Depressors of Interferon Synthesis: Further Studies on the Production, Action and Properties of the So-called Enhancer

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(Accepted 3 March 1969)

SUMMARY

Mode of action, production and some properties of a viral growth-enhancing factor (enhancer) which was detected in allantoic fluids of eggs infected with parainfluenza type 1 virus were investigated. The factor markedly depressed interferon synthesis induced by u.v.-irradiated Newcastle disease virus in chick embryo cells, but did not inhibit the action of exogenous interferon. It had no effect on the multiplication of Newcastle disease virus in chick embryo cells. The depression of interferon synthesis by the factor was unlikely, therefore, to be due to an inhibition of the early steps of virus cell interaction. On the basis of its biological properties the factor should be termed 'interferon depressor'. The appearance of the factor in allantoic fluids of eggs infected with parainfluenza type 1 virus could be determined by selectively destroying the interferon also contained in the materials. The activity of the factor reached a peak 24 hr after virus inoculation and remained stationary thereafter. Present data on the chemical nature of the factor are compatible with the view that it is a carbohydrate.

INTRODUCTION

Kato, Okada & Ohta (1965a, b) described the appearance of an enhancer of virus multiplication in the allantoic fluids and chorioallantoic membranes of chick embryos infected with parainfluenza type 1 (HVJ) or with influenza virus (PR8). A further analysis of this phenomenon was undertaken and some parts of this work have been reported in preliminary form (Kato & Ohta, 1965, 1966; Ohta & Kato, 1966; Ohta, 1967). The present communication describes in detail the production, properties and action of the enhancer. The so-called enhancer depresses the synthesis of interferon, but does not inhibit interferon action. It seems more appropriate, therefore, to use the term 'interferon depressor' (ID) rather than 'enhancer'.

METHODS

Viruses. The Nagoya 1/60 strain of parainfluenza type 1 virus (haemagglutinating virus of Japan or Sendai virus) (Kato et al. 1961) and the Miyadera strain of Newcastle disease virus were grown in the allantoic cavity of 11-day-old eggs. Allantoic fluids
were harvested 48 hr after virus inoculation. Sindbis virus was propagated in chick embryo cell cultures.

**Cultures of pieces of chorioallantoic membranes.** Roller tube cultures of pieces of shell with chorioallantoic membranes attached were prepared as previously described (Kato, Okada & Ohta, 1965a). Six tubes with one piece in each tube were used for each experimental group.

**Cultures of chick embryo cells.** Monolayer cultures of chick embryo cells in 60 mm. plastic Petri dishes were prepared from 11-day-old embryos by trypsinization (Kraus & Schäfer, 1963). When used after overnight incubation the cell sheet in a dish contained approx. $4 \times 10^6$ cells. Lavit medium (Kraus & Schäfer, 1963) without serum was used as maintenance medium.

**Infectivity and haemagglutinin titrations.** Infectivity assays in chick embryos, haemagglutinin (HA) titrations (Kato, Okada & Ohta, 1965a) and the plaque assay (Kato & Eggers, 1969a) were made as described previously. Infectivity titres were expressed as EID$_{50}$ (50% embryo infectious dose).

**Virus antiserum.** Virus particles of Sendai were purified from infected allantoic fluids by one cycle of differential centrifugation. Virus suspensions containing 10,240 HA units/ml. were inactivated by u.v. irradiation as described previously (Kato, Okada & Ohta, 1965b). Two ml. of u.v.-inactivated virus were injected into chickens three times at intervals of 3 to 4 days. The first two injections were given intra-muscularly and the last was given intravenously. The chickens were bled 7 to 10 days after the last injection. The sera were inactivated by heating at 56°C for 30 min. The antiserum thus obtained contained 2560 HA inhibition units/ml. against 4 HA units.

**Preparation of interferon depressor.** Allantoic fluids harvested from 11- to 12-day-old chick embryos 20 hr after inoculation with approx. $10^5$ EID$_{50}$ of Sendai were used for preparation of interferon depressor (ID) as described before (Kato, Ohta & Okada, 1966). Pools of the allantoic fluids were centrifuged at 80,000 g for 1 hr to remove haemagglutinin. The supernatant fluids contained some residual infectious virus which was completely inactivated by heating at 60°C for 30 min. before use. This material did not contain any detectable interferon activity. Allantoic fluids from 11- to 12-day-old uninfected embryos were used as controls.

