PROPERTIES OF BOVINE INTERFERONS

by

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A THESIS

IN

MEDICAL MICROBIOLOGY

Submitted to the Graduate School of
Texas Tech University Health
Sciences Center in Partial
Fulfillment of the
Requirements for
the Degree of

MASTER OF SCIENCE

Approved

Accepted

December, 1982

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ACKNOWLEDGMENTS

I wish to express my most sincere gratitude to Dr. Stanley S. Lefkowitz for his patient guidance throughout my graduate studies at Texas Tech University Health Sciences Center.

My appreciation is also expressed to Dr. Shanti Bilimoria and Dr. John M. McKenna for their suggestions and academic advice.

I am also grateful to those persons in the laboratory for their technical assistance and especially for their friendship. Also, my gratitude is directed towards the Department of Microbiology for providing me with research facilities, financial assistance, and friendship.

I am most grateful also to my family for their moral support throughout my years of schooling.

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CHAPTER I

INTRODUCTION

Statement of the Problem

Interferons are proteins made by cells in response to certain viral and nonviral stimuli. Interferons in turn stimulate the production of new antiviral proteins which protect other cells from virus infections. Some interferons have other effects on cells in addition to their antiviral properties such as inhibition of cell growth, modulation of immune responses, enhancement of interferon production, etc. Many of these responses involve the induction of intracellular enzymes.

Since interferons were discovered it has been widely accepted that they have species-specificity. A number of exceptions have been reported in recent years. It was found that interferon preparations from both bovine and porcine sources have antiviral activity on human cells; thus, it became practical to search for an interferon of nonhuman origin which could display high antiviral activity on human cells. It was hoped that these interferons could be used to treat human diseases.

Clinical studies have been limited by the amount of interferon available as well as the expense incurred in

producing it. Until recently only a few laboratories could produce human interferon in any significant amounts. As noted above, bovine interferon (IFN) could "theoretically" be used to protect humans from viral diseases. The number of cattle slaughtered each year in the U.S. could provide enormous quantities of cells which could produce enough IFN for many long term clinical studies.

To date, there have been few reports on the biological and physical properties of the bovine interferons. In addition, if bovine interferons are to be used in clinical trials and veterinary medicine it is essential to know their physicochemical properties. Furthermore, there is currently no consistent nomenclature system for the different bovine interferons. The old nomenclature of leukocyte, fibroblast, and immune interferons is still in use. This same nomenclature will be used throughout this thesis.

The purposes of this research were to elucidate some of the properties of bovine IFNs with the hope of discriminating between the classes of IFNs, and to see if they have similar properties to interferons of other mammalian species.

Background

Historical Survey

In 1957, Alick Isaacs and Jean Lindenmann discovered a protein which conferred resistance to viral replication in normal cells. The protein, which they found in fluids from virus-infected cell cultures, was termed "interferon" (48,80).

Interferon has been defined as ". . . a protein which exerts virus non-specific antiviral activity at least in homologous cells, through cellular metabolic processes involving synthesis of both RNA and protein" (144,145).

Since interferon was initially described, a vast amount of literature on the area has accumulated (8,117,118,143). A number of interferon types has been described suggesting considerable heterogeneity. The different interferons have been characterized on the basis of physical, chemical, and biological properties (113,136,147). Initially, interferons were classified according to their inducers and the cell types producing them. Traditionally, the two major interferon classes had been called type I and type II interferons (45,94,113), which are referred to as "virus" and "immune" types, respectively. "Virus" type interferons are normally induced by viruses or synthetic polynucleotides

(45,150,168,171,175), whereas "immune" type interferons are normally induced in lymphocytes by specific antigens or mitogens (62,84,94,96,113,153). Further, virus type interferons are heterogeneous and consist of two major antigenic types. They are referred to as leukocyte and fibroblast interferons.

Recently a new system was devised for the classification of interferons (144,145). The nomenclature was established for both human and murine interferons. These are the best characterized of all interferons. The type designations are alpha, beta, and gamma, which correspond to the former designations of leukocyte, fibroblast, and immune interferons respectively. Interferon alpha is represented by a multigene family which may code for various interferon molecules with similar primary amino acid sequences (100,111,154). Also, each interferon is identified according to the species of origin (144,145). For instance, human interferon is expressed as HuIFN, mouse as MuIFN, and bovine as Bov-IFN.

Since there are insufficient data about interferons from other animal species, the above nomenclature cannot be applied to them. Investigators studying the interferons from nonhuman and nonmurine origin should attempt classification on the basis of antigenic homologies

using specific antisera. Unfortunately, there are no specific antibody preparations available to characterize bovine interferons.

Properties of Interferons

Owing to their proteinaceous nature, interferons can be destroyed by proteolytic enzymes, but not by nucleases. Some interferons have carbohydrate moieties in their structure, and the function of such regions is unclear. It has been suggested that there is a relationship between glycosylation of the molecule and the "species barrier" (15). Human fibroblast interferon is extensively glycosylated and its activity on heterologous cells is at least one thousand-fold less than human leukocyte interferon (81). Also, other leukocyte interferons of porcine origin have an antiviral activity several-fold higher when measured on human cells than on their homologous cells (16). On the other hand, interferon obtained from animal fibroblast cells does not show crossspecies activity; these interferons are highly glycosylated (26). The cross-protection ability of different interferon preparations may be helpful in separating alpha interferon from beta interferon. Also a model was proposed for the existence of specific receptors on cells which recognize specific carbohydrate moieties on

the IFN molecule (20). However, this model is still subject to question. After the removal of the carbohydrate from the polypeptide it has been observed that interferon maintains its activity (149). Also, the same observation was made with IFN produced by recombinant DNA methods, which suggests that the carbohydrates may not be necessary for the biological activity of interferons.

In terms of the chemical properties of the different interferons it has been established that virus-induced or alpha and beta interferons are generally stable in an acid pH of 2 (48,94,113,145,173), whereas immune or gamma interferons are labile at pH 2 (48,94,113,145,173,176). For many years, before the discovery of gamma interferons, pH 2 stability was a parameter used to classify a virus inhibitor as interferon. Now, stability at low pH can be used to separate alpha and beta interferons from gamma interferons. In the bovine system the pH stability of interferons is not known. Contradictory reports have appeared in the literature, even from the same laboratory, describing differences in pH 2 stabilities for virus-induced interferons (2,25,55,130,132).

In addition to pH, temperature stability has been used as a property to separate IFN classes. Virus-

induced interferons and immune interferons differ in their temperature stability at 56 degrees centigrade. Human immune type is more stable at that temperature (48,145,173) than alpha or beta.

Another chemical property that descriminates among alpha, beta, and gamma interferons is the instability of the molecules in the presence of ionic detergents and reducing agents. Human alpha interferon is more stable, beta interferon is partially stable, and gamma interferon is unstable (110,143,146,165).

One of the most important properties of interferons relative to their separation is their distinct antigenic specificity (176). Virus-induced interferons and immune interferons are antigenically different (173). Further, alpha and beta interferons were shown to be antigenically distinct (69,165). It was originally shown that antisera made against leukocyte interferon can neutralize both alpha and beta interferons, whereas antiserum made against fibroblast interferon can only neutralize beta interferon. It was later found by Havel and his colleagues (69) that in the case of human leukocyte interferon preparations, there were two interferon populations, both of which were antigenically distinct. It is clear today that there is little cross-reaction antigenically between the classes of interferon.

The very high biological activity of IFN is one of the most striking properties of interferon. Friedman (48) expresses the specific activity of mouse alpha and beta interferons as being between 2×10^8 to 2×10^9 units/mg of protein. This theoretically means that one molecule of interferon can protect one cell. He also expresses this activity another way in that interferons are active at a concentration of 10^{-15} M.

