Interferon Production by Myxoviruses in Chick Embryo Cells

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(Accepted 27 August 1969)

SUMMARY

Both fowl plague and Newcastle disease viruses failed to produce interferon in chick-embryo cells because of the inhibitory effect of infection on host-cell protein synthesis. When this inhibitory effect was destroyed by ultraviolet irradiation or by hydroxylamine inactivation, interferon was formed. No virus RNA synthesis could be detected in the interferon-producing cells and it was concluded that the virus single-stranded RNA was the stimulus for interferon formation.

INTRODUCTION

Inducers of interferon formation may be divided into three broad classes: the viruses, the synthetic polynucleotides and a group of substances which includes bacteria, bacterial endotoxin, and a number of synthetic polyanions. Members of the first two groups of substances induce interferon formation both in tissue culture cells and in whole animals, whilst members of the third group are generally effective only in animals. In the last, formation of interferon is not susceptible to inhibitors of RNA and protein synthesis, and has been described as a ‘release’ process (Ho et al. 1966), while interferon formation induced by viruses is sensitive to low doses of inhibitors of RNA and protein synthesis and probably involves a derepression event (Burke, 1966) followed by the normal pathway of protein synthesis. Polynucleotide-induced interferon production is less sensitive than the virus-induced process to inhibitors of protein and RNA synthesis (Finkelstein, Bausek & Merigan, 1968), and it is not yet clear whether the mechanism of these two processes is identical. However, in both cases the inducer is the nucleic acid; double-stranded RNA isolated from a number of viruses (Lampson et al. 1967; Tytell et al. 1967; Field et al. 1967b) was active in inducing interferon formation. In contrast, single-stranded viral RNA was inactive (Lampson et al. 1967), and it was argued that double-strandedness was the essential characteristic of an interferon inducer, although the possibility could not be excluded that the single-stranded RNA molecules were inactive because of their destruction by ribonuclease. Investigation of interferon formation induced by Semliki Forest virus showed that viral RNA synthesis was necessary for interferon formation and it was suggested that, in this case too, the inducer was the double-stranded viral RNA synthesized in the infected cells (Skehel & Burke, 1968b). The double-stranded synthetic polynucleotides (Field et al. 1967a) are highly active as interferon inducers, although there are reports that synthetic single-stranded polynucleotides are also weakly active (S. Baron and co-workers, personal communication, E. D. De Clercq & T. C. Merigan, personal

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communication). Thus interferon formation appears to be a cellular response to polynucleotides, but it is not certain if induction is restricted to double-stranded polynucleotides.

U.v.-irradiated myxoviruses have been known to be good inducers of interferon for some years (Burke & Isaacs, 1958; Ho & Breinig, 1965; Youngner et al. 1966). Unirradiated virus is inactive in chick embryo cells but on irradiation a non-infective virus which induces interferon is formed, and on further irradiation this capacity is lost. This system was investigated in order to determine whether single- or double-stranded RNA was the inducer. The results demonstrate that the interferon inducer is single-stranded RNA, and provide an explanation for the failure of unirradiated virus to produce interferon.

**METHODS**

**Materials.** Actinomycin D was given by Merck, Sharp and Dohme, Ltd, and gliotoxin by Lederle Laboratories, Pearl River, New York, U.S.A. [5-3H]-uridine (30 μCi/mole), [3H]-DL-valine (250 μCi/mole) and [32P]-sodium phosphate were obtained from the Radiochemical Centre, Amersham, Bucks.

**Media, cells and viruses.** These were previously described by Walters, Burke & Skehel (1967) and Long & Burke (1970).

**Interferon production and assay** were previously described by Walters et al. (1967), except that Semliki Forest virus was used to challenge. An internal standard, included in all assays, gave a 50% depression of the control plaque count (PDD50) of 1.84 log10 units with a standard deviation of 0.052 log10 units (eight determinations). The assay was 2.6 x less sensitive than when Chikungunya virus was used to challenge but was considerably quicker.

**Growth and purification of radioactive virus.** Fowl plague virus, labelled with 32P, was prepared as described by Barry & Davies (1968). The virus was purified by differential centrifugation followed by sucrose density gradient centrifugation, as described by Skehel & Burke (1969), and the RNA was extracted by the method of Barry & Davies (1968).

