Interferon induction in pigs and porcine cell cultures with viral and synthetic inducers

by

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TABLE OF CONTENTS

INTRODUCTION

The interferon system has become recognized as a major natural defence mechanism against viral diseases. It is believed to play an important role in the recovery of host animals from viral infections and serves to limit virus spread through the blood stream (11, 54). Interferon promises to become a major clinical tool against viral diseases. In recent years increasing attention has been directed at elucidating the mechanism of production and protective action of interferon.

Interferon has been induced in many animal species and in cell cultures by viral and certain non-viral stimuli (41, 54). Despite the recent interest in synthetic interferon inducers, virus-induced interference continues to be widely studied. Viruses are responsible for the interferon formation *in vivo* and thus in studying virus-induced interferon formation we are looking at a process which occurs under the normal physiological conditions.

The ability of the pig to produce interferon in response to various stimuli has not yet been extensively studied. V. Torlone *et al*. (149) reported the successful induction of interferon in pigs infected with hog cholera virus (HCV). There has also been a report of interferon production by suspension cultures of swine peripheral blood leucocytes in response to phytohemagglutinin (133).

The purpose of this study was to investigate the inter-

feron-producing capacity of swine and swine cell culture in response to various inducers. The inducers used included polyriboinosinic-polyribocytidylic acid (Poly I:C) a synthetic double-stranded ribonucleic acid, swine influenza virus, a single-stranded enveloped RNA virus, and pseudorabies virus, a double-stranded enveloped DNA virus.

The synthetic ribonucleic acid and swine influenza virus are known to be good interferon inducers (41, 54) while pseudorabies virus is considered to be a poor inducer of interferon (78).

LITERATURE REVIEW

The role of a specific protein called interferon in the resistance of cells to viral infection was observed and described by Isaacs and Lindenmann (87) in 1957. They incubated cells from chorio-allantoic membranes with inactivated influenza virus and found an extracellular substance that interfered with the replication of live influenza virus. They concluded that the extracellular product had been produced in response to the inactivated influenza virus and that this substance could confer viral immunity on other cells.

Subsequent work by many investigators in virology, biochemistry and molecular biology has added much information about interferon. It is known to be a protein of cellular origin which is capable of initiating non-specific intracellular inhibition of virus replication (41, 87).

Interferon Inducers

Originally only viruses were thought to be capable of inducing the production of interferon (87). In 1963 evidence was presented that nucleic acids, provided they were foreign to the cells, stimulated production of interferon (138). Subsequently various other agents have been shown to elicit the interferon response. Gram negative bacteria and bacterial endotoxins (24, 80), rickettsiae (17, 78), chlamydia (17, 78), plant extracts (40), fungi and fungal extracts (24, 78),

protozoa (10, 17), antibiotics (17, 78), phytohemagglutinin (133), synthetic materials (2, 50, 51, 111) as well as different live and inactive (65, 66) viruses have been shown to be interferon inducers. With the exception of viruses and certain synthetic materials, most of these inducers are not highly effective (78).

Viruses of all the major virus groups can act as interferon inducers (13, 24, 30, 58, 85, 99, 115, 158, 161). The most effective inducers are members of the myxo, paramyxo and arbovirus groups (54). In general, enveloped RNA-containing viruses seem to be the best inducers of interferon (75), but DNA-containing viruses also induce interferons (61, 99, 135, 136). Ability to induce interferon may vary greatly among strains of the same virus (76, 144). It has been observed that poor viral stimulators of interferon production are usually also relatively insensitive to interferon action (78). However, Newcastle disease virus (NDV) is an exception (78). It is a very good interferon inducer but is relatively resistant to its action.

Influenza viruses

Influenza virus was successfully used as an interferon inducer in chicken embryo (76, 87, 158), chickens (15), rats (15), mice (86, 144), mouse tissue culture (63) and humans (54). Swine influenza virus, used as a nasal spray, significantly reduced human influenza in comparison with a control

group (54). Mice, infected intranasally with influenza virus did not produce detectable interferon in serum but challenging with Bunyamwera virus, the difference with control mice was highly significant (86). It is possible that a small amount of interferon could account for the infection interference (76).

Soloviev and Gutman (144) checked the capacity of 85 strains of the Influenza A2 virus to induce interferon in mice following intravenous inoculation. They found that 29 strains were of high activity, 32 were intermediate and 24 did not induce interferon. After ten successive passages in chick embryos or mice these differences were gradually lost and the negative strains became interferogenic. The authors observed that the interferogenic strains were less pathogenic to man and had higher neuraminidase activity. During the chicken embryo passages the difference in neuraminidase activity diminished, the neuraminidase activity in the negative strains increasing more rapidly.

Herpesvirus group

Most of the interferon work with herpes-group viruses has been done with the herpes simplex virus (HSV).

Herpes simplex virus is a poor interferon inducer (93) but low levels of interferon are produced in host systems in which this virus replicates (55, 58, 93, 99), as well as in abortively-infected cell cultures (6). Non-infectious HSV also

readily induces the formation of interferon. Thus, HSV inactivated by ultraviolet light and inoculated into the allantoic cavity of chick embryos produced interferon which was released into the allantoic fluid (58). Interferon has been produced in rabbits by intradermal inoculation of HSV (55).

Chicken pox or herpes zoster induces high titers of interferon in vesicles at different stages of development (5). Interferon was found in the medium of mouse cytomegalovirus (MCMV) infected mouse embryonic fibroblast cultures (120) but other workers stated that MCMV does not induce interferon production in vivo or in mouse embryo cell cultures (121). There are reports of unsuccessful attempts to induce interferon in human cell cultures with human cytomegalo virus (64).

Calves, inoculated intravenously with infectious bovine rhinotracheitis (IBR) virus rapidly produced high levels of interferon (136). Peak titers were reached in 1 to 2 days post inoculation and detectable interferon persisted as long as 7 days (136). Intravenous inoculation induced production of higher levels of interferon than were induced by intramuscular, or intrtracheal inoculation using the same dose of inducer (136).

The sensitivity of the herpes-group viruses to interferon is controversial (99). It is usually assumed, perhaps in the absence of sufficient data that the varicella-zoster (99), herpes simplex (12, 13, 19, 30, 33, 35, 51) and Marek's disease

viruses (117) are rather resistant to interferon.

Poly I:C induced interferon had minimal therapeutic effect in herpes simplex keratitis in rabbits (30, 35, 93) and the effect was even less in man (35). On human embryo kidney cells Poly I:C induced interferon was not effective against HSV (51). Interferon used for herpes simplex encephalitis treatment was of questionable value (19). Humans and mice were protected only against very low doses of HSV inoculated intracerebrally (33). Human cytomegalovirus is relatively insensitive to the antiviral action of interferon in vitro and in vivo (64). Mouse cytomegalovirus was responsive to exogenous interferon in vitro and in vivo in mice (121). Human (129) and mouse cytomegalovirus (120) could be inhibited by interferon in vitro according to other reports. NDV induced interferon in calves was reported to be active against IBR (135). Statolon-or NDV-induced interferon protects mice against PRV (114).

Poly I:C induced interferon was reported to be active in vitro against HSV (70) and Herpesvirus saimiri (7). In rabbits (70, 123, 126) and mice (70, 123), interferon enhances recovery from keratitis and keratoconjunctivitis of HSV origin. Poly I:C administered locally significantly reduces human herpetic keratitis (116) and it is very effective in early and late superficial lesions (68).

J. O. Oh (119) observed a distinct difference between HSV

types 1 and 2 in sensitivity to Poly I:C induced interferon which may partially explain the differences noted in the efficacy of Poly I:C by various investigators.

Many workers agree that HSV as well as other herpes viruses require relatively large doses of interferon to inhibit replication (16, 63, 85, 91).

Synthetic inducers

The demonstration of interferon induction by synthetic anionic polymers with defined structure has suggested a series of inducers which might be suitable for clinical purposes (40).

The synthetic double-stranded RNA copolymer Poly I:C (polyinosinic-polycytidylic acid) is one of the most potent inducers of interferon and has high antiviral activity in vivo and in vitro (1, 4, 21, 24, 30, 35, 50, 51, 111, 112, 153, 154). The amount of Poly I:C required to induce resistance to viruses is correlated with interferon sensitivities of the viruses (147).

Some common structural requirements of the synthetic inducers must be kept in mind, namely, a sufficiently high molecular weight (41, 52, 75, 97, 111), regular and dense sequences of negative charges on a long-chain backbone (41) and a stable primary (carbon to carbon) or secondary (inter-strand base pairing) structure (41). Double or multi-strandedness does appear to be a factor necessary for interferon induction (52, 111, 153). It has however been reported

that single-stranded synthetic RNA can be active in inducing resistance to viruses when used in high concentrations (12, 36, 107). Two possible explanations are available, namely, the possibility of contamination with extraneous double-stranded material (51) or the fact that a high concentration of single strands can force formation of double-stranded secondary structures (40).

The sugar moiety is important because DNA or DNA-RNA hybrid polymers produce no or very low levels of interferon (52). Resistance to nuclease action appears to be important for interferon induction (71, 111).

Recently it was reported (39) that priming of seven tested cell cultures with Poly I, followed by treatment with Poly C, gave a consistently greater antiviral activity than does Poly I:C complex itself. The surprisingly high antiviral activity obtained with successive administration of Poly I and Poly C to cell cultures can be explained assuming that the homopolymers form a double-stranded complex at the outer cell membrane or within the cell, or that the two strands do not reunite but act independently on the cell. The fact, that separate administration of the individual homopolymers only partially restores the interferon inducing capacity of Poly I:C <u>in vivo</u>, could be explained assuming that the homopolymers are probably going to different cells and do not react with the same cell and partial antiviral effect obtained with Poly I and Poly C <u>in vivo</u>, when

injected in rapid succession, may be ascribed to an association of the two homopolymers and to activity of the double-stranded complex.

Poly I:C is a potent interferon inducer *in vivo* but there are reports about pyrogenicity (75, 104, 118, 134) and toxicity (1, 71, 75, 134) of the compound which limits its wide use.

