PRODUCTION AND CHARACTERIZATION OF BOVINE INTERFERONS

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

I. Statement of the Problem

Interferons are a heterogenous group of proteins manufactured by almost all nucleated animal cells. The property which differentiates this substance from other cellular proteins is its characteristic antiviral effect. More recently, a number of non antiviral properties have been described. Much effort has been exerted in order to evaluate the clinical efficacy of interferon on viral diseases.

For many years, interferon was considered species specific. Recent discoveries regarding the cross-species antiviral activities of interferons have stimulated research on the interferon systems of other species. The possibility of finding an animal interferon that has profound antiviral activity on human cells would provide an economic way of producing non-human interferon for human medical use. Among animal interferons tested, bovine interferons have been found to impart protection to certain types of human cells. The reverse is also true, i.e., human interferons also protect bovine cells. In light of these findings and the fact that studies on bovine interferon have been limited in scope, our laboratory has studied certain properties of bovine interferons.

Our studies consisted of two parts. Part I was concerned with *in vitro* kinetics of bovine interferon production. In this part, bovine cell types were tested for their responses to interferon inducers. Part II

was concerned primarily with the basic characteristics and properties of bovine interferon.

II. Background

A. History. Interferons, like antibodies, are proteins induced in animal cells in response to "foreign" substances. These interferons have the ability to inhibit viral replication. For a time, viruses were thought to be the sole agent capable of inducing antiviral activity (12, 55). It is now known that interferon production can be elicited by synthetic double stranded polynucleotides (33, 45, 47, 52), mitogens (98), antigens (100), and enterotoxin (58).

The term "interferon" is often used to suggest that more than one molecular form of interferon is produced in response to different stimuli. These interferons were found to be different in terms of molecular weights, chemical and physical properties, as well as biological activities (2, 19, 20, 28, 84, 85, 86, 100). Since the discovery of a mitogen type and an immune-recognition type interferon (79, 98), it is possible to categorize interferons into two major types based on the nature of the inducers and certain properties of the interferons (52). All interferons produced in response to stimulation with either live or inactivated viruses and synthetic polynucleotides are classified as type I interferons to distinquish them from those produced as a result of either immune response to specific antigens or stimulation of non-sensitized lymphocytes by mitogens, including staphylococcal enterotoxin. These interferons are classified as type II. More recent data have suggested that there may be subclasses of type I and type II interferons. For example, virus induced leukocyte

interferon is biochemically different from virus induced fibroblast interferon (84, 85, 86). Also, B-cell mitogens induce interferon in lymphocyte cultures which differs from that of T-cell mitogen induced interferon (99). The terms "RNA induced or RNA type" and "mitogen induced or mitogen type" interferons will be used throughout this thesis to denote the types of interferon we have studied. However, the terms type I and type II interferon will still be used, where appropriate, to coincide with the terminology used in some references. A number of laboratories have published reviews on the production and properties of murine and human interferons. The mechanism of induction, the efficiency of the inducers, the regulation of production, and the purification and characterization of interferons are well summarized by Finter (35), Ho and Armstrong (52), and Bean (6).

B. Mechanism of Interferon Action. The binding of interferon molecules to the cell surface is the initial step for antiviral action (3, 61). The site of binding seems to be specific since trypsin treatment of monkey-mouse hybrid cells destroys the cells' sensitivity to primate interferon (16). Specific affinity of interferon for the carbohydrate side chain of cell membrane gangliosides has been shown (8, 9, 15). Furthermore, many substances of known affinity for gangliosides inhibit action of interferon. These include cholera, thyrotropin, and human chorionic gonadotropin (37, 69). Although these reports suggest the presence of specific interferon receptors on the cell surface, their existence remains hypothetical until demonstrated directly.

The amount of interferon bound to cells is only a small fraction of that applied to the cells (7, 83). Interferon is not by itself the direct

antiviral agent. Cells treated with actinomycin D after exposure to interferon have been shown to be susceptible to virus (23), suggesting that interferon acts by triggering a process involving the synthesis of another "antiviral protein" (AVP). Large quantities of AVP could be produced by the translation of its mRNA induced by interferons. This amplification scheme explains why interferon has a high biological activity and only a few molecules are needed to protect a cell.

The intracellular events following activation of cell surface receptors by interferon leading to the derepression of the genes for AVP are not known at present. Recent data on interferon action have been reviewed by Joklik (60). Undoubtedly, the clarification of this system would help to understand the action of interferon as well as certain aspects of genetic regulation.

C. Interferon Induction.

Type I interferon production. In general, two mechanisms of interferon production have been described: (1.) de novo interferon synthesis which involves the derepression of interferon genes followed by transcription and translation, and (2.) interferon may be released as preformed endogenous interferon proteins (51, 59). However, this hypothesis still remains to be challenged (70, 87).

It is now known that the best inducers for type I interferons in vivo and in vitro are live or inactivated double stranded RNA viruses and synthetic double stranded RNA such as polyriboinosinic-polyribocytidylic acid (poly I:C). Injection of these inducers into animals usually resulted in the production of large quantities of classical type I interferons (48, 50, 95).

It is also well recognized that cattle are capable of producing interferon in response to viral infection as well as in vivo injection of poly I:C (72, 73, 74, 91). Usually, very low serum titers of interferon were obtained. Titers ranging from 2 to 48 units/ml of serum were reported by Rosenquist (72) when he inoculated calves with New Castle Disease Virus (NDV), Vaccinia virus, and parainfluenza-3 virus (PI-3). Kinetics of production in these experiments showed that the production of type I interferon in vivo was a rapid process, with peak titer occurring during the first 6 hours post inoculation. Highest titers of interferon were obtained in animals injected intravenously (i.v.) while longest persistence of antiviral activities found in sera were associated with intramuscular (i.m.) injection of the viral inducers. In spite of the production of interferon in vivo, bovine serum contains excessively large amounts of albumin and other proteins, making the isolation and purification of bovine serum interferon a difficult process.

In vitro production, therefore, should offer a means to eliminate the above problems. It is well established that bovine tissue cells could produce interferon upon exposure to viruses. Calf kidney cells have been used in many studies, and among the agents used to induce interferon in these cells are PI-3 (46, 71, 94); influenza A virus (34, 41, 90); foot and mouth disease virus (26, 27); NDV (25, 46); poly I:C (33); and infectious bovine rhinotracheitis virus (IBR)(73). Other bovine tissues reported to be capable of producing interferon are tracheal organ cultures, thyroid, tongue epithelium, leukocytes and testicle. A review by Rosenquist (75) has summarized these data.

Type II interferon production. The production of type II or immune-specific interferon was first observed by Green (42) when he exposed sensitized human lymphocytes to specific antigen. Stinebring and Absher (89) infected mice with Bacille-Calmette-Guerin (BCG) and later injected them with purified protein derivative (PPD) and obtained high levels of circulating interferon. Salvin, et al. (77, 78, 79) also found a high level of serum interferon in BCG infected mice injected with old tuberculin (OT). The latter authors were the first to recognize major differences between immune and classical interferons.

Production of type II interferon is not restricted to immune-specific reactions. Researchers have shown that interferon production also occurred when non sensitized lymphocytes were stimulated by mitogens, such as phytohemagglutinin (PHA) (98), pokeweed mitogens (PWM) (36), concanavalin A (con A) (97), antilymphocyte serum (ALS) (31), streptolysin O (36), and staphylococcal enterotoxin (SEA) (58). The mechanisms by which interferon is produced by mitogen stimulation is not known at the present time. The types of interferon produced in leukocyte cultures in response to mitogen stimulation, and those produced by sensitized lymphocytes upon challenge of the specific antigens have been traditionally referred as type II to distinquish them from the type I interferon produced by numerous cell types in response to viruses or synthetic polyribonucleotides. However, the types of interferon produced by lymphocytes upon stimulation is not always predictable, and can be influenced by the type of inducer used, the cell subpopulation, and the immune status of the blood donor (29).

One result of the interaction of mitogen with lymphocytes is the transformation of lymphocytes accompanied by an increase in DNA synthesis.

Lazary, et al. (64) showed that bovine lymphocytes did exhibit blastogenesis when stimulated by mitogens such as PHA, con A, and lipopolysaccharide. In his experiments he did not look for the production of interferon. Fulton (38) reported that only very low titers of interferon were produced by bovine lymphocytes upon exposure to PHA.

Stimulation by IBR and PPD antigens led to the production of interferons within 24 hours in bovine peripheral blood lymphocytes obtained from IBR virus and tuberculin immunized cattle (4). The separation of T-lymphocytes suggested that T cells were the antigen-specific cell for immune interferon production. The levels of interferon produced were increased about 2- to 10-fold when lymphocytes were cocultivated with autologous macrophages. The substances released from macrophages which had potentiated the effect on lymphocyte interferon production were not identified, but physical contact between macrophages and lymphocytes was required for the enhancement of interferon production. Although the authors claimed that the interferons produced were of the immune-specific type, no further characterization of these interferons were done.

D. Properties of Interferon. Properties of interferon from various species of animals can be generalized as follows: All interferons are protein or glycoprotein and are destroyed by various proteases but not by nucleases (32). The molecular weights usually range from 20,000 to 100,000 daltons. These molecules are non-dialysable and are not sedimented by 10,000 g for 2 hours. The isoelectric points of interferons usually lie between pH 6.5 and pH 7.5. Stability of type I interferons is usually maintained within the range of pH 2 to pH 10 and is relatively stable at 37°C.

It is established that type I interferons are stable at pH 2 but labile at 56°C (12), while type II is labile at pH 2 but stable at 56°C (100). However, the stability of interferons to extreme pH and to heat is greatly influenced by the degree of impurities present in the crude preparations (80). For example, interferon activity of a virus-treated mouse tissue culture (type I interferon) was found to be stable at 60°C for 60 minutes (39), while Rytel and Kilbourne (76) noted that type I mouse serum interferon lost 50 percent of its activity even at 37°C. Lockart has suggested that pH stability was not a satisfactory criterion for characterizing interferons (66). In the area of bovine interferon, some controversy still surrounds the issue of pH 2 stability. For example, Kono (62) and Fulton (38) both reported pH 2 inactivation of bovine type I and bovine leukocyte interferons. Bearing these facts in mind, the assessing of the pH and heat stability of bovine interferons was performed in our laboratory.

Because low pH and high temperature stability are not in themselves satisfactory criteria for characterizing interferons, other parameters have been used including: (1.) Rate of antiviral protein induction (24). It was found that murine and human type I interferons induced the antiviral state in their homologous cells earlier than the type II interferons. (2.) Cross species protection (4). It was shown that bovine immune type interferon protects heterologous cells better than bovine type I interferon. Besides having protective effects on human cells, bovine interferons have also been shown protective in porcine cells (1), rhesus monkey kidney cells (90), monkey cells BSC (93), rabbit RK-13, Equine E-derm,

canine DK, and porcine ESK cells (4). Except for porcine ESK cells, which were protected by bovine immune interferon to a greater extent than that on homologous cells, the other cells were only partially protected. (3.) Anticellular activity of interferon which slows the growth of many cells in cultures has also been used for interferon characterization. For example, the growth of osteosarcoma lines is restricted more by human fibroblast interferon than by leukocyte and lymphoblastoid interferons (13). (4.) Antitumor effects of interferons have been evaluated (18, 53). Preliminary reports from Glasgow, et al. (40) have shown that in the murine osteogenic sarcoma system, type II interferon was more effective as an antitumor agent than type I.

Immunoregulation by the two types of interferon emerged recently as one of the more important non-antiviral properties of interferon. It is known that type I mouse interferon inhibits in vitro antibody responses to T-dependent and T-independent antigens, blastogenesis of lymphocytes, and allograft rejection (57). Also type I interferon modulates the secondary antibody response as well as the primary response to a variety of antigens (10, 11, 17, 56). It was found that type II murine interferon is 100 to 1,000 times more immunopotent than type I interferon (Sonnenfeld, 1977, unpublished data). Moreover, interferon can be immunopotentiating or immunosuppressive depending on the dose of interferon and the time of application relative to antigen (67).

In our studies, we have employed low pH stability, rate of heat inactivation, rate of cellular reactivity leading to AVP production, and degree of cross species antiviral activity to characterize the types of

bovine interferon which were prepared. No studies were carried out in the area of immunoregulation, antitumor, or anticellular activity.

