The Production of Interferon in Chick Cells Infected with DNA Viruses: A Search for Double-stranded RNA

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Double-stranded RNA has been widely regarded as the inducer of interferon formation, both in virus-infected cells and in cells treated with synthetic polynucleotides (Colby, 1971). It is not difficult to see how infection with RNA viruses may lead to the formation of double-stranded RNA, but how do DNA viruses induce interferon formation? Colby & Duesberg (1969) have isolated a double-stranded RNA from chick cells infected with vaccinia virus, and shown that this double-stranded RNA induced interference against a virus challenge. However, they did not measure interferon production, either by virus infected cells or by the cells treated with double-stranded RNA, and since we found that chick cells infected by vaccinia virus did not produce interferon, there was some doubt about their explanation.

An alternative explanation for interferon production in cells infected with adenoviruses has been advanced by Pusztai et al. (1969), who have suggested that the penton antigen of the virus is responsible for the production of interferon by human adenoviruses in chick cells (Beladi & Pusztai, 1967). In view of these different explanations, we investigated further the relationship between interferon production and double-stranded RNA formation in chick cells infected with vaccinia virus, and looked for double-stranded RNA in chick cells infected with adenovirus.

Chick embryo cells were prepared as previously described (Walters, Burke & Skehel, 1967), and chick kidney cells from 20-day-old chick embryos. HEp-2 cells were cultured in Eagles Minimal Essential Medium with 7% calf serum. Adenovirus type 12 (HUIE strain) and vaccinia virus (EVANS vaccine strain) were grown in HEp-2 cells, the avian adenovirus (GAL) in chick kidney cells, and Semliki Forest virus in chick embryo cells. All were stored at −70°C. Vaccinia virus used for infection of chick cells was partially purified by centrifuging through 36% sucrose, while vaccinia virus used for polymerase assays was more rigorously purified, as described by Joklik (1962), to give a product of specific activity $8 \times 10^8$ p.f.u./mg protein. This value compares well with that obtained by others (see summary in Pirie et al. 1971). Infectivity was measured by plaque assay in chick cells for vaccinia and Semliki Forest virus, and by c.p.e. in HEp-2 cells for adenovirus, infectivity titre being calculated by the formula of Reed & Muench (1938).

Interferon was produced by infection of chick cells with adenovirus type 12 (0.01 to 0.03 TCD 50/cell), GAL virus (0.02 TCD 50/cell), or with vaccinia virus (0.3 to 2 p.f.u./cell). Infected cells were incubated for 3 hr at 37°C, followed by washing and further incubation at 37°C. The fluids were harvested after 44 hr, traces of virus removed by heating at 56°C for 1 hr, and interferon assayed by the dye uptake method (Finter, 1969), or by plaque reduction (Walters et al. 1967) using Semliki Forest virus as challenge. The results are expressed as reference units of chick interferon. Double-stranded RNA was prepared from chick cells infected with vaccinia virus, by adding [3H]-uridine (100 μC/culture to $10^8$ cells) immediately after infection, extracting the nucleic acids 6 hr later, treating with nuclease as described by Colby & Duesberg (1969) and chromatography on a 6% agarose column (1.5 × 70 cm). It was prepared from adenovirus infected cells by a similar procedure except that the nuclei were not removed before extraction of nucleic acid. Vaccinia virus polymerase was assayed...
Fig. 1. The fractionation of double- and single-stranded RNA oligonucleotides from (a) uninfected, (b) vaccinia virus infected, and (c) adenovirus infected chick embryo cells. The double-stranded RNA is the small peak at fraction 5 or 6.

as described by Kates & Beeson (1970) using $2 \times 10^7$ p.f.u. of virus/assay, which had been pre-treated with Triton X-100 and 2-mercaptoethanol, as described by Szilagyi & Pennington (1971). The polymerase assay mixture was incubated for 30 min. at 37°; controls were either incubated at 0° or the reaction was stopped at zero time. Ultraviolet irradiation was done as described by Long & Burke (1970) using a dose of 23.5 ergs/mm.$^2$/sec. Agarose was purchased from Bio-Rad, Micro-Bio Labs., 46 Pembroke Road, London, W. 11. [5-3H]-Uridine (24c/m-mole), [14C]-thymidine (62mc/m-mole) and [5-3H]-uridine-5-triphosphate, ammonium salt (14c/m-mole) were purchased from the Radiochemical Centre, Amersham, Buckinghamshire.

Double-stranded RNA was readily detected in cells infected with vaccinia virus as a peak in the exclusion volume of the column (Fig. 1 b) which was very small in uninfected controls (Fig. 1 a). The material in this peak was isolated by ethanol precipitation and treated with ribonuclease and deoxyribonuclease as described by Colby & Duesberg (1969). In three experiments the material was found to be 68, 97 and 100% resistant to the effect of nucleases. These values are similar to those given by Colby & Duesberg (1969), who showed that this material was virus specific. The material which appeared in the exclusion volume when the nucleic acids from uninfected cells were fractionated was 64% resistant to the effect of nucleases, as was found by Colby & Duesberg (1969).

However, despite the demonstrations of the presence of double-stranded RNA in infected cells, no interferon (titres < 4) was formed over a range of multiplicities of infection (0.3 to 2). Interferon was only formed when chick cells were treated with u.v. irradiated vaccinia virus, when rather low titres (16 to 32) were produced. This material showed the usual characteristics of an interferon: it was stable to pH2, destroyed by treatment with 0.01% crystalline pepsin, not sedimented at 100,000 g and inactive in mouse L cells.