Tilorone hydrochloride, the orange water-soluble dihydrochloride salt of 2, 7 bis [2-(diethylamino) ethoxy] fluoren-9-one, was reported to be an orally active antiviral agent and was found to fulfil the biological criteria to be classified as an interferon (96, 109). Tilorone hydrochloride is active against different RNA and DNA viruses (96, 148) and has anti-tumor *in vivo* and *in vitro* effects (2). The post-inoculation interferon duration time in sera is longer than interferon induced by Poly I:C in mice (148).

Other synthetic substances such as pyran copolymers, polyacrylic acid and polyvinyl sulfate have recently been reported to induce interferon (111).

Host Responses to Interferon Inducers

Interferon has been produced by and shown to be active as an antiviral system in the living host or cells of man (54, 78), rabbits (4, 50), mice (78, 86, 144), rats (15), monkeys (78, 147), hamsters (78, 147), guinea pigs (78, 147), chickens (15, 81), cattle (134, 135), horses (78, 147), tortoises (78, 147), bats (78, 147), fish (9), sheep (122), in dogs (6) and in swine

(133, 149). Viral inhibitor substances resembling interferon have been described in plants (130, 131).

It was observed that NDV stimulates production of about equal amounts of interferon in the mouse and rabbit and less in the monkey (95). Poly I:C induced production of higher titers of interferon in rabbits than in mice (78). Cell lines vary also in their capacity to produce interferon (44, 105) and the amount of interferon released is under Mendelian factor control (43). Hybrid mouse-hamster cells produced ten times more hamster interferon than the parent hamster cell line, indicating that the presence of the mouse cell genome in some way allowed for a better expression of the information carried in the hamster genome (32). Individuals, even from the same species, were found to differ considerably in production of serum interferon (42, 143).

Chick embryo cells, aged for several days, produced more interferon after virus induction (10, 31, 72, 78, 92). The amount of interferon in serum of mice after induction by NDV decreased with increasing age (42).

The spleen, bone marrow, lung and to a lesser extent liver and peripheral lymphocytes are the most important sites of interferon formation <u>in vivo</u> following I.V. injection of inducers (40, 54, 78). The relative importance of these tissues may differ for different inducers, animal species and routes of injection (40).

There are apparent differences in the effect of interferon

on different viruses. It is not certain whether the differences are due to the inherent properties of the virus, to the species of the interferon or to the interferon antagonists produced by the cell strain in the assay system. It is shown that the viruses, which are relatively sensitive to the interferon from one animal species, may well be resistant to the interferon from the other species (59).

Mechanism of Interferon Production

The mechanism of interferon production is not yet understood and two hypotheses for the mechanism of induction have been proposed. One involves the de novo synthesis of interferon and the other the release of preformed interferon (81, 107, 152, 156). Living agents such as viruses appear to induce synthesis of interferon de novo by derepression of host to form a mRNA for synthesis of the corresponding interferon molecule (107, 152). Biological extracts such as endotoxins and synthetic polyanions have been thought to release preformed interferon (152, 154, 156). It is not yet clear whether interferons which can be released are present as interferon molecules and need only to be unbound or whether they exist as some type of precursor molecule and require transformation to become active as an interferon (107). There are suggestions that both de novo production and release of preformed interferon is probably occurring (81, 156) with any inducer. The distinction between the two patterns of induction has been based on rela-

tively indirect arguments such as a different sensitivities to inhibitors of RNA and protein synthesis and different kinetics of interferon production (41). A likely possibility is that each type of response requires de novo synthesis but that during viral induction additional steps occur such as uncoating of virions and formation of double-stranded RNA, which are more sensitive to metabolic inhibitor than interferon itself (40). The time needed in the cell for the removal of the protein coat from the virion might explain the late appearance of virus-induced interferon. Previously uncoated reovirus type 3 produced early type interferon in rabbits (151). These findings suggest that the differences in kinetics for interferon production are more closely related to a secondary factor such as delivery of the inducer to the cell receptor site (41).

The derepression concept of interferon production has been based on the demonstration that the virus-induced synthesis of interferon could be blocked by a compound like Actinomycin D which preferentially inhibits DNA-directed RNA synthesis, or by puromycin or p-fluoruophenylalanin which specifically interfere with the translation of mRNA and protein synthesis (54). Virus-induced interferon production was generally unaffected (159) but Poly I:C-directed interferon synthesis increased (152) when Actinomycin D was added after interferon production has begun. This could be explained as a suppression of the synthesis of an inhibitor for the formation of interferon and

its synthesis could be more sensitive to the action of Actinomycin D than the synthesis of interferon itself (41).

The derepression hypothesis of interferon production is widely accepted but it is not proven. None of the genetic elements of the interferon system have been isolated or characterized and likewise the molecule which triggers the derepression of the host genome is not known for certain (107). The high interferon-inducing capacity of double-stranded RNA of both viral and synthetic origin in contrast to the inactivity of single-stranded RNA and double-stranded DNA was the basis for the hypothesis that the double-strandedness of RNA provides the final derepression for formation of mRNA and interferon (52, 73, 98). In 1969 Colby and Duesberg (37) demonstrated the presence of a virus-specific double-stranded RNA in chick cells infected with vaccinia virus which could explain the stimulation of interferon by DNA-containing viruses. Lockart (107) stated that he could find no evidence indicating that double-stranded RNA or viral protein is responsible for induction of cells to produce interferon. He believes that the cell itself provides inducer molecules to a wide range of stimuli (107).

Factors Influencing the Induction and Production of Interferon

The interferon response may be altered by various factors. Stress (142) and hormones (10, 55, 127, 142) have an effect. Corticosteroids given in amounts above the usual physiological

level inhibited the interferon response to endotoxin (127) and virus (101, 142).

Production of interferon occurs within definite temperature ranges. Generally it is inhibited at 4 C (54). Optimal temperatures for interferon production vary with different inducers and host systems (139, 140).

Immune recognition increases interferon production and may initiate production. Lymphocytes from persons sensitized with bacterial toxoids produced interferon on exposure to the appropriate antigens in vitro, whereas cells from non-immune donors did not (67).

Development of tolerance or refractoriness to repeated stimulations by inducers is an important phenomenon in animals (16, 47, 78, 80) and cell cultures (18, 20, 78, 152). Stimulators, be they virus, endotoxins or Poly I:C cause tolerance in animals which then remain tolerant to subsequent injections for about 4 to 13 days after which they are again sensitive (23, 78).

The refractoriness mechanism is obscure. Some authors feel that interferon production somehow produces a negative feedback for its own production (78). A humoral factor has been suggested as an explanation of the tolerant state following injection of endotoxin in rabbits but has not been demonstrated in mice (41, 82). Vilcek (152) thinks that an endogenous cellular inhibitor participates in the development

of a cellular refractory state to repeated interferon stimulation. Borden and Murphy think that refractoriness is caused by a newly synthesized compound which blocks interferon synthesis (23). They showed that the kinetics of the development of refractoriness were clearly separable from the kinetics of interferon production in vivo and in vitro. It was observed that following release of interferon, if cells are allowed to divide, they rapidly regain reproducibility and if multiplication is inhibited in deficient medium, they remain refractory for life (23, 124). Youngner and Hallum (155) have shown that treatment with large dose of interferon suppressed the subsequent inductions of interferon with Poly I:C in L cells. This raised the possibility that the refractory state to repeated interferon induction is mediated by interferon (156). In contrast it was observed that L-cell culture could produce high amount of interferon by MM virus, which usually doesn't induce interferon after pretreatment with small doses of interferon (146). This effect was reported also with subsequent stimulation with Poly I:C (18, 108) and NDV (23, 145) in cell cultures. This priming effect is expressed as an earlier release and higher titer of interferon, or an induction of interferon by viruses which failed to induce it without pretreatment (3, 8, 146).

The hyporeactivity and priming is thought to be a result of combining effect of a first exposure to interferon or an

interferon inducer on the synthesis of the interferon mRNA and of hypothetical cellular repressor of interferon synthesis (8).

Properties of Interferon

Physico-chemical

The physical and chemical properties of interferon have been determined by studying impure preparations and the extent to which impurities in the preparations have contributed to the observed properties is as yet unknown (48).

The protein nature of interferon was first described by Isaacs and Lindenmann (87). Interferon is retained by dialysis membranes (77, 87, 103), it is inactivated by trypsin, chymotrypsin, pepsin and papain (77, 87, 103), but not by nucleases or ether (48, 87, 103) which suggests the absence of nucleic acids and lipids as essential part of interferon. Only little is known about the chemical composition and it is generally agreed that interferons are or at least contain protein (48, 77, 87) and a carbohydrate moiety (27, 48). The exact chemical composition and amino acid chain sequences are not known yet since interferons have not been purified adequately for definitive chemical analysis (48).

Interferons are stable over a wide range of pH ranging from pH 2 to 12 (48, 77, 87), they can't be sedimented by centrifugation at 100,000 g (77, 87, 103) and they are relatively heat stable at 70 C (77, 87).

Interferons are heterogeneous with regard to molecular weight and the presence of multiple molecular species in a single interferon preparation has been reported (48, 100). The range of molecular weights of interferon is very wide and varies from 28,000 to 160,000 (48). Molecular weight depends on the nature of the inducer and the host system (48). Virus-induced interferons usually have lower molecular weights than interferons obtained after non-viral stimulation (48). High molecular weight interferon could be an aggregate of the smaller species found for example in urine (94).

The isoelectric point of interferon varies from pH 5.0 to 10.0 (48) depending on its origin, inducer and host system (48).

Disulfide bonds are required for interferon activity (113). Some, but not all, interferons are inactivated by urea (113).

Biological properties

Several properties are shared by all substances which have been called interferon. Their mode of biological action is essentially species specific (54, 87). There are a few exceptions to the species specificity rule, for example, interferon produced by Rhesus monkey kidney cells is active in human cells (30, 88). Cross reactivity was observed among species as different as mice and chickens (11, 61). Pig produced interferon was reported to be active in primary cultures of calf kidney cells (149). One way full-scale crossing of species

barrier was reported of human interferon in rabbit cells (46), and interferon in rabbit cells could have even higher titer (45).