**Assay of interferon depressor.** Interferon depressor was assayed by determining its activity in enhancing the production of HA in cultures of shell-membrane pieces infected with Sendai (Kato, Okada & Ohta, 1965a, b). In order to arrive at a reliable and reproducible assay, several dilutions of an ID sample were tested for enhancement of the mean HA yields ($\log_2$) between ID-treated (V) and control cultures ($V_o$). When $V - V_o$ was plotted against the dilution series ($\log_2$) of ID preparations (Fig. 1) an approximately linear relationship was found in the HA range 1 to 5 ($\log_2$). Furthermore, the curves of the 4 ID preparations with different potency (Fig. 1) ran approximately parallel in this range. At smaller values of $V - V_o$, the curves tailed off. In view of these results, the activity of an ID preparation could be reliably expressed as the reciprocal of the dilution which produced an HA yield fourfold higher than that of the control. This dose was designated the HED $\times$ 4 (fourfold HA-enhancing dose). In the experiments described below, ID activity will be expressed in HED $\times$ 4 values. When even undiluted preparations caused no or less than fourfold enhancement of the HA yield, their HED $\times$ 4 is designated 0.

**Preparation of interferon.** Interferon was produced in monolayer cultures of chick
embryo cells by treatment with u.v.-irradiated Newcastle disease virus (NDV u.v.) (Kato & Eggers, 1969a). It was confirmed that heating at 65° for 1 hr did not inactivate interferon but destroyed residual haemagglutinating or interfering activity of NDV.

Interferon assay by the plaque-inhibition method. The plaque-inhibition assay for interferon was performed with Sindbis virus (Kato & Eggers, 1969a). The interferon titres were expressed as PDD50 values (the dose to give a 50% depression of the plaque count in the control) (Lindenmann & Gifford, 1963).

![Graph](image)

Fig. 1. Enhancement of multiplication of Sendai virus by ID preparations.

Single-cycle growth of Sindbis virus. To obtain single-cycle growth curves of Sindbis virus, embryo cells were inoculated with an input multiplicity of 15 p.f.u./cell. After an adsorption period of 1 hr at 37° the inoculum was removed, the cultures were washed twice with phosphate buffered saline (Dulbecco & Vogt, 1954), 4 ml. of maintenance medium were added, and the cultures were incubated at 37°. At various periods after virus inoculation, the infectivity titre of the medium was determined for two cultures.

RESULTS

Effect of physical and chemical treatments on ID activity

Heat. Samples of an unheated preparation were heated for 30 min. at 60, 70, 80, 90, 100 and 120° respectively. Heating at 120° was performed in an autoclave. An unheated sample was treated with chicken antiserum to Sendai virus at a final dilution of 1/100 to neutralize residual infectious virus. The HED×4 values of these preparations were
determined (Fig. 2). The ID activity was stable at 90° for 30 min., but was decreased by treatment at higher temperatures.

**Dialysis.** Ten ml. samples of an ID preparation were dialysed overnight at 4° against 500 ml. of 0.01 M-phosphate-buffered saline, pH 7.2 (BS). The HED × 4 values of the original and dialysed preparations were determined. The activity of ID was not dialysable.

**Ether.** Two volumes of an ID preparation and one volume of ether (Squibb) were mixed and agitated on a magnetic stirrer at room temperature for 1 hr. The mixture was centrifuged at 3000 rev./min. for 15 min. and the aqueous phase was collected. Excess ether was allowed to evaporate. There was no loss of ID activity.

![Fig. 2. Effect of heat on the activity of ID.](image)

**Enzymes.** Samples of an ID preparation were incubated for 2 hr at 37° with trypsin (2 × crystalline, Nutritional Biochemicals Corp.), α-chymotrypsin (crystalline, Kowa Co.), deoxyribonuclease (DNase; noncrystalline, Sigma Chemical Co.) and ribonuclease (RNase; 5 × crystalline, Nutritional Biochemicals Corp.) each at a final concentration of 0.01%. At the end of the incubation period all the treated preparations were heated at 90° for 30 min. For control, samples of BS were mixed with the same concentration of each enzyme and subsequently heated at 90° for 30 min. The HED × 4 values of the untreated and treated preparations and of the control fluids were determined. None of the enzymes caused a decrease in the activity of ID. None of the control fluids exhibited any enhancing activity.