III. Objectives

The objectives of our studies were: (1.) to devise a workable system for the production of large quantities of bovine type I and type II interferons *in vitro*, and (2.) to characterize certain parameters which would help classify the types of bovine interferons.

CHAPTER II

MATERIALS AND METHODS

Animals. A healthy Holstein steer, age 16 months, was the principal blood donor. This steer had been vaccinated with a bovine rhinotracheitis virus-bovine diarrhea virus-parainfluenza-3 virus triple vaccine (IBR-BVD-PI-3) 10 months before blood was drawn for experimental use. Non-lactating dairy cows were also used as sources of blood lymphocytes for cultures. All cattle involved were kept at the Texas Tech Farm, and were tended by experienced personnel.

Chemicals and reagents. Poly I:C purchased from P-L Biochemicals (Milwaukee, Wisconsin) was used as a type I interferon inducer. A stock solution of poly I:C at 200 µg/ml was prepared in phosphage buffered saline (PBS). This stock solution was subsequently diluted in Eagle's minimal essential medium (EMEM) to the desired concentrations just prior to use.

Bovine triple vaccine containing IBR-BVD-PI-3 was obtained from Diamond Laboratories (Des Moines, Iowa). The vaccines were reconstituted in 10 ml of PBS per vial and dilutions were made with EMEM before being added to lymphocyte cultures for interferon induction.

Phytohemagglutinin-P (PHA-P) (Difco, Detroit, Michigan) was reconstituted with 5 ml of sterile Millipore filtered water. Dilution of 1:50 of this reconstituted PHA was made. The stock mitogen was stored at -20°C until used. Concanavalin A (con A) was purchased from Difco (Detroit, Michigan). The mitogen was dissolved in 5 ml of sterile 1 M NaCl. One

ml was diluted 1:10 with RPMI-1640 resulting in a final concentration of 1,000 µg/ml. All con A preparations were kept frozen until used.

Buffer solutions with pH values from 2 to 6 were made according to the method of McIlvaine (68). Stock solutions of 0.1 M citric acid and 0.2 M disodium phosphate were prepared and stored separately. Buffer solutions were made from these two stock solutions just before use.

Anti-human leukocyte interferon and anti-mouse fibroblast interferon globulins in lyophilized form were donated by Dr. June Dunnick of the National Institute of Allergy and Infectious Diseases. The powder was redissolved in 1 ml of sterile PBS and stored at -20°C.

Cell cultures. Bovine kidney cells (BK) were cultivated in our laboratory from kidney tissue obtained from local slaughter houses. Primary kidney cultures were set up according to standard tissue culture procedures (54). Briefly, the capsule was removed, and medullary tissue were excised and minced under aseptic conditions. Finely cut tissues were trypsinized with 0.25 percent trypsin (Gibco, Grand Island, New York) for 25 minutes at 37°C. Epithelial cells were selected for culture by passing trypsinized tissue suspensions through sterile guaze. Bovine kidney cells were cultured in tissue culture flasks in EMEM supplemented with 10 percent Fetal Calf Serum (FCS) (Sterile Systems, Logan, Utah), 100 units/ml penicillin, and 100 µg/ml streptomycin. Confluent BK cells were employed in interferon production. Bovine embryonic kidney cells (BEK) (Microbiological Associates, Bethesda, Maryland) were also used for interferon production. The primary cells were cultivated in our laboratory for three passages before being used for interferon production.

Splenic lymphocytes were obtained from spleens of healthy cattle slaughtered locally. The outer capsules of spleens were removed aseptically. Tissues from the middle portion of a spleen were excised, minced, and teased in 3x antibiotics supplemented PBS. Tissue suspensions were allowed to sediment at 1x g at 4°C for 20 minutes. Suspended lymphocytes were aspirated and washed twice with PBS by two consecutive centrifugations at 1,000 rpm for 5 minutes. The washed lymphocytes were resuspended in RPMI-1640 medium supplemented with 10 percent heat inactivated FCS. Final pH of the media was adjusted to 7.2 with $NaHCO_3$. Cell concentration was adjusted to $5x10^6$ cells/ml.

Bovine blood was collected from animals by jugular venipuncture with 60 cc syringes containing 5 ml of 4 percent disodiumethylene-diamine tetraacetate (EDTA). Buffy coat lymphocytes were collected by centrifuging 7 ml volumes of whole blood at 2,000 rpm for 20 minutes. Buffy coats were harvested and diluted in equal volume of PBS. Four ml aliquots of diluted buffy coat were overlaid carefully on top of 3 ml of Ficoll-Paque (Pharmacia, Piscataway, New Jersey) solution. All samples were centrifuged at 1,500 rpm for 25 minutes at 25°C. Harvested lymphocytes were washed twice with PBS and resuspended in RPMI-1640 medium supplemented with 10 percent heat inactivated FCS. Final pH was adjusted to 7.2 with sodium bicarbonate. RPMI-1640 media used in experiments involving long term cultivation (i.e., more than 48 hours) of lymphocytes with mitogen was further supplemented with 0.025 M N-2-hydroxyethylpiperagine-N'-2'-ethanesulfonic acid (HEPES) buffer. In either case the resuspended cells were counted in a Coulter Counter and the final cell concentration was adjusted to $5x10^6$ ml.

A number of other cell strains and lines were also employed in cross species antiviral activity experiments. The cells used are listed on Table I.

RNA type interferon induction. A blood sample was taken from the steer before injection. Two doses of bovine triple vaccine were injected both i.v. and i.m. At 6, 24, 48, and 72 hours as well as 1 and 2 weeks post injection, blood samples were drawn and sera separated by centrifugation. One and a half months later, the steer was reinoculated i.v. with the vaccine and interferon levels were again measured.

Attempts were made to induce classical type I or fibroblast interferon in BK, BEK, and MDBK cells. These three cell types were grown to confluency on tissue culture plates. Poly I:C at 20 µg/ml was added to each plate. The cells plus the inducer were incubated for two hours, then washed and refed with fresh medium. After refeeding, induced cells were incubated overnight. The ability of each cell type to produce interferon was evaluated.

BEK cells were chosen later for the kinetic studies. In these experiments, BEK cells were grown to confluency on 30 mm tissue culture dishes. Poly I:C at concentrations of 5, 10, and 20 µg/ml medium were added to the cells in 2 ml volumes. Triplicate plates for each concentration were set up and the poly I:C was allowed to adsorb to the cells for 2 hours at 37°C. After which time, the media were aspirated and the cells washed twice with PBS then refed with EMEM maintenance media followed by an additional incubation period. At intervals from 1 to 24 hours post refeeding, small aliquots of media were removed from the cultures for interferon assay.

TABLE I

SUMMARY OF CELL CULTURES UTILIZED IN EXPERIMENTS

Cell Type	Passage	Source
MDBK Bovine Stable Cell Line	>200	Dr. William Stewart, II Sloan Kettering Cancer Institute, New York
L-929 Mouse Fibroblasts	>200	Dr. William Stewart, II Sloan Kettering Cancer Institute, New York
HFF Human Foreskin Fibroblasts		Dr. John Morrow Texas Tech University School of Medicine
WI-38 Human Embryonic Lung	30-35	Microbiological Associates Bethesda, Maryland
	26-30	Dr. William Stewart, II Sloan Kettering Cancer Institute, New York
HEK Human Embryonic Kidney	3-7	Microbiological Associates Bethesda, Maryland
MK Monkey Kidney		Microbiological Associates Bethesda, Maryland
PK Porcine Kidney		Flow Laboratories Rockville, Maryland
Hela Human Carcinoma		Dr. William Stewart, II Sloan Kettering Cancer Institute, New York
CEC Chick Embryo	3-6	Microbiological Associates Bethesda, Maryland
RK-13 Rabbit Kidney		Microbiological Associates Bethesda, Maryland
Rat Rat Embryo (Fischer)	50	Microbiological Associates Bethesda, Maryland

In order to potentiate interferon production in MDBK cells, 100 µg of DEAE-Dextran per ml of medium (MW $2x10^6$, Pharmacia, Uppsala, Sweden) was used together with poly I:C (22, 30). Interferon levels were determined after overnight incubation.

Lymphocytes isolated from bovine blood and splenic tissues were used for interferon production. Bovine triple vaccine and poly I:C were the inducers used. After reconstitution, the vaccine was diluted with RPMI-1640 medium to 10^{-1}, 10^{-2}, and 10^{-3}. Volumes of 0.1 ml were added to cultures containing 3 ml with $5x10^6$ cells/ml. These cultures were incubated under 5 percent CO_2 at 37°C. Tube cultures were incubated at an angle of about 10°. On days 1, 2, and 3, cultures were removed, and irridated with UV light for 5 minutes at 15 cm distance, and the fluids were assayed for interferon activity.

Poly I:C at 10 to 40 µg/ml cells was added to tube cultures containing 3 ml of cell suspension. The tubes were incubated for 2 hours at 37°C. After incubation, cells were centrifuged, washed, then refed with RPMI-1640 medium, then reincubated. At intervals of 4 to 24 hours after refeeding, samples were withdrawn from each culture and assayed.

Mitogen type interferon induction. Blood and splenic lymphocytes were exposed to either PHA-P or con A. PHA-P at concentrations from 4 to 20 µg/10^6 cells were used, while the concentrations of con A used varied from 10 to 30 µg/10^6 cells. After addition of the mitogens, interferon levels in cultures were monitored at 24 hour intervals. The mitogen of choice used in the kinetic studies was con A. The optimal level was 10 µg/10^6 cells which was added to 5 ml of medium containing $5x10^6$ lymphocytes/

ml. The tubes were incubated and small aliquots of tissue culture fluid were taken from each tube at intervals for interferon assay.

In order to determine the effect of the length of mitogen-cell contact on interferon production, some cultures were given a pulse induction with con A for only 2 hours. After the first two hours of incubation with con A, the cells were centrifuged, washed, and refed with fresh RPMI-1640. These cultures were then reincubated and interferon titers were determined.

Interferon assay. Vesicular stomatitis virus (VSV) Indiana strain was cultivated in mouse L-929 cell line. After harvest, the virus stock was stored at -70°C until used for interferon assay. The assay employed was the viral plaque inhibition method (14) with slight modifications. Briefly, bovine MDBK cells were grown to confluency on Micro Test II plates (Falcon, Oxnard, California) in three-fold serially diluted media preparations containing interferon. The cells were challenged with fixed amounts of VSV for one hour. Cells were then overlaid with 0.5 percent methylcellulose in EMEM. Interferon titer was expressed as the reciprocal of the interferon dilution that reduced 50 percent of the viral plaques.

Quantitatively, the exact titer was calculated according to the following equation:

$$\text{Interferon titer} = \frac{[(1/2 \text{ plaque on control}) - (\text{plaque number of low neighbor})]}{(\text{difference in plaque number})} \times (\text{difference in dilution}) + \text{low dilution}$$

Interferon characterization. Trypsin and ribonuclease, which inactivate interferons and poly I:C respectively, were used to confirm that the substances which imparted antiviral activity to the assay system were protein in nature and not "carried-over" poly I:C (in the poly I:C induced

interferon samples). Trypsin (Gibco, Grand Island, New York) at 0.05 mg/ml sample and ribonuclease (Sigma, St. Louis, Missouri) at 100 µg/ml were incubated with interferon samples picked at random from both the RNA induced and mitogen induced interferon preparations. After incubation of the interferon samples with either trypsin or ribonuclease at 37°C for 30 minutes, the interferon titers were determined and compared to control samples. Methods used were those suggested by Baron (5) and Lockart (66).

Both RNA and mitogen induced interferons were characterized according to their pH stability. Interferons made during the first 6 hours post induction of BEK cells by poly I:C were designated as early RNA induced interferon (ERIF) and those produced at the 18-hour or overnight incubation were designated as late RNA induced interferon (LRIF). Mitogen induced interferons were also divided into early (EMIF) and late (LMIF) types. These interferon samples were dialysed against a range of pH's using McIlvaine's citric acid sodium phosphate buffers. Before dialysis, all dialysis tubing was boiled in 1 mM EDTA for 30 minutes and the treated bags were then transferred to 0.1 mM EDTA solution for storage. Samples in 0.4 ml volumes of all four groups of interferons were placed in individual EDTA treated dialysis bags. The bags with their contents were dialysed overnight against at least 100 times their volume of buffers at pH 2 through 6. This was followed by dialysis with PBS overnight to bring the dialysed samples back to physiological pH. Treated samples were assayed for interferon activities on MDBK cells. The amount of residual interferon activity was expressed as the percent reduction in titer compared to samples dialyzed at pH 7.2 only.