Chick cells, infected with adenovirus, produced much higher titres of interferon (128 to 512). However, when the total nucleic acids were extracted from infected cells and fractionated on agarose columns, no double-stranded RNA could be detected (Fig. 1 c). A similar result was obtained when the isotope was present between 2 and 8 hr after infection and between 12 and 18 hr after infection. It was concluded that double-stranded RNA formation could not be detected in this system.

Since the double-stranded RNA in vaccinia virus infected cells is synthesised by the virus particle DNA-directed RNA polymerase, the effect of u.v. irradiation on the virus particles
The enzyme was investigated to see if there was any correlation with interferon formation. Purified vaccinia virus was irradiated, infectivity and RNA polymerase activity determined and then interferon production measured in chick cells.

The results of two experiments are shown (Fig. 2) while a third, in which the virus sample had a lower polymerase activity, gave similar results. In all three experiments, infectivity was lost after 2 min. u.v. irradiation. Polymerase activity was lost much more slowly than infectivity (cf. a similar situation for NDV; Meager & Burke, 1972), and no interferon was formed until polymerase activity was almost completely destroyed. Interferon titres were low but there was no evidence of any loss of interferon capacity on long irradiation periods as in the case of the RNA viruses (cf. Gandhi & Burke, 1970). It is clear that virus which had no detectable polymerase activity could still induce interferon formation.

Metabolic inhibitors were also used in an attempt to dissociate the processes of virus multiplication and interferon production. Iodo deoxyuridine (IUDR; 1000 μg./ml.) was without effect on interferon formation by the avian adenovirus GAL, despite a profound inhibitory effect on virus multiplication (Table 1). This dose of inhibitor strongly inhibited DNA synthesis (measured by incorporation of [3H]-thymidine) without any effect on RNA synthesis (measured by incorporation of [3H]-uridine). Another inhibitor of DNA synthesis, cytosine arabinoside, also had no effect on interferon production by adenovirus, although it completely inhibits virus multiplication in HEP-2 cells (Table 2). It was concluded that neither virus nor cellular DNA synthesis was necessary for interferon production.

These experiments failed to demonstrate any correlation between interferon formation and double-stranded RNA synthesis in chick cells infected with either vaccinia virus or adenovirus: in vaccinia infected cells double-stranded RNA but not interferon was formed, while in adenovirus infected cells, interferon but not double-stranded RNA was formed. However, u.v.-irradiated, non-infective vaccinia virus did induce interferon formation and in this respect it resembled adenovirus (Pusztai et al. 1969). The vaccinia particle polymerase (Kates & McAuslan, 1967) provided a more sensitive method of examining the relationship between virus-directed RNA synthesis and interferon formation with u.v. irradiated vaccinia virus.
Short Communications

Table 1. The effect of IUDR on interferon production by avian adenovirus GAL

<table>
<thead>
<tr>
<th>Dose of metabolic inhibitor (µg./ml.)</th>
<th>Interferon titre</th>
<th>Virus titre (TCD 50/0.1 ml.)</th>
<th>Effect of inhibitor on RNA synthesis as % of control</th>
<th>Effect of inhibitor on DNA synthesis as % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>256</td>
<td>$10^5$</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>100</td>
<td>256</td>
<td>$10^5$</td>
<td>100</td>
<td>33</td>
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<tr>
<td>1000</td>
<td>512</td>
<td>0</td>
<td>100</td>
<td>1.5</td>
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</tbody>
</table>

* As measured by the rate of incorporation of [3H]-uridine and [14C]-thymidine respectively: the results are corrected for the effects of the inhibitors on the TCA soluble pools.

Table 2. The effect of cytosine arabinoside on interferon production by adenovirus

<table>
<thead>
<tr>
<th>Dose of metabolic inhibitor (µg./ml.)</th>
<th>Pretreatment†</th>
<th>Interferon yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<tr>
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</tr>
<tr>
<td>100</td>
<td>Yes</td>
<td>630</td>
</tr>
</tbody>
</table>

† Pre-treatment was for 3 hr before infection as well as during infection.
Both doses of inhibitor caused a 99 % reduction in virus yield in HEp–2 cells.

It was found that interferon was only formed when the vaccinia particle polymerase activity had been completely inactivated, suggesting that it was very unlikely that formation of virus coded RNA plays an important role in the induction of interferon in this system. It is, of course, not disputed that treatment of cells with double-stranded RNA causes interference and induces interferon formation: but our results suggest that the double-stranded virus RNA observed in vaccinia-infected cells does not play any role in the production of interferon, and that there is no evidence for the participation of double-stranded RNA in interferon production in cells infected with adenovirus.

We conclude that the nature of the inducer in DNA virus induced interferon formation is not yet known. It is clear that virus DNA synthesis is not necessary and the results obtained with vaccinia virus suggest that virus RNA synthesis is unnecessary. However, Pusztai et al. (1969) have shown that the interferon inducing capacity of adenovirus was destroyed by long periods of u.v. irradiation, while Ustacelebi & Williams (1972) have isolated its mutants of adenovirus which do not induce interferon at the non-permissive temperature. These results indicate that some virus function, as yet unidentified, is necessary for interferon production.

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REFERENCES


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