BLUETONGUE VIRUS-INDUCED INTERFERON SYNTHESIS

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ABSTRACT


INTRODUCTION

Viruses from practically every major group have been found to induce interferon synthesis in suitable host or cell systems. The efficiency of induction can, however, vary greatly between different viruses and different strains of the same virus (Sellers, 1964; Lockart, 1965). The basic requirement for the induction of interferon synthesis appears to be the presence of double- or multistranded ribonucleic acid (RNA) (Field, Tytell, Lampson & Hilleman, 1967; Lampson, Tytell, Field, Nemes & Hilleman, 1967). In the case of the double-stranded RNA-containing reovirus, the viral genome itself is an active inducer of interferon (Tytell, Lampson, Field & Hilleman, 1967). In single-stranded RNA viruses the double-stranded replicative form of RNA formed in the cell may be the active principle.

Bluetongue virus contains double-stranded RNA which is very similar to that of reovirus (Verwoerd, 1969). It was therefore of interest to study the interferon inducing characteristics of this virus.

The purpose of this paper is to report the kinetics of interferon synthesis in primary mouse embryo (ME) cells and in mice. Different strains of bluetongue virus differed in their ability to induce interferon. Interferon production in ME cells commences after a 5 hour lag phase and the cells continue to produce interferon for 20 hours. Isolated double-stranded bluetongue virus RNA was found to induce maximum titres of interferon in mice approximately 4 hours earlier than was the case with whole virus.

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Preparation of double-stranded RNA

Owing to the low yields of virus obtained after purification, double-stranded RNA was extracted from virus-containing cells without prior purification of the virus.

A cytoplasmic fraction was prepared from the virus-containing cells according to the method of Bellamy, Shapiro, August & Joklik (1967). Cells were allowed to swell at 4°C for 15 min in RSB buffer (0.01 M NaCl, 0.0015 M MgCl₂, 0.01 M Tris, pH 7.4) and disrupted with 15 strokes of a tight-fitting Dounce homogenizer. Nuclei were spun down at 1000 g for 2 min. RNA was extracted from the cytoplasmic fraction using the hot phenol—SDS method (Scherrer & Darnell, 1962). Residual phenol was removed by two ether extractions and the RNA precipitated overnight with two volumes of cold ethanol at −20°C. Precipitates were dissolved in SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.4) and treated with 5 μg/ml pancreatic ribonuclease (RNase) at 37°C for half an hour. Magnesium chloride was added to a final concentration of 0.02 M and the solution treated with 10 μg/ml deoxyribonuclease (DNase) at 37°C for 15 min.

Double-stranded RNA was further purified by separation on a methylated-albumin-kieselguhr (MAK) column. MAK was prepared according to the method of Mandell & Hershey (1960). A column of 1.5 × 25 cm was packed in 0.1 M NaCl and the RNA eluted with 300 ml of a linear 0.2 M NaCl to 1.2 M NaCl gradient in 0.01 M Tris buffer at pH 7.0. Degraded RNA and nucleotides eluted with the buffer front

Received for publication on 16 July 1969. — Editor
at 0.2 M NaCl and double-stranded RNA as a heterogenous peak at about 0.69 M NaCl. Double-stranded RNA was precipitated with two volumes of ethanol at -20°C. Precipitates were dissolved in PBS-buffer and stored at -20°C.

**Assay of interferon-activity**

A plaque reduction method similar to the one developed by Wagner (1961) was used. Two-fold dilutions of interferon were made in serum-free Eagle's medium. L-cell monolayers in 60 mm petri dishes were incubated overnight at 37°C with 1.5 ml of each of the dilutions. The cells were subsequently washed with serum-free Eagle's medium and challenged with approximately 60 PFU of echovirus. A 1.0 per cent suspension of agarose in Earle's saline was used as an overlay. Plaques were counted after 3 days. The reciprocal of the dilutions at which the plaque count was 50 per cent of that of the controls was taken as the interferon titre and was expressed in interferon units (IU). When greater accuracy was required the titre was calculated from the linear relationship between log interferon concentration and percentage plaque inhibition (Gifford, Toy & Lindemann, 1963).