The stability of interferon at various temperatures was evaluated. In these experiments, crude interferon samples from the late production phase were subjected to either 45 or 56°C for periods of 15, 30, and 45 minutes. At each of these incubation periods, small aliquots were withdrawn and assayed for interferon. The titers obtained were compared to the titers of controls not exposed to heat.

The rate of AVP production was measured following addition of RNA induced and mitogen induced interferons. MDBK cells were grown to confluency on Micro Test II microtiter plates. Both types of interferon at concentrations of approximately 2,000 units/ml were serially diluted in wells above the cell sheets. Care was taken not to disturb cell sheets. The plates were then incubated for 1 to 24 hours. At intervals, cells incubated with each type of interferon were challenged with fixed amounts of VSV after washing off the unadsorbed interferon twice with PBS. Antiviral activities on the plates were assayed.

The neutralization of interferon by specific antisera is one of the ways to identify interferon as being the cause of antiviral activity. Unfortunately, there is no antisera to bovine interferons. Anti-human leukocyte interferon globulins (Anti HLeIF) and anti-mouse fibroblast interferon globulins (Anti MoIF) were tested for activity against bovine interferons. The lyophilized anti HLeIF and anti MoIF were reconstituted in 1 ml of PBS and dilutions of 1:50, 1:100, 1:200, and 1:400 were made. Each dilution of human or mouse anti interferon in 0.2 ml volumes was added to equal volumes of crude bovine interferon. PBS was added to controls instead of antibody. The samples were incubated at 37°C for 30 minutes to allow

completion of antibody-antigen reactions. Residual antiviral activities were assayed on MDBK cells.

One method of characterizing interferons is the range which interferon will protect. A battery of cells were obtained from various sources. Table 1 shows the types of cells used and their sources. These cells were used along with MDBK cells for interferon assays. Each interferon sample was assayed on MDBK cells as well as cells from other species. Thus cross-species antiviral activity of a particular interferon preparation could be measured and compared.

CHAPTER III

RESULTS

I. Production of Interferons

A. Type I (RNA type) Interferon. Injection of viral vaccines in vivo resulted in the production of moderate levels of interferon. Following the first 2 doses of triple vaccine, the circulating level of interferon peaked 6 hours post injection (Figure 1). The titer decreased to less than the background level after 24 hours then increased again to a maximum titer of 200 which was obtained by 48 to 72 hours. After the first week post injection, serum interferon had already dropped to pre-injection levels. It should be noted that the prebleeding serum from this steer had a titer of 70 units/ml of serum. One and a half months later, the prebleeding serum prior to a second i.v. injection of the triple vaccine showed a background interferon level of about 15. Six hours after i.v. injection of vaccine, a slow increase in interferon titer occurred (Figure 2). This level increased to a maximum titer of 40 by the third day.

Poly I:C at 20 µg/ml was used to induce interferon in BK, BEK, and MDBK cells in vitro. Using these procedures, the highest titers of interferon were made by BEK cells (Table 2) after 24 hours of incubation. These cells were used for the kinetic studies reported below. No antiviral activity (interferon) was made in MDBK cells, even when cultures were supplemented with DEAE-Dextran.

High titers of interferon were observed following exposure of BEK cells to poly I:C. Figure 3 shows that with all concentrations of poly I:C

Figure 1. Interferon production in vivo. Two doses of bovine triple vaccine were administered i.v. and i.m. Serum interferon levels were monitored at intervals. Each value represents an average of two or more assays.

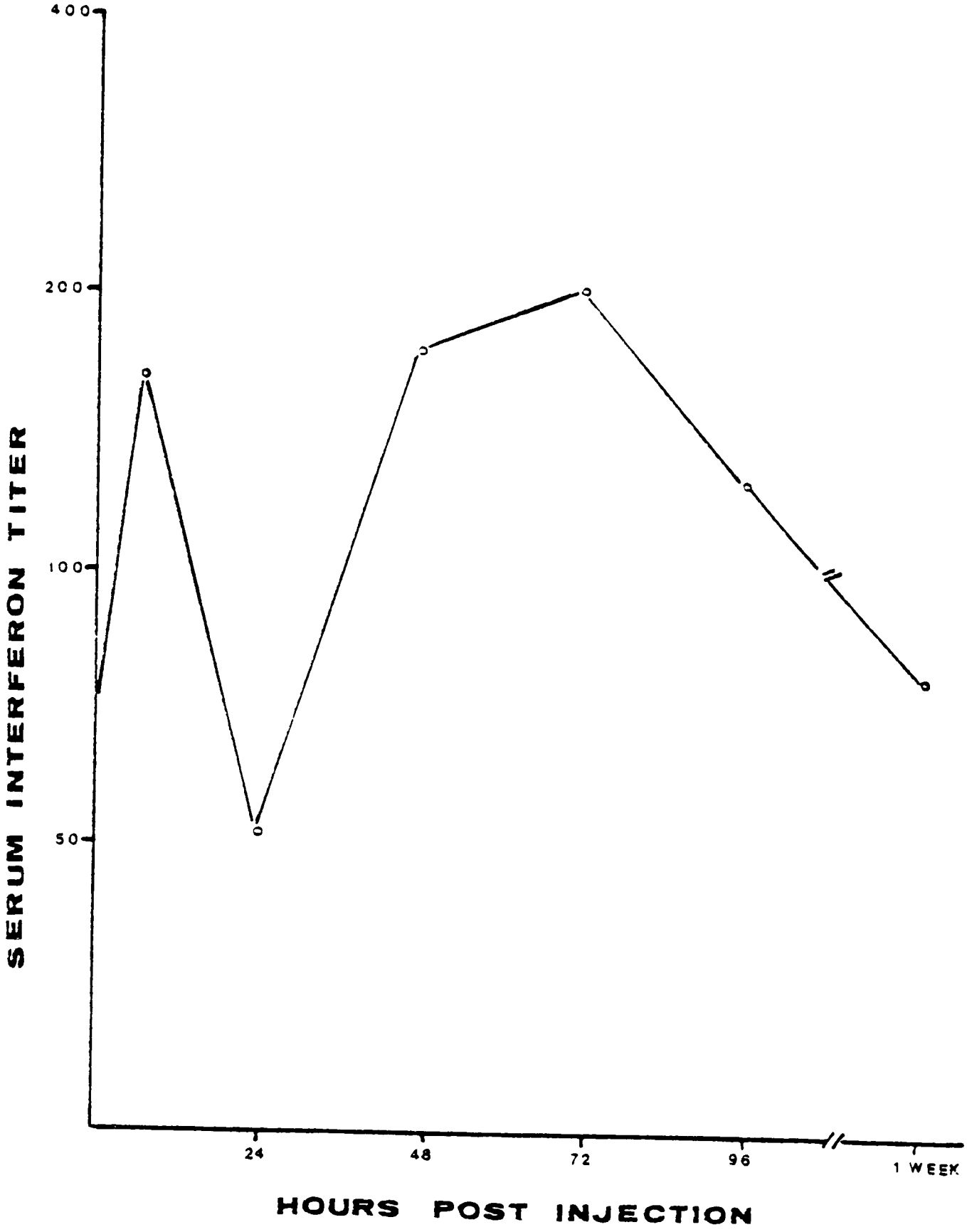

400
200
100
50
SERUM INTERFERON TITER
24
48
72
96
1 WEEK
HOURS POST INJECTION

Figure 2. Interferon production in vivo. Single booster dose of bovine triple vaccine was given i.v. one and a half months after the first vaccination. Each value is an average of two or more assays.