It appears that interferon and antibody production are both highly specific functions of the lymphoid and reticulo-endothelial (RE) system and not merely consequences of non-specific stimulation of the RE system (40). Although interferons are proteins in origin, they are poor antigens (125).

Interferons are active in a broad variety of viral infections (41, 87, 103, 107) and a large number of different viruses, both RNA and DNA are susceptible to the interferon action. Interferon has been observed to have protective effect against phylogenetically higher organisms (41, 132).

Interferon may act as a primer for its own production. Pretreatment of cells with interferon prior to viral induction results in earlier release and higher titers of interferon by viruses which fail to induce without pretreatment (3, 8, 146).

Mechanism of Action

The mode of action of interferon has been studied in viral infections and it was observed that interferon does not act on the virus directly (57, 74, 103, 107, 157). Interferon does not prevent virus attachment to the cell or penetration or release from the cell (57, 74, 103). Instead, it blocks the synthesis of new virus within the cell (74). Cells infected with virus and treated with interferon may still be destroyed

but virus replication is suppressed and other cells are saved from viral infection (60).

Necessity of interferon binding to cells for development of antiviral activity is reported (57). The amount of interferon bound to the cells must be a small portion of the interferon applied since no significant loss of the applied interferon activity could be demonstrated in the overlying medium (25).

Experiments with protein or RNA synthesis inhibitors suggest that interferon might induce the synthesis of another protein by derepression of the host genome and transcription of the specific mRNA (28, 57, 74, 90). This second protein translation inhibitory protein (TIP) prevents the synthesis of new virus (14, 74, 90). Interferon or the hypothetical secondary antiviral protein (TIP) may act at the ribosomal level by interupting the synthesis of viral-coded proteins which are required for virus multiplication (74, 90, 102).

Ribosomes of interferon-treated cells have not been observed by most workers to form polysome complexes with the viral mRNA (74, 90). Translation of the viral mRNA is suppressed, whereas translation of the host cell mRNA is preserved (74, 90). Despite extensive analysis minimal or no evidence has yet been obtained for the existence of new protein synthesis in interferon-treated cells (74) so far, the TIP remains largely a hypothetical concept, and if the protein is produced during exposure of the cells to interferon it appears

to be only in minute amounts (74).

Several materials have been described which antagonize the activity or production of interferon and therefore stimulate virus growth (89, 137, 150). One of the inhibitors, namely the "blocker", was found in the allantoic fluid of eggs infected with NDV and influenza virus (89). Interferon and the "blocker" could be separated by purification. The mode of action of these inhibitors is even more obscure than the action of interferon (137).

The antiviral activity of interferon is related to concentration of interferon and exposure time of cells to interferon (141). There is a saturation level beyond which an increase in the concentration of interferon fails to effect a significant increase in antiviral activity (141). Antiviral activity also depends on the pH of interferon during induction, lower pH being less efficient. A pH level of 7.2 was reported to be the optimal (141).

Biological Role

It is widely assumed that interferon is a major factor in the recovery from viral infections (9, 10, 71). Measurable antibody appears later and is more active in inhibiting the late spread of virus through the organism or in preventing reinfection (75). Interferon limits virus spread through the blood stream (9, 10). It appears that interferon and antibody complement each other in their function in the living

organism (62). Titers of interferon do not always correlate with the clinical course of the disease (34).

The action of interferon is limited by its rapid clearance from the circulation. In the serum of different mammals, intravenous injected exogenous interferon has a half life of 7 to 11 minutes (83). The rapid loss of interferon from the circulation can be attributed to metabolism by cells and excretion (83). The liver is not of major importance in the inactivation of serum interferon (22). Interferon, while broad-spectrum in its action against different viruses, does require a close phylogenetic relationship between the host species used to produce the interferon and the host species in which it is to be used (75). All these limitations to the use of exogenous interferon in antiviral therapy suggest that endogenous stimulation of interferon may be the best approach in practice (75). To be considered for application in clinical medicine the interferon inducers should be highly active, non-infectious, free of toxicity and sufficiently non-antigenic to allow repeated use (71). Synthetic polyanions and especially Poly I:C were found to be potent interferon inducers (1, 4, 21, 24, 50, 51, 111, 112, 153, 155), but because of pyrogenicity (75, 104, 118, 134) and toxicity (1, 71, 75, 134) only low doses have been used in clinical trials (71).

The new synthetic oral interferon inducer Tilorone hydrochloride (96, 110) looks promising but has not been studied sufficiently to warrant its clinical use.

MATERIALS AND METHODS

Cell Lines

MDBK cells

The Madin-Darby bovine-kidney cell line (MDBK) was used for the cloning of pseudorabies virus (PRV) strains, for the serum-virus neutralization tests and for interferon assays.

The cells were propagated in 250 ml plastic tissue-culture flasks[a] in Eagle's basal medium (BME) with Earle's salts, supplemented with 10 per cent newborn calf serum[b] and 1.1 per cent sodium bicarbonate. After trypsinization with a 0.2 per cent trypsin-versene solution (see later) the cells were diluted 1:3 in growth medium and 1 ml was inoculated into disposable glass tissue-culture tubes[c], 2 ml per well into 35x10 mm plastic tissue-culture plates[d] and 20 ml into 150 ml plastic tissue-culture flasks. Monolayers usually developed in 24 to 48 hours.

PK_{15} cells

A porcine kidney cell line (PK_{15}) was used for the interferon production and assay, and for PRV, vesicular stomatitis (VSV) and vaccinia virus propagation and titration.

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[a]Falcon Plastics, Los Angeles, California.

[b]Grand Island Biological Company, Grand Island, New York.

[c]Kimble Glass Company, Toledo, Ohio.

[d]Linbro Chemical Co., New Haven, Connecticut.

The cells were grown in BME supplemented with 10 per cent newborn calf serum and 1.1 per cent sodium bicarbonate using the same vessels described for MDBK cells.

BHK cells

The baby hamster kidney (BHK) cell line was used for the interferon assay. The cells were grown in BHK-21 Medium[a] supplemented with 10 per cent tryptose phosphate broth, 10 per cent newborn calf serum and 1.1 per cent sodium bicarbonate.

All three cell lines were grown at 37 C in a humidified atmosphere containing 5 per cent CO_2.

Trypsin-Versene Solution

A trypsin-versene solution was used to remove cells from the surface of the flasks after propagation of the MDBK, PK_{15} and BHK cell lines. The formulation was as follows:

Trypsin[b]	2 g
Na Cl	8 g
KH_2PO_4	0.2 g
Na_2HPO_4	1.15 g
H_2O	1 liter
Ethylenediamine	

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[a]Grand Island Biological Co., Grand Island, New York.

[b]Difco Laboratories, Inc., Detroit, Michigan.

Tetraacetic acid, disodium salt (EDTA) 1 g

Water

The water used in all procedures and solutions was glass distilled and deionized. It routinely contained less than .05 ug/ml of sodium chloride equivalents as measured with a conductivity meter[a].

Agar-Overlay Medium

A double concentration of Eagle's basal medium with Earle's salts and supplemented with 10 per cent newborn calf serum and 2.2 per cent sodium bicarbonate was combined with an equal volume of 1.8 per cent Noble Agar[b]. This was used as an agar-overlay medium giving a final concentration of .9 per cent agar, 5 per cent newborn calf serum and 1.1 per cent sodium bicarbonate. Ten thousand units of penicillin and ten thousand micrograms of streptomycin was used per 100 ml of agar-overlay medium.

Neutral Red Agar-Overlay Medium

After two days incubation a second agar-overlay, containing 1:10,000 neutral red was added to the cell monolayer in the 35x10 mm plastic tissue-culture plates to differentiate

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[a]Barnstead Company, Boston, Massachusetts.

[b]Difco Laboratories, Inc., Detroit, Michigan.

the virus plaques more clearly. The following formulation was used:

Neutral red	0.1 g
Noble-agar	10.0 g
Water	qs 1 liter

Maintenance Medium

Eagle's basal medium with Earle's salts and 2 per cent newborn calf serum and 1.1 per cent sodium bicarbonate was used for the maintenance of the cell monolayers, for the cell washing, as a diluent in the interferon titration and as a diluent for Poly I:C.

Pucks saline solution G (128) was used as a virus diluent and as an inoculum in experiment 4. It was made up as follows:

Water	1 liter
Glucose	1.1 g
Phenol red	5 mg
Na Cl	8 g
K Cl	.4 g
Na_2HPO_4 .7 H_2O	.29 g
K H_2PO_4	.15 g
Mg SO_4 .7 H_2O	.15 g
Ca Cl_2 .2 H_2O	16 mg

Physiological Saline Solution

Physiological saline solution was used as an inoculum in experiment 2 and in the interferon's sensitivity to the proteolytic enzymes experiments. It was made up as follows:

Na Cl	8.5 g
Water	qs 1 liter

Buffer Solution

A .01 M phosphate buffer (pH 7.5) was used as a dialysis solution for the serum and the cell culture fluid samples and as a diluent in the hemagglutination test.

Eight and one half ml of 1M Na_2HPO_4 and 1.5 ml of 1 M NaH_2PO_4 were mixed and made up to 1 liter with physiological saline solution.

A 0.1 M K Cl - H Cl buffer (pH2) was used as a dialysis solution for serum and cell culture fluid samples. This buffer was prepared by mixing 0.1 M K Cl and 0.1 M H Cl until the desired pH2 was obtained.

Virus Titration

Tenfold serial dilutions of the virus were made in saline G. One half ml of each dilution of the virus was inoculated into each of two wells of the 35x10 mm plastic tissue-culture plates, containing PK_{15} cell monolayers. After an adsorption period of one hour at 37 C the virus was aspirated and the monolayers were overlayed with the agar-overlay medium 2 ml per

well. These plates were incubated until the plaques were seen. The neutral red agar overlay was then added. The titers of the viruses were expressed in plaque forming units (pfu) per ml. This technique was used to determine titers of pseudorabies, vesicular stomatitis and vaccinia virus.