**Inactivation of interferon without loss of ID activity**

As reported previously (Kato, Okada & Ohta, 1965a), allantoic fluids taken 24 hr after infection with Sendai contain interferon in addition to ID. Interferon inhibits HA production by Sendai in cultures of chorioallantoic membranes (Kato, Ohta & Okada, 1966). To obtain a true curve of the appearance of ID, it was necessary to
Interferon depressors

inactivate interferon contained in the ID preparations without loss of ID activity. As described above, ID is stable to treatment with trypsin followed by heating at 90° for 30 min. On the other hand, it has been reported that chick interferon is partially inactivated by digestion with trypsin (Lindenmann, Burke & Isaacs, 1957). Therefore, the effect on interferon by treatment with trypsin followed by heating at 90° for 30 min. was investigated. Allantoic fluids harvested 84 h after inoculation with Sendai virus were used, because at this time the interferon titre has reached a maximum level (Kato et al. 1965a). The material was centrifuged at 80,000 g for 1 hr. Crystalline trypsin was added to a concentration of 0.04 %, and the preparation was incubated at 37° for 6 hr. Subsequently, it was heated at 90° for 30 min. Finally, soy-bean trypsin inhibitor was added to a concentration of 0.02 % to complete the inactivation of trypsin. Preparations heated at 60° for 30 min. were used as untreated controls.

Interferon was assayed by the HA inhibition method using the same cultures of chorioallantoic membranes as used for the assay of ID. The procedure of interferon assay was as follows. Cultures of shell-membrane pieces were preincubated for 24 hr with the material to be tested. The cultures were then washed, infected with 1 HA unit of Sendai virus, and 1 ml. of YLH medium (Kato et al. 1965a) was added to each culture. After 84 hr incubation at 37° the HA was titrated in the culture fluids. The content of interferon was measured in terms of inhibition of HA production as compared to the controls. ID activity was assayed as described above. It was found that trypsin treatment followed by heating at 90° for 30 min. destroyed interferon almost completely, but did not reduce ID activity. Therefore, this procedure could be employed to inactivate interferon selectively.

Appearance of ID in allantoic fluids of eggs infected with Sendai virus

Allantoic fluids were harvested at various times after inoculation with 10⁶ EID₅₀ of Sendai virus. After ultracentrifugation the supernatants were treated with trypsin and heat to inactivate interferon. Treated and untreated fluids were assayed for ID activity (Fig. 3). The ID activity of treated samples increased linearly with time until it reached a maximum 24 hr after virus inoculation, and remained stationary thereafter. In contrast, untreated fluids showed a marked reduction in ID activity after 36 hr. As shown before (Kato et al. 1965a), the reduction in ID activity in the later stages of infection was due to increasing amounts of interferon masking the ID activity actually present.

Effect of ID on interferon synthesis in chick embryo cell cultures

ID was obtained in the following way. Allantoic fluids were harvested 12 and 22 hr after inoculation of Sendai virus. For control, allantoic fluid from uninfected eggs was used. After ultracentrifugation the allantoic fluids were treated with trypsin and heat to inactivate interferon. Monolayer cultures of chick embryo cells were incubated at 37° for 4 hr with 1 ml. of these materials. Three cultures were used per variable. At the end of the pretreatment period the fluids were removed, the cultures were washed once with phosphate buffered saline, and interferon synthesis inducible by NDV u.v. was determined (Table I). Pretreatment with allantoic fluids from infected embryos depressed interferon synthesis markedly; the 22 hr sample was more active than the 12 hr sample. Allantoic fluid from uninfected embryos exhibited no significant interferon-depressing effect.
Effect of ID on the multiplication of Newcastle disease virus in a single growth cycle

Monolayer cultures were pretreated with ID or control fluids for 4 hr at 37°C. The cultures were then inoculated with Newcastle disease virus at an input multiplicity of 20 p.f.u./cell. After an adsorption period of 1 hr at 37°C the cultures were washed twice with phosphate buffered saline and incubated with 4 ml of maintenance medium. The medium was harvested 20 hr after virus inoculation, at which time virus multiplication had reached a peak. The yield of Newcastle disease virus in the ID-treated samples was $3.5 \times 10^6$ p.f.u./ml, in the control $3.3 \times 10^6$ p.f.u./ml. Thus, pretreatment of cells with ID did not affect the multiplication of Newcastle disease virus. The depression of NDV u.v.-induced interferon synthesis by ID was therefore unlikely to be due to an inhibition of the early steps of virus cell interaction.

![Graph](image)

Fig. 3. Kinetics of the development of ID activity in allantoic fluids of eggs infected with Sendai virus. O, Treated with trypsin and heat to inactivate interferon; o, untreated.