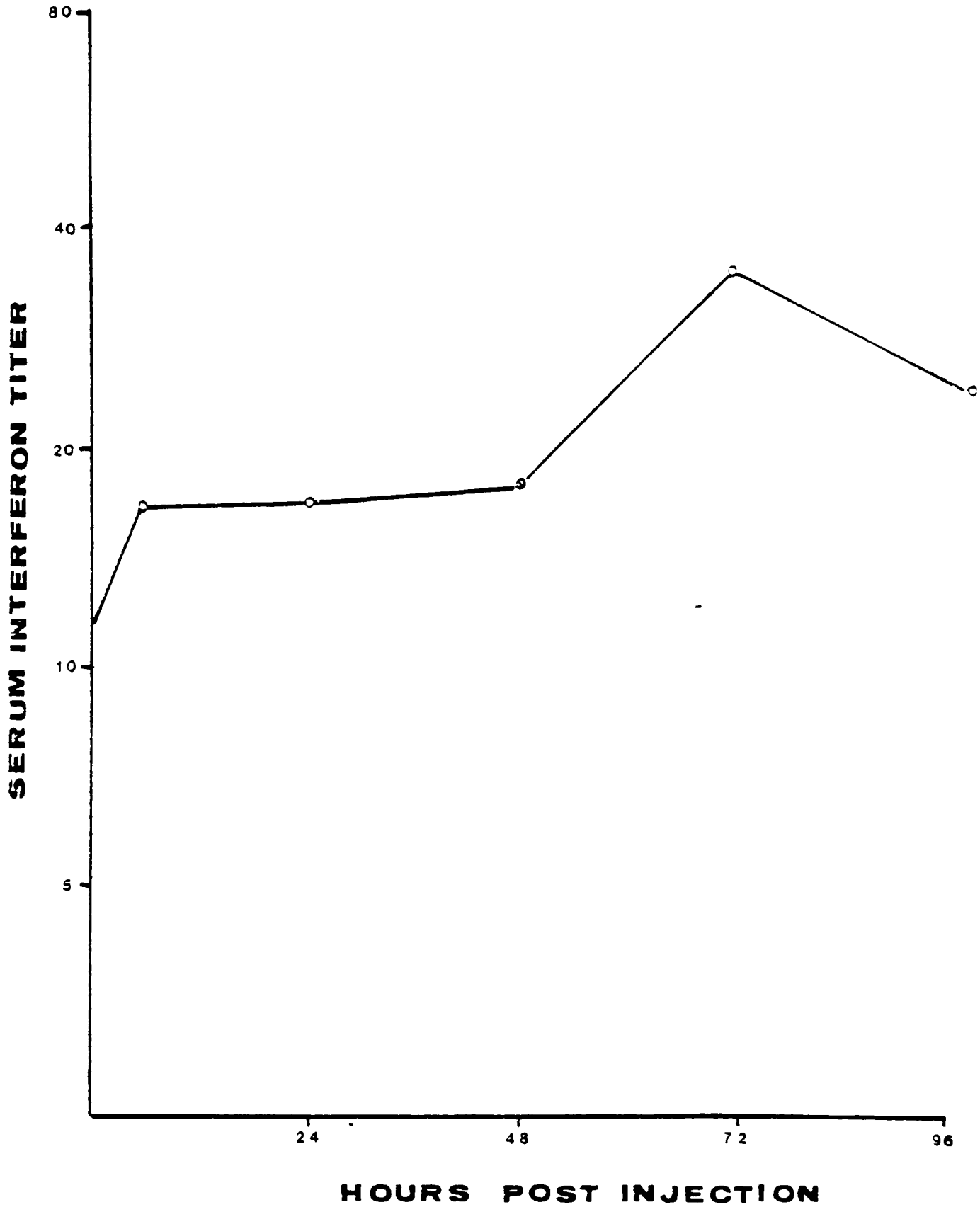

SERUM INTERFERON TITER
80
40
20
10
5
24
48
72
96
HOURS POST INJECTION

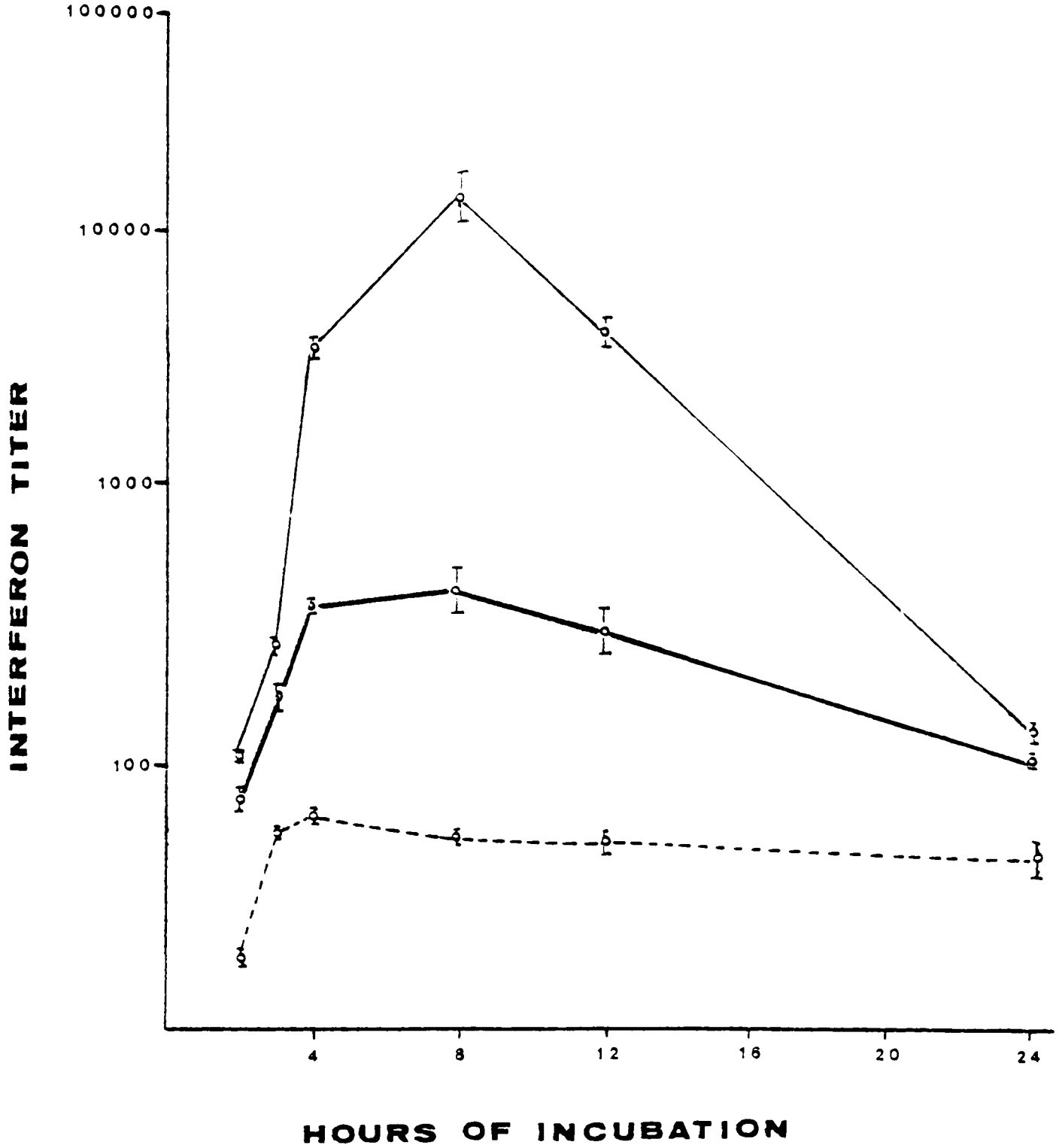
TABLE 2

RESPONSES OF BOVINE TISSUE CELLS TO POLY I:C

Cell Types	Inducer	Interferon Titers[a]
BEK	poly I:C 20 µg/ml	80-100
BK	poly I:C 20 µg/ml	20-30
MDBK	poly I:C 20 µg/ml	0
MDBK	poly I:C 20 µg/ml + DEAE-Dextran 100 µg/ml	0

[a]Titer expressed as units of interferon/ml medium.

Figure 3. RNA-type interferon production in BEK cells. Poly I:C at 5 ▬▬, 10 ——, and 20 ----, μg/ml medium was used to induce BEK cells. Results are expressed as mean values ± S.E. of three or more experiments.


100000
10000
1000
100
INTERFERON TITER
4
8
12
16
20
24
HOURS OF INCUBATION

used, peak titers were observed between 4- to 8-hour post induction. This was followed by a rapid disappearance of detectable interferon in the media. Ten µg poly I:C/ml were found to be the optimal dose followed by 5 µg/ml. Twenty µg/ml were toxic to cells as revealed by visible cytopathic effects.

When poly I:C at 10, 20, and 40 µg/ml was added to blood lymphocytes and incubated without removal of the inducer, low levels of interferon were produced. Figure 4 reveals that the rate of induction was faster in cultures stimulated by 20 µg poly I:C/ml than those stimulated by 10 µg/ml. Peak titers were observed on day one following induction with 20 µg/ml, but cultures with 10 µg/ml produced the maximum amounts of interferon 72 hours after induction.

Peripheral blood lymphocytes were used to illustrate the kinetics of interferon production. The response of lymphocytes to various amounts of poly I:C is shown in Figure 5. Highest interferon titers were observed in cultures induced by 20 µg poly I:C/ml. The curves in this figure also reflect that detectable interferon levels in the media declined rapidly within hours after the peak titers. Cultures induced by 40 µg/ml of poly I:C produced less interferon with the peak titers occurring several hours after the optimal dose.

Blood lymphocytes from an animal injected with viral vaccine were challenged *in vitro*. Viral dilutions of 10^{-2} and 10^{-3} of the reconstituted vaccine did not induce interferon. Both 10^{-1} and undiluted vaccine were slightly more effective than more diluted vaccine (Figure 6). Furthermore, the level of interferon continued to rise during incubation in cultures exposed to the undiluted vaccine.

Figure 4. Responses of peripheral lymphocytes to poly I:C induction in vitro. Poly I:C at 10 —— and 20 ---- μg/ml medium was co-incubated with lymphocytes. Results are expressed as mean values of pooled samples from four cultures.

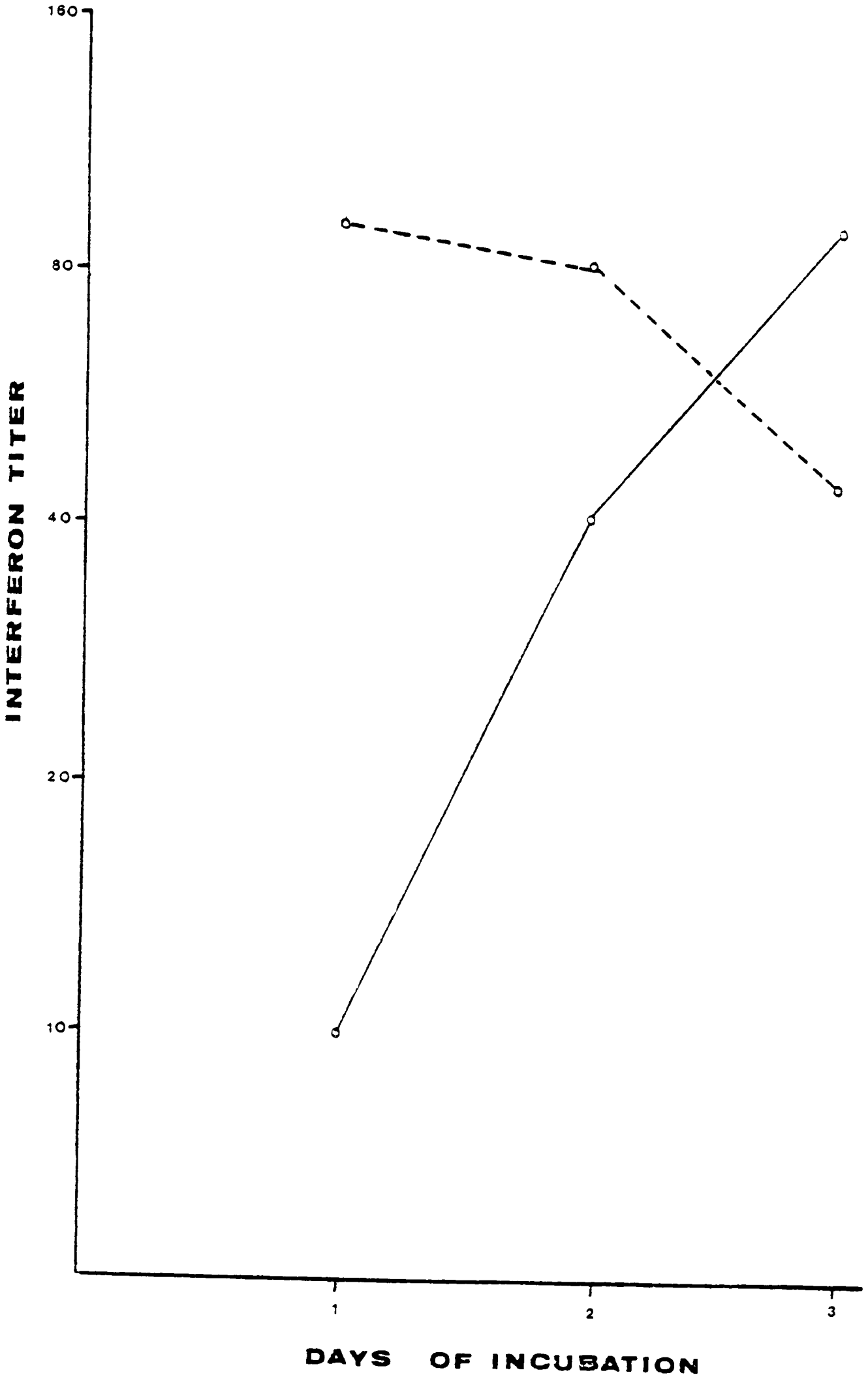

160
80
40
20
10
INTERFERON TITER
1
2
3
DAYS OF INCUBATION

Figure 5. Kinetics of leukocyte interferon production. Peripheral lymphocytes were exposed to poly I:C at 20 ——— and 40 ---- µg/ml medium for two hours. Results represent the mean values ± S.E. of three experiments.