Production of Interferon

Interferons can be produced by most nucleated cells when exposed to the appropriate inducer. It is difficult to pinpoint a chemical structure that is responsible for interferon induction since a wide spectrum of agents can induce interferons both in vitro and in vivo.

The first inducers described were viruses (7,80). All, single- or double-stranded RNA or DNA, viruses induce interferon (143). Some viruses are better inducers than others. In most production systems paramyxoviruses are used. The viruses frequently employed for the production of bovine interferons include Sendai virus (141), Newcastle disease (71,132), parainfluenza-3 (72,130), foot and mouth disease (40), infectious bovine rhinotracheitis (25,54,55,56,131,158), and bovine viral diarrhea (127).

For some time it has been thought that viruses were

good interferon inducers because of their ability to form replicative intermediates involving double-stranded RNA. This notion became more acceptable when certain forms of double-stranded RNA were found to be excellent inducers of interferon (45). However, interferons have been found to be induced by agents that do not seem to involve any double-stranded RNA.

As stated by Stewart (143), the first nonviral inducer reported was probably endotoxin, which was found to protect mice against virus diseases (59,60). Among the bacterial genera which have been reported to be involved in the induction of interferon are *Brucella* (10,177), *Escherichia* (73), *Haemophilus* (29), *Salmonella* (152), *Serratia* (152), *Staphylococcus* (29), and *Corynebacterium* (86). Besides intact bacteria, bacterial products have been implicated in interferon induction (63,85,151,152,174). Other organisms reported to be involved in interferon induction include fungi (5,6,93,139), chlamydia (82,89,107), rickettsiae (68), mycoplasma (22,23,126,128), and protozoa (137,138).

In addition to the naturally occurring inducers, there are a number of other interferon inducers which are of synthetic nature. One of the most widely used is a synthetic double-stranded RNA molecule referred to as polyinosinic:polycytidylic acid or poly I:C (45). In the

search for a molecule less toxic than poly I:C and superior to it for the induction of interferon a number of chemical modifications have been made to poly I:C (9,27,123). The results so far have not yielded a more active compound. Other synthetic inducers that have been reported include anionic polymers (28,36,106,125), tilorone (30,58,91), cationic dyes (57), and propane-diamine (75).

As stated above, all nucleated cells are capable of producing interferon. Viruses can induce virtually any cell to synthesize the molecules, whereas antigens or mitogens act as inducers only in T-lymphocytes. Fibroblasts do not respond to either mitogens or antigens (172). Knowledge of the inducing agents and the cells capable of producing interferon allows the researcher the option of synthesizing the molecule in vivo or in vitro.

The above discussion indicates that the number of inducers are quite varied. One characteristic that they have in common is their ability to alter protein synthesis in the cell (172).

For in vivo production of bovine interferon cattle have been injected with viruses (25,56,130,131,132,158) and poly I:C (129,132). The levels of circulating interferon induced in calves were

frequently low (131,132). The maximum titer of circulating interferon was about 100 units/ml. The route of administration has been shown to be important. The highest interferon titers were obtained when the virus was administered intravenously, whereas intramuscular administration resulted in low but persistent activity (131). Rosenquist and Loan (131) observed that after intravenous injection of infectious bovine rhinotracheitis, the peak titers in the animal were reached within the first day post-inoculation and detectable serum interferon levels persisted as long as 7 days.

High levels of interferon have also been detected in the nasal secretions of calves after intranasal administration of IBR virus (25,158). The peak titers of interferon were detected anywhere between 24 and 40 hours post-inoculation, depending on the dosage and the strain of the virus used. Again, interferon was detected as long as 7 days after induction.

Due to the toxicity of the inducers, the low titers obtained, and the difficulty in purifying both sera and nasal interferons, it does not appear to be practical to produce bovine interferon in vivo. In vitro production would be more practical. In vitro synthesis of interferons by bovine cells has been achieved quite successfully (40,54,55,130,141). Calf kidney cells have generally

been the cells of choice. Agents used to induce interferon were infectious bovine rhinotracheitis virus (25,55), parainfluenza-3 virus (71,130), Newcastle disease virus (71), foot and mouth disease virus (40), and poly I:C (45) among others. Besides kidney cells, cells from other bovine tissues have employed for the production of interferon, among which are cells of tracheal origin (53), lung (133), testicular (121), leukocytes (54,55,121,144), and spleen (55).

If interferon is to be potentially useful for treating human and animal diseases, it would be important to produce large amounts of the substance. Three methods were suggested by Friedman (48) to obtain higher quantities of interferon: a) improving the methods now used, b) chemically synthesizing the molecules, and c) cloning the genes.

Most of the interferon preparations that have been tested have come from the stimulation of leukocytes with paramyxoviruses. The two main sources of these alpha interferon preparations have been the laboratory of Dr. Kari Cantell in Helsinki, Finland, and Wadley Institutes of Molecular Medicine in Dallas, Texas. A good source of human alpha interferon is the Namalva cell line, which is a lymphoblastoid line derived from a Burkitt's lymphoma. The latter source of interferon is not used in

this country due to the possible presence of occult human viruses. Beta interferon preparations have also been produced but they have the tendency to stay at the site of injection and do not enter the circulation. An elimination of the carbohydrate moieties could alter this property.

Alpha and beta human interferons have been sequenced (3,35,111,154), which opens the possibility for the chemical synthesis of interferon molecules. Gamma has also been sequenced. It is possible to synthesize DNA sequences coding for interferon molecules (48).

The cloning of the interferon genes has already been accomplished. The recombinants containing the sequences for human leukocyte interferon were isolated by Maeda et al. (103) and Nagata et al. (112). Other groups were responsible for isolating the recombinants containing the sequences for human fibroblast interferon (35,61,156). The cloning of interferon genes will make available large amounts of pure interferons for various clinical studies.

Mechanisms of Interferon Synthesis

Since interferon is an induced protein, its gene must be derepressed. The induction process takes several hours. The exact mechanism of induction is not known. It is known that the synthesis of interferon requires the

derepression of interferon gene(s) resulting in the synthesis of interferon mRNA (46). Prior to the transcription process there must be binding, uptake, and processing of the inducer (143).

Production of interferon resembles the synthesis of other secreted glycoproteins. It involves the transcription of mRNA, its translation, posttranscriptional processing of the protein, and secretion of the interferon molecule (48).

The existence of the mRNA has been confirmed by experiments in which interferon mRNA obtained from cells of one species was translated in cells of a heterologous species (17,34,90). Also, interferon has been synthesized in cell-free protein synthetic systems using mRNA obtained from induced human cells (13,119,120).

Interferon synthesis starts soon after induction in most types of cell cultures. Peak titers occur within 24 hours followed by a gradual decline of interferon synthesis (74). The presence of a negative control for the production of interferon has been speculated (155,163,167). It has been suggested that there is an inhibitor synthesized which is responsible for the shutting-off of interferon production (166). As stated by Yabrow (172), The inhibitor has not been isolated. It seems to be proteinaceous in nature (166), and it may

exert its control at a posttranscriptional level (64,124,140,164,166).

One other control mechanism for interferon production addressed by Friedman (48) is the presence of different gene loci related to the inducer. It has not been answered as to why there are so many genetic loci associated with interferon induction. This and other controls of interferon synthesis need to be defined.

Mechanism of Interferon Action

Interferon itself is not sufficient to produce the antiviral state. It cannot prevent virus replication if inhibitors of transcription and translation are present (51,157). This indicates that interferon acts as an inducer of the antiviral protein(s).