<table>
<thead>
<tr>
<th>Material tested</th>
<th>Yield of interferon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maintenance medium</td>
<td>70</td>
</tr>
<tr>
<td>Normal allantoic fluid</td>
<td>64</td>
</tr>
<tr>
<td>Allantoic fluid harvested 12 hr after inoculation of Sendai virus</td>
<td>12</td>
</tr>
<tr>
<td>Allantoic fluid harvested 22 hr after inoculation of Sendai virus</td>
<td>2.4</td>
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Table 1. Effect of ID on interferon synthesis
Lack of direct effect of ID on the action of interferon

Monolayer cultures of chick embryo cells were divided into four groups. Each group of cultures was incubated at 37° for 4 hr with 1 ml. of the following mixtures, consisting of equal volumes of ID + interferon, ID + maintenance medium, interferon + maintenance medium, and maintenance medium alone. The interferon preparation used was produced by NDV u.v. in chick embryo cells. It contained 170 PDD 50/ml. After removal of the fluids, the cultures were washed with phosphate buffered saline and infected with Sindbis virus. Single cycle growth curves of the virus in each group of cultures were obtained (Fig. 4). Interferon reduced the virus yield by approx. 99%.

Fig. 4. Single cycle growth curves of Sindbis virus in chick embryo cells pretreated with ID (○--○), interferon (△--△), ID plus interferon (△--△). Control cultures (●--●) were preincubated with maintenance medium. The input multiplicity of the virus was 15 p.f.u./cell.
ID did not significantly affect the inhibitory effect of interferon. Virus replication in cultures pretreated with ID alone was quite similar to that in control cultures. ID therefore had no direct effect on the action of interferon; nor did it enhance the multiplication of Sindbis virus during one cycle of replication. This finding implies that during one cycle of Sindbis virus multiplication no interferon is synthesized. This assumption was experimentally verified. Interferon was assayed in medium harvested at various times after virus inoculation. No interferon production was detectable for at least 7 hr after virus inoculation, when virus multiplication had already reached a peak \((6 \times 10^9 \text{ p.f.u./ml.})\). Interferon was detectable only after virus multiplication had been completed. For this reason ID cannot be expected to affect a single cycle of Sindbis virus replication.

**DISCUSSION**

Interferon depressor (ID), formerly called 'enhancer', has the following physical and chemical properties. It is stable at 90° for 30 min., not dialysable, and stable to ether. Trypsin, \(\alpha\)-chymotrypsin, DNase and RNase cause no decrease in ID activity. By exclusion, it appears likely that the material exhibiting ID activity is associated with a polysaccharide. This interpretation is strengthened by the fact that a chick allantoic mucopolysaccharide was also found to depress interferon synthesis without affecting the action of interferon (Kato & Eggers, 1969 b).

A central finding was the demonstration that ID depresses interferon synthesis. This effect does not seem to be due to an inhibition of the early steps of virus cell interaction. In addition, ID does not inhibit the action of exogenous interferon.

In the allantoic cavity of eggs, infected with Sendai virus, apparently several processes take place. Besides virus multiplication, ID becomes demonstrable and reaches a peak in activity 24 hr after virus inoculation. In addition, interferon does appear which in the later stages of infection masks ID activity. At present, it is not known whether one and the same cell or different cells are responsible for the appearance of ID and interferon. Furthermore, it is not clear whether during infection ID is synthesized *de novo* or whether it is perhaps converted from an inactive into an active form. Most important, it remains to be seen whether the observed phenomena are of biological significance in natural infection.

Isaacs, Rotem & Fantes (1966) described a 'blocker' of interferon production which was found in allantoic fluids of eggs infected with fowl plague virus, Newcastle disease virus or influenza virus B/England/939/59. The stability of this blocker to some physical and chemical agents is quite similar to that of ID described in the present paper. The blocker could be dissociated from interferon by purification of interferon or by treatment with trypsin or pepsin. Furthermore, it was found to exert no effect on the action of interferon. In view of these facts, the blocker and ID appear to be quite similar. Similar factors capable of depressing interferon synthesis were also found in other virus cell systems (Ohta & Kato, 1966; Ohta, 1967; Paucker & Boxaca, 1966, 1967).

ID described in this paper differs in activity and its properties from antagonists of interferon. These antagonists prevent the antiviral activity of interferon but not the production of interferon. They are sensitive to digestion by trypsin (Brailowsky & Chany, 1965; Chany & Brailowsky, 1965, 1967; Truden, Siegel & Dietrich, 1967).
We wish to thank Miss Erica Waidner for excellent technical assistance.
N. Kato was in receipt of a Dozentenstipendium from the Alexander von Humboldt-Stiftung. Permanent address: Department of Bacteriology, Nagoya University School of Medicine, Nagoya, Japan.

REFERENCES


(Received 18 November 1968)