Viruses

Vesicular stomatitis virus

Vesicular stomatitis virus, New jersey strain (VSV-NJ) at the third passage level on the MDBK cells[a] was propagated on PK_{15} cells in 250 ml tissue-culture flasks. The cultures were harvested when the cytopathic effect (CPE) was nearly complete. Viral suspensions were frozen, thawed and centrifuged at 300 g in order to remove the cell debris, and then stored at -90 C. VSV at the second and the third passage levels on the PK_{15} cells was used for the interferon assay.

Swine influenza virus

The Shope strain of the swine influenza virus[b] (SIV) of the undetermined passage level was used. The virus was propagated by allantoic-cavity inoculation of 12-day-old chick embryos. The allantoic fluid was harvested at 96 hours post-inoculation from the dead or live eggs. The virus was titrated

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[a]Obtained from Dr. R. Dierks, Veterinary Medical Research Institute, Iowa State University.

[b]Obtained from Dr. W. P. Switzer, Veterinary Medical Research Institute, Iowa State University.

by the hemagglutination test using 0.5 per cent chicken erythrocytes. SIV was used for interferon induction experiments.

Vaccinia virus

A stock strain of the Vaccinia virus originally obtained from Dr. C. H. Cunningham[a] in 1949 was used. This virus has subsequently undergone a number of chick embryo passages. The vaccinia virus at the second passage on PK_{15} cells was used for interferon characterization experiments.

Pseudorabies virus

The pseudorabies virus Pa strain (PRV-Pa) was isolated[b] from a steer which died of pseudorabies. The virus was isolated by the inoculation of the brain tissue subcutaneously into a rabbit. After the death of the rabbit, the brain tissue was inoculated into the bovine testicle monolayers and passaged three times. The virus was then passed twelve times on the MDBK cells. During these passages the virus was cloned three times in order to establish viral purity. PRV-Pa at the second and the third passage levels on PK_{15} cells was used for interferon characterization and interferon induction experiments.

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[a]Dr. C. H. Cunningham, Michigan State University, East Lansing, Michigan.

[b]Dr. L. N. Brown, Iowa Veterinary Diagnostic Laboratory, Iowa State University.

Pseudorabies virus DR strain (PRV-DR) was isolated[a] on MDBK cells from the brain of a calf which showed typical pseudorabies symptoms. PRV-DR was cloned three times on MDBK cells and the eleventh passage was used as an inoculum to infect PK_{15} cells. PRV-DR at the second passage level on PK_{15} cells was used for interferon induction and interferon characterization experiments.

Pseudorabies Be strain (PRV-Be) was isolated[b] on MDBK cells from the brain of a dog that had died of pseudorabies. PRV-Be was cloned three times prior to use and the eighth passage level on the MDBK cells was used as the inoculum onto PK_{15} cells. PRV-Be at the second passage level on PK_{15} cells was used for interferon induction and interferon characterization experiments.

All three strains were identified as PRV by the serum-virus-neutralization test. PRV-Pa and PRV-DR were neutralized by the PRV-Shope[c] antiserum and PRV-Be was neutralized by the PRV-DR antiserum.

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[a]Dr. C. J. Maré, Veterinary Medicine College, Iowa State University.

[b]Dr. C. J. Maré, Veterinary Medicine College, Iowa State University.

[c]Obtained from Dr. W. Stewart, National Animal Disease Laboratory, Ames, Iowa.

Polyriboinosinic-Polyribocytidylic Acid

Poly I:C[a] was stored at 4 C in vials until used. Poly I:C, serial number 77360, was used as the interferon inducer in PK_{15} cell cultures (experiment 1) and serial number 76139 was used as the interferon inducer in pigs (experiment 2) at the doses indicated later.

Experimental Pigs

Pigs used in experiments 2 and 4 were of mixed breed and obtained from a private pig producer. The pigs used in experiment 6 were of the Hampshire breed and were obtained from the respiratory disease free swine herd maintained at the Veterinary Medical Research Institute, Iowa State University.

The pigs were of both sexes and ranged in weight from 10.4 kg to 19.4 kg at the start of the experiments. The pigs were housed individually in 2.5x2.5x3 foot isolation cages, with airtight doors and virus filters on the intake and exhaust ports. The pigs were allowed to adjust to their new environment for seven days prior to the start of the experiments.

Virus Propagation in Chick Embryos

Fertilized eggs were obtained from the specific-pathogen-free flock maintained at the Veterinary Medical Research Institute, Iowa State University. The twelve-day-old chick

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[a]Microbiological Associates, Bethesda, Maryland.

embryos were inoculated by the allantoic cavity route with 0.1 ml of SIV inoculum. Inoculated eggs were incubated in a humidified 37 C incubator. As soon as death occurred or at 96 hours postinoculation, allantoic fluid was harvested, pooled and the virus was titrated by hemagglutination.

Purification and Concentration of PRV Strains

Three strains of PRV were grown in 250 ml plastic tissue-culture flasks on PK_{15} cells until cytopathic effect was nearly complete. The viral suspensions were partially purified by centrifugation at 300 g for 10 minutes, followed by 2,000 g for 10 minutes, to remove cellular debris. The virus then was pelleted by centrifugation at 30,000 g for 90 minutes. The pellet was re-suspended in saline G and titrated on PK_{15} cells.

Serum-Virus-Neutralization Tests

Serum-virus-neutralization tests were conducted to show that the experimental pigs were free of PRV antibodies.

Pre-inoculation serum samples were obtained from the pigs used in experiment 4. The serum was inactivated for 60 minutes at 56 C and a standard type neutralization test (constant serum, decreasing virus method) was run on MDBK cells, grown in disposable glass tissue-culture tubes.

Tube Hemagglutination Test

Serial twofold dilutions of the allantoic fluid containing SIV were made in PBS (pH 7.5) and equal volumes of 0.5 per cent

chicken erythrocytes in PBS (pH 7.5) were added to the virus dilutions and thoroughly mixed. The test was incubated for 60 minutes at the room temperature. A positive hemagglutination pattern consisted of a uniform thin layer of erythrocytes covering the bottom of the tube. The negative pattern consisted of a round "button" of sedimented cells. The reciprocal of the highest dilution of the allantoic fluid which gave positive agglutination was considered to represent the titer of the virus.

Serum Preparation for Interferon Assay

Blood samples were collected by the orbital sinus bleeding technique (84) and after firm clots were formed at room temperature the blood was kept overnight at 4 C. The sera were harvested after centrifugation for 15 minutes at 2,000 rpm. The sera and the tissue culture supernatant fluids were dialyzed in cellulose tubing at 4 C against 50 to 100 volumes of 0.1 M KCl-HCl buffer (pH2) for 24 hours and then against 50 to 100 volumes of PBS solution (pH 7.5) for 24 additional hours. Precipitation developed in some specimens after dialysis and was removed by centrifugation at 30,000 g for 60 minutes at 4 C. The supernatant fluids were collected, filtered through 300 nm Millipore filters[a] and stored at 4 C until assayed for interferon activity.

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[a]Millipore Filter Corporation, Bedford, Massachusetts.

Interferon Assay

Serial two-fold dilutions of the dialyzed and centrifuged fluids were made in maintenance medium. One ml of each dilution was inoculated into each of two wells of 35x10 mm plate containing PK_{15} monolayers and incubated at 37 C for 18 to 22 hours. The control wells were inoculated with 1 ml of the maintenance medium and were incubated for the same period of time. At the end of the incubation period the fluids were aspirated, the plates were washed twice with the maintenance medium and 0.5 ml of VSV-NJ, vaccinia or PRV (calculated to contain 50 to 150 plaque forming units) was added to each well. After adsorption of the virus at 37 C for 1 hour, the excess viral fluids were aspirated and the cultures were covered with an agar-overlay medium. After the incubation until the visible plaques were seen, a neutral red agar-overlay was added. The interferon titers were expressed as a reciprocals of the dilutions which produced 50 per cent reduction in the number of viral plaques when compared with the number in the control plates.

Characterization of Interferon

Acid stability and dialyzability

All samples were dialyzed for 24 hours against 50 to 100 volumes of 0.1 KCl-HCl buffer at 4 C cellulose dialyzer tubing[a] in an Oxford Multiple dialyzer. This was followed by a similar

[a]Arthur H. Thomas Company, Philadelphia, Pennsylvania.

dialysis against PBS (pH 7.5) for 24 additional hours. The final samples were assayed for interferon activity as described previously.

The representative interferon-containing samples, whether produced in PK_{15} cells or in pigs, were tested for species specificity, activity against different viruses, heat stability, trypsin sensitivity and the effects of ultracentrifugation, as described below. Suitable controls were included with each test.

Host species specificity

The interferon-containing samples produced on PK_{15} cells and in pigs were tested for their activity on MDBK and BHK cells. The plaque reduction assays were performed as previously described.

Activity against different viruses

The interferon preparations were tested for their lack of viral specificity challenging with different viruses (VSV-NJ, PRV-DR, PRV-Pa, PRV-Be and vaccinia viruses) on PK_{15} cells.

Heat stability

Heating of sera was performed in a water bath, adjusted to the temperature indicated below. After the required length of time, sera were immediately diluted in a maintenance medium and assayed for interferon.

Trypsin sensitivity

Equal volumes of interferon-containing serum and a solution of trypsin[a] containing 2 mg/ml in physiological saline were mixed. This mixture was incubated for two hours at 37 C. After the incubation, serial two-fold dilutions of the interferon were made and the interferon was assayed for antiviral activity against VSV-NJ on PK_{15} cells. The number of plaques was compared with the controls which consisted of the equal volumes of the interferon and physiological saline solution incubated for two hours at 37 C.

Ultracentrifugation

The samples were ultracentrifuged in an International B60 ultracentrifuge[b] at 100,000 g for 60 minutes and the supernatant fluid was assayed for antiviral activity using VSV-NJ.