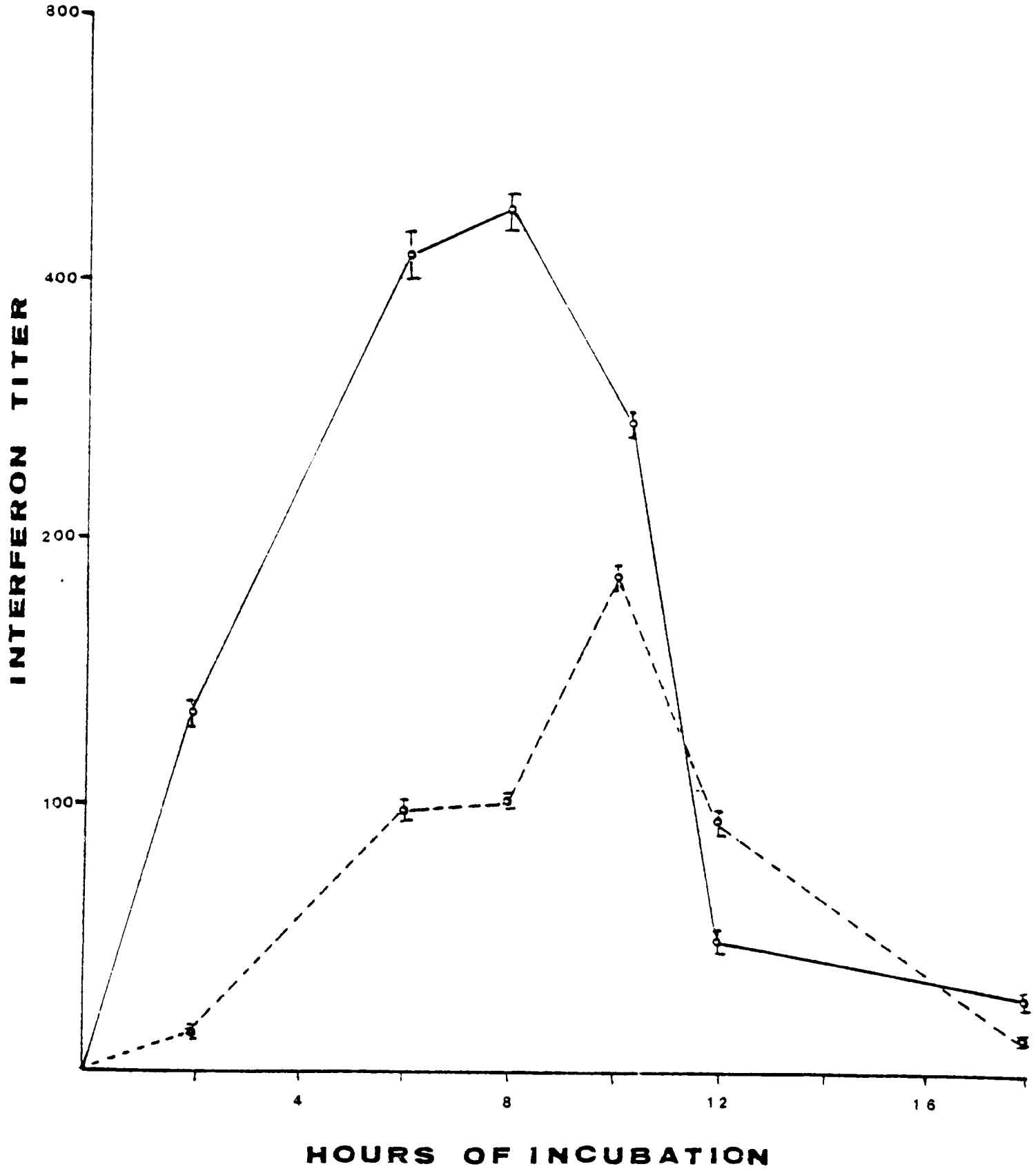
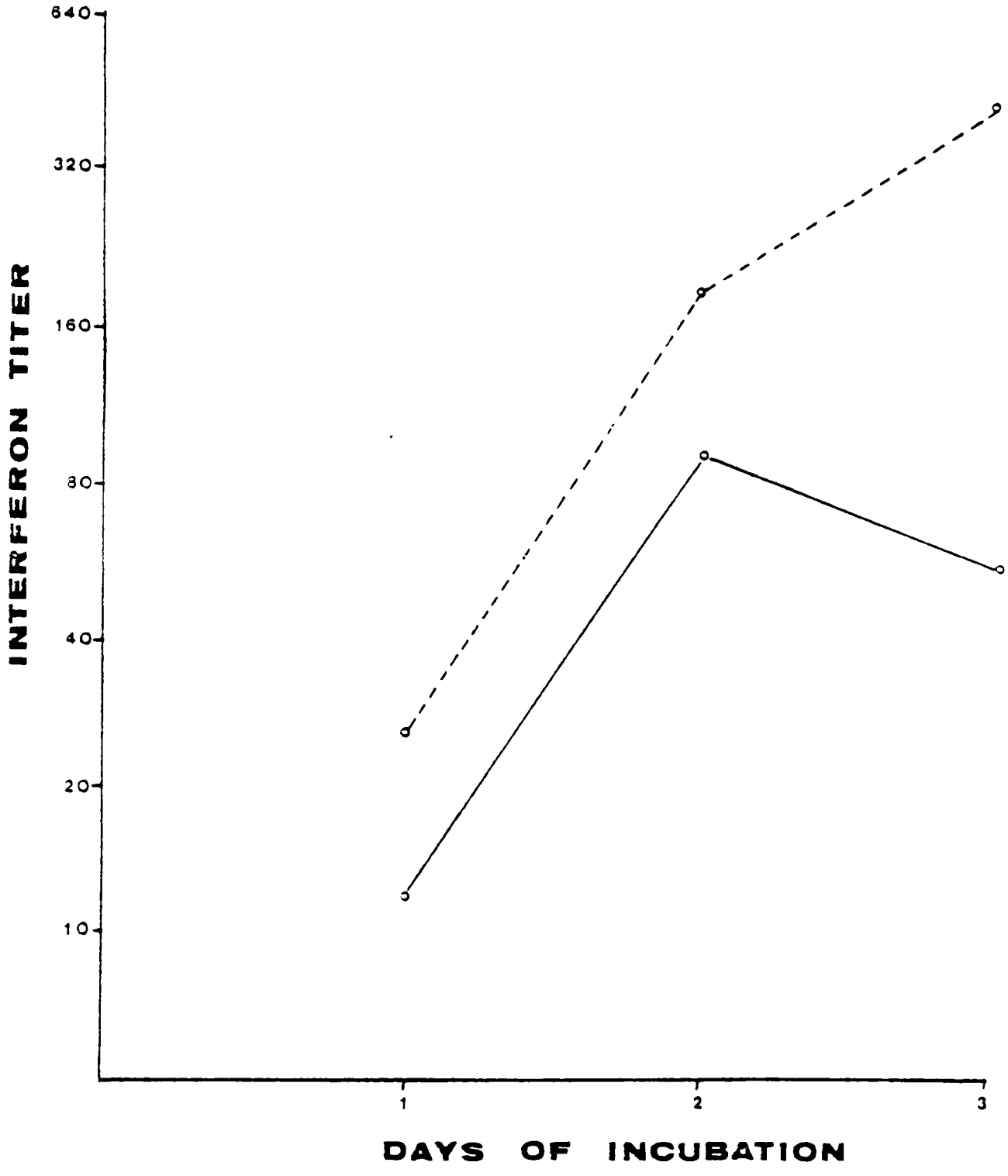

800
400
200
100
INTERFERON TITER
4
8
12
16
HOURS OF INCUBATION

Figure 6. Responses of peripheral lymphocytes to bovine triple vaccine. Lymphocytes from an animal sensitized to bovine triple vaccine were co-cultivated with the same vaccine *in vitro*. Undilute ---- and 10^{-1} ——— of the vaccine were used. Each value represents the titer of a pooled sample from three tubes.


INTERFERON TITER
640
320
160
80
40
20
10
1
2
3
DAYS OF INCUBATION

B. Mitogen Type Interferon Induction. Peripheral blood lymphocytes were tested for their responses to PHA-P and con A. Figure 7 shows that PHA-P at 4 μg/10^6 cells did not induce as much interferon as 20 μg/10^6 lymphocytes. The maximum titer of approximately 800 on day 3 of incubation was obtained with the higher dose of PHA while a titer of only 200 was obtained in clutures stimulated with the lower amounts of the mitogen during the same incubation period.

Figure 8 shows the production of interferon by peripheral lymphocytes induced by 10 and 30 μg con A/10^6 cells. Both concentrations induced high interferon titers, but 30 μg of con A had a higher toxicity to lymphocytes. Toxicity was measured by viable cell counts using a trypan blue dye uptake exclusion count. Table 3 shows that higher concentrations of con A were toxic to lymphocytes. The lower dose, 10 μg/10^6 cells, was used throughout the remainder of these studies.

Ten μg con A/10^6 cells were added to splenic lymphocyte cultures, and the production of interferon was monitored. Figure 9 shows that, like peripheral lymphocytes, the interferon level was still rising on day 3 of incubation. However, the level of interferon produced was much lower than that in the lymphocyte cultures.

With regard to con A, lymphocytes continued to produce interferon up to three days provided that the inducer was present during the entire incubation period (Figure 10). A biphasic-induction curve was characteristically noted. The first induction phase was observed usually in the first 10 hours of incubation which was followed by a decrease in interferon titer. This drop in titer occurred at approximately 10 to 12 hours followed by an increase through 72 hours.

Figure 7. Responses of peripheral lymphocytes to PHA-P. Lymphocytes were co-cultivated with 4 ---- and 20 —— µg PHA/10^6 cells. Results represent the values of pooled samples from three tubes.

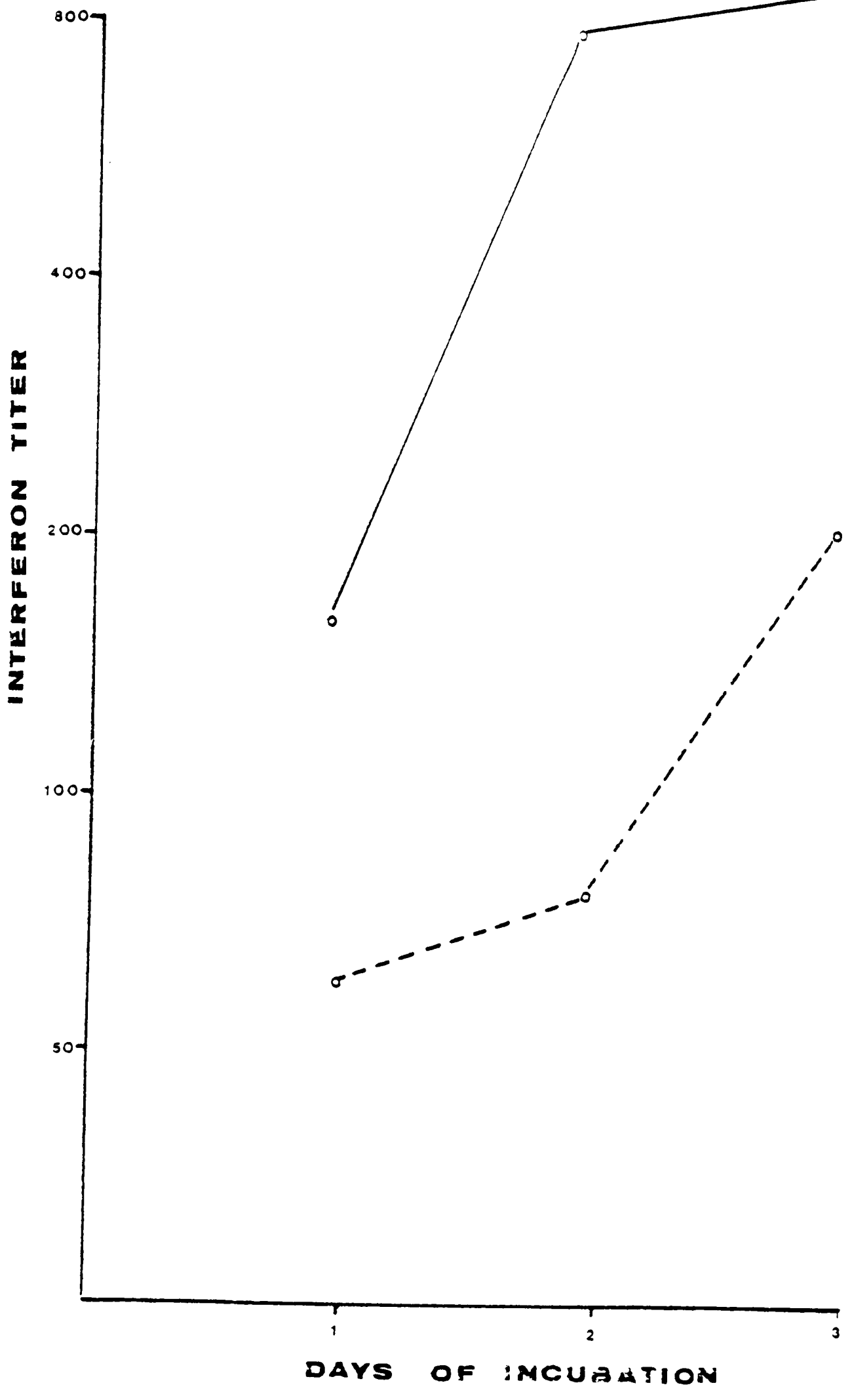

INTERFERON TITER
800
400
200
100
50
1
2
3
DAYS OF INCUBATION

Figure 8. Responses of peripheral lympohcytes to con A. Lymphocytes were cultivated with 10 ---- and 30 —— µg con A/10^6 cells. Results are expressed as the average values of pooled samples from triplicate tubes.

