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Interferon induction by influenza type C

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SUMMARY

The presence of a heat-stable interferon-like inhibitor in allantoic and amniotic fluids collected from chick embryos infected with type C influenza virus was determined. This inhibitor was characterized as an interferon and the ability of both live and ultra-violet-irradiated influenza type C virus to induce the substance was examined under various conditions.

INTRODUCTION

In a previous communication (Jennings & Freeman, 1972), the production of infectious virus and virus haemagglutinin by influenza type C/1233 in the chick embryo allantoic cavity was examined and the possibility of using this system as a means for investigating some of the biological properties of influenza C discussed.

A property of many myxoviruses and paramyxoviruses is the ability to induce the formation of interferon, a substance able to inhibit the growth of most viruses, probably by indirectly disrupting the synthesis of viral proteins (Marcus & Salb, 1966) in suitable host-cell systems. Interferon was first detected with a member of the myxovirus group, a type A influenza virus, which had been inactivated by heat-treatment (Isaacs & Lindenmann, 1957) and since then many related viruses, inactivated either by heat or by ultra-violet (UV) irradiation (Burke & Isaacs, 1958*a*; Henle, Henle, Deinhardt & Bergs, 1959; Cantell, 1961), as well as live myxoviruses and paramyxoviruses (Burke & Isaacs, 1958*b*; Youngner, Scott, Hallum & Stinebring, 1966) have been found to induce interferon formation.

The ability of type C influenza virus to induce interferon has not been examined in detail, although Cantell *et al.* (1965) were able to demonstrate the presence of an interferon-like viral inhibitor in pooled allantoic fluids from chick embryos infected with influenza C. The present study confirms the appearance of a heat-stable viral inhibitor following both the intra-allantoic and intra-amniotic inoculation of live influenza C/1233 into embryonated hens' eggs, describes the characterization of the substance as an interferon and determines the effect of UV-irradiation on the ability of the virus to induce interferon.

MATERIALS AND METHODS

Viruses

The 1233 strain of influenza type C (C/1233) was used in the present study after 21 to 30 passages in the chick embryo allantoic cavity at 32° C.

The influenza B/Lee virus used to make a working standard preparation of interferon was kindly supplied by Professor K. Cantell, State Serum Institute, Helsinki, Finland. It was propagated in the allantoic cavity of 11-day-old chick embryos, using an inoculum concentration of 10^3 or 10^4 egg infectious doses (EID 50) at 37° C. for 72 hr.

Semliki Forest virus (SFV), kindly provided by Dr N. B. Finter, I.C.I. Research Laboratories, Alderly Edge, Cheshire, was used as challenge virus in the assays for interferon. It was propagated in chick embryo fibroblast (CEF) tissue cultures.

Infectivity and haemagglutination (HA) titrations

These were performed by methods described elsewhere (Jennings & Freeman, 1972). Assays for infectious virus were carried out by the intra-allantoic route, using virus dilutions prepared in chilled Dulbecco 'A' phosphate-buffered saline (PBS), pH 7·3.

Preparation of standard interferon

A working standard of interferon was prepared in 11-day-old chick embryos using B/Lee influenza virus as described by Cantell *et al.* (1965). The crude interferon was partially purified by the method of Lampson, Tytell, Nemes & Hilleman (1963), omitting the column chromatography and all subsequent steps. This partially purified material contained 1280 units of interferon/ml. when assayed by the method of Fantes (1967).

Virus inactivation checks

All samples of allantoic fluid to be tested for interferon activity were heated at 60° C. for 1 hr. in a water-bath to inactivate the virus. The absence of live virus was determined by 3 serial passages in the allantoic cavity of 8-day-old chick embryos. After incubation at 32° C. for 72 hr. at each passage the embryos were chilled and the allantoic fluids tested for virus haemagglutinin. Several samples were tested similarly, by intra-amniotic inoculation into 10- or 11-day-old embryos and incubation at 35° C. for 48 hr.