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[a]Trypsin 1:250, Difco Laboratories, Detroit, Michigan.

[b]Int. Equipment Co., Boston, Massachusetts.

EXPERIMENTAL PROCEDURES AND RESULTS

Experiment I - Procedure

Interferon induction in PK_{15} cell cultures by polyriboinosinic-polyribocytidylic acid (Poly I:C).

One-day-old PK_{15} cell-culture monolayers grown in 30 ml plastic tissue-culture flasks were used. Poly I:C was dissolved in maintenance medium to the dilutions indicated below and the cell monolayers were covered with 0.8 ml of Poly I:C per flask and incubated for 1 hour at 37 C. They were then thoroughly washed with the maintenance medium and replenished with 5.0 ml of the maintenance medium. The culture fluids were collected, dialyzed in cellulose tubing at 4 C against 0.1 M KCl-HCl buffer (pH2) for 24 hours and then against PBS (pH 7.5) for 24 hours, centrifuged, filtered and assayed for antiviral activity against VSV-NJ on PK_{15} cells.

Kinetics of interferon production

A solution of Poly I:C containing 50 µg/ml was incubated for 1 hour on PK_{15} cells. Sufficient cell culture flasks were used so that the fluids could be collected at 2, 4, 6, 8, 10, 12 and 24 hours after the treatment and assayed using VSV-NJ virus as described previously

Induction of interferon by various concentrations of Poly I:C

Poly I:C concentrations of 0.625 to 320 µg/ml were used to induce interferon production. The culture fluids were

collected 6 hours after treatment and assayed using VSV-NJ virus.

Characterization of interferon

Poly I:C at a concentration of 320 µg/ml was used to induce the production of interferon on PK_{15} cells. The culture fluids were collected 6 hours after exposure and VSV-NJ was used for interferon assay.

Experiment I - Results

Kinetics of interferon production

Low levels of interferon were detected in cell culture fluids collected at 2 hours postexposure. The maximum titer of 16 was detected at 6 hours and decreased titer of 8 was found at 24 hours. The results are summarized in Fig. I.

Induction of interferon by various concentrations of Poly I:C

Poly I:C concentrations of 0.625 to 2.5 µg/ml did not induce any detectable interferon formation in PK_{15} cell culture. Poly I:C at a concentration of 5 µg/ml induced a low level of interferon. A positive correlation was observed between Poly I:C concentration and the titer of interferon induced in PK_{15} cell culture. The results are presented in Table 1.

Characterization of interferon

The viral inhibitor produced on PK_{15} cells by Poly I:C stimulation (320 µg/ml) was characterized as being acid-re-

sistant, non-dialyzable, non-sedimentable at 100,000 g for 60 minutes, trypsin sensitive, relatively heat stable and active against several different viruses. It also possessed species specificity. These characteristics justify the conclusion that this antiviral substance was interferon. The results are given in Table 2.

Table 1. Effect of Poly I:C concentration on interferon titer in PK_{15} cells

Concentration of Poly I:C µg/ml	Interferon titer/ml
0	0
0.625	0
1.25	0
2.5	0
5	2
10	8
20	8
40	8
80	32
160	128
320	256

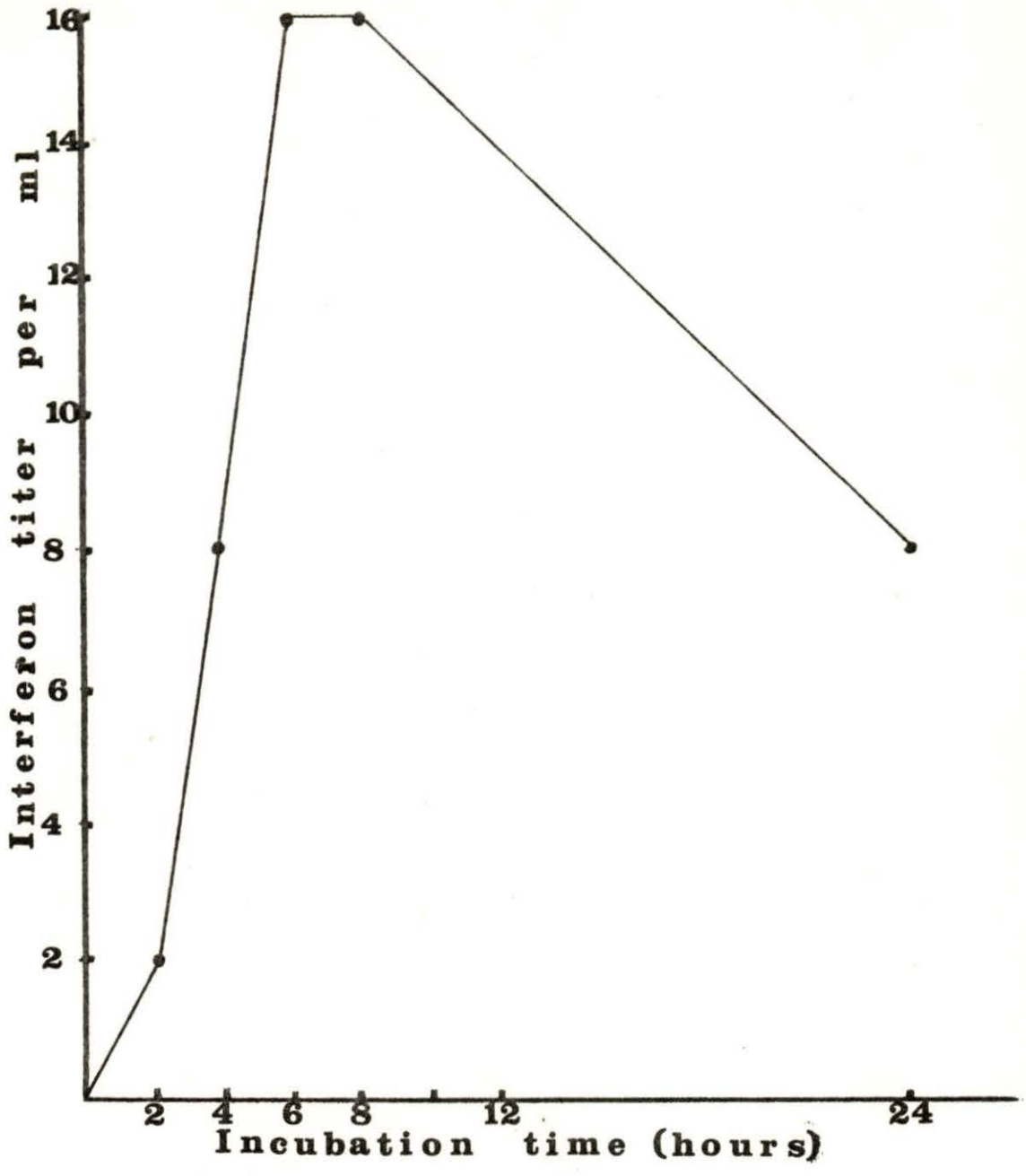

Fig.1 Induction of interferon in PK_{15} cell culture by Poly I:C (50ug/ml)

Table 2. Properties of interferon induced by PK_{15} cells in response to Poly I:C

Treatment	Results
1. Dialysis for 48 hours at 4 C	Non-dialyzable
2. Acidification to pH2	Activity retained
3. Activity against different viruses	Broadspectrum activity
Virus	Titer*
VSV-NJ	256
PRV-DR	32
PRV-Pa	16
PRV-Be	32
Vaccinia	128
4. Ultracentrifugation	Non-sedimentable
Titer before ultracentrifugation	256
Titer after ultracentrifugation	128
5. Heat stability	Relatively stable
Control titer	256
Titer after heating 37 C for 6 hours	256
Titer after heating 37 C for 12 hours	128
Titer after heating 56 C for 30 minutes	64
6. Trypsin sensitivity	Activity destroyed
Control titer	256
Trypsin (2mg/ml) for 2 hours at 37 C	0

*Interferon titer is expressed as the reciprocal of the dilution which produced 50 per cent reduction in the number of viral plaques.

Table 2 (Continued).

Treatment	Results
7. Activity against VSV-NJ in heterologous cells	Not active
Titer on PK_{15}	128
Titer on BHK	2
Titer on MDBK	0

Experiment II - Procedure

Interferon induction in pigs by polyriboinosinic-polyribocytidylic acid (Poly I:C).

The experimental pigs were inoculated intravenously with Poly I:C using the ear veins. The dosage levels per kilogram of body-weight are indicated in the Table 3. The blood samples were collected from the orbital sinus of each pig before inoculation and at 2, 6, 12, 24, 48, and 72 hours post-inoculation. The serum was harvested and the interferon titers were determined using VSV-NJ on PK_{15} cells. The rectal temperatures were recorded twice daily.

Pigs number 2 and 3 were restimulated with Poly I:C administered intravenously on the seventh day after the first stimulation. Pig number 4 was used as a control. The dosage levels per kilogram of body weight are indicated in the Table 4. The blood samples were collected at 0, 2, 6, 12, 24, 48

and 72 hours postinoculation.

Interferon was characterized using the tests which were described before.

Experiment II - Results

In the pigs injected intravenously, circulating interferon was detected and was found at maximal titer by postinoculation hour 2. (Table 3). Interferon was not detected or the titers of the serum interferon were reduced by the 6th hour post-inoculation. The magnitude and the duration of the response does not appear to be dose related within the dose range of the drug tested. Pigs number 2 and number 3 restimulated by the intravenous administration of Poly I:C on the seventh day after the first stimulation produced lower interferon titers than after the first induction (Table 4). Neither pyrogenicity nor toxicity of intravenously injected Poly I:C for the pigs was observed.

The viral inhibitor, produced in the pigs by Poly I:C stimulation was characterized as being acid-resistant, non-dialyzable, heat stable, trypsin sensitive and active against different viruses (Table 5). It was thus considered to be interferon.

Experiment III - Procedure

Interferon induction in PK_{15} cell cultures by pseudo-rabies virus.

