An Interferon Produced by Foot and Mouth Disease Virus (FMDV) in Calf Kidney Cells.* (27370)

Z. DINTER AND L. PHILIPSON
Institute of Virology, University of Uppsala, Uppsala, and The State Veterinary Institute, Stockholm, Sweden

Various virus-cell systems have been shown to produce substances capable of inhibiting multiplication of related and unrelated viruses. These inhibitors appear to comprise a class of substances with similar properties irrespective of the virus-cell system producing them. They have been given the name of interferons or interferon-like substances according to Isaacs(1). These agents are produced by myxoviruses(1,2), vesicular stomatitis(3), polio(4), measles(5) and polyoma virus(6). Interferon appears to act intracellularly(1) and an uncoupling of the oxidative phosphorylation(7) of the cells by interferon has been suggested. It was therefore of interest to investigate whether foot and mouth disease virus (FMDV) could produce an interferon, since multiplication of this virus appears to be only slightly affected by compounds(8,10) or conditions(9) which will uncouple oxidative phosphorylation.

The presence, characteristics and properties of an interferon-like agent in FMDV-infected calf kidney cultures are described.

Material and methods. Cell cultures. Primary cultures of calf kidney cells were prepared from trypsinized kidneys of newborn calves. Growth medium was Hanks' salt solution with 0.5% lactalbumin hydrolysate (Hanks' + Lah) enriched with calf serum. The same medium was used in tests, but without serum. Primary monkey kidney cultures were prepared similarly, and the same growth medium was used. In tests growth medium was replaced by Parkers medium 199.

Viruses. FMD-virus of subtype O_2 and subtype A_5 was used. Stocks of virus were obtained from calf kidney cultures. Parainfluenza virus type 3, strain 23, and pseudorabies virus, a Yugoslavian strain Z, were used as stocks from calf kidney cultures. A strain of bovine enterovirus(20/61) isolated in this laboratory, was also used as stock from calf kidney cultures. ECHO type 7, Wallace strain, and Polio type 1 (a Swedish strain 1423/53) were used as stocks from monkey kidney cultures. All these viruses are included in the strain collection of this laboratory.

Assay of virus. Serial 10-fold dilutions were inoculated in 0.1 ml amounts in each of 3-5 tube cultures. Infectivity titers are expressed as log 10 units of TCD_{50}/0.1 ml.

Plaque assays were performed as previously described(11), except that Falcon plastic petri dishes 60 × 15 mm were used instead of bottles and incubation was carried out in incubators with approximately 5% CO_2 in air.

Antisera. FMDV antisera were obtained from cattle hyperimmunized against the respective subtypes of virus.

Ultracentrifugation was performed in a Spinco L-preparative centrifuge. The rotor SW 39 L was used.

Assay of interferon. Routine assays of interferon were done with FMDV subtype A_5 as challenge virus. Serial 2-fold dilutions in Hanks' + Lah of the interferon preparation were added in 1 ml amounts to 2 tubes per dilution. The cells were allowed to react with the interferon preparation for 24 hours and subsequently 1000 TCD_{50} of challenge virus was added. Control cultures were destroyed by FMDV subtype A_5 at this concentration within 24 hours. The interferon titer was expressed as the highest dilution, which in both cultures clearly diminished the CPE. In some experiments the interferon was assayed by the plaque reduction technic as described by Wagner(12) with the modification that the interferon preparation was allowed to react with the cells for 24 hours

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prior to addition of challenge virus.

In some experiments other viruses, as indicated, were used as challenge virus.

Experiments and results. Preparation of interferon. Interferon was prepared from culture fluids of calf kidney cells infected with FMDV subtype O_2 for 48 hours. A multiplicity of 10^{-5} was used. To obtain an appreciable interferon titer the collected culture fluids were pooled and concentrated 5-6-fold, either by evaporation at room temperature or dialysis against powder of polyethylene glycol (PEG 20M). Subsequently infectivity was inactivated by dialysis against citrate-buffer pH 2.5 for 6 hours or by addition of hyperimmune serum. Interferon was then, before testing, repeatedly dialyzed against Earle's salt solution with 0.5% lactalbumin hydrolysate. There was no difference in interferon titers obtained by the different methods used. Most preparations were obtained by concentration with PEG 20M and subsequent treatment at pH 2.5.