Interferon must interact with the cell membrane for production of antiviral proteins (49,142). The existence of membrane receptors for interferon was initially postulated in 1973 by Chaney et al. (21). It has been shown that proteins that bind to ganglioside receptors can inhibit the action of interferon (50,88), which would indicate that gangliosides are essential constituents of the interferon receptor. Chaney suggested that when interferon interacts with the receptor, it triggers the mechanism of action of the molecule (20). It is unclear whether interferon has to enter the cell for the antivi-

ral state to be established or not. It is not known what kind of a message is sent from the membrane to the nucleus of the cell, or how that message reaches the nucleus.

Historically the mode of action of interferon has variously been ascribed to interference with virus attachment, uncoating, transcription, maturation, or release. Later, it was shown that it did not prevent virus attachment, uncoating, or release of virus particles (108,162,169). Most studies have been directed towards the possible role of interferon in altering viral RNA transcription and protein synthesis. The possibility of the influence of interferon on both transcription and translation of viral mRNA was initially suggested by Metz et al. (109) and Oxman (114).

Recent evidence indicates that there are at least two proteins that appear to be linked to the antiviral state induced by interferon. These two proteins are a protein kinase and 2',5' oligoadenylate synthetase (48,99,105,). The protein kinase phosphorylates the small subunit of the protein synthesis initiation factor (eIF-2), which would explain the inhibition of viral protein synthesis in interferon-treated cells. The 2',5' oligoadenylate synthetase is responsible for the production of 2',5' oligoadenylates which activate an endonu-

lease which in itself degrades viral mRNA, thus preventing elongation of nascent chains of viral proteins. A summary of the action of interferon was reported by Marx (105).

Nonantiviral Functions of Interferon

Besides their antiviral effects, interferons have been reported to have a wide variety of other actions. These include immunomodulation, inhibition of cell growth, alteration of cellular structure, regulation of its own production, inhibition of tumor cells, etc.

Interferons have stimulatory as well as inhibitory effects on various aspects of the immune response (32,143,172), affecting both humoral and cellular immunity. For instance, interferon has been shown to regulate the antibody response, depending on the dose of interferon and the time of administration (48). When interferon was given in small amounts at the time of immunization, it stimulated the production of antibody (11). On the other hand, if interferon is given in high doses prior to immunization it inhibits antibody production (12,83). Interferon also affects allograft survival (78), delayed type hypersensitivity (31,33), macrophage functions (76,97,101), histamine release (77), and natural killer cell activity (70).

Interferon also inhibits the growth of both tumor

and normal cells (65). It slows the rate of cell division (66). Interferon treatment of newborn mice causes a marked inhibition of growth and development (67). In man, neonates treated with interferon for congenital cytomegalovirus infection develop an adverse effect on their normal growth pattern requiring cessation of therapy (48).

Other effects of interferon on the cell involve alteration of the cell surface and alteration of the cytoskeleton. Interferon has been shown to increase the negative charges on the membrane of the cell (87), increase the number of intramembrane granules and change the membrane density (19). Pfeffer et al. (122) reported that the actin-containing cytoplasmic fibers are increased in size and number, and the cell surface fibronectin is arranged in arrays of long filaments which cover the surface of interferon treated cells.

Another nonantiviral function of interferon is priming. Cells pretreated with it may alter their production of interferon in three ways (148): a) interferon production may be increased, b) the lag period between the addition of the inducer and interferon synthesis may be shortened, or c) production of interferon may be inhibited. The ability of interferon to prime for its own production was originally described by Isaacs and

Burke (79). They noticed that chick cells exposed to interferon were able to produce more interferon than nonexposed cells when challenged by a virus. Some cells which are unable to produce interferon, when infected with a virus, can produce interferon if they are treated with interferon prior to induction (1,148). Pretreatment of cells with high concentrations of the molecule(s) can have an opposite effect on interferon production, which will be inhibited (47,102). It has also been reported that primed cells produce more interferon than do normal cells when poly I:C is used as an inducer (148). It has been suggested that the enhanced production of interferon in primed cells may be due to an enhanced efficiency of translation of the interferon mRNA's (1).

Bovine Interferon Potential

Bovine interferons have potential applications in controlling a wide variety of viral diseases common in cattle. Although attenuated viral vaccines are available against a number of viral pathogens, there are viruses for which no vaccines have been, or are likely to be, developed. As stated by Rosenquist (132), a good candidate for the application of bovine IFN is the syndrome of shipping fever. This is an acute respiratory disease in cattle, in which a multiplicity of viruses are

involved. For these and other reasons an understanding of bovine interferons has both practical and theoretical applications.

CHAPTER II

MATERIALS AND METHODS

Chemicals and Reagents

Phosphate buffered saline (PBS) was prepared as described by Van Wezel (161). It was made up in water purified by a millipore system. The solution was later filter-sterilized. Each liter of solution contained 8.0 gr of NaCl, 0.2 gr of KCl, 2.0 gr of Na HPO₂, and 0.4 gr of KH₂PO₄. The salts were purchased from Fisher Scientific Co. (Fair Lawn, NJ).

Media employed in cell cultures included RPMI-1640 and Eagle's minimal essential media (MEM) (Gibco, Grand Island, NY). Both were supplemented with 10% heat inactivated fetal bovine serum (FCS) acquired from Sterile Systems, Inc. (Logan, UT), and 0.25% gentamycin. The final pH of the media was adjusted to 7.2 with sodium bicarbonate (MCB Reagents, Cincinnati, OH). RPMI-1640 media was further supplemented with 0.025 M HEPES (N-2-hydroxyethyl piperazine-N'-2'-ethanesulfonic acid) purchased from Sigma (St. Louis, MO).

Concanavalin A (con A) was obtained from Sigma (St. Louis, MO). A stock solution of the mitogen was prepared at a concentration of 1 mg per ml in sterile

PBS. The stock solution was subsequently diluted in RPMI-1640 medium to the desired concentration, just prior to use.

Polyribonucleosinic-polyribocytidylic acid (poly I:C) was purchased from P-L Biochemicals (Milwaukee, WI) in lyophilized form. A stock solution of poly I:C was prepared by adding 1 mg of the compound to 1 ml of sterile PBS which was agitated gently until completely dissolved. This stock solution was diluted to the appropriate concentration in MEM immediately prior to use.

A stock solution of ribonuclease A (Sigma) was prepared at a concentration of 1 mg/ml immediately prior to use.

Trypsin (Difco, Detroit, MI) was prepared at a concentration of 0.25% in PBS. The solution was supplemented with 0.02% EDTA and 0.0015% phenol red. The solution was later filter-sterilized.

The stains used were trypan blue (Eastman Kodak, Rochester, NY) and crystal violet (Harleco, Philadelphia, PA). Trypan blue was employed at a concentration of 1% in PBS. Crystal violet was prepared at a concentration of 1% in 80% methanol (Fisher, Fair Lawn, NJ)

Viruses used were Sendai, Newcastle disease (NDV), and vesicular stomatitis (VSV). Sendai virus and NDV were grown in embryonated eggs in this laboratory. These

viruses were titrated using direct hemagglutination of human O erythrocytes (135). VSV (Indiana strain) was grown in cultures of bovine cells in this facility. The virus was titrated by plaque formation on MDBK cells (24).

Cell Cultures

Confluent cultures of bovine kidney cells (MDBK) were the source of fibroblast interferon. They were also used in the interferon assays. Bovine cell strains employed included bovine embryonic kidney (BEK), embryonic bovine tracheal cells (EBTr), and bovine turbinate (BT). A complete list of cells used in the experiments is contained in Table 1. The cell lines were subcultured according to standard methods (92,116).

Non-lactating dairy Holstein cows were used as the source of bovine leukocytes. The animals were maintained at the Texas Tech University dairy facility at New Deal.