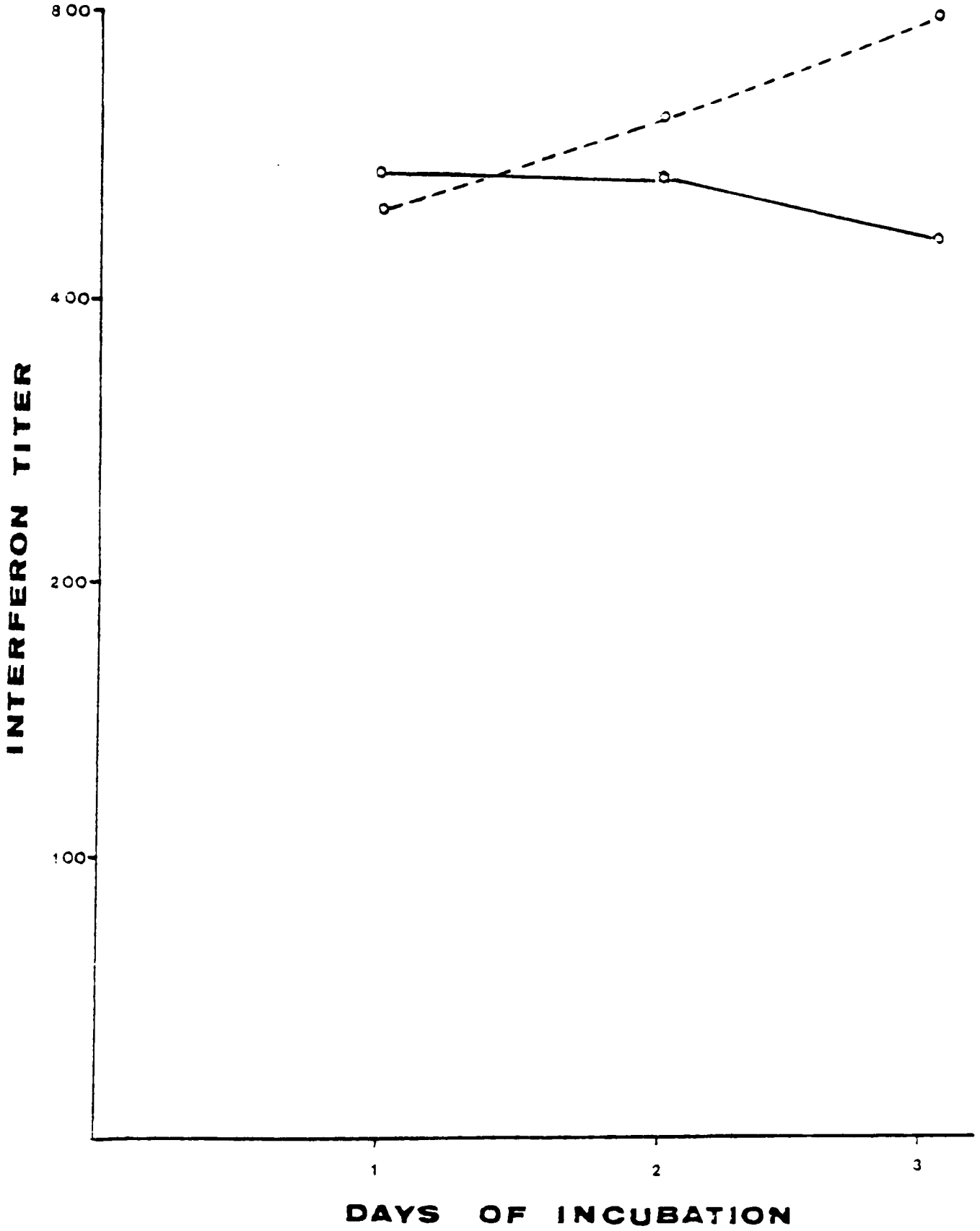

800
400
200
100
INTERFERON TITER
1
2
3
DAYS OF INCUBATION

TABLE 3

CYTOTOXICITY OF CONCANAVALIN A TO PERIPHERAL LYMPHOCYTES

Hours of Incubation	Controls	Con A/10^6 cells	
		10	30
4	100.0[a]	55.9	7.9
8	72.7	37.4	7.6
10	64.0	18.9	4.1
24	68.2	18.8	3.2
48	19.9	4.1	1.4

[a]Percent of viable cells assessed by trypan blue uptake.

Figure 9. Responses of splenic lymphocytes to con A. Splenic cells were co-cultivated with con A at 10 μg/10^6 cells. Results are expressed as mean values ± S.E. of three experiments.

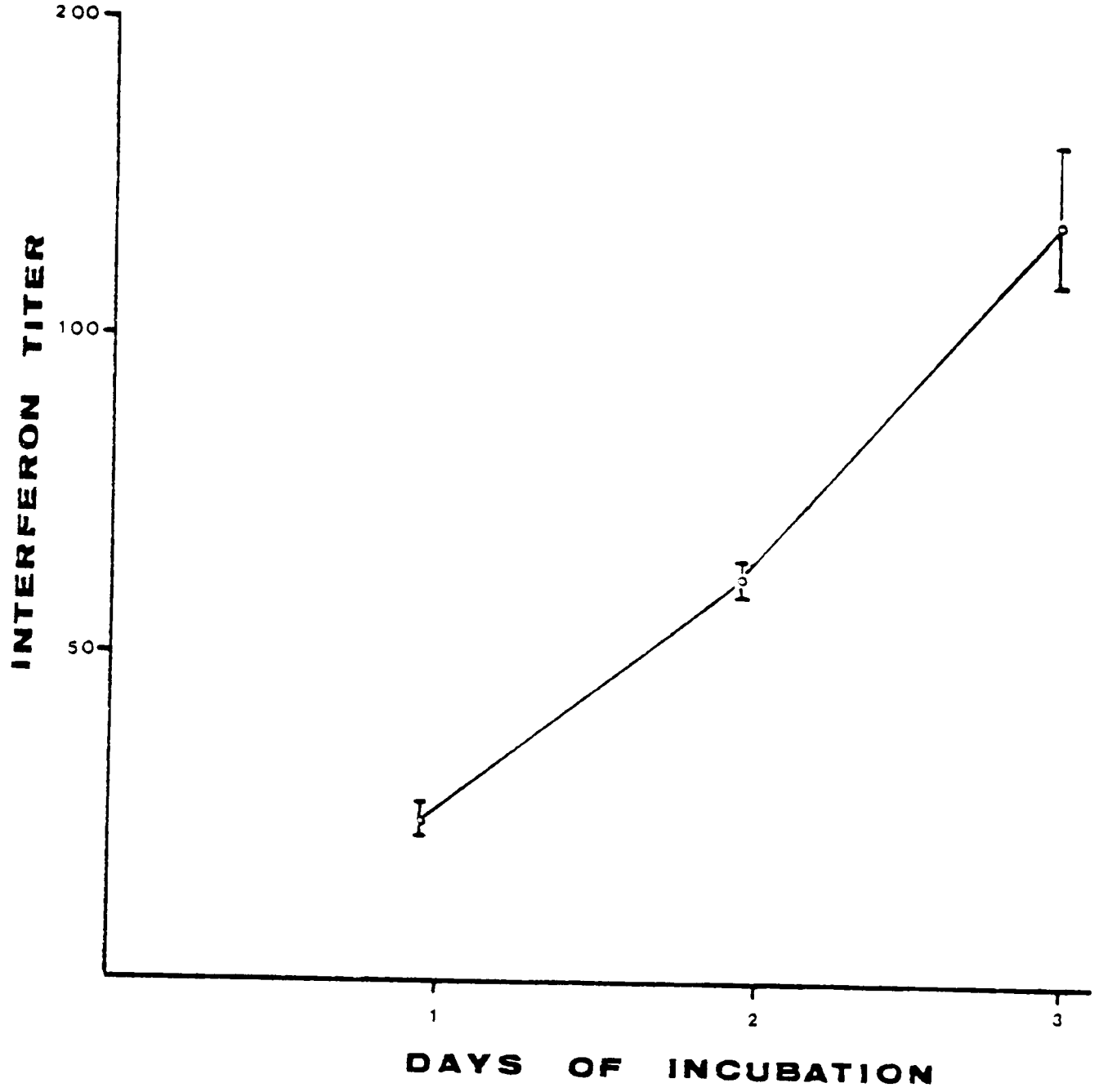
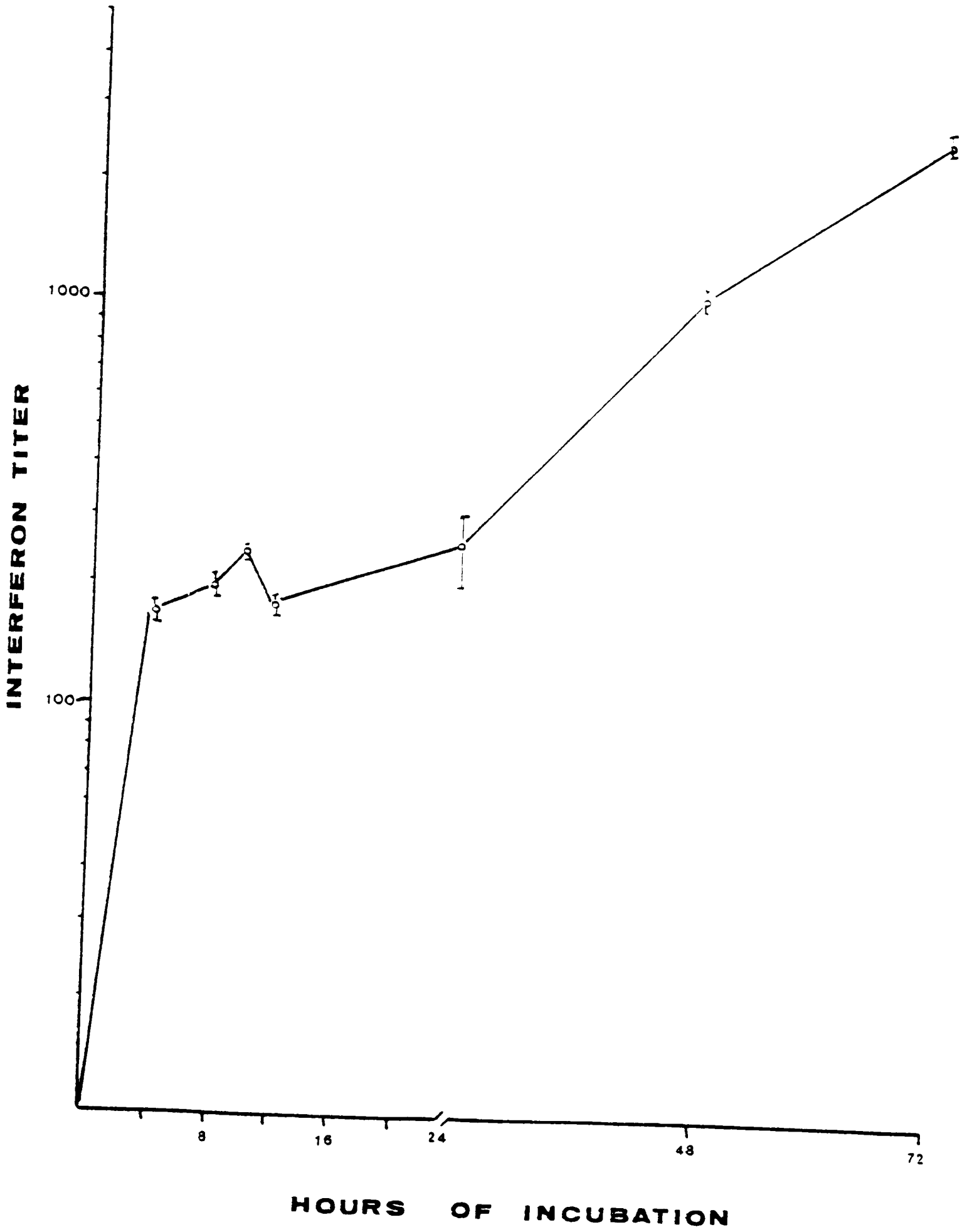

200
100
50
INTERFERON TITER
1
2
3
DAYS OF INCUBATION

Figure 10. Kinetics of mitogen-type interferon production in vitro. Peripheral lymphocytes were co-cultivated with 10 µg con A/10^6 cells. . Results expressed as mean values ± S.E. of three experiments.


INTERFERON TITER
1000
100
8
16
24
48
72
HOURS OF INCUBATION

Only a single phase of interferon production was observed in lymphocyte cultures when the inducer, con A, was removed from the cultures after a 2-hour incubation. Figure 11 shows the kinetics of this induction. An average peak titer of 300 in the cultures was compatible in timing with the peak titer of the first induction phase shown in Figure 10.

