether it would produce interferon. Treatment of MEL allantoic fluid with 0·02 per cent formaldehyde at 37° overnight led to loss of infectivity, and after dialysis against Earle's buffer the virus (8000 agglutinating doses per membrane) was incubated with chorio-allantoic membranes for 3 hr. at 37°. After incubation with buffer overnight the membranes were challenged with live MEL virus and the haemagglutinin yield measured after 24 hr. There was no interference established in the membranes and no interferon was produced. This extends the correlation, previously noted with heat and ultra-violet inactivated virus, between the ability of different viruses to induce interference and to produce interferon.

Incomplete virus.—Incomplete virus has previously been shown to produce some interferon on the chick chorion (Burke and Isaacs, 1958) and it was of interest to compare it with inactivated viruses in whole chorio-allantoic membranes. Virus of infectivity $10^{6.6}$ ID_{50}/ml. (4000 haemagglutinating doses per membrane) was used and the production of interferon was measured after dialysis at pH 2 to destroy intact virus. Very little interferon was produced under these conditions.

Production of interferon by live virus

Interferon was not found to be produced by incubation of live influenza virus on the chick chorion during a 24 hr. incubation period (Burke and Isaacs, 1958) but an observation by Dr. D. A. J. Tyrrell of the Common Cold Unit, Salisbury (personal communication) prompted us to re-examine the production of interferon during longer periods of incubation. Dr. Tyrrell found that a cyclical production of influenza virus in tissue cultures of bovine kidney was associated with cyclical production of interferon in these cultures. In confirmation of his findings we have found that interferon is produced *after* the first 24 hr. incubation but not *during* the first 24 hr., when live virus (0·05 ml./membrane haemagglutinin titre 2400) is incubated with chorio-allantoic membranes over a period of 3 days. The fluids were dialysed at pH 2 before testing. The results are shown in Table III.

In many experiments of this kind it has been found that during the early stages of growth of live MEL virus in chorio-allantoic membranes interferon is not produced. The possibility was considered that when interference had been induced in the chorio-allantoic membrane the virus might behave differently in

TABLE III.—*Production of Interferon by Live Virus*

Time	Virus haemagglutinin produced	Interferon as shown by virus yield after challenge: $-\log_2$ HA titre	Interferon as shown by virus yield after challenge: Per cent control group
First 24 hr.	12,800 A.D.	6·6	100
Second 24 hr.	8,000 ,,	2·9	11
Third 24 hr.	160 ,,	4·3	29
Buffer	—	6·0	—

A.D. = Agglutinating doses.

regard to interferon production. This experiment had to be carried out with heated MEL, since even small doses of ultra-violet inactivated virus led to the production of interferon in the second 24-hr. period.

Six whole chorio-allantoic membranes were incubated for 3 hr. at 37° with shaking, with heated MEL (56°), while four whole membranes were incubated with buffer. After washing, the membranes were incubated with buffer overnight and the fluids retained for testing. The membranes which had been treated with heated MEL were again incubated for 3 hr. with (*a*) heated MEL, (*b*) MEL allantoic fluid diluted in buffer and (*c*) buffer, while the buffer-treated membranes received inocula (*a*) and (*b*). After washing, the membranes were incubated with buffer overnight and the fluids removed and dialysed against buffer and tested for the presence of interferon, with the results shown in Table IV.

TABLE IV.—*Production of Interferon After Challenge of Membranes in which Interference has been Induced*

Group No.	First inoculation	Interferon as shown by virus yield after challenge: $-\log_2$ HA titre	Interferon as shown by virus yield after challenge: Per cent control yield	Second inoculation	Interferon as shown by virus yield after challenge: $-\log_2$ HA titre	Interferon as shown by virus yield after challenge: Per cent control yield
1	Heated MEL	0·9	2·5	Buffer	4·8	35
2	,, ,,	1·0	2·5	Heated MEL	2·1	5·5
3	,, ,,	0·3	1	MEL	2·8	9
4	Buffer	6·0	82	,,	5·2	47
5	,,	6·3	100	Heated MEL	3·1	11

Two membranes per bottle were incubated with 3800 agglutinating doses of heated MEL in 6 ml. buffer, or with buffer, and interferon was released into 6 ml. buffer. For the second inoculation 2900 agglutinating doses of heated MEL, 380 agglutinating doses of live virus in buffer or buffer alone were used.