Leukocytes were separated from whole blood by means of a selective hypotonic lysis procedure developed in this laboratory (98). Briefly, blood was collected from non-lactating dairy cows by jugular venipuncture with 60 cc syringes. The syringes contained 5 ml of 4% disodium-ethylene-diamine tetraacetate (EDTA) which acted as an anticoagulant. The blood was then mixed with distilled water containing 0.3% galactose in a 1:1.5 ratio of

Table 1

LIST OF CELL LINES EMPLOYED IN THE EXPERIMENTS

Cell Type	Passage	Source
EBTr Embryonic bovine trachea	29	American Type Culture Collection (ATCC) Rockville, Maryland
BT Bovine turbinate	18	ATCC Rockville, Maryland
MDBK Bovine kidney	91	ATCC Rockville, Maryland
MRC-5 Human embryonic lung	18	Flow Laboratories Inglewood, California
L-929 Murine connective tissue		University of Texas Medical Branch Galveston, Texas
Wish Human amnion		ATCC Rockville, Maryland
HEp-2 Human epidermoid carcinoma	370	Dr. Radmila Mirkovic Texas Tech Univ. HSC Lubbock, Texas
CT Cat tongue		Dr. Joseph Cummins Texas A&M Experimental Sta. Amarillo, Texas
Vero Green monkey kidney		same as above

blood to galactose solution respectively. The suspension was mixed vigorously for 30 seconds and then allowed to stand at room temperature for 3 minutes. Then 1.5 parts of cold (4 degrees C) buffered saline gelatin (BSG) at pH 7.2 were added to the mixture. BSG was added to restore isotonicity. The suspension was centrifuged at 1,200 rpm for 10 minutes at 4 degrees C to separate the leukocytes from the hemolyzed erythrocytes. The leukocyte cell pellet was then resuspended in 50 ml of cold BSG and vortexed. The cells were centrifuged and washed again. Finally, the cells were suspended in RPMI-1640 medium. The cells were counted in a Coulter counter and the final concentration was adjusted to 5×10^6 cells per ml of culture.

When only the lymphocyte population was needed, 4 ml aliquots of mixed leukocytes, obtained by the "lysis" procedure described above, were carefully overlaid on top of 3 ml of Ficoll-Paque (Pharmacia, Piscataway, NJ). The samples were centrifuged at 400 rpm for 40 minutes. The lymphocytes obtained from the interface of the two solutions were washed with PBS, centrifuged, and resuspended in RPMI-1640 media plus supplements. The lymphocytes were adjusted to the aforementioned concentration.

Induction of Interferon Synthesis

For the production of virus-induced interferon from

leukocytes, virus was added and allowed to adsorb for one hour. At the end of the incubation period, the cells were washed twice with PBS and centrifuged at 1,200 rpm for 10 minutes. The cell pellet was resuspended in RPMI-1640 medium and incubated in culture tubes for 24 hours at 37 degrees centigrade and 5% CO₂. The tubes were placed in the incubator at a 15 degree angle. At the end of the incubation time the fluids were collected. The samples were irradiated for 15 minutes at 12 cm from an ultraviolet light (15 watts) source to eliminate viral infectivity.

Immune-type interferon was obtained from lymphocytes induced with con A. The lymphocyte population was obtained as previously described. The mitogen was added at a concentration of 0.01 mg/10 cells or 0.05 mg/ml. The cultures were established in tubes as described above. The fluids, collected at 48 hours post-induction, were frozen at -20 degrees C. until assayed.

For the production of fibroblast interferon four bovine cell lines were employed: BEK, MDBK, EBTr, and BT. The cells were grown to confluency on 25 sq cm tissue culture flasks from Corning (Corning, NY). Triplicate cultures of each cell line were established. The virus (NDV or Sendai) was added to washed cell monolayers at a concentration of 40 HA units/ml. The virus was

allowed to adsorb for one hour and then the excess virus was decanted. The monolayers were washed with PBS and 10 ml of MEM were added. The cells were allowed to incubate for 24 hours. At the end of the incubation period the fluids were collected and the virus inactivated as above. When poly I:C was the inducer used, it was added at a concentration of 0.01 mg/ml of cell suspension. DEAE-dextran at a concentration of 0.1 mg/ml was added along with the poly I:C.

Priming of Interferon Production

For this phase of the study, leukocyte cultures were established in triplicate in tissue culture tubes. The IFN primers were added to the cultures and allowed to incubate for 2 hours. At the end of the incubation period, the primers were removed, the cells washed, and induced. The culture fluids were collected and treated as above.

The MDBK cultures were seeded using tissue culture plates containing 96 wells (Costar, Cambridge, MA). The plates were divided into areas to allow for a high number of replicates per sample. The MDBK cells were allowed to form monolayers before the primers were added. Small amounts of 10 or 100 units/ml (1-10 units per well) of interferon were added to the cells and allowed to adsorb for 3 hours. The cells were then washed

twice with PBS, and the inducers were added and allowed to adsorb for 1 hour. At the end of the incubation period, the inducers were removed and the cells were refed with maintenance medium. The fluids were collected at various intervals post-induction.

Interferon Assay

To titrate the bovine interferon preparations a microplaque reduction assay was employed (14). This assay can be described as follows: interferon preparations were serially diluted in the 96-well tissue culture plates. MDBK cells were added at a concentration of 2.5×10^5 cells per 0.1 ml. The plates were incubated overnight at 37 degrees C under 5% CO₂. The cells were challenged with a concentration of VSV that would instigate from 30 to 50 plaques. The virus was allowed to adsorb for one hour. The cells were then overlaid with 0.5% methylcellulose in MEM, and incubated again for 20-24 hours followed by staining with crystal violet.

The interferon titer was calculated as the reciprocal of the dilution causing a 50% reduction in the number of plaques. Each interferon assay had a virus control and a standard interferon control. The exact titer of each preparation was calculated according to the formula described by Epstein (45):

IFN Titer

$$= \frac{(1/2 \text{ C} - \text{l.n.}) \times (\text{dilution e.p.} - \text{dilut. l.n.})}{(\text{plaque \# e.p.} - \text{plaque \# l.n.})} + \text{l. dil.}$$

l.n.= low neighbor

C = control

e.p.= end point

l. dil.= low dilution

Example:

Plaque # at dilution Plaques at control Mean 1/2 control

1:16	1:32	1:64	55	52	26
0	12	27	50		
			51		

$$\text{IFN titer} = \frac{(26-12) \times (64-32)}{(27-12)} + 32 = 62$$

Various tests were performed to confirm that the reduction of the number of plaques was caused by interferon added to the assay system and not by endogenous production in the assay plate caused by "carried-over" inducer. One test involved the use of ribonuclease (0.1 mg/ml), which would degrade any RNA potentially present in the test fluids. Another test was to assay a mock interferon preparation consisting of a suspension of a

virus inducer which had been UV irradiated for 15 minutes at 12 cm from the UV lamp. Trypsin was employed also to verify that the antiviral activity was sensitive to digestion by proteolytic enzymes. IFN preparations were incubated with trypsin at 37 degrees centigrade for 30 minutes.

Rate of Development of Antiviral Proteins

The rate of antiviral protein synthesis was assayed in a manner similar to that reported by Dianzani et al. (37). Briefly, several microtiter (96-well plates) were seeded with MDBK cells. When the cells had attached and formed a monolayer, the culture fluids were removed and the cells washed twice with PBS. Interferon samples were serially diluted two-fold directly in the plates. the plates were incubated at 37 degrees C under 5% CO₂. At various intervals, plates were removed from the incubator, the fluids decanted, and the cells challenged with VSV.