Table 3. Serum interferon titers of pigs given injection of Poly I:C intravenously

Pig No.	Sex	Body weight kg	Poly I:C dose mg/kg	Physiological saline dose ml	Interferon titer hours postinoculation						
					0	2	6	12	24	48	72
1	M	13.6	.3	0	0	4	0	0	0	0	0
2	M	17.2	.1	0	0	128	2	0	0	0	0
3	F	14.5	.3	0	0	64	4	0	0	0	0
4	F	16.8	.1	0	0	4	0	0	0	0	0
5	M	19.4	.1	0	0	16	2	0	0	0	0
6	M	15.0	0	2	0	0	0	0	0	0	0

444

Table 4. Serum interferon titers of pigs restimulated with Poly I:C intravenously

Pig No.	Sex	Body weight kg	Poly I:C dose mg/kg	Physiological saline dose ml	Interferon titer hours postinoculation						
					0	2	6	12	24	48	72
2	M	19.8	.1	0	0	16	2	2	0	0	0
3	F	18.6	.1	0	0	32	0	0	0	0	0
4	F	19.0	0	2	0	0	0	0	0	0	0

Table 5. Properties of serum interferon induced by Poly I:C in pigs

Treatment	Results
1. Dialysis for 48 hours at 4 C	Non-dialyzable
2. Acidification to pH2	Activity retained
3. Activity against different viruses	Broadspectrum activity
Virus	Titer
VSV-NJ	32
Vaccinia	16
4. Heat stability	Heat stable
Control titer	16
Titer after heating 56 C for 30 minutes	16
5. Trypsin sensitivity	Activity destroyed
Control titer	16
Trypsin (2mg/ml) for 2 hours at 37 C	0

One-day-old PK_{15} cell-culture monolayers grown in 30 ml plastic tissue-culture flasks were used. Three strains of Pseudorabies virus of the following titers were used:

PRV-DR	1.6×10^8 pfu/ml
PRV-DR	1.6×10^2 pfu/ml
PRV-Pa	9.1×10^7 pfu/ml
PRV-Pa	9.1×10^2 pfu/ml

PRV-Be	2.0×10^8 pfu/ml
PRV-Be	2.0×10^2 pfu/ml

The cell monolayers were inoculated with 1 ml of virus per flask which was allowed to adsorb for 60 minutes at 37 C. The inoculum was then poured off and cells were thoroughly washed with 5.0 ml of the maintenance medium. A sufficient number of cell culture flasks was used to allow collection of cell culture fluids at 2, 6, 12, 24 and 48 hours after exposure. After dialysis, centrifugation and filtration, cell culture fluids were assayed for interferon activity using VSV-NJ as indicator virus.

Experiment III - Results

All three strains of pseudorabies virus at the two concentrations tested failed to induce the production of detectable interferon in PK_{15} cell cultures. Cytopathic effect started after 16 to 24 hours and the experiment was stopped after the 48 hours postinoculation, since almost 100 per cent of the cells showed cytopathic effects.

Experiment IV - Procedure

Interferon induction in pigs by pseudorabies virus.

The experimental pigs were inoculated intravenously into the ear veins with PRV-Pa containing 3.7×10^7 pfu/ml. The dosage levels are indicated in Table 6. Prior to inoculation and at 2, 6, 24, 48, 73, 96, 120, 144 and 168 hours post-

inoculation, blood samples were collected from the orbital sinus. The sera were harvested and the interferon concentrations were determined using VSV-NJ as indicator virus. Rectal temperatures recorded immediately before inoculation and every day afterwards. The interferon was characterized using the tests which have been described before.

Experiment IV - Results

A detectable low level of interferon was produced by 6 hours postinoculation only in a pig number 7. All the pigs, except the control, produced interferon by 24 hours postinoculation. Due to electrical failure, resulting in an inadequate air circulation in the isolation units, pigs number 9 and 10 died from suffocation at 48 hours postinoculation. Maximal interferon titers were reached in pigs number 8 and 11 at 72 hours postinoculation. Pig number 7 reached the peak titer at 24 hours. The serum interferon levels diminished thereafter but in pig number 11 titers remained at detectable levels through 144 hours postinoculation. Pigs number 7 and number 8 had detectable interferon in their sera until 96 hours postinoculation (Table 6). Both died displaying typical symptoms of pseudorabies infection on the 5th day after the inoculation of virus.

The serum interferon, induced by PRV-Pa in pigs, was nondialyzable, resistant to pH2, trypsin sensitive and lacked viral specificity as shown by activity against VSV-NJ and vaccinia

viruses. It also possessed species specificity. The activity was reduced by heating to 56 C for 30 minutes. Results are given in Table 7.

All infected pigs showed a febrile response at the different times postinoculation of a virus. No positive correlation between febrile responses and serum interferon titers was observed.

Experiment V - Procedure

Interferon induction in PK_{15} cell cultures by swine influenza virus (SIV).

One-day-old PK_{15} cell monolayers grown in 30 ml plastic tissue-culture flasks were used. One ml of swine influenza virus with a titer of 320 hemagglutinating units per ml was inoculated per flask and allowed to adsorb for 1 hour at 37 C. The cells were then thoroughly washed with maintenance medium and replenished with 5.0 ml of maintenance medium. The fluids were collected at 2, 4, 6, 12, and 24 hours after exposure. After dialysis, centrifugation and filtration, the cell culture fluids were assayed for the interferon activity using VSV-NJ as indicator virus.

The interferon was characterized using the tests which were described before.

Table 6. Serum interferon titers of pigs given injections of PRV-Pa intravenously

Pig No.	Sex	Body weight (kg)	PRV-Pa dose ml	Saline G dose ml	Interferon titer hours postinoculation									
					0	2	6	24	48	72	96	120	144	168
7	F	11.3	1.0	0	0	0	2	64	32	32	32	Died	-	-
8	F	10.4	1.0	0	0	0	0	64	64	128	32	Died	-	-
9	F	12.3	0	1.0	0	0	0	0	Died	-	-	-	-	-
10	F	13.6	1.0	0	0	0	0	16	Died	-	-	-	-	-
11	F	11.8	1.0	0	0	0	0	4	4	64	16	2	2	0

Table 7. Properties of interferon induced by PRV-Pa in pigs

Treatment	Results
1. Dialysis for 48 hours at 4 C	Non-dialyzable
2. Acidification to pH2	Activity retained
3. Activity against different viruses	Broadspectrum activity
Virus	Titer
Vaccinia	32
VSV-NJ	32
4. Heat stability	Heat stable
Control titer	32
Titer after heating 56 C for 30 minutes	16
5. Trypsin sensitivity	Activity destroyed
Control	16
Trypsin (2mg/ml) for 2 hours at 37 C	0
6. Activity against VSV-NJ in heterologous cells	Not active
Titer on PK_{15}	16
Titer on MDBK	0

Experiment V - Results

A low level of interferon was detected in cell culture fluids, collected at 2 hours postexposure. A maximal titer of 32 was detected at 6 hours. The results are presented graphically in Fig. 2.

The viral inhibitor, produced on PK_{15} cells by SIV was considered to be an interferon since it was acid-resistant, non-dialyzable, non-sedimentable at 100,000 g for 60 minutes, trypsin sensitive, relatively heat stable, active against different viruses and possessed species specificity. The results are summarized in Table 8.

Experiment VI - Procedure

Interferon induction in pigs by swine influenza virus.

Experimental pigs were inoculated intravenously with SIV-containing allantoic fluid with a titer of 320 hemagglutinating units per ml. A control pig was inoculated with allantoic fluid from uninfected 13-day-old chick embryos. The dosage levels are indicated in Table 9. Before inoculation and at 2, 6, 12, 24, 48, 72, 96 and 168 hours postinoculation, blood samples were collected from the orbital sinus and interferon assayed using VSV-NJ. Rectal temperatures were recorded each day.

Pigs number 14 and 15 were restimulated with SIV administered intranasally on the 14th day after the first stimulation. Pig number 12 not previously exposed to virus also received SIV intranasally. Dosage levels are indicated in Table 10. Blood samples were collected at 0, 6, 12, 24, 48, 72 and 120 hours postinoculation. The antiviral substance was characterized as interferon using the same tests which were described before.

Experiment VI - Results

Interferon was not detectable in the serum of pig number 14 which received SIV. Pig number 16 produced a low level of interferon (titer of 2) only at 6 hours postinoculation. Low levels of the interferon were detectable in sera collected at 6, 12, 24 and 48 hours after inoculation of the pig number 13. The peak titer was reached at the 12th postinoculation hour. Pig number 15 produced higher titers of an interferon, reaching a maximum titer of 8 at 24 hours postinoculation. Detectable interferon was found on the third postinoculation day (72 hours). The results are given in Table 9.

Intranasally injected SIV virus induced low levels of detectable interferon in pig number 12. Interferon was detected in sera harvested at 6, 12 and 24 hours postinoculation. Pigs number 14 and 15, restimulated with SIV by the intranasal route, produced low levels of interferon which was found at 12, 24 and 48 hours postinoculation (Table 10).

The interferon was characterized as being acid-resistant, non-dialyzable and trypsin-sensitive. It did show an antiviral activity after the ultracentrifugation. The results are summarized in Table 11.

All intravenously injected pigs showed a slight febrile response. A positive correlation between febrile response and a serum interferon titer was not observed.