**Incorporation of radioactive precursors.** This was done as previously described (Skehel et al. 1967).

**Extraction and fractionation of virus RNA.** Virus RNA was extracted from infected cells with phenol and sodium dodecyl sulphate, as described by Cartwright & Burke, (1970), and precipitated with two volumes of ethanol at -20°. It was then fractionated by polyacrylamide gel electrophoresis and radioactivity determined, as described by Cartwright & Burke (1970), using 2.5% polyacrylamide gels for fowl plague virus RNA and 2.2% for Newcastle disease virus RNA.

**Virus inactivation.** Virus was inactivated with hydroxylamine, as described by Skehel & Burke (1968a), and with u.v. light, as described by Long & Burke (1970). The lamp emitted 6.1 x 10⁸ ergs cm⁻² sec⁻¹.

**Other assays.** Neuraminidase and complement-fixing antigen titres were measured as described by Long & Burke (1970). The serum used for complement fixation contained no anti-haemagglutinin activity.
RESULTS

Infection of cells with partially inactivated fowl plague virus

When virus which had been inactivated for different times with u.v. light was used to infect chick cells and the yield of various virus-induced products measured, it was observed (Fig. 1) that the capacities to produce these products were lost at different rates. This effect was first observed by Scholtissek & Rott (1964), using ethylene iminoquinone inactivation, and our results are similar to theirs, except that they found the production of complement fixing antigen to be more stable than neuraminidase production. This difference could be due to the different methods used for assaying complement-fixing antigen or to different specificities of the antisera employed. The capacity to

![Graph 1](Fig.1.png)

![Graph 2](Fig.2.png)

![Graph 3](Fig.3.png)

Fig. 1. The effect of u.v.-irradiation of fowl plague virus on the yield of various virus-induced properties in infected cells. The multiplicity of infection was 100 and the virus received 5 hits/15 sec. irradiation as determined by the loss of infectivity. Symbols as in Fig. 3.

Fig. 2. The effect of hydroxylamine inactivation of fowl plague virus on the yield of various virus-induced properties in infected cells. The virus preparation received 4 hits/hr of inactivation. Symbols as in Fig. 3.

Fig. 3. The effect of u.v.-irradiation of Newcastle disease virus on the yield of various virus-induced properties in infected cells. The virus preparations received 5 hits/15 sec. irradiation. Yields of infectivity (□), haemagglutinin (□) and interferon (■) were measured 24 hr after infection, the yield of neuraminidase (□) and complement-fixing antigen in the infected cells (■) 8 hr after infection and "cut-off" (□) was measured by pulsing with [3H]-valine between 7 and 8 hr after infection.
produce infectious virus was lost most rapidly, then that to produce haemagglutinin, followed by complement-fixation antigen synthesis, then by neuraminidase and finally by 'cut-off', which was the most resistant of all. 'Cut-off' refers to the reduction in the rate of protein synthesis which is a consequence of fowl-plague infection (Scholtissek, 1965), and which has been shown (Long & Burke, 1970) to be dependent on the synthesis of a virus-directed protein. It was measured by pulsing with $[^{3}H]$-valine between 7 and 8 hr after infection. Interferon yields at 24 hr after infection were also measured, and it can be seen that no interferon was produced until the capacity to cut-off host-cell protein synthesis had been lost because interferon is a cellular rather than a virus protein. Over-irradiation destroyed the capacity to produce interferon. Hydroxylamine-inactivated fowl plague virus gave a similar result (Fig. 2), the sequence again being infectivity, haemagglutinin, complement-fixing antigen, neuraminidase and finally 'cut-off'. Again no interferon was formed until the capacity to cut-off host-cell protein synthesis had been destroyed, and again prolonged treatment led to loss of the capacity to produce interferon. Since the primary effect of u.v. irradiation and of hydroxylamine is on nucleic acid, it was concluded that viral nucleic acid was the stimulus for the production of viral proteins and also for the production of interferon.