Production of interferon. To investigate at what time during the multiplication cycle of virus interferon was produced, cultures of calf kidney were inoculated with a multiplicity of 10^{-3} with FMDV subtype O_2. The cultures were washed after one hour's incubation at 37°C and subsequently at different intervals; samples were taken and infectivity and interferon titers determined. As seen from Fig. 1 increase in interferon titer parallels increase in infectivity and both activities reach a peak at 36 hours after infection.

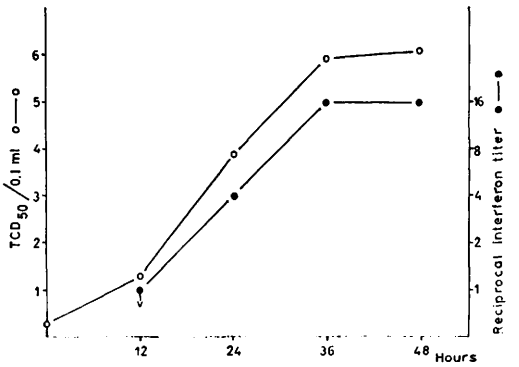


FIG. 1. Production of infectious virus particles compared with production of interferon in calf kidney cells infected with FMDV subtype O_2.

Mode of action. It was demonstrated in several experiments that interferon did not inactivate the virus. After 6 hours' incubation with interferon at 37°C virus titer of a pool originally containing 5.9 TCD_{50} was 5.3 TCD_{50} compared to 5.5 TCD_{50} in the controls.

The period necessary to keep interferon in contact with cells to give protection against challenge virus was next investigated. Plates with confluent layers of calf kidney cells were exposed to the interferon preparation for varying periods. Interferon was then washed away and 50 PFU of challenge virus in 0.5 ml inoculated, which was allowed to attach for one hour at 37°C. Plates were then overlaid and plaques read on the second day. As seen in Fig. 2 a preincubation period of at least 8 hours for interferon and cells prior to challenge was necessary to develop maximal resistance.

It was further investigated whether cells rendered resistant by interferon attached the challenge virus normally. Plates pretreated with interferon for 24 hours and control plates were inoculated with 0.5 ml of FMDV subtype A_5 and at different intervals 0.1 ml was removed and assayed for unattached virus. As seen in Fig. 3 no clear difference in attachment rate of challenge virus could be observed for the resistant and the normal cells. In similar experiments it was also demonstrated that attachment of FMDV subtype A_5 in presence of interferon exhibited the same rate of attachment as that observed in its absence.

Spectrum of interference. To obtain further information on the characteristics of FMDV-interferon, several viruses were tested for inhibited multiplication in interferon treated cells. Calf kidney and monkey kidney cultures were pre-treated with FMDV-interferon for 24 hours at 37°C. Subsequently challenge viruses were titrated in such cultures and in control cultures.

Table I gives the difference in titer obtained in interferon treated cells and control cells. It is clear that widely different viruses are inhibited but to a varying extent as previously demonstrated(4). Parainfluenza virus

TABLE I. Inhibition by FMDV-Interferon of Viral CPE or Hemadsorption for Several Different Viruses.

Virus	Infectivity titer*			
	Calf kidney cultures		Monkey kidney cultures	
	Interferon treated	Control cultures	Interferon treated	Control cultures
Parainfluenza type 3	<2.5	6.7	3.5	6.7
Pseudorabies	2.5	3.9	n.d.	n.d.
Bovine enterovirus	<2.5	6.7	n.d.	n.d.
ECHO type 7	n.d.	n.d.	7.2	7.5
Polio type 1	n.d.	n.d.	7.2	8.2

* TCD_{50}/0.1 ml. n.d. = not done.

type 3, pseudorabies and a bovine enterovirus are inhibited by interferon. By comparison of the inhibition of parainfluenza virus type 3 in both monkey and calf kidney cells it appears that interferon prepared in calf kidney cultures acts more effectively in calf kidney than in monkey kidney cultures. This may account for the slight effect of interferon on polio type 1 and ECHO type 7 virus in monkey kidney cultures.

Properties of FMDV-interferon. FMDV-interferon was inactivated by a 30-min. exposure to trypsin at pH 7.4 at a concentration of 1 mg/ml of the enzyme. RNase and DNase had no activity at 100 μg/ml for 30 min. at 37°C. A one hour exposure to ether (50% v/v) at room temperature completely destroyed inhibitory activity. Treatment with chloroform (20% v/v) and fluorocarbon (trifluorotrichlorethane 33% v/v) at room temperature for 15 min. inactivated the inhibitory effect. When sensitivity of FMDV-interferon to heat was investigated it was found that exposure to 56°C for 30 min. did not inactivate the inhibitory activity. Table II gives a comparison of the susceptibility of interferon and virus infectivity of FMDV to different chemical and physical treatments.