Interferon assays

Interferon assays were carried out in chick embryo cells challenged with SFV (Fantes, 1967). The cells used were filtered through gauze after trypsinization and finally suspended in modified minimum essential medium (MEM) (MacPherson & Stoker, 1962) supplemented with 5% calf serum. Samples to be tested for interferon activity were serially diluted in minimum essential medium without calf serum and each dilution mixed with an equal volume of chick embryo cell suspension containing $3{\cdot}0 \times 10^6$ cells/ml. before seeding into tubes. Three tubes were used

for each dilution. After 20–24 hr. at 37° C. cell sheets were challenged with 100 TCD 50 of SFV and the results read after incubation at 37° C. for a further 48 hr. The end-point was taken as the highest dilution of the sample that completely inhibited SFV cytopathic effect. The working standard of interferon was titrated with each assay.

RESULTS

Production of an interferon-like inhibitor in the allantois of chick embryos infected with influenza C

In an attempt to detect the presence of interferon in allantoic fluids from chick embryos infected with influenza C/1233 at different ages and subsequently incubated at different temperatures, groups of ten embryos were inoculated with $10^{3 \cdot 0}$ EID 50 of the virus prepared in PBS. Each embryo was inoculated intra-allantoically with 0·2 ml. of virus dilution and two groups, one of 8-day-old, the other of 10-day-old chick embryos, were incubated at 32° C. and the remaining two groups, similarly 8- and 10-day-old, incubated at 35° C.

After 72 hr. eggs were chilled, the allantoic fluids harvested and equal volumes pooled. Four pools, corresponding to the groups inoculated, were prepared and tested for haemagglutinin, infectivity and, after heating at 60° C. for 1 hr., for interferon. A substance able to inhibit the appearance of SFV cytopathic effect in chick embryo tissue culture was detected in three pools (Table 1) but was not observed in the pool prepared from chick embryos inoculated at 10 days and incubated at 35° C. No such inhibition was produced by heat-treated allantoic fluids from uninfected chick embryos tested at similar dilutions in the same way.

The highest titre of this material was found in fluids from eggs infected at 8 days and incubated subsequently at 32° C., and this pool also contained the greatest amount of virus as determined by both HA and infectivity titrations, and there appeared to be a positive correlation between the yield of C/1233 virus and the titre of the interferon-like inhibitor.

This correlation was examined further as shown in Table 2 which includes the results of three separate experiments. In each experiment groups of 8-day-old chick embryos were inoculated intra-allantoically with C/1233 virus diluted in PBS. Each embryo was inoculated with 0·2 ml. of virus dilution and, after incubation for 72 hr. at either 32° or 35° C., embryos were chilled, individual allantoic

Table 1. *The production of a viral inhibitor in the allantois of chick embryos infected with influenza C*

Embryo age *at inoculation*	Incubation temperature *(°C)*	Haemagglutinin *titre*	Infectivity *(titre/ml.)*	Inhibitor *titre*
8 days	32	1600	$10^{8 \cdot 70}$	80
	35	400	$10^{7 \cdot 35}$	20
10 days	32	400	$10^{7 \cdot 94}$	40
	35	25	$10^{5 \cdot 99}$	<10

All titres expressed as reciprocals of the end-point dilution.

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fluids harvested and tested for virus haemagglutinin. Fluids with relatively high haemagglutinin titres, indicating good virus growth, were pooled in equal volumes and similar pools prepared from fluids with low haemagglutinin titres. Fluids of intermediate haemagglutinin titre were not included in the pools. Samples of each pool were stored at −70° C. and subsequently titrated for virus haemagglutinin, infectivity and viral inhibitor.

The results show that those pools with high haemagglutinin and EID50 titres also gave good yields of the interferon-like inhibitor and in fact this substance was not detected in pools containing relatively small amounts of C/1233 virus. This was observed after incubation at either 32° or 35° C.

Production of an interferon-like inhibitor in the amnion of the chick embryo

To determine if an interferon-like inhibitor could be induced by influenza C/1233 in the amniotic sac of the chick embryo, two groups of 10-day-old embryos were inoculated intra-amniotically with 0·1 ml. amounts of $10^{4·5}$ EID50 of allantoically propagated virus. After incubation for 48 hr. at either 32° or 35° C., the embryos were chilled, the amniotic fluids collected and pooled. Eight-day-old embryos, inoculated intra-allantoically with the same virus dose and incubated at similar temperatures for 72 hr. were set up for comparative purposes, and used to prepare allantoic fluid pools.