The results with group 1 in Table IV show that interferon production was essentially complete in the first 24 hr., while from groups 2 and 5 it can be seen that heated MEL led to the production of interferon in membranes in which interference had been established and also in untreated membranes. The results with group 3 show that live virus, which was unable to induce the production of interferon in the time used (group 4) led to the production of a second crop of interferon. Live and heated MEL were then almost equally effective in inducing

the production of interferon in membranes in which interference had already been induced.

Use of inactivated fowl plague, Newcastle disease and influenza B viruses

It was previously found that interferon can be prepared from both the MEL and PR8 strains of influenza A (Lindenmann, Burke and Isaacs, 1957) and several other viruses have now been examined for their ability to produce interferon. The viruses were purified by chick red cell adsorption-elution (LEE strain of influenza B) or by high speed centrifugation (Newcastle disease virus and fowl plague), inactivated by ultra-violet irradiation and used to prepare interferon in the normal way. All three viruses gave interferon ; the interferon from Newcastle disease virus and from fowl plague like that from the MEL strain of influenza was stable at pH 2 but was destroyed by incubation with 0·001 per cent pepsin at pH 2 at 37° for 1 hr. The test used to detect the presence of interferon shows only the presence of material which will interfere with influenza virus, and we have not yet been able to determine by serological or other tests whether the interferons prepared from these different viruses are identical.

Production of interferon in different cell systems

Attempts were made to prepare interferon from inactivated influenza virus cultured in other cell systems. Fully grown cells were first washed, then treated with ultra-violet inactivated virus at a high multiplicity in medium 199 for 3 hr. at 37°. After washing, the cultures were incubated overnight at 37° in fresh medium without shaking. The yields of interferon are shown in Table V.

TABLE V.—*Production of Interferon from Different Cell Systems*

Experiment No.	Cell system	Challenge virus	Interferon as shown by virus yield after challenge: $-\log_2$ HA titre	Interferon as shown by virus yield after challenge: Per cent control group
1	Monkey kidney	Sendai	0·9	13
	Buffer	,,	3·8	100
2	Human amnion	MEL	6·9	70
	HeLa cells	,,	6·1	40
	Buffer	,,	7·4	100

The interference shown by the HeLa cell interferon is barely significant while the monkey kidney gave a low yield of interferon. A second experiment with monkey kidney cells and human amnion cells gave the same result. Dr. D. A. J. Tyrrell has also found that interferon is produced in cultures of bovine kidney cells (personal communication). We have previously found that both the chorionic and the allantoic surfaces of the chorio-allantoic membrane, which differ in their ability to synthesise live influenza virus nevertheless produce interferon equally well (Lindenmann *et al.*, 1957).

Miscellaneous

Dr. R. M. Franklin (personal communication) working with fowl plague virus grown in chick fibroblasts has shown that proflavine inhibits the production of

infectious virus and haemagglutinin at much lower doses than those which inhibit production of S-antigen, a nucleoprotein, and this effect may be due to a blockage of cytoplasmic protein synthesis by proflavine. At Dr. Franklin's suggestion we investigated the effect of proflavine on the production and action of interferon in chorio-allantoic membranes on the hypothesis that the production or the action of interferon might depend on cytoplasmic protein synthesis. However there was no inhibition of interferon production when this was carried out in the presence of 5·3 μg. proflavine/ml. and no inhibition of interferon action when tested in the presence of 8 μg. proflavine/ml. Higher concentrations than this could not be tested because of their toxicity under these conditions, although in Dr. Franklin's system there was significant inhibition of infectious virus and haemagglutinin production at these concentrations. Dr. Franklin also suggested that interferon might be produced from inactivated virus which remained on the cell surface. To test this possibility, viral antiserum was added to chorio-allantoic membranes one and 2 hr. after the addition of inactivated virus. There was no effect on interferon production, suggesting that it is produced within the cells of the membrane.