Stability of Bovine Interferons

The pH stability of the different interferons was evaluated. This was done by dialyzing the IFN preparations against 100X their volume of pH 2 buffer prepared according to the methods of Rovozzo and Burke (134). After dialysis for 24 hours, the samples were subsequently redialyzed against pH 7.2 buffer for 24

hours. The above samples were compared to the titers of samples dialyzed against pH 7.2 buffer only.

Temperature stabilities of the different interferon samples were also determined. Interferon preparations were subjected to temperatures of 45 and 56 degrees centigrade for different time periods after which the interferons were assayed along with control preparations.

Cytostatic Effects of Interferons

The ability of different interferons to inhibit cell growth was evaluated. Methods used were essentially those of Evinger and Pestka (44) with certain minor modifications. Interferon preparations were added to cell suspensions which were cultured in 60 mm Petri dishes. Interferon samples were added to the dishes at a concentration of 50 units/ml. Nine dishes were established for each interferon type being assayed. The controls were nine dishes which had culture medium from non-induced MDBK cells and nine more dishes which had culture fluids from non-induced leukocytes. The dishes were seeded with a low concentration of cells and then they were allowed to incubate for 1, 2, and 3 days. On each day, 3 representative dishes of each group were harvested, the cells trypsinized and counted in a hemocytometer using the trypan blue (dye) exclusion method.

Cross-Species Protection of Bovine Interferons

The activity of IFN on heterologous cells was determined for each of the major types. To test the cross-reactivity of the various bovine interferons samples were assayed on heterologous cells, which are listed in Table 1. The IFN titers obtained on the various cells were compared to titers obtained using MDBK cells. Prior to the assays, the stock VSV was titered on each cell type to determine the amount of virus required for the production of 30-50 plaques per well (cell monolayer).

CHAPTER III

RESULTS

Kinetics of Interferon Production

A reference IFN preparation was used in each assay. It was prepared using poly I:C induction of BEK cells. The purpose was to assess the reproducibility of the assay. Random "control" titers from assays of this material were run over a period of 18 months and are compiled in Table 2. It can be seen that the titer of the samples was relatively consistent, with a deviation of plus or minus "one dilution."

Leukocyte and Immune Interferons

The inducers used for the production of leukocyte IFN were NDV, Sendai virus, and poly I:C. The efficacy of these inducers was compared in Table 3. It can be seen that NDV was the best inducer of bovine leukocyte interferon and was the principal inducer used in these studies. Inducers of immune interferon included Con A and PHA. Con A was more effective than PHA (Table 3) and was used throughout these studies.

The rates of IFN synthesis were compared using NDV, poly I:C, and Con A (Figure 1). It can be seen that the peak of leukocyte IFN was obtained within 24 hours post-induction, whereas maximum production of immune interfe-

Table 2
CONSISTENCY OF IFN ASSAY^a

End-point ^b	Titer ^c
9	4195
10	6400
9	3723
8	2593
9	7480
8	4320
9	3801
9	5880
9	3987
8	2337
8	3938

Mean: 4421 ± 1564 S.D.

(a) Fibroblast

(b) Well number

(c) Units/ml

Table 3

EFFECTIVENESS OF IFN INDUCERS ON LEUKOCYTES

Inducer	Titer		
	High	Low	Average (c)
^a NDV	11627	351	5881±2953
^a Sendai	2100	160	1800± 866
^a Poly I:C	4907	38	1683±1400
^b Con A	756	20	269± 192
^b PHA	189	15	80± 62

(a) Inducers of leukocyte IFN

(b) Inducers of immune IFN

(c) Mean of 10 or more samples ± standard
deviation

Figure 1. Comparison of inducers of interferon synthesis by leukocytes. Leukocytes were exposed to 10 micrograms of Con A/ml ●—● , 10 micrograms of poly I:C/ml ▲—▲ , and 40 HA units of NDV/ml ○—○ . Results represent the mean values of 4 experiments.

INTERFERON TITER (Units/ml)

ron took longer than 70 hours. Leukocyte interferon titers were always higher than immune interferon titers with NDV as the most effective inducer.

Priming

The relative ability of fibroblast and leukocyte interferons to prime the production of immune interferon by leukocytes is presented in Table 4. It was observed that fibroblast interferon was a better (4x) primer of immune IFN synthesis than leukocyte IFN. When the rate of immune interferon synthesis was compared between primed and unprimed leukocytes (Figure 2), it was noted that peak titers of IFN synthesis were achieved by primed leukocytes much earlier than by unprimed cells. The peak titers of primed leukocytes were achieved within 24 hours post-induction, whereas unprimed leukocytes had not peaked by 70 hours post-induction.

A comparison of the ability to produce immune interferon was made between mixed leukocyte and lymphocyte populations (Table 5). It was noted that lymphocytes were about 5.5 times more effective inducers of immune IFN when they were separated from the other leukocytes.

Fibroblast Interferon

The ability of various bovine fibroblasts to produce fibroblast IFN is presented in Table 6. Three different inducers were individually tested on each cell

Table 4
PRIMING OF IFN PRODUCTION BY LEUKOCYTES^a
IFN Primers^b

Control ^c Titer	Leukocyte IFN ^d Titer	F. E.	Fibroblast IFN Titer	F. E.
15	20	1.3	528	35.2
20	212	10.6	120	6.0
160	576	3.2	544	3.4
71	305	4.3	959	13.5
118	307	2.6	850	7.2
0	34	34.0	203	203.0
57	68	1.2	234	4.1
54	76	1.4	378	7.0
53	101	1.9	493	9.3
56	179	3.2	207	3.7
40	492	12.3	132	3.3
0	36	36.0	160	160.0
0	38	38.0	106	106.0
Mean		11.5		43.2

(a) Inducer was 0.05 mg Con A/ml

(b) Primer at 100 units/ml

(c) In units/ml

(d) Fold Enhancement

Figure 2. Rate of interferon production by primed leukocytes. Leukocytes were exposed to 100 units of leukocyte IFN/ml , 100 units of fibroblast IFN/ml , and culture medium prior to addition of the inducer. Results express the mean values of three experiments.

Table 5

COMPARISON OF IMMUNE IFN PRODUCTION^a BETWEEN^b
 "MIXED" LEUKOCYTES AND ISOLATED LYMPHOCYTES

Leukocyte	Lymphocyte
<u> ^c</u>	<u> </u>
320	640
80	640
40	320
147	533

(a) The inducer was Con A at 0.05 mg/ml

(b) 5×10^6 cells/ml

(c) Titers in units/ml

Table 6

COMPARISON OF BOVINE FIBROBLAST IFN PRODUCTION BY
DIFFERENT CELL-INDUCER COMBINATIONS

Cell Line	Inducer		
	<u>NDV (a)</u> c	<u>Sendai (a)</u>	<u>Poly I:C (b)</u>
BEK	640	20	120
BT	1280	20	80
EBTr	465	320	67
MDBK	6542	2500	2300

(a) 40 HA units/ml

(b) Poly I:C 0.01 mg/ml

(c) units IFN/ml

line. When the different cell lines were compared on their ability to make fibroblast IFN, it was noted that MDBK cells were the best producers regardless of the inducer. As with the leukocyte IFN, NDV was a better inducer than the others used regardless of the cell type.

Figure 3 illustrates the rates of fibroblast IFN synthesis by three different cell lines. The three cell lines employed displayed similar patterns of IFN production but differed in the levels of IFN produced. Figure 3 also reflects the effect of UV irradiation on the inducer (NDV). When the virus was irradiated for 1 minute at 12 cm from the light source, a considerable reduction in its inducing ability was noted.

The effects of inducer level on the synthesis of fibroblast IFN were studied. As shown on Figure 4a, there was a dosage effect caused by the level of the inducer. The high and the medium doses caused similar patterns of production, whereas with the low dose, cells reached their peak production sooner and then gradually declined.