**Production of interferon**

(a) *In vitro* production: Confluent ME cell monolayers of about 5 x 10^6 cells were washed with serum-free Eagle's medium and inoculated with a known multiplicity of virus. After an adsorption period of 1 hour unadsorbed virus was removed and 50 ml serum-free medium was added. After incubation at 37°C for 24 hours, the medium was removed and assayed for interferon. Virus in the supernatant was inactivated by adjusting the pH with HCl to 2.5 for 2 hours.

(b) *In vivo* production: Mice from an inbred colony maintained at Onderstepoort were used. They were injected intravenously with a known amount of virus or isolated viral RNA and blood was collected at the intervals indicated under results. Before being assayed for interferon activity, serum was diluted in a citric acid buffer at pH 2.5, centrifuged at 100,000 g for 2 hours and dialyzed against PBS buffer.

**Concentration of interferon**

Virus in the interferon-containing medium was inactivated by adjusting the pH to 2.5 with HCl. Cell debris was removed by centrifugation at 1,000 g for 30 min and the interferon concentrated by two successive zinc acetate precipitations at pH 6.5 as described by Lampson, Tytell, Nemes & Hilleman (1963). Zinc ions were removed by dialysis against a solution of 0.9 per cent NaCl in diluted HCl, pH 2.5, for 24 hours followed by dialysis against PBS buffer.

**RESULTS**

**Production of interferon in vitro**

L-strain mouse fibroblast cells, Ehrlich ascites carcinoma cells (Verwoerd et al., 1967), primary mouse kidney cells and primary mouse embryo cells were investigated for possible induction of interferon synthesis by bluetongue virus. The L-cells and kidney cells produced no detectable amounts of interferon. The Ehrlich ascites cells produced small amounts of an uncharacterized interferon-like inhibitor and the ME cells produced high titres of interferon under optimum conditions. ME cells were, therefore, used in all *in vitro* experiments.

(a) **Kinetics of interferon production in vitro:** The kinetics of bluetongue virus-induced interferon production in ME cells were studied by infecting ME cell-cultures with virus and determining the interferon concentration in the medium at different intervals.

Ten confluent monolayers of ME cells in Roux flasks were infected with bluetongue virus Type 10A at an input multiplicity of 20 PFU/cell. At intervals from 1 to 28 hours after a 1 hour adsorption period 0.2 ml samples were removed from each flask, pooled and assayed for interferon activity. The results are indicated in Fig. 1.

Three different stages are observed. After a lag phase of 5 hours, when no interferon is produced, interferon synthesis commences and the titre increases linearly for 20 hours. Following this production stage, a stationary phase is reached when no further synthesis takes place.

(b) **Effect of input multiplicity of different bluetongue virus strains on interferon production:** No attempt was made to compare the interferon inducing ability of all existing bluetongue virus strains. A few strains were, however, compared and some notable differences were observed. Confluent monolayers of ME cells were infected with bluetongue virus Types 10A, 8A, 1A and 1V at different input multiplicities for all four strains. Interferon concentrations in the medium were determined after 24 hours. The results are shown in Fig. 2.

An initial input multiplicity of at least one PFU/cell was needed to induce interferon in ME cells in detectable amounts. From 2 PFU/cell the interferon titre increases linearly with increase in input multiplicity. Between 10 and 20 PFU/cell, depending on the bluetongue strain used for induction, this linear relationship is no longer observed and the interferon titre increases only very slowly
with increase in input multiplicity. If a satisfactory yield of interferon is to be obtained cultures should be inoculated with an input multiplicity of at least 15 PFU/cell.

The results further indicate that different serotypes of the same virus can show a considerable variation in their interferon-inducing ability. Blue-tongue virus Type 8A induces significantly higher interferon titres in ME cells than type 10A, whereas Types 1A and 1V appear to induce only relatively low titres. It should be noted that it was difficult to obtain a high titre virus stock solution of Types 1A and 1V in comparison to 8A and 10A which presented no difficulty. The induction of interferon in ME cells may therefore be related to the ability of the inducing virus strain to infect and replicate in the cell.

(c) Passage level of the ME cells: In order to test whether the number of passages for which the ME cells were maintained in vitro had any effect on the cell's ability to produce interferon, ME cells were passaged weekly for 13 weeks. Cultures obtained in this way were designated ME (13) to differentiate them from the primary ME (1) cultures.