DISCUSSION

Interferon has been found to be produced by a number of myxoviruses in several cell systems and it has already been shown to be active against a number of different viruses, including both DNA and RNA viruses (Isaacs, Burke and Fadeeva, 1958). It has yet to be shown that the preparations of interferon produced by different viruses and in different cell systems are identical, but the results to date suggest that interferon is involved in some fundamental part of the viral multiplication cycle common to different viruses and host cells.

With all these viruses a general correspondence has been noted between the degree of interference induced and the amount of interferon produced. This gives more weight to the idea that interference in this system is mediated through interferon. In cultures infected with live virus, no interferon was detected during the early stages of virus growth when maximal virus yield was occurring. After 24 hr. it is likely that incomplete virus and virus inactivated at 37° may accumulate in the culture and be responsible for the production of interferon. It is possible that this interferon may then play a role in the spontaneous termination of virus production which occurs in this and other systems, although it is not known whether interferon and virus are being simultaneously produced in the same cells or in different cells during the later stages of infection.

One hypothesis which could explain the results of the experiment shown in Table IV is that following incubation with heated virus the synthetic ability of the cells is directed towards the synthesis of interferon rather than that of virus or virus intermediates ; as a result, superinfection of these membranes with live virus leads to further synthesis of interferon. The possibility that this is part of the mechanism of interference is being further investigated.

The use of a single dose of lightly irradiated virus for the production of interferon over prolonged periods of time is of practical importance and simplifies its production in large amounts. The fact that production of interferon after a single dose of ultra-violet inactivated virus continues for 3 days is a further argument in favour of the hypothesis that interferon is synthesised in the membrane following cell-virus interaction.

SUMMARY

The production if interferon by influenza virus treated in different ways was investigated. In general there was a correspondence between the interfering activity of a preparation and its ability to produce interferon. Following a single inoculum of ultra-violet irradiated virus, chick chorio-allantoic membranes produced good yields of interferon over a period of 3 days.

Live influenza virus did not produce interferon in the first 24 hr. incubation, *i.e.* during the period of peak virus multiplication. After the first 24 hr., when virus production was falling off interferon accumulated in the cultures. However live virus produced interferon within the first 24 hr. after incubation with membranes previously treated with heated influenza virus.

Interferon was produced by incubating chick chorio-allantoic membranes with irradiated influenza B, fowl plague and Newcastle disease viruses, and in small amounts, by irradiated influenza A virus incubated with monkey kidney and HeLa cells.

We should like to thank Miss V. Coker for her able technical assistance.

REFERENCES

BURKE, D. C. AND ISAACS, A.—(1958) *Brit. J. exp. Path.*, **39**, 78.
HENLE, W.—(1950) *J. Immunol.*, **64**, 203.
ISAACS, A. AND EDNEY, M.—(1950) *Aust. J. exp. Biol. med. Sci.*, **28**, 219.
Idem AND LINDENMANN, J.—(1957) *Proc. Roy. Soc.* (B), **147**, 258.
Idem, BURKE, D. C. AND FADEEVA, L.—(1958) *Brit. J. exp. Path.*, **39**, 447.
LINDENMANN, J., BURKE, D. C. AND ISAACS, A.—(1957) *Ibid.*, **38**, 551.
VON MAGNUS, P.—(1951) *Acta path. microbiol. scand.*, **28**, 278.
TYRRELL, D. A. J. AND TAMM, I.—(1955) *J. Immunol.*, **75**, 43.