Priming

The rates of interferon synthesis by MDBK cells primed with leukocyte IFN are illustrated in Figure 4b. The amount of IFN used as a primer has a marked effect

Figure 3. Synthesis of interferon by bovine fibroblasts. 40 HA units of NDV/ml were used to induce IFN synthesis in MDBK cells , EBTr cells , and BT cells . Also, 40 HA units of UV irradiated NDV were added to MDBK cells . Results represent the mean of four experiments.

Figure 4a. Effect of inducer level on interferon synthesis by MDBK cells. NDV at 2.5 ●—●, 10 ■—■, and 40 ▲—▲ HA units/ml of medium was used to induce MDBK cells. Results are expressed as mean values of three experiments.

Figure 4b. Rate of interferon synthesis by MDBK cells primed with leukocyte IFN. MDBK cultures were exposed to 10 units of leukocyte IFN/ml of medium and 2 hours later they were induced with 2.5 ●-----● , 10 ■-----■ , and 40 ▲-----▲ HA units of NDV/ml of medium. Other MDBK cultures were primed with 100 units of leukocyte IFN/ml of medium and after 2 hours of incubation , the cells were induced with 2.5 ○-----○ , 10 □-----□ , and 40 △-----△ HA units of NDV/ml of medium. Results express the mean of three experiments.

on the synthesis of interferon by the treated cells. In general, cells primed with 100 units/ml of leukocyte IFN produced considerably higher amounts of IFN than unprimed cells (Figure 4a). Cells primed with only 10 units/ml of leukocyte IFN also yielded higher levels of interferon than unprimed cells. The level of inducer also affected IFN titers of the primed cells. A concentration of 40 HA units of NDV/ml induced more IFN than 10 HA units. The latter concentration was also better than 2.5 HA units/ml, which was the lowest used.

The rates of IFN synthesis by MDBK cells primed with fibroblast and immune interferons were also investigated. As illustrated in Figures 4c and 4d, cells primed with fibroblast and immune IFNs showed basically the same patterns of production as cells primed with leukocyte IFN. Priming was initiated by immune IFN when the cells were primed, induced, and allowed to incubate for at least 24 hours.

MDBK cultures were primed with each of the three classes of IFN. The cells were later induced and allowed to incubate 24 hours before any samples were collected. The IFN samples from primed cells were compared with IFN preparations obtained from unprimed cells. It was observed that all three IFN classes were capable of priming the production of fibroblast IFN (Table 7). It was noted

Figure 4c. Rate of interferon synthesis by MDBK cells primed with fibroblast IFN. MDBK cells were primed with 10 units of fibroblast IFN/ml of medium and later they were induced with 2.5 ●-----● , 10 ■-----■ , and 40 ▲-----▲ HA units of NDV/ml of medium. Other MDBK cell cultures were primed with 100 units of fibroblast IFN/ml of medium and were later induced with 2.5 ○-----○ , 10 □-----□ , and 40 △-----△ HA units of NDV/ml of medium. Results express the mean value of three experiments.

INTERFERON TITER (Units/ml)

Figure 4d. Rate of interferon synthesis by MDBK cells primed with immune IFN. Before inducing the MDBK cells with 40 HA units of NDV/ml of medium, the cells were primed with 10 ▲-----▲ and 100 ▲———▲ units of immune IFN/ml. Results express the mean values of 2 experiments.

Table 7
PRIMING OF FIBROBLAST IFN PRODUCTION

Control b	IFN Primers ^a					
	Leukocyte-IFN ^c		Fibroblast-IFN		Immune-IFN	
	Titer	F. E.	Titer	F. E.	Titer	F. E.
6542	11112	1.7	24205	3.7	28131	4.3
4654	N.D.	N.D.	18616	4.0	20943	4.5
3117	7169	2.3	37404	12.0	34287	11.0
104	2028	19.5	2350	22.6	11113	10.7
171	4908	28.7	1197	7.0	547	3.2
117	2785	23.8	995	8.5	784	6.7
67	1059	15.8	603	9.0	1173	17.5
80	1168	14.6	600	7.5	1200	15.0
74	592	8.0	829	11.2	1421	19.2
73	1146	15.7	628	8.6	1314	18.0
Mean		14.4		9.4		11.0
(a)	100 units/ml					
(b)	In units/ml					
(c)	Fold Enhancement					

that leukocyte IFN was a slightly better primer than the other two IFN types although these differences may not be significant. On the average, bovine leukocyte IFN was found to enhance the production of fibroblast IFN 14.4 fold. Fibroblast and immune IFNs were found to enhance the synthesis 9.4 and 11.0 fold, respectively.

Development of Antiviral Proteins

The rates of antiviral protein synthesis were also monitored on MDBK cells treated with virus-induced interferons. The IFN types employed were leukocyte IFN and fibroblast IFN. A difference in terms of antiviral protein induction was observed between the two interferon classes. As shown in Figure 5, cells treated with fibroblast IFN developed the antiviral state sooner than did cells treated with leukocyte IFN. The cells treated with fibroblast IFN displayed 40% of the maximum antiviral activity after 4 hours of contact with IFN. On the other hand, cells treated with leukocyte IFN, after the same period of contact, only showed 6% of the maximum antiviral activity. After 8 and 10 hours of contact, the difference in the rates of antiviral state induction by the two different IFNs was still quite noticeable. By ten hours, the cells treated with fibroblast IFN were displaying 90% of the maximal antiviral activity, whereas cells treated with leukocyte IFN displayed only 30%

Figure 5. Rate of synthesis of the antiviral proteins. MDBK cell cultures were exposed to 100 units of leukocyte IFN/ml ▲—▲ and 100 units of fibroblast IFN per ml of medium ●—●. The cell cultures were challenged at various intervals after the addition of the IFN. Results represent the mean values of three experiments.

of the final titer. By 12 hours, both IFN classes exhibited their maximal activity.

It should be pointed out that the antiviral state of cells treated with fibroblast IFN was developed in a gradual fashion. On the other hand, cells which were treated with leukocyte IFN slowly synthesized antiviral proteins up to the tenth hour of contact. After that, there was a sharp increase in synthesis until the maximal antiviral state was developed.

Stability of Bovine Interferons

Acid pH

The stability of the three types of IFN under acidic conditions was assessed by dialyzing the IFN preparations against a pH 2 buffer for 24 hours. After the preparations were dialyzed and brought back to physiological pH, as described above, it was observed that the three IFN types had different stabilities at low pH. As presented on Table 8, leukocyte and fibroblast IFNs were found to have similar pH stability. Both were relatively stable at low pH. Immune IFN was unstable at pH 2. The latter IFN class lost over 90% of its activity.

Temperature

The temperature stability of the bovine interferons was assessed by exposing preparations to temperatures of 45 and 56 degrees C. As seen on Table 8, leukocyte IFN

Table 8

IFN Type	pH 2	STABILITY OF BOVINE INTERFERONS ^a			Trypsin ^c	Ribonuclease ^d
		45 degrees C	56 degrees C			
	^b					
Leukocyte	65	100± 9	90±30		2±0.8	98±2.3
Fibroblast	78	59±16	53±13		4±1.2	99±1.2
Immune	4	N.D.	15± 9		2±0.6	97±0.9

(a) Percent of the control titers

(b) Average of three or more experiments

(c) 0.05 mg of trypsin/ml of sample. Incubated at 37 degrees C/30 min.