ME (13) and ME (1) cultures were infected with an input multiplicity of 20 PFU/cell of bluetongue virus types 8A and 10A. Interferon yields and the virus yield/cell were determined. The results are summarized in Table 1. They indicate that the ME cells that have been maintained in vitro for the indicated number of passages have to a large extent lost their ability to induce interferon. The percentage reduction in interferon titre again depended on the virus serotype used for induction. Induction of interferon in ME (13) cells with Type 10A produced yields of less than 8 per cent of the yield on primary ME (1) cultures, whereas with Type 8A the yield on ME (13) was approximately 25 per cent of that on ME (1).

With Type 10A the lower interferon yield may be related to the lower virus yield per cell. With Type 8A the virus yield per cell is roughly equivalent in the two cultures.

### Table 1. Influence of passaging of ME cells on interferon yield

<table>
<thead>
<tr>
<th>Bluetongue virus strain</th>
<th>Interferon yield (IU/ml)</th>
<th>Virus yield (PFU/cell)</th>
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<tbody>
<tr>
<td></td>
<td>ME(1)</td>
<td>ME(13)</td>
</tr>
<tr>
<td>10A</td>
<td>130</td>
<td>&lt;10</td>
</tr>
<tr>
<td>8A</td>
<td>310</td>
<td>80</td>
</tr>
</tbody>
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Production of interferon in vivo

Mice were injected with $4 \times 10^4$ PFU of partially purified bluetongue virus Type 10V and bled in groups of five at various intervals until 28 hours after infection. Serum was treated and assayed for interferon activity as described under methods. The results summarized in Fig. 3 indicate that interferon synthesis commences 4 hours after infection. The titre reaches a maximum between 8 and 12 hours, declining thereafter until all circulating interferon had disappeared after 24 hours.

As these results were obtained with partially purified virus the experiment was repeated using purified bluetongue virus Type 10A and the results were compared with the kinetics of interferon production using isolated double-stranded RNA from Type 10A virus as an interferon inducer. Mice were injected with 8 µg double-stranded RNA and bled at intervals from 2 to 14 hours after injection. The results obtained with purified virus (Fig. 4) compare favourably with the results indicated in Fig. 3. Isolated double-stranded RNA induces interferon in mice after a lag phase of about 2 hours. Maximum interferon titres are obtained approximately 6 hours after injection of the RNA. This maximum precedes that of virus-induced interferon synthesis by about 4 hours.
Physico-chemical properties of the produced interferon

The following tests were performed to determine the characteristics of the inhibitory factor studied, and to identify it as interferon by means of accepted criteria.

(a) Enzyme sensitivity: 95 per cent of the activity of an interferon solution was lost after treatment with 100 μg trypsin/ml for 5 hours at pH 8. No activity was lost after treatment with RNase and DNase.

(b) Temperature stability: The interferon was stable at 55°C for 1 hour but after incubation at 70°C for 1 hour, 90 per cent of the activity was lost.

(c) pH stability: The interferon was found to be stable at pH 2.5 and room temperature for at least 18 hours.

(d) Molecular weight and size: No molecular weight determination was carried out but gel-filtration through a column of sephadex G 100 indicated a molecular weight lower than 50,000. The molecule was also found to be non-dialysable and non-sedimentable at 10,000 g for 1 hour.

(e) Precipitation with zinc acetate: The procedure used for concentrating the interferon with two successive precipitations with zinc acetate is described under methods. The recovery of interferon activity after the two precipitations was found to be very variable. In the first zinc acetate precipitation, the percentage yield varied from 60 to 90 per cent and in the second precipitation from 30 to 60 per cent. More quantitative precipitation of interferon was obtained at pH 6.5 than at pH 6.0.

FIG. 4.—Kinetics of interferon production in mice induced by purified bluetongue virus Type 10A and by its isolated double-stranded RNA

SUMMARY

Bluettongue virus was found to induce interferon in mouse embryo cells (ME cells) and in mice. Different strains of bluetongue virus differed in their ability to induce interferon. Interferon production in ME cells commences after a 5 hour lag phase and the cells continue to produce interferon for 20 hours. Isolated double-stranded bluetongue virus RNA was found to induce maximum titres of interferon in mice approximately 4 hours earlier than the virus itself.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. D. W. Verwoerd for advice and guidance during the investigation.
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