It was also shown that the interferon titer was not affected by treatment with hyperimmune antisera from cattle infected with FMDV subtype O_2.

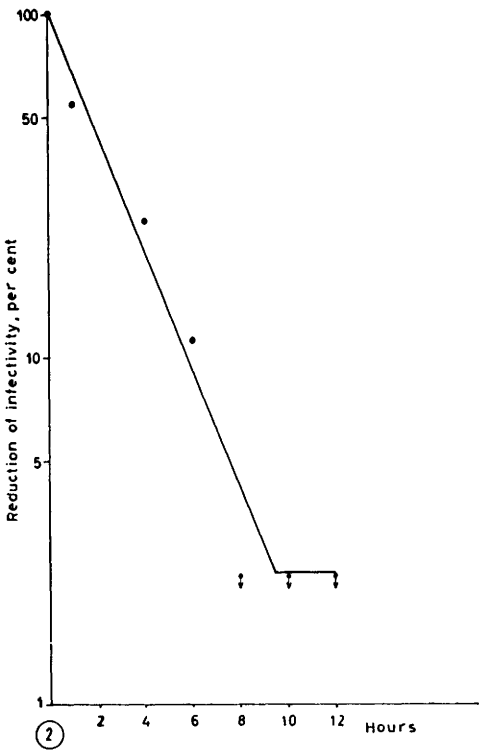
Discussion. The data provide information on the nature and properties of an inhibitor of viral activity appearing in FMDV-infected calf cultures. Production and release of this inhibitor is coincident with that of infectivity of the virus. The virus particle can be distinguished from the inhibitor. The relationship of the inhibitor with the infective particle or virus associated complement fixing antigen is unlikely as neutralizing antibodies have no effect on the interferon.

Its mode of action is still unclear, but as it does not combine directly with virus nor is attachment of virus to cells affected, it appears likely that it acts intracellularly as has been demonstrated for other interferons(1,4). Interferon from FMDV-infected cultures is, as shown for all interferon preparations previously described(1-5), sensitive to trypsin. It is also sensitive to ether like influenza virus interferon(13), but contrary to measles virus interferon(5). The chemical and physical properties examined agree in general with those found for other interferon preparations. It therefore appears reasonable to assume that these inhibitors are similar although not identical substances. An inhibitor from FMDV-infected cultures has previously been described in a preliminary way(14), although it was not clearly identified as interferon. The chronic infection of calf kidney cells observed with FMDV subtype O_2 when the virus multiplication was suppressed during the first cycle either by antibodies(15) or high concentration of normal serum(16) cannot, however, be explained solely by the presence of interferon since there is no correlation between interferon titer and virus titer

TABLE II. Some Physical and Chemical Properties of FMDV-Interferon as Compared with Those of Infectious Particles.

		Interferon	Infectivity
Inactivated by:	Trypsin	+	+
	RNAase	—	—
	DNAase	—	—
	Ether	+	—
	Chloroform	+	—
	Fluorocarbon	+	—
Inactivated after 30 min. at 56°C		—	+
Sedimented after 2 hr at 100,000 *g*		±*	+
Sensitivity to viral neutralizing antibodies		—	+

* A 2-fold reduction of interferon titer in the supernate.



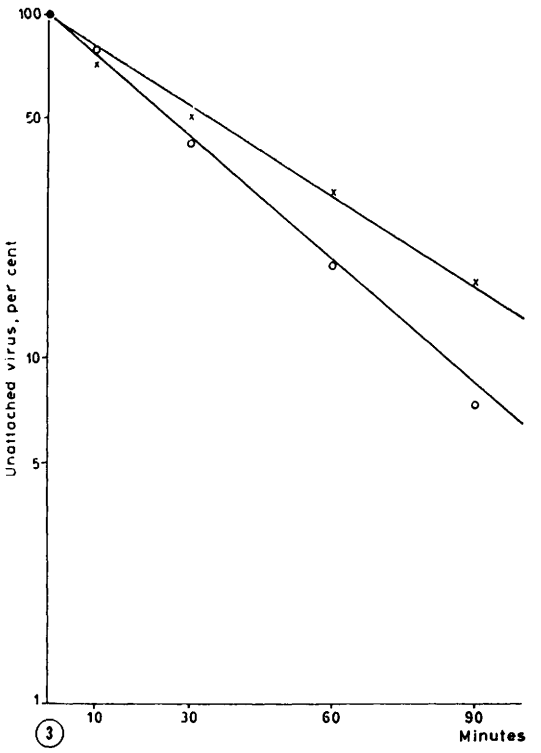


FIG. 2. Influence of preincubation of cells with interferon on reduction of infectivity of challenge virus.