II. Characterization and Properties of Bovine Interferons

When poly I:C induced and mitogen induced interferons were subjected to digestion by trypsin, almost complete destruction of interferon was observed in samples of both interferon types (Table 4). Data in this table also show that ribonuclease did not significantly reduce the interferon titers.

The stability of bovine interferons at low pH was tested. Table 4 shows that both types of bovine interferons was unstable at pH 2. A slightly greater effect was noted on the mitogen induced interferon. Interferons induced by both poly I:C and mitogen were tested for their pH stability. Figure 12 shows the percent reduction of the late phase RNA induced interferon (LRIF) and late phase mitogen induced interferon (LMIF) after dialysis against various pH's. Partically, all antiviral activities of the two types of interferon were destroyed by pH 2. At pH 3, mitogen induced interferon activity was less stable than that of the poly I:C induced interferon, but the difference was not found to be significant. When the samples were subjected to pH 4 treatment, it was found that this pH inactivated about 80 percent of the mitogen activity but only 8 percent of the poly I:C activity. At pH 5, little effect was noted on LRIF, but

Figure 11. Pulse induction of peripheral lymphocytes by con A. Ten μg con A/10^6 cells was incubated with lymphocytes for 2 hours. After washing and refeeding induced lymphocytes, interferon levels were measured at intervals up to 24 hours. Results expressed as mean values ± S.E. of three experiments.

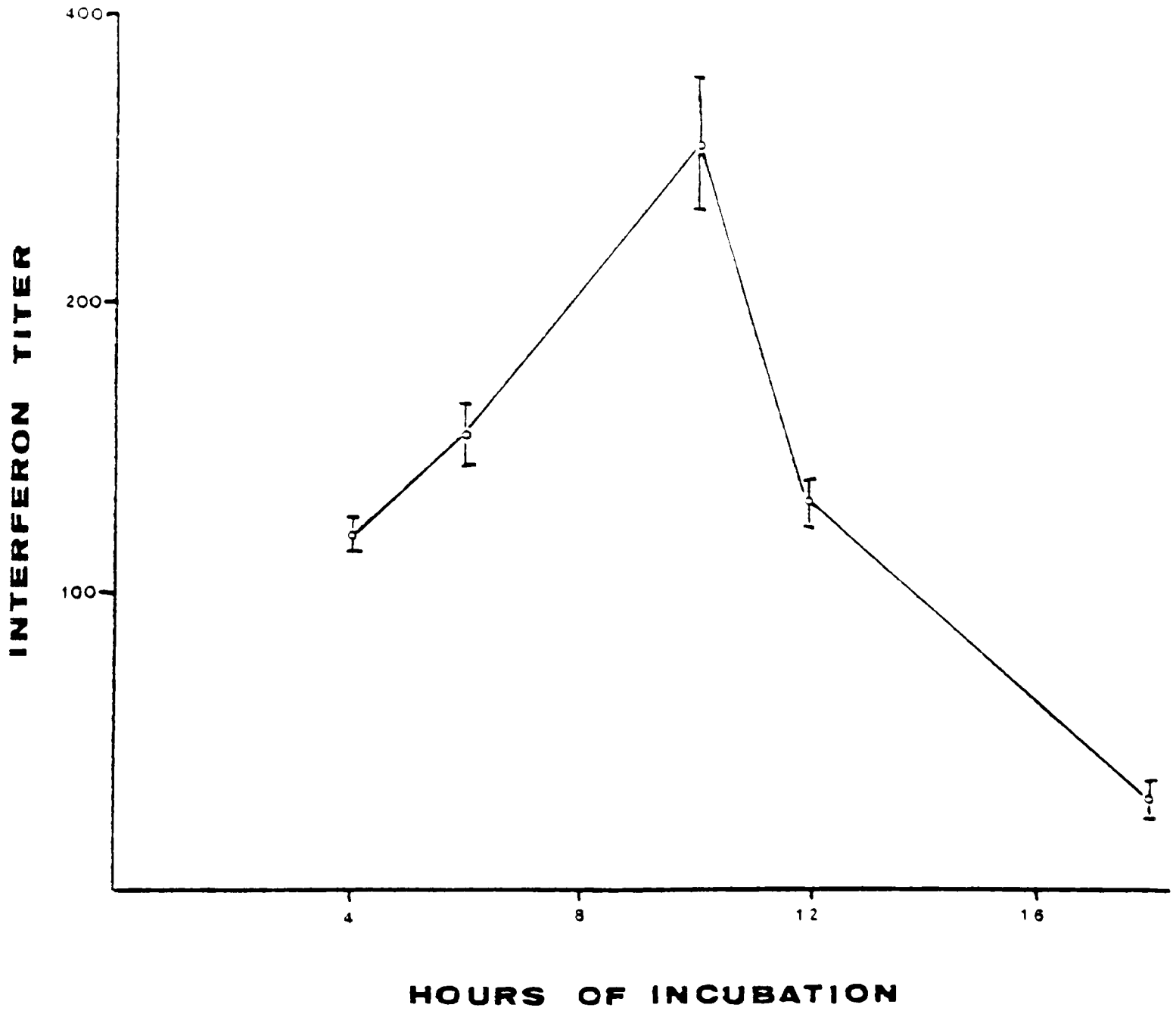

400
200
100
INTERFERON TITER
4
8
12
16
HOURS OF INCUBATION

TABLE 4

EFFECTS OF VARIOUS TREATMENTS ON BOVINE INTERFERON

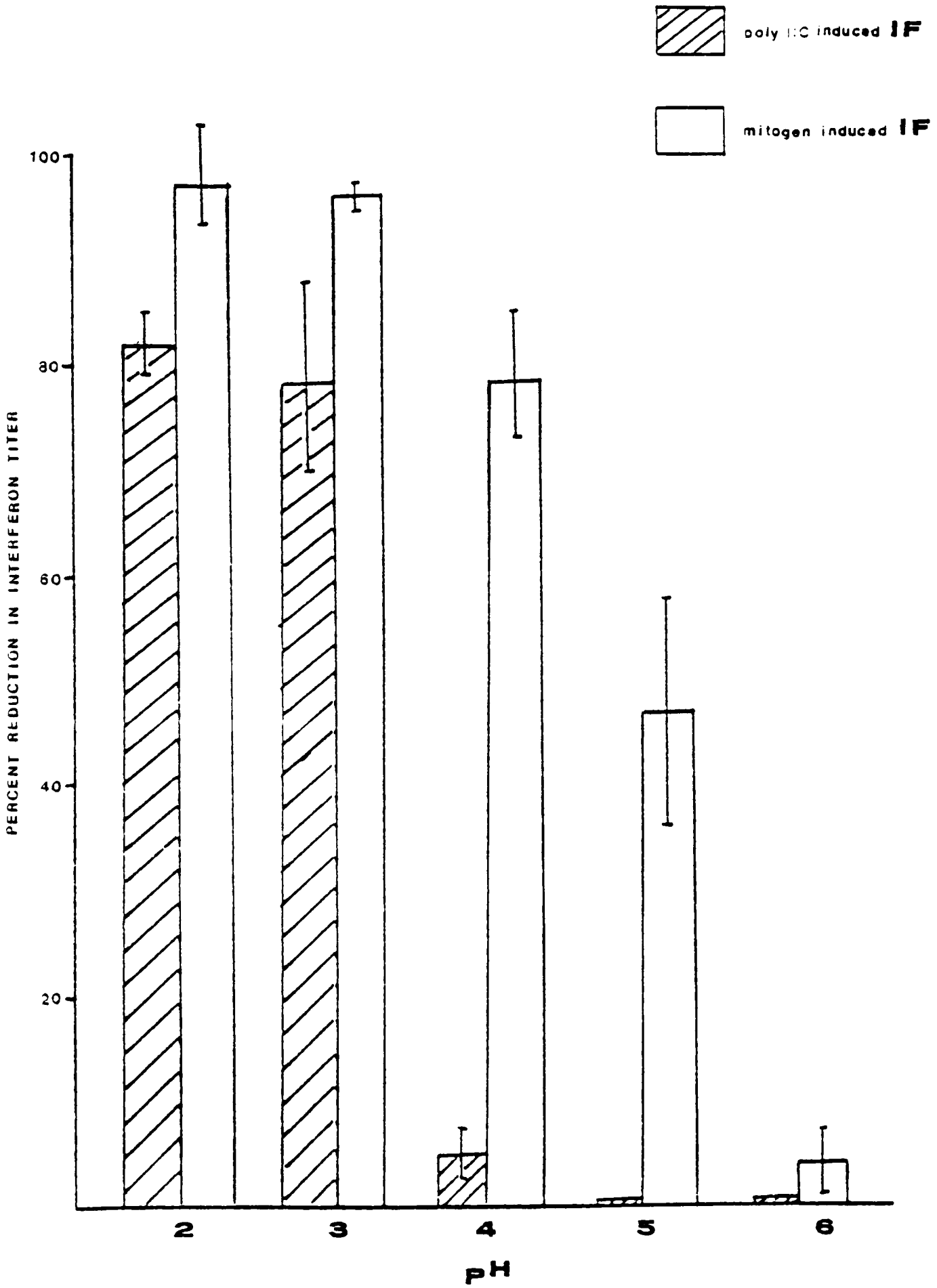
Treatments	Percent Reduction in Titer	
	RNA Induced Interferon	Mitogen Induced Interferon
pH 2[a]	90 ± 5.7	98 ± 4.1
Trypsin[b]	96 ± 0.2	98 ± 0.7
Ribonuclease[c]	3	ND

[a]Interferon samples were dialyzed against 100x volumes of buffer at pH 2.

[b]Trypsin at 0.05 mg/ml was incubated with interferon samples at 37°C for 30 minutes.

[c]Ribonuclease at 100 µg/ml was incubated with interferon samples at 37°C for 30 minutes.

Figure 12. Stability of bovine interferons at low pH. Results expressed as mean values ± S.E. of ten or more experiments.


poly I:C induced IF
mitogen induced IF
PERCENT REDUCTION IN INTERFERON TITER
100
80
60
40
20
2
3
4
5
6
pH

the antiviral activity of LMIF was reduced up to 50 percent. Dialysis at pH 6 was found to have little effect on either LRIF or LMIF.

Low pH was employed as a means to differentiate possible heterogeneity between the interferons produced during early and late phases of production. ERIF, LRIF, EMIF, and LMIF were subjected to dialysis at pH 4. Reductions in interferon titers were observed primarily in the EMIF and LMIF samples, as shown in Figure 13. There was approximately a 63 percent reduction in the early phase interferon, but an average of 89 percent reduction in late interferon samples. ERIF and LRIF were not inactivated by pH 4 significantly. Most samples of ERIF were totally unaffected, and only 5 percent reduction in activity was observed in the LRIF.

Temperatures of 45 and 56°C were used to assess the stability of bovine interferons. The higher temperature (56°C) was found to completely inactivate all interferon samples even when the samples were subjected to that temperature for 10 minutes. At 45°C, RNA induced bovine interferon was more labile than the mitogen induced type. Figure 14 shows that approximately 50 percent of the RNA type interferon activity was lost in the first 15 minutes at 45°C. This same period of incubation did not reduce the mitogen induced type significantly. Both 30 and 45 minutes at 45°C inactivated about 80 percent of the RNA type interferon preparations, but under the same thermal conditions only about 5 percent of the mitogen induced interferon activity was destroyed.

A difference in the rate of production of AVP was observed in cells treated with each type of bovine interferon. A rapid rate of AVP induction was noted in cells treated with RNA induced as compared to mitogen

Figure 13. Stability of early and late interferons at pH 4. Results represent the mean values ± S.E. of four or more experiments.