Infection of cells with partially inactivated Newcastle disease virus

When a similar experiment was carried out with partially inactivated Newcastle disease virus, it was found that the capacity to produce infectivity, haemagglutinin, neuraminidase and 'cut-off' were lost at about the same rate (Fig. 3), and that interferon was only formed after the capacity to cut-off protein synthesis had been lost. Since cut-off capacity was destroyed much more rapidly than in the case of fowl plague virus, interferon production was stimulated by much more lightly irradiated virus. However, the capacity to induce interferon formation was destroyed by longer periods of irradiation at about the same rate as in the case of fowl plague virus.

RNA synthesis in virus-infected cells

U.v.-irradiated fowl plague and Newcastle disease viruses induced interferon production even though their capacity to synthesize virion proteins had been lost. It was, however, possible that this irradiated virus was still able to synthesize double-stranded virus RNA which was the inducer of interferon, and the effect of u.v. irradiation on virus-RNA synthesis in infected cells was therefore investigated. Fowl plague virus RNA synthesis was readily detected by polyacrylamide gel electrophoresis of $[^{3}H]$-uridine-labelled RNA extracted from infected cells which had been treated with actinomycin 4 hr after infection. A control experiment with actinomycin-treated uninfected cells, followed by polyacrylamide gel electrophoresis for 30 min. showed that incorporation of $[^{3}H]$-uridine was restricted to 4S RNA and smaller species (Fig. 4). When electrophoresis was carried out for 1 hr this low molecular weight material was electrophoresed out of the gel. Electrophoresis of the RNA extracted from fowl plague virus infected cells for 1 hr showed that at least four, and probably five, components could be distinguished (Fig. 6a). These components were identical to those obtained by extraction of the RNA from the purified virion (Fig. 5). Duesberg (1968a) also resolved five species of viral RNA in infected cells by polyacrylamide gel electrophoresis and showed that they corresponded to the RNA species found in the
virion, and Pons & Hirst (1968) found five species in influenza virus RNA and in the replicative form purified from infected cells. There was very little double-stranded material present since the virus RNA was completely degraded by treatment with a low concentration of ribonuclease (Fig. 6a). Duesberg & Robinson (1967), using similar conditions, found that ribonuclease-resistant RNA comprised about 15\% of the total labelled virus RNA. When fowl plague virus which had been irradiated for 180 sec. was used to infect cells, no viral RNA synthesis could be detected (Fig. 6b).

RNA synthesis in chick embryo cells infected with Newcastle disease virus was studied by measurement of [\textsuperscript{3}H]-uridine incorporation between 6 and 8 hr after infection, after addition of actinomycin (5 \mu g./ml.) 5½ hr after infection (Bratt & Robinson, 1967). Electrophoresis of the extracted RNA in 2.2 \% polyacrylamide gels (Fig. 7) demonstrated the presence of a number of species of viral RNA, including the 57 s virus particle RNA at fraction 10 (Duesberg, 1968b) and a number of other species whose electrophoretic mobility corresponds to sedimentation constants between 18 and 35 s (Bratt & Robinson, 1967). All the species were sensitive to low concentrations of ribonuclease, indicating the absence of double-stranded RNA. When virus which had been irradiated with ultraviolet light for 30 sec. was used for infection, only a very small amount of viral RNA synthesis could be detected, which was undetectable using virus which had been irradiated for longer periods. Since virus preparations which gave maximum interferon yields did not stimulate any detectable viral RNA synthesis in infected cells, it was concluded that viral RNA synthesis was not essential for interferon production in this system.
Miscellaneous

Gliotoxin has been shown to selectively inhibit the synthesis of polio virus and Coxsackie virus RNA in infected cells (Miller, Milstrey & Trown, 1968; Trown, 1968), and if interferon production by u.v.-irradiated fowl plague virus required the synthesis of virus RNA then interferon production might be selectively inhibited by gliotoxin. However, gliotoxin inhibited fowl plague virus multiplication only at doses which also inhibited cellular RNA synthesis, and thus showed no selective effect against this virus.