FIG. 3. Attachment of FMDV subtype A_5 to interferon treated ×—× and untreated calf kidney cultures ○—○.

in the chronically infected cells. Another mechanism seems to operate to explain this phenomenon. Interference observed in chronically infected cells appears to involve an impairment of attachment for challenge virus by a sedimentable factor(16). The interferon preparations described here seem to be slightly contaminated with this factor because a small effect on attachment of challenge virus (Fig. 3) and partial sedimentation of the interfering capacity at 100,000 *g* for 2 hours (Table II) were observed.

It is of interest, however, that FMDV has been shown to propagate normally in calf kidney cultures in the presence of concentration of dinitrophenol(8,10) effective in uncoupling oxidative phosphorylation. Since an uncoupling of oxidative phosphorylation by interferon has been a suggested mode of action for its viral inhibitory effect(7), the presence of an interferon produced by FMDV would not agree with this theory.

Summary. An interferon from FMDV-infected newborn calf kidney cultures is described. The interferon appears to be distinct from virus and virus-associated antigens. It is not virucidal, nor does it significantly affect attachment of virus to cells, but it reduces the cytopathic effect as well as the quantity of virus produced.

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Fluorescent Antibody Studies in Tissue Culture of Parainfluenza 3 Infection.* (27371)

HUNEIN F. MAASSAB AND PHILIP C. LOH† (Introduced by Thomas Francis, Jr.)
Department of Epidemiology and Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor

Fluorescent antibody has been applied by a number of investigators to study the sites of synthesis of the various units of animal viruses in infected cells. Studies on the multiplication of myxoviruses have shown that some, like influenza and fowl plague viruses, have their nucleoproteins and hemagglutinin components synthesized in the nuclei and the cytoplasm respectively(1,2,3); others like mumps, Sendai, and Newcastle disease viruses have not been found by fluorescent antibody to produce specific antigens in the nucleus at any stage of the development cycle(4). Because of recent interests in the newer myxoviruses, an investigation was undertaken to characterize parainfluenza 3 or hemadsorption type I, to study its growth in a continuous line of cells and to make a dynamic approach to the events occurring during viral biosynthesis.

Materials and methods. Conjugation procedure. Rabbit antiserum against parainfluenza 3 was labeled with trimethyl rhodamine isothiocyanate (TMRITC), and its gamma globulin component isolated by the cellulose anion exchange method of Riggs *et al.*(8). The conjugated preparations were successively absorbed with liver powder, from monkey and man, to reduce non-specific staining.

Staining procedures. 1) Fluorescent antibody. Infected cells on glass coverslip were stained with the labeled specific gamma globulin using the direct method(9). Uninfected cells stained with labeled antibody and infected cells treated with unlabeled antibody prior to staining, served as controls. In each instance, the specificity of the labeled serum was indicated, *i.e.,* uninfected cells showed no specific fluorescence and infected cells pretreated with the unlabeled antibody blocked the binding of the labeled one. 2) Acridine orange. The method of Armstrong and Niven(10) was followed for the acridine orange fluorochrome technic.

Microscopy. A standard Zeiss GF425 microscope with an Osram HBO 200 lamp as a light source was used. A UG2 filter and OG4 filter in combination with a GG4 were used as exciter and barrier filters, respectively. All photomicrographs were taken with a 35 mm Zeiss camera and super Anscochrome daylight film (ASA 100).

Tissue culture. The line of continuous monkey kidney (LLC-MK_2) cells used was obtained from Dr. Robert Hull of Eli Lilly research laboratories. As routine, the cultures were grown in synthetic mixture 199(5) with 1% inactivated horse serum. The maintenance medium used throughout during virus multiplication was composed only of the mixture 199.

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† Present address: Dept. of Microbiology, University of Hawaii, Honolulu.