(d) 0.10 mg of ribonuclease/ml of sample. Incubated at 37 degrees C/30 min.

retained all of its activity after exposure to 45 degrees C for 30 minutes. Fibroblast interferon retained slightly over a half of its original titer when exposed to 45 degrees C for a half hour. When the three IFN classes were exposed to 56 degrees C for 30 minutes, it was observed (Table 8) that leukocyte IFN was the most stable of the three, losing only 10% of its original activity. Fibroblast IFN was found to retain about half of its initial titer. Immune IFN was the least stable retaining only about 15% of its activity. Figure 6 illustrates the rates of inactivation of the three IFN classes at 56 degrees C for up to one hour. Both virus-induced interferons were observed to gradually lose their antiviral activity when they were exposed to that temperature. Fibroblast IFN lost 41% of its activity within the first 15 minutes of exposure. Immune IFN lost 85% of its activity during the initial 15 minutes to over 95% by one hour.

Trypsin Treatment

The different IFN preparations were characterized as to their susceptibility to trypsin. They were subjected to 0.05 mg of trypsin in 1 ml of PBS for 60 minutes. Trypsin treatment reduced the titers of all three IFN types to less than 5% of their original activity (Table 8).

Figure 6. Stability of bovine interferons at 56°C. The different interferon samples were exposed to a temperature of 56°C for different time intervals, and later the samples were assayed. The IFN samples tested were leukocyte IFN , fibroblast IFN , and immune IFN . The results represent the mean of 3 or more experiments.

Ribonuclease Treatment

The three IFN types were exposed to 0.1 mg of ribonuclease per ml of PBS. None of the IFN types were susceptible to treatment with ribonuclease (Table 8). In all three cases the antiviral activity remained intact.

Cytostatic Effects of Bovine Interferons

The cell growth-inhibitory effects exerted by the virus-induced interferons on normal cells are reflected in Figure 7. MDBK cells grew at a linear rate when they were exposed to fluids from non-induced fibroblast and leukocyte cultures. When small amounts (50 units/ml) of leukocyte and fibroblast interferons were added to the growth medium the cells showed a minimal amount of growth during the incubation period. It should be mentioned that the points in the curves represent the number of viable cells, whereas the numbers of dead cells were consistently low.

Cross-Species Protection by Bovine Interferons

Leukocyte and fibroblast interferons were compared for their ability to protect heterologous cells. Interferon produced by fibroblasts protected heterologous cells to a greater extent than leukocyte IFN (Table 9). Of the bovine cell lines used to assay bovine IFNs, EBTr cells displayed only 43% of the total activity of leuko-

Figure 7. Cell growth inhibition caused by bovine interferons. Various MDBK cultures were incubated with either of the following: 50 units of leukocyte IFN/ml ○—○ , 50 units of fibroblast IFN per ml □—□ , leukocyte culture fluids ●—● , or fibroblast culture fluids ▲—▲ . Each point on the curves represents 3 samples. The results express the average of 2 experiments.

Table 9
CROSS-SPECIES PROTECTION

Cell Line	Relative Protection by Bovine IFNs ^a	
	Leukocyte-IFN	Fibroblast-IFN
^b MDBK	^c 100	100
^b EBTr	43±19	100± 7
^b BT	80±11	95± 8
^d CT	78±14	77±17
^e Vero	0	0
^f Wish	5± 3	80± 9
^f MRC-5	7± 2	63± 7
^f HEp-2	2±1.3	55±16
^g L-929	34±14	84±11

(a) Average of 3 or more experiments

(b) Bovine

(c) Percent of the titer on control MDBK cells

(d) Feline

(e) Primate

(f) Human

(g) Murine

cyte IFN which had been titered on MDBK cells. Cat tongue cells were well protected by both IFN types. Vero cells were the negative controls since they failed to develop antiviral activity when exposed to the bovine IFNs. Human and murine cells were protected by fibroblast IFN, whereas leukocyte IFN induced minimal antiviral activity on these cells.

CHAPTER IV

DISCUSSION

The currently available methods to assay interferon involve an indirect measure of the effects that IFNs have on cells (143). The present studies were dependent upon a "microplaque reduction assay" (14), which in turn depends on the ability of viruses to form plaques. The assay is subject to a variety of factors which may alter it, thereby requiring the samples to be run in duplicate and triplicate. Data obtained indicate that the assay was consistent and reproducible between experiments.

It should also be mentioned that the interferon preparations used were not purified. The samples were basically raw tissue culture fluids. This suggests that some of their effects involving more antiviral activity could be enhanced by factors other than pure IFN. All three IFN types met the first criterion for an antiviral agent to be classified as an interferon, that is, to be a protein (102). The three IFN classes lost all of their activity after they were exposed to trypsin. Also, the activity of all three IFN classes remained intact after they were subjected to ribonuclease treatment. This indicates that none of their activity was due to "carried-over" RNA inducer into the assay system.

Newcastle disease virus has consistently been used

to induce IFN synthesis in a number of systems (143). In the present studies NDV was found to elicit the highest IFN titers in bovine leukocytes. Sendai virus and poly I:C were not as effective inducers as NDV. Possibly, this difference in interferon induction may be related to the presence of specific receptors on the cell and/or the amount of "replicative" intermediates formed during replication of the virus. The latter are generally believed responsible for the induction of IFN (45).

Concanavalin A was noted to be a better inducer of bovine immune IFN than PHA. This is in agreement with the observation of Langford et al. (95) who found Con A to be a better inducer of human immune IFN than PHA.

When the rates of synthesis of leukocyte IFN and immune IFN were compared, it was observed that leukocyte IFN was produced earlier than immune interferon. The time required for immune IFN synthesis to peak agrees with the report of Johnson et al. (84) who explored the human immune IFN. Also, Babiuk and Rouse showed that bovine immune IFN synthesis peaks after 48 hours post-induction (4). The present study, however, differed from that of Babiuk and Rouse, who induced immune IFN with an antigen (IBR virus). The inducer employed in this lab was a mitogen (Con A).

The ability of interferon to prime its own synthesis is one of its nonantiviral properties. Priming is currently employed in various IFN production systems (117). Normally, during production of interferon, the same "species" is used to prime. Little attention has been paid to the priming ability of one IFN class on the synthesis of a different IFN class. This study included a comparison of leukocyte and fibroblast IFNs as primers of immune IFN synthesis. The enhancement of immune IFN primed with fibroblast IFN and with leukocyte IFN were 43.2 fold and 11.5 fold, respectively. The basis for these differences is not understood. Perhaps, fibroblast IFN affects transcriptional and translational events in a fashion which is different from that of leukocyte IFN. It has been shown that there is more IFN mRNA in primed than in nonprimed cells (52). It has also been demonstrated that IFN mRNA is produced faster following induction in primed cells than in nonprimed cells (148). Abreu et al. (1) observed that IFN-primed cells produced IFN earlier than nonprimed cells. A similar observation was made in the current study when the kinetics of immune IFN production by primed and non-primed cells were compared.

Babiuk and Rouse (4) found that bovine immune interferon was derived from T-lymphocytes. They also

found that when the T-lymphocytes were combined with autologous macrophages there was a 2- to 10-fold increase of immune interferon synthesis. Epstein (42) confirmed the above study by using a combination of mouse macrophages and human leukocytes. The mixed culture upon induction with PHA produced an immune IFN which had specific antiviral activity in human cells and not in mouse cells. In the present study the higher titers of immune IFN were produced by lymphocytes alone. A mixed population of leukocytes was induced for immune IFN production in hopes that the presence of macrophages would generate higher IFN titers than would lymphocytes alone. The mixed culture of leukocytes produced lower levels of IFN than those produced by lymphocytes alone. It is possible that one of the other leukocyte subpopulations might have been producing an inhibitor(s) (i.e., prostaglandins) of IFN production. Another possibility could be that the amount of IFN could relate directly to the number of T-lymphocytes and therefore simply reflects the number of cells capable of making IFN.