Fig. 6. Polyacrylamide gel electrophoresis of the RNA extracted from cells infected with (a) unirradiated fowl plague virus (●—●), (b) fowl plague virus irradiated for 180 sec. (●—●). Actinomycin (5 µg./ml.) was added 4 hr after infection and [3H]-uridine (50 µc/culture) was present between 4½ and 6½ hr after infection. The extracted RNA was also treated with 1 µg./ml. of ribonuclease for 10 min. in 2 x SSC before a second phenol extraction and electrophoresis (■—■). [14C]-Ribosomal RNA ( ○—○) was added as a marker. The marker peak positions are arrowed.

Scholtissek & Rott (1969) have shown that the synthesis of virus protein in cells infected with fowl-plague virus is more sensitive to increased temperatures than is cellular protein synthesis. Since the ‘cut-off’ of the host-cell protein synthesis in virus-infected cells is due to a virus directed protein (Long & Burke, 1970), its synthesis might be more temperature-sensitive than that of cellular protein and unirradiated fowl plague virus should produce interferon at these higher temperatures. However, even at 45° some depression of protein synthesis in infected cells was observed and no interferon was produced by unirradiated virus at 45° or at lower temperatures.
DISCUSSION

Neither fowl plague nor Newcastle disease viruses produced interferon in chick embryo cells, confirming the earlier reports of Burke & Isaacs (1958) for influenza and Ho & Breinig (1965) and Youngner et al. (1966) for Newcastle disease virus. When fowl-plague virus was inactivated by u.v.-irradiation or by hydroxylamine, yields of various virus-specified products were lost in a stepwise manner. This phenomenon was first observed by Scholtissek & Rott (1964), and is probably because influenza virus RNA exists as several pieces, the different pieces functioning independently in viral protein synthesis.

Newcastle disease virus, on the other hand, contains a single piece of RNA and all virus-specified functions were inactivated at the same rate. Using either virus, no interferon was produced until no detectable virus products were formed. Since in the case of fowl plague virus the capacity to depress host-cell protein synthesis ('cut-off') was the most resistant property to u.v.-irradiation of those measured, it is likely that the virus cut-off inhibits the formation of the cellular protein, interferon. Unirradiated Newcastle disease virus reduced the rate of the protein synthesis by 40% and produced no interferon, while virus irradiated for 15 sec. reduced the rate by only 15% and produced some interferon. Similarly, fowl plague virus irradiated for 60 sec. reduced the rate by 31% and produced no interferon, while virus irradiated for 120 sec. reduced...
the rate by only 3% and produced some interferon. These results would suggest that interferon production in chick embryo cells is relatively sensitive to reduction in the rate of total protein synthesis.

Interferon production by u.v.-irradiated fowl plague virus reached a maximum with virus that had received 60 hits, and production was still detectable after the virus had received 120 hits. It is highly unlikely that such heavily irradiated virus could code for any virus product, and the synthesis of viral RNA in infected cells was undetectable using either virus preparation. It was concluded that interferon production in chick embryo cells did not depend on prior virus RNA synthesis, and that single-stranded viral RNA was the stimulus. This conclusion appears to conflict with that of Skehel & Burke (1968b) who, using Semliki Forest virus as an interferon inducer, demonstrated a requirement for functional viral RNA, and concluded that viral RNA synthesis was essential for interferon formation. However, they were using a multiplicity of infection of 5 to 10 p.f.u./cell while in the present experiments the multiplicity was about 100. We have found that, at multiplicities above 10 p.f.u./cell, Semliki Forest virus can induce interferon formation without RNA synthesis (B. Lomniczi & D. C. Burke, unpublished), and it appears that, at high multiplicities, the single-stranded RNA of the input virus is a sufficient stimulus for interferon production, while at lower multiplicities there is insufficient input virus and newly synthesized virus RNA is the stimulus.

It is not known why the interferon-producing capacity of the myxovirus falls on long periods of irradiation. Measurements of virus adsorption to cells showed that it was not due to failure of heavily irradiated virus to adsorb. The most likely explanation is that the inducer RNA molecule is itself broken down and further experiments to test this hypothesis are in progress.

We thank the Medical Research Council for grants which supported this work; and Messrs C. Gibb and B. Pirie for technical assistance.

REFERENCES


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(Received 7 July 1969)