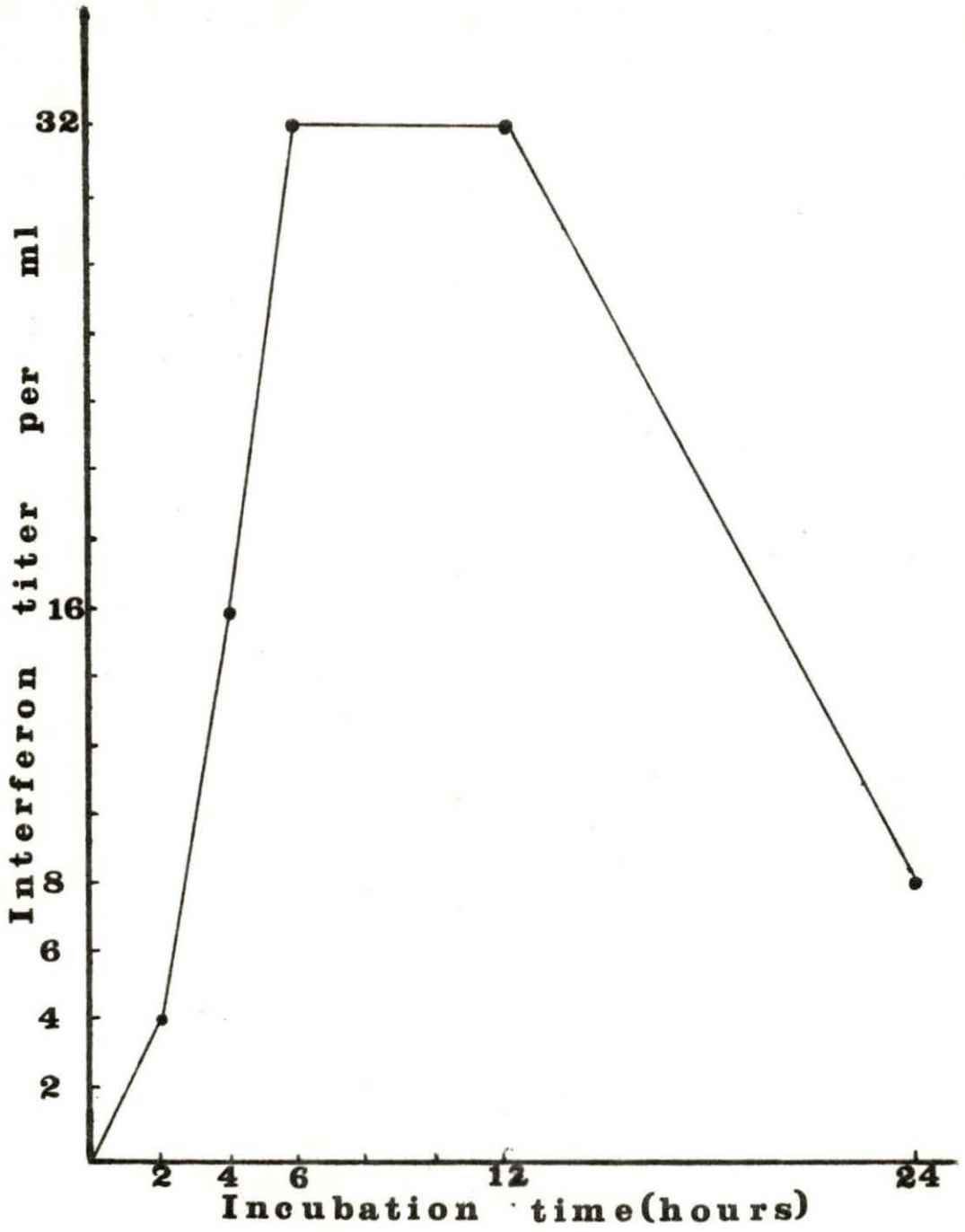

Fig.2 Induction of interferon in PK_{15} cells by treatment with SIV

Table 8. Properties of PK_{15} cell culture interferon induced by SIV

Treatment	Results
1. Dialysis for 48 hours at 4 C	Non-dialyzable
2. Acidification to pH2	Activity retained
3. Activity against different viruses	Broadspectrum activity
Virus	Titer
VSV-NJ	32
Vaccinia	8
PRV-DR	2
4. Ultracentrifugation	
Titer before ultracentrifugation	16
Titer after centrifugation	16
5. Heat stability	Relatively stable
Control titer	16
Titer after heating 37 C for 6 hours	16
Titer after heating 37 C for 12 hours	4
Titer after heating 56 C for 30 minutes	4
6. Trypsin sensitivity	Activity destroyed
Control titer	16
Trypsin (2mg/ml) for 2 hours at 37 C	0
7. Activity against VSV-NJ in heterologous cells	Not active
Titer on PK_{15}	32
Titer on BHK	0
Titer on MDBK	0

Table 9. Serum interferon titers in pigs given injections of SIV intravenously

Pig No.	Sex	Body weight kg	SIV dose ml	Normal allantoic fluid ml	Interferon titer hours postinjection								
					0	2	6	12	24	48	72	96	168
12	F	14.5	0	1.5	0	0	0	0	0	0	0	0	0
13	M	14.8	1.0	0	0	0	2	4	2	2	0	0	0
14	M	14.5	2.0	0	0	0	0	0	0	0	0	0	0
15	M	13.7	2.0	0	0	2	2	4	8	4	4	0	0
16	M	13.2	1.0	0	0	0	2	0	0	0	0	0	0

Table 10. Serum interferon titers in pigs inoculated with SIV intranasally

Pig No.	Sex	Body weight kg	SIV dose ml	Interferon titer hours postinoculation						
				0	6	12	24	48	72	120
12	F	20.2	1.0	0	2	2	2	0	0	0
14	M	20.3	1.0	0	0	2	4	4	0	0
15	M	19.8	1.0	0	0	2	4	2	2	0

Table 11. Properties of interferon induced by SIV in pigs

Treatment	Results
1. Dialysis for 48 hours at 4 C	Non-dialyzable
2. Acidification to pH2	Activity retained
3. Trypsin sensitivity	Activity destroyed
Control titer	4
Trypsin (2mg/ml) for 2 hours at 37 C	0
4. Ultracentrifugation	Non-sedimentable
Titer before ultracentrifugation	4
Titer after centrifugation	4

DISCUSSION

A synthetic double-stranded polyribonucleotide complex, Poly I:C, has previously been shown to be an effective inducer of interferon in cell cultures (50, 51, 56, 154) in mice (26, 75, 112, 117, 154), rabbits (49, 50, 93, 104), calves (134) and man (53, 116). No report of interferon induction in swine systems in response to Poly I:C has been found in the literature.

In this study Poly I:C has been successfully used to induce interferon both in PK_{15} cell cultures and in pigs. Detectable interferon levels were produced in cell cultures by the second hour postinoculation. Maximal titers were reached by the sixth hour and lower titers were still found after 24 hours. This decrease in titer could be explained as a partial decrease in interferon activity after 24 hours exposure to 37 C. A positive correlation was observed between the concentration of Poly I:C and the titer of interferon produced in PK_{15} cell cultures (Table 1). No plateau was reached using even 320µg/ml concentration of Poly I:C. A minimum Poly I:C concentration of 5 µg/ml was required to induce a detectable interferon level in PK_{15} cell cultures. This concentration of Poly I:C is much higher than that required for interferon induction in primary rabbit kidney cell cultures (51, 75), dog kidney and human embryonic kidney (51) cell cultures. It is known that cell cultures vary in their sensitivity to an inducer with respect

to their species of origin. Variation in sensitivity also exists between primary and line cells and even stable line cells show variable sensitivity the cause of which is not known (51). One variable which should not be overlooked is the fact that the source of Poly I:C used by different investigators may have been different and variations in sensitivity of cell systems may be due to this fact.

An antiviral agent, produced in PK_{15} cells in response to Poly I:C was found to fulfill the biological criteria to be classified as an interferon (Table 2). It was a macromolecule of relatively small size as indicated by its lack of sedimentation at 100,000 g for 1 hour and the fact that it was non-dialyzable. It possessed stability over a broad range of pH and sensitivity to trypsin which indicated that the active antiviral substance was protein in nature. The antiviral property was partially inactivated by heating at 56 C for 30 minutes. Broad-spectrum antiviral activity was demonstrated by inhibition of VSV-NJ, PRV-DR, PRV-Pa, PRV-Be and vaccinia viruses. Differences in interferon activity against the different viruses was observed. All three strains of pseudorabies virus were less sensitive to interferon action than VSV-NJ or vaccinia virus. This is probably due to the inherent properties of the virus (59).

A narrow host species specificity was shown by treating heterologous BHK and MDBK cell cultures with the antiviral

substance produced on PK_{15} cells. No antiviral activity was shown on BHK cells even though it is known that these cells are capable of producing an active inhibitor of virus replication in a response to interferon (51, 75). No antiviral activity was shown on MDBK cells after treatment with the antiviral substance produced on PK_{15} cells.

The intravenous injection of Poly I:C into pigs resulted in the appearance of an antiviral substance in the serum (Table 3). This substance has been identified as an interferon as it is non-dialyzable, pH stable, sensitive to trypsin, possess broad antiviral spectrum and is heat stable (Table 5).

The response of the pigs to Poly I:C by the production of circulating interferon, resembles that of the rabbit, mouse, man, calf and rat and is unlike the response of the monkey, dog and guinea pig which failed to develop detectable circulating interferon following intravenous injection of Poly I:C (53).

The peak of interferon production in all five pigs occurred at 2 hours after intravenous injection of Poly I:C. The circulating interferon decreased rapidly and was detected at low titers in only 3 out of 5 pigs at 6 hours after the drug administration. By 12 hours postinoculation no interferon was found. This rapid appearance and early disappearance of the Poly I:C-induced inhibitor is similar to the response seen in rabbits (50) and calves (134). The serum interferon did not

reach the high concentrations reported for mice and rabbits (50, 155). In vitro, PK_{15} cell cultures yielded a higher titer of interferon than in vivo and the production was dose-related. The magnitude and the duration of the response in vivo did not appear to be dose-related within the dose range of the drug used. It is possible that the optimal intravenous dose of Poly I:C was not reached. This may explain why the interferon titer in vivo was not as high as in vitro. The interferon titer in pigs ranged from 4 to 128 following the intravenous Poly I:C injection. It appears that a host sensitivity to the inducer varies widely within the species.

The pigs given Poly I:C in repeated injections on the seventh day after initial stimulation were capable of interferon reinduction. However, the interferon titers were lower than after the first stimulation in both pigs (Table 4). Unfortunately, insufficient data was obtained to define the amount of Poly I:C required to bring hyporesponsiveness or to determine the duration of the refractory period. From these results it appears that refractoriness in pigs is of relatively short duration, less than seven days.