Table 2. *Relationship between the growth of influenza C and viral inhibitor in the allantois of the chick embryo*

Incubation temperature	32° C.						35° C.			
Virus dose inoculated (EID50)	$10^{3·0}$		$10^{3·5}$		$10^{4·5}$		$10^{3·0}$		$10^{3·5}$	
No. of embryos inoculated	10		29		37		10		29	
No. of fluids in pool	3	3	5	6	8	8	2	3	5	12
Haemagglutinin titre	3200	10	1200	10	1600	25	2400	10	800	5
Infectivity titre	$10^{8·9}$	$10^{6·7}$	$10^{7·0}$	$10^{6·0}$	$10^{7·1}$	$10^{7·2}$	$10^{7·2}$	$10^{5·7}$	$10^{7·2}$	$10^{5·7}$
Inhibitor titre	20	<10	20	<5	20	<5	80	<10	5	<5

All titres expressed as reciprocals of the end-point dilution.

Table 3. *Production of a viral inhibitor in the chick embryo amniotic cavity*

Site of inoculation	Amnion		Allantois	
Embryo age (days)	10	10	8	8
Incubation time (hr.)	48	48	72	72
Incubation temperature (°C.)	32	35	32	35
Haemagglutinin titre	3200	1600	400	200
Infectivity titre/ml.	$10^{8·9}$	$10^{8·1}$	$10^{8·0}$	$10^{7·3}$
Inhibitor titre	64	16	8	<4

All titres expressed as the reciprocal of the end-point dilution.

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All the pools were tested for infectious virus, virus haemagglutinins and viral inhibitor and the results are shown in Table 3. A substance able to inhibit the appearance of SFV cytopathic effect in chick embryo tissue cultures was detected in both heat-inactivated allantoic and amniotic fluid pools. The titre of this substance was significantly greater in pools prepared from infected amniotic fluids as compared to infected allantoic fluids.

Characterization of the interferon-like inhibitor induced by C/1233

The heat-stable, virus-inhibiting substance present in influenza C-infected allantoic fluids was characterized as an interferon in the following manner. Samples of a pool of allantoic fluid containing the inhibitor, after low-speed centrifugation and heating at 60° C. for 1 hr., were treated in various ways including dialysis against 0·1 M glycine/HCl buffer (pH 2·1) for 24 hr. at 4° C.; reaction to a final concentration of 0·5 mg./ml. of crystalline trypsin for 1 hr. at 37° C.; treatment with specific influenza C/1233 antiserum and centrifugation at 150,000 *g* for 1·5 hr. Only treatment with trypsin, which reduced the titre of the inhibitor to an undetectable level, had any significant effect.

As a further test, a sample of inhibitor-containing allantoic fluid was subjected to ultracentrifugation at 100,000 *g* for 1·5 hr. and the resulting supernatant fluid and deposit (after resuspension in PBS) assayed for infectious virus and interferon. The results are shown in Table 4 and it can be seen that the deposit from the ultracentrifugation, although containing a considerable amount of infectious virus, contained no detectable interferon, all of which was recovered in the supernatant.

In addition, the crude, heated, inhibitor-containing allantoic fluid, although able to protect chick embryo cell monolayers against challenge by 100 TCD 50 of SFV, failed to protect primary calf kidney cell monolayers against a similar dose of the same virus.

Temporal relationship between virus growth and interferon production

The relationship between the production of infectious virus, virus haemagglutinin and interferon with time was examined by inoculating 8-day-old chick embryos intra-allantoically with $10^{3\cdot5}$ EID 50 of virus diluted in chilled PBS. Each embryo was inoculated with 0·2 ml. of virus dilution and incubated at 32° C. for 72 hr. Groups of five embryos were chilled at intervals, the allantoic fluids collected, pooled in equal volumes and stored at −70° C. prior to assay for virus and interferon.

Table 4. *Lack of relationship between viral inhibitor and infectious virus particles*

Material tested	Titre of viral inhibitor	Titre of infectious virus
Original	8	$10^{8\cdot7}$
Supernatant from ultracentrifugation	8	$10^{4\cdot0}$
Resuspended deposit from ultracentrifugation	<2	$10^{6\cdot0}$

Inhibitor titre expressed as the reciprocal of the end-point dilution.