Bovine kidney cell cultures have been employed in various studies for the production of bovine fibroblast IFN. Of the four cell types tested MDBK cells were the best producers. MDBK cells have previously been used for the production of bovine fibroblast IFN (41). In the

present study, the rates of IFN synthesis by different bovine cells were compared and MDBK cells showed a slightly faster rate than the other cell types. The basis for the differences of IFN production among the three cell lines may depend on the presence of cellular receptors for the inducers. It is also possible that MDBK cells synthesize more IFN mRNA than the other bovine cell types. Another possibility is that the three cell types have different control mechanisms which allow MDBK cells to produce more IFN before the repressor is produced. It cannot be ruled out that IFN levels could simply reflect differences in cell densities inherent in each cell type depending on culture conditions.

Three agents were compared for their abilities to act as inducers of fibroblast IFN. Again, NDV was the best inducer of the group. It was noted that the level of IFN produced was directly related to the level of inducer added to the cell cultures. This could have been expected since there exists a direct relationship between the number of cells challenged and the amount of IFN produced.

It was observed that primed MDBK cells produced more IFN than nontreated cells. This agrees with previous reports by Stewart (1, 148). It was also noted that cells pretreated with 100 units/ml (10 units/well)

of IFN upon induction, produced more IFN than cells primed with only 10 units/ml (1 unit/well) of IFN. This agrees with previous findings in this laboratory. Tyring et al. (160) reported that murine cells (L-cells) which were pretreated with 500 units/ml of IFN (alpha-beta or gamma) produced more IFN than cells pretreated with lower concentrations of IFN. As stated above, the IFN pretreatment may alter the efficiency of transcription of IFN mRNA or its translation in the primed cells. It was found in these studies that all three IFN types could enhance the production of fibroblast IFN. Leukocyte IFN was a slightly better primer than the other two. However, this difference was not significant since the mean fold-enhancement caused by the three IFN types were similar to each other, whereas the priming of immune IFN with leukocyte and fibroblast IFNs showed a noticeable difference.

Dianzani et al. (39) found that cells treated with immune IFN develop the antiviral state at a much slower rate than cells treated with leukocyte and fibroblast IFNs. This property allows differentiation between immune and virus-induced IFNs. It was thought that perhaps there might be differences between the two virus-induced bovine IFNs in terms of the development of the antiviral state in the bovine cells. It was observed

that cells treated with fibroblast IFN developed the antiviral state sooner than cells treated with leukocyte IFN. Both IFN types induced 100% of their original titer at about the same time. Dianzani and Baron (37) reported that these differences in development of the antiviral state induced by different IFN types may be accounted for by different derepressional mechanisms.

Human and murine IFNs have been characterized by their pH and temperature stabilities (104,170,176). There have been conflicting reports on the stability of bovine IFNs when exposed at pH 2 and 56 degrees C. (2,25,55,130,132).

Stability at pH 2 has been used to separate immune IFN from virus-induced IFNs. Maehara et al. (104) noted that fibroblast and leukocyte IFNs retained about 90% of their original activity after pH 2 treatment. Conversely, immune IFN retained only 8 to 12 percent of its original titer after dialysis against pH 2 buffer. Similar observations were reported by Wheelock (170) and Youngner and Salvin (176). The observations made in this study agree with the above reports. Bovine leukocyte and fibroblast IFNs were both much more stable than bovine immune IFN at pH 2. The former two retained about 70% of their original titer after dialysis against a pH 2 buffer, whereas the latter retained about 4% of its

original activity.

The three IFN types were shown to differ in their temperature stabilities. Of the three IFN classes, leukocyte IFN was the most stable at 56 degrees C. and immune IFN was the least stable. There have been other reports in the literature indicating similar results (18,170). Wheelock found a greater reduction in titer of human immune IFN, after incubation at 56 degrees C./hour, than in the titer of the virus-induced IFN, which remained stable under the same conditions (170). In the present study, leukocyte and fibroblast IFNs were found to differ in their stability at 45 degrees C. Leukocyte IFN was completely stable at that temperature, whereas fibroblast IFN lost 40% of its original titer under the same conditions.

A kinetic study of inactivation at 56 degrees C. was performed employing all three IFN types. This study illustrated the differences in inactivation among the IFN types. After 15 minutes of exposure, leukocyte IFN lost only 3% of its activity, fibroblast IFN lost 41% of its original titer, and immune IFN lost 85% of its titer. In the absence of antibody specific to these IFNs this parameter may allow the consistent separation of the three IFN types.

Paucker et al. (115) first reported the cytostatic

effects of interferon preparations on cells in culture. In light of the similarities between leukocyte IFN and fibroblast IFN, it was hoped that they could be separated on the basis of cytostatic effects on normal cells. The cytostatic effects observed by both IFN types were similar on normal cells. Both IFN types retarded the growth rate of cells. On the basis of these limited studies, it is difficult to conclude anything other than some factor provided in the inbred cultures, probably IFN, inhibited cell division.

It was reported by Jankowski et al. (81) that human fibroblast IFN cross reacts less than human leukocyte IFN does. Similar observations have been made in IFNs from other mammalian species (16,26). In this phase of the present studies, bovine leukocyte and fibroblast IFNs were tested for their abilities to cross-protect heterologous cells. However, the results obtained were opposite of Jankowski's. Bovine fibroblast IFN was more effective in protecting heterologous cells than leukocyte IFN. It is believed that the extent of glycosylation may determine species specificity. It is not known the extent of glycosylation between the various bovine IFNs. It is possible that the bovine fibroblast IFN is less glycosylated than fibroblast IFN from other species and bovine leukocyte IFN itself.

The objectives of the present study were partially achieved since it became evident that the various bovine IFNs have different properties. Bovine immune IFN was found to be the least stable at both pH of 2 and 56 degrees C. Also, it was found that leukocyte and fibroblast IFNs have a number of distinct properties. The parameters that may be used to separate the latter two IFN classes include cross-species protection and development of the antiviral state.

It is important to repeat these studies with highly purified IFN samples. For that purpose it is essential to clone the genes for the various bovine IFNs. It is our understanding that cloning of the bovine genes has already been accomplished.

The knowledge of the properties of the various bovine IFNs can be beneficial in veterinary medicine. It may become possible to treat various bovine viral diseases for which there are no treatments at the present time. It is also possible that bovine IFN could be used as a therapeutic agent in other mammalian species (i.e., human).

CHAPTER V

SUMMARY AND CONCLUSION

The production of various bovine interferons (IFN) was implemented in these studies. Bovine peripheral leukocytes were induced with either Newcastle disease virus (NDV) or concanavalin A (Con A) for the production of bovine leukocyte IFN and immune IFN, respectively. Fibroblast IFN was obtained from MDBK cells induced with NDV virus.

Various physical and biological properties of the three interferon classes were investigated. Priming, a non-antiviral property of IFN, was effective as a method to obtain high IFN yields. Bovine fibroblast and leukocyte IFNs were shown to prime the production of a different IFN class, immune IFN. All three IFN classes primed the production of fibroblast-type IFN.

Bovine leukocyte and fibroblast IFNs were found to differ in their development of the antiviral activity as well as their protection of heterologous cells. Fibroblast interferon induced the development of the antiviral state more rapidly than leukocyte IFN. Also, fibroblast IFN appeared to protect heterologous cells (other species) more than leukocyte IFN.

The three interferon types were found to have dif-

ferent stabilities when exposed to pH 2 and 56 degrees centigrade. Immune IFN was the least stable of the three when exposed to these conditions. Leukocyte and fibroblast interferons differed in their temperature stability. Leukocyte IFN was the most stable of the three IFN types.

It was concluded from these studies that various bovine interferon classes exist which may be separated on the basis of their biophysical properties. To obtain a more definitive answer in terms of their properties, it will be necessary to use highly purified samples for future studies.

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