Neither pyrogenicity nor toxicity was observed in pigs in response to intravenous inoculation of Poly I:C. This is in contrast to reports about the effect of intravenous inoculation of Poly I:C in calves (134), man (53), dogs (75) and rabbits (104). Apparently pigs are less sensitive to I.V. inoculation

of Poly I:C. Pyrogenicity was reported in man after IV administration of Poly I:C at .002 to .4 mg/kg (53). Rabbits showed a febrile response after receiving .0005 mg/kg IV (104). Toxicity and pyrogenicity was observed in calves which were inoculated with .01 to .92 mg/kg IV. (134). Poly I:C at a dosage of .25 mg/kg given to dogs by inhalation was toxic (75).

Interferon production in response to PRV *in vivo* or *in vitro* in a swine or any other species has not been reported. It is generally thought that DNA-containing viruses are poor interferon inducers. Nevertheless, some of them, such as vaccinia (54), Herpes simplex (6), IBR (136) and adenoviruses (54) induce detectable interferon levels in cell cultures or *in vivo*.

In this study all three strains of pseudorabies virus at the two concentrations tested failed to induce production of interferon *in vitro*. An observable cytopathic effect (CPE) started after 16 hours following PRV inoculation at high (10^7-10^8 pfu/ml) concentration. By 36 hours all the cells showed CPE. Observable CPE started later using PRV strains at low (10^2 pfu/ml) concentrations, but by 48 hours complete CPE was observed. Since PK_{15} cells produced interferon in response to other inducers (Poly I:C, SIV) they obviously possess the genetic capability for interferon production. Results of this study confirm the earlier findings (51, 64, 93, 121) that Herpes group viruses are poor interferon inducers *in vitro*.

Pseudorabies virus did however induce circulating interferon in vivo in pigs (Experiment 4). The most acceptable explanation for the failure of PRV to induce interferon in PK_{15} cell cultures would be that the virus rapidly shuts off the host RNA synthesis. Apparently the PK_{15} cells were damaged by PRV-replication prior to interferon synthesis.

In this study it was found that PRV-Pa readily induced the formation of interferon in pigs. All four pigs inoculated intravenously with the PRV-Pa produced detectable interferon. Interferon could be detected as early as 6 to 24 hours after the pigs were inoculated and persisted in serum until their death (in pigs number 7, 8, 10) or as long as 144 hours post-inoculation in pig number 11 which recovered from PRV infection (Table 6). The peak of interferon production occurred at 24 to 72 hours after intravenous inoculation and it diminished afterwards. All the injected pigs showed a febrile response at the different times postinoculation. No positive correlation between febrile response and serum interferon titers was observed. The serum antiviral substance was identified as an interferon based on the parameters described before (Table 7).

The titer of PRV-induced interferon varied from 16 to 128 in different pigs and the titers ranged from 4 to 128 in response to Poly I:C. Surprisingly PRV-Pa appeared to be an effective interferon inducer in pigs. The persistance of virus-induced interferon was much longer than Poly I:C induced

interferon. This can be explained by the fact that virus is able to replicate in swine and stimulates the interferon-producing cells for a longer period of time. Since Poly I:C has only one-step activity, its interferon-inducing capacity is limited to the initial dose.

The enveloped, single-stranded RNA-containing viruses of the Myxovirus group are considered to be good interferon inducers (54, 87, 144, 158). For this reason swine influenza virus was used in this study.

A low level of interferon was detected in PK_{15} cell culture fluids collected at two hours postexposure to SIV. The maximal titer of 32 was reached at six hours postexposure (Figure 2). Interferon was identified by the usual criteria (Table 8).

In contrast to what one would expect swine influenza virus was found to be a very poor interferon inducer in pigs. One of the four pigs which were infected intravenously with SIV did not produce any detectable interferon in its serum. Pig number 16 produced a low level (titer of 2) at 6 hours post-inoculation. Pig number 13 produced a detectable interferon level at 6 hours postinoculation a titer which lasted until 48 hours postinoculation. In pig number 15, interferon appeared as early as 2 hours and lasted until 72 hours postinoculation. Peak titers of 4 and 8 respectively were reached at 12 to 24 hours postinoculation (Table 9).

The poor interferon inducing ability of SIV <u>in vivo</u> may

be a characteristic of the virus strain. It has been reported in the literature that different myxovirus strains possess variable interferon inducing ability (76, 144). Soloviev and Gutman (144) found that influenza A2 virus strains which did not induce interferon may become interferogenic after ten successive passages in chick embryos or in mice.

The Shope strain of swine influenza virus used in this study was of an undetermined passage level in chicken embryos. The passages may have altered the interferon-inducing ability of the virus or this particular strain may be a poor interferon inducer. Since interferon was induced in PK_{15} cell culture using the same virus strain, another possibility is, that the dose administered was not optimal. However, 640 hemagglutinating units of SIV inoculated intravenously into pig number 14 did not induce detectable interferon while the same dose in pig number 15 induced the highest interferon titer in vivo (titer of 8) (Table 9). SIV at a concentration of 320 HA units induced low levels of circulating interferon in both pigs inoculated. Apparently the Shope strain of SIV is a poor interferon inducer in vivo.

Even though all three pigs inoculated intranasally produced detectable interferon, it is not possible to draw the conclusion that the respiratory route of infection with SIV is more efficient than I.V. inoculation for interferon production at the virus dose levels used. Pig number 14, which did not pro-

duce interferon after intravenous inoculation with SIV, when restimulated intranasally on the 14th day, produced a low level of circulating interferon. This can possibly be explained by assuming that an undetectable level of interferon was produced after the first stimulation and that this earlier interferon induction had a priming effect on the pig. Pig number 12, not previously exposed to the virus, produced a very low level of interferon (titer of 2) which was detected in the serum from 6 to 24 hours postinoculation. Pig number 15, restimulated intranasally, produced an interferon response which was of lower titer and shorter duration than the interferon resulting from initial IV stimulation. No priming effect was observed.

Isaacs, A., et al. (89) observed that pretreatment of chicken embryo fibroblast cell cultures with a large dose of crude interferon inhibits production of interferon in response to avian influenza virus. They suggested that the inhibition was due to a "blocker". It is possible that the decreased response to restimulation observed in pig number 15 is the result of the action of a "blocker" produced during the IV stimulation. A more likely explanation is that the response observed reflects the individual characteristics of the experimental pig.

The results of these investigations show that pigs and porcine cell culture can produce interferon in response to several inducers. A synthetic ribonucleic acid (Poly I:C) and

SIV produced detectable interferon on PK_{15} cell cultures, whereas, pseudorabies virus appears to be a poor inducer producing no detectable interferon *in vitro*. Poly I:C was found to be a potent interferon inducer in pigs. In contrast to what one would expect PRV-Pa was found to be a better interferon inducer than SIV-Shope strain *in vivo*.

The interferon system is recognized to play an important role in recovery from viral infections (9, 10, 71). Exogenous and especially endogenous interferons look very promising for clinical use. The present studies have demonstrated that it is feasible to stimulate significant levels of endogenous interferon in pigs.

Hopefully, potent viral and artificial interferon inducers will be developed in the near future to help us in controlling viral diseases in man as well as in his animals.

SUMMARY

The production of interferon by pigs and porcine cell cultures (PK_{15}) in response to several inducers was studied. The inducers used included synthetic polyriboinosinic-polyribocytidylic acid (Poly I:C), swine influenza virus (SIV) and pseudorabies virus (PRV). Following exposure to these inducers cell culture fluids and pig sera were examined for interferon by the plaque-reduction method on PK_{15} cell cultures using VSV-NJ as the challenge inoculum.

The synthetic double-stranded ribonucleic acid Poly I:C and the SIV (Shope) induced production of interferon on PK_{15} cell cultures, whereas, all three strains of PRV virus at the two concentrations tested failed to induce production of interferon *in vitro*.

Detectable interferon levels were produced in cell cultures in response to Poly I:C and SIV by the second hour postinoculation and maximal titers were reached by the sixth hour postinoculation. A positive correlation was observed between the concentration of Poly I:C and the titer of interferon produced in PK_{15} cell cultures. The synthetic double-stranded Poly I:C, SIV (Shope) and PRV-Pa all induced circulating interferon when injected IV into pigs. The Poly I:C was found to be a potent interferon inducer. In contrast to what one would expect PRV-Pa was found to be a better interferon inducer than SIV *in vivo*.

The peak of circulating interferon in response to Poly I:C occurred at 2 hours after I.V. injection. Serum interferon either was not detected or was reduced in titer by 6 hours postinjection. The magnitude and the duration of the response did not appear to be dose-related within the dose range of the drug used. Neither pyrogenicity nor toxicity was observed in pigs in response to I.V. inoculation of Poly I:C. Apparently pigs are less sensitive to I.V. inoculation than man, rabbits, dogs and calves. The pigs restimulated intravenously with Poly I:C on the seventh day after initial stimulation were capable of interferon reinduction. It appears that refractoriness in pigs is of relatively short duration, less than seven days.

All four pigs inoculated intravenously with PRV-Pa produced detectable interferon. Interferon could be detected as early as 6 to 24 hours after the pigs were inoculated and persisted in serum until their death or as long as 144 hours postinoculation. All infected pigs showed febrile responses at the different times postinoculation. No positive correlation between febrile responses and serum interferon titers was observed.

Swine influenza virus (Shope) was found to be a very poor interferon inducer in pigs either by intravenous or intranasal inoculation.

One of the four pigs which were infected I.V. with SIV did not produce a detectable level of interferon. Another pig

produced a very low interferon level of short duration. The other two pigs produced detectable interferon at 2 to 6 hours postinoculation. Maximal titers were reached at 12 to 24 hours and circulating interferon was detected as long as 48 to 72 hours postinoculation.

The interferon produced in cell cultures and in pigs was identified as an interferon by the fact that it was pH stable, non-dialyzable, sensitive to trypsin, non-sedimentable, it possessed broad-spectrum antiviral activity and host species specificity and it was heat stable.

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