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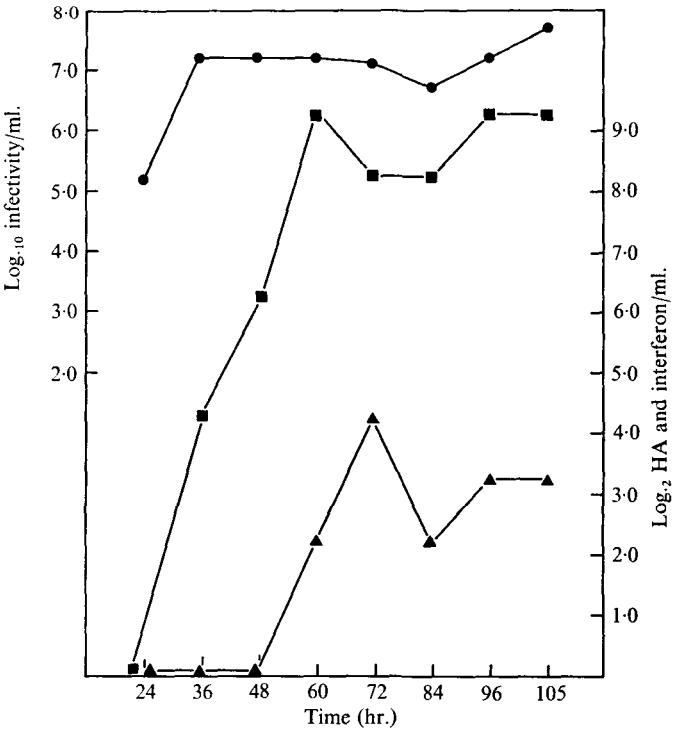

Fig. 1. Relationship between the production of infectious virus, virus haemagglutinin and interferon by C/1233 in the chick embryo allantois. ●—●, Infectious virus. ■—■, Virus haemagglutinin. ▲—▲, Interferon.

The results of these assays can be seen in Fig. 1. Interferon did not become detectable in allantoic fluid pools until 60 hr. after virus infection, and appeared considerably later than both infectious virus and virus haemagglutinin. The maximum amount of interferon, 20 units/ml., was observed at 72 hr. after infection.

Effect of ultra-violet light on the interferon-inducing capacity of influenza C

To determine whether influenza C/1233 virus was able to induce the formation of interferon following UV-irradiation, a pool of C/1233-infected allantoic fluid with an infectivity titre of $10^{8\cdot2}$ EID 50/ml. and containing 1280 haemagglutinating units/ml. was clarified by centrifugation at 2000 rev./min. for 10 min., and then dialysed against PBS, pH 7·3, for 48 hr. at 4° C. Samples of the resulting material were subjected to irradiation with UV-light for varying periods of time up to 2 min., in 5 cm. Petri-dishes 7 cm. away from the light source, a Hanovia 'Chromatolite' lamp with maximum emission at 2537 Å. The approximate thickness of the 3 ml. samples under irradiation was 1·5 mm. and they were gently agitated during exposure.

Assays for infectious virus and virus haemagglutinin were performed at all stages of the experiment and the irradiated samples tested for their ability to induce interferon. Each sample was diluted 1/100 in chilled PBS and inoculated, in 0·2 ml. amounts, intra-allantoically into groups of 8-day-old chick embryos. After incuba-

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tion at 32° C. for 72 hr. the allantoic fluids were collected, pooled and assayed for interferon.

The results are shown in Table 5. No detectable interferon was induced by influenza C/1233 after UV-irradiation under these conditions although the non-irradiated control virus after clarification and dialysis, procedures which resulted in a considerable decrease in the amount of infectious virus and haemagglutinin, was able to do so.

Further experiments to induce interferon with irradiated influenza C, using both higher and lower inoculum concentrations and samples exposed to UV-light for shorter and longer periods, failed to elicit the appearance of interferon in the chick embryo allantoic cavity.