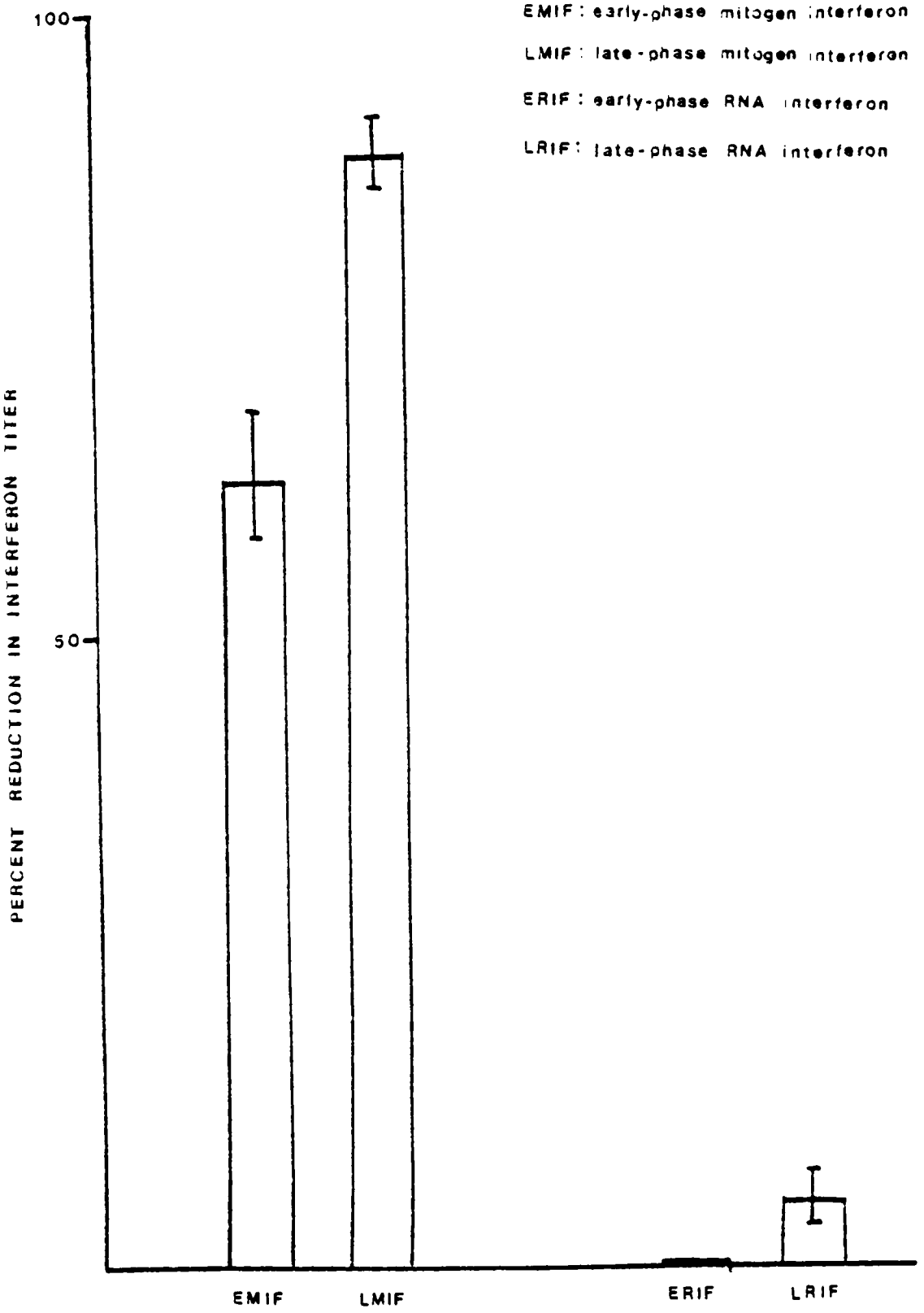
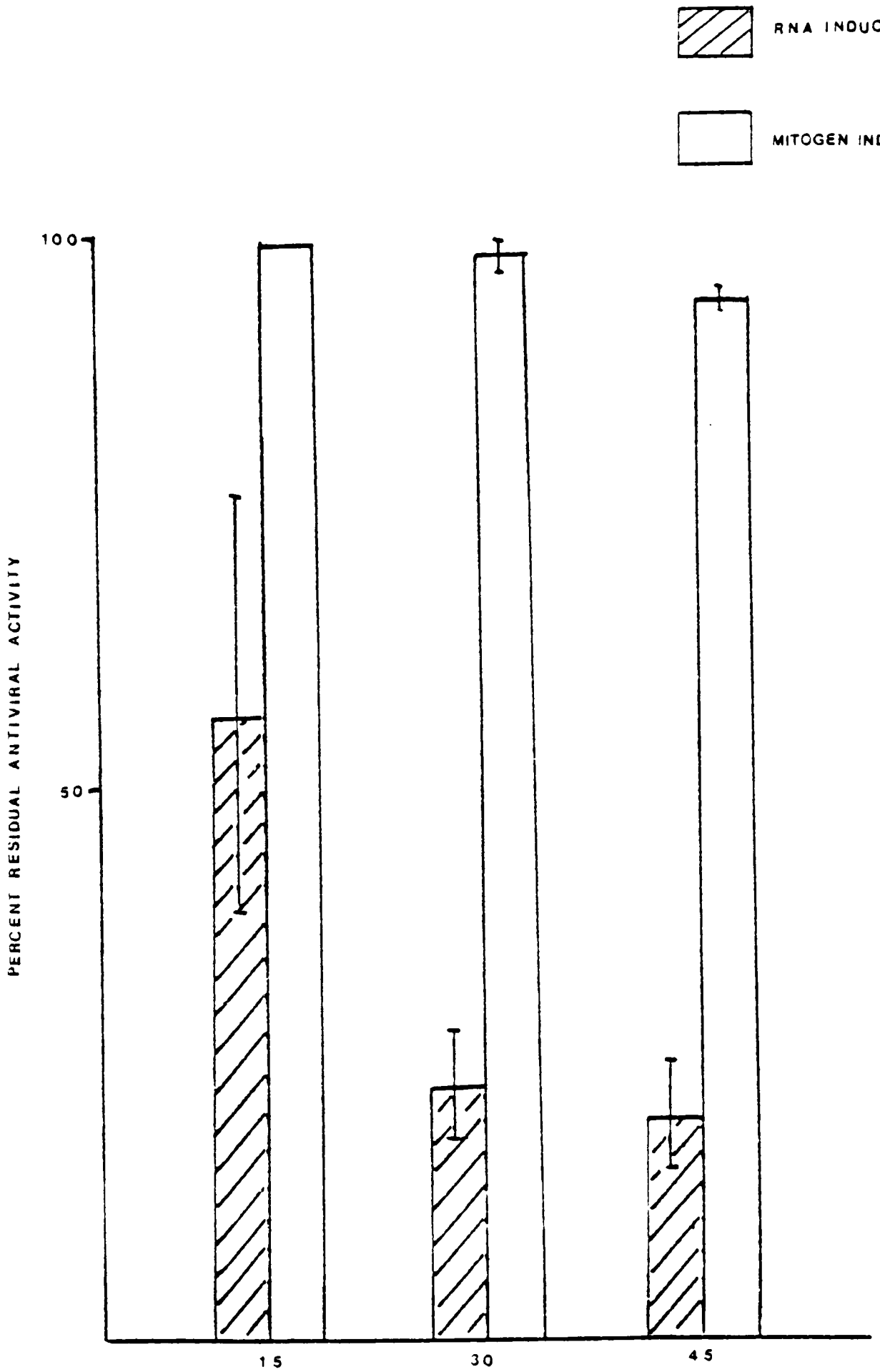

EMIF : early-phase mitogen interferon
LMIF : late-phase mitogen interferon
ERIF : early-phase RNA interferon
LRIF : late-phase RNA interferon
PERCENT REDUCTION IN INTERFERON TITER
100
50
EMIF
LMIF
ERIF
LRIF

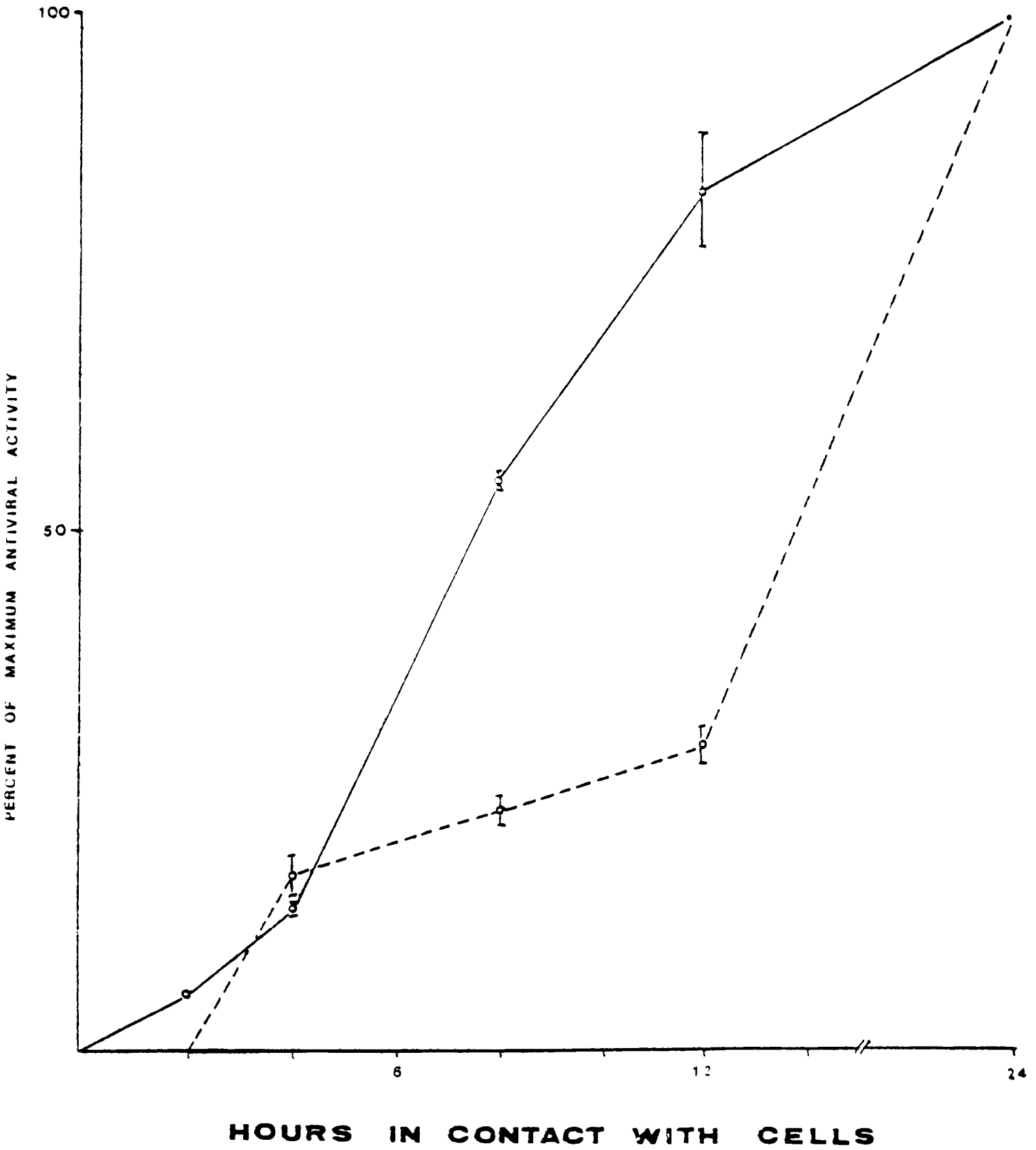
Figure 14. Stability of bovine interferon at 45°C. Results expressed as mean values ± S.E. of three or five experiments.


RNA INDUCED IF
MITOGEN INDUCED IF
100
50
PERCENT RESIDUAL ANTIVIRAL ACTIVITY
15
30
45
MINUTES OF INCUBATION
AT 45°C

induced interferon. When the two types of interferon were allowed to interact with MDBK cells for two hours, about 100 units of antiviral activity, equivalent to 5 percent of maximum level, were induced by RNA type interferon but no antiviral activity was found in cells treated with mitogen type interferon (Figure 15). After four hours of incubation, significant levels of antiviral proteins were induced by both types of interferon. At this time, the antiviral activities induced by both types of interferon were almost equivalent, with less than 5 percent difference. The increase in antiviral activity in cells treated with RNA induced interferon was proportional to the time in which the interferon was in contact with the cell. The increase of AVP in cells treated with mitogen induced interferon was also proportional to the duration of interferon-cell contact, but the level of antiviral activity was lower. After 12 hours of incubation, the cells treated with RNA induced interferon expressed high levels of antiviral activity when compared