DISCUSSION

The ability of live influenza virus to induce the formation of interferon in cells of the chick embryo chorioallantoic membrane was first demonstrated by Burke & Isaacs (1958*b*), using influenza A/Melbourne. The induction of interferon by a type B influenza virus, B/Lee, in the chick embryo allantois was reported subsequently (Hahnemann & Reinicke, 1965) and since then the appearance of interferon in the allantoic fluid of chick embryos inoculated with live influenza viruses has been observed by many workers (Wagner, 1961; Vaczi, Hadhazy & Horvath, 1963; Cantell *et al.* 1965; Fantes, 1967). Most of these studies were carried out with influenza type A or B viruses, but Cantell *et al.* (1965) did find low levels of an interferon-like inhibitor in allantoic fluids from chick embryos infected with influenza type C/1233. Earlier observations (Hirst, 1950) showed that live C/1233 virus could interfere with the growth of both Newcastle disease virus in the chick embryo allantois and Western equine encephalitis virus in the mouse brain.

The present report demonstrates the appearance of a heat-stable inhibitor, able to prevent the cytopathic effect produced by SFV in CEF tissue cultures, in both allantoic and amniotic fluid pools collected from embryos infected with influenza C/1233 virus. This viral inhibitor, subsequently characterized as an interferon, could be detected after the incubation of infected chick embryos at either 32° or

Table 5. *Effect of UV-irradiation on the interferon-inducing ability of influenza C*

Treatment	Titre of infectious virus/ml.	Haemagglutinin titre	Titre of induced interferon
Centrifugation, 2000 r.p.m. for 10 min.	$10^{6\cdot2}$	50	NT
Dialysis × PBS for 48 hr. at 4° C.	$10^{5\cdot2}$	<10	5
UV-irradiation for 10 sec.	$10^{4\cdot0}$	<10	<2
UV-irradiation for 20 sec.	$10^{3\cdot7}$	<10	<2
UV-irradiation for 40 sec.	$10^{2\cdot5}$	<10	<2
UV-irradiation for 80 sec.	$<10^{1\cdot0}$	<10	<2
UV-irradiation for 120 sec.	$<10^{1\cdot0}$	<10	<2

All titres expressed as the reciprocal of the end-point dilution. NT = Not tested.

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35° C. In both allantoic and amniotic fluid pools, however, the titre of the inhibitor appeared to be highest at the lower temperature, and it is at this incubation temperature, 32° C., at least in the chick embryo allantois, that the greatest yields of influenza type C/1233 are produced (Deichman, 1958; Jennings & Freeman, 1972).

This finding is in apparent contrast to that of other workers (Vaczi *et al.* 1963), who observed the induction of interferon by influenza A/PR8 to be greatest at temperatures higher than the optimum growth temperatures of the virus. However, by preparing pools from chick allantoic fluids containing relatively large amounts of virus it was possible to demonstrate high interferon titres induced by influenza C after incubation at 35° C., whereas, after incubation at 32° C., allantoic fluid pools with low virus titres contained no detectable interferon. Interferon induction by live influenza C virus thus depends, in part, on the amount of virus replication, which is greatest, and affected least by egg to egg variations (Jennings & Freeman, 1972), at 32° C.

The viral inhibitor induced by influenza C/1233 in the chick embryo allantois possesses many properties of an interferon. It is non-sedimentable, non-dialysable and unaffected by acid pH and by heat at 60° C. for 1 hr., but is destroyed by trypsin. It is not active in primary calf kidney tissue cultures and is not identical with infectious virus particles. Interferon induced by influenza C is only detected in chick embryo allantoic fluids at a late stage of virus growth, after the appearance of infectious virus and virus haemagglutinin and this is similar to interferon formation following the infection of chick embryos with live influenza A or B viruses (Wagner, 1961; Vaczi *et al.* 1963; Cantell *et al.* 1965; Hahnemann & Reinicke, 1965).

Some myxoviruses and paramyxoviruses, after inactivation either by heat or by UV-irradiation, are able to induce good yields of interferon in chick chorioallantoic membranes (Burke & Isaacs, 1958*a*, *b*). Type C influenza virus on the other hand failed to induce any detectable interferon after inoculation into the chick embryo allantoic cavity after UV-irradiation. Failure of irradiated influenza C/1233 to induce interferon was observed when the virus infectivity was either partially or completely destroyed by the UV treatment. The reason for the inability of UV-irradiated C/1233 to induce interferon is unknown, but the 'interferon-inducing factor' in the virus may be highly sensitive, and hence rapidly destroyed by UV-light, or else may fail to reach a site in the cell where it can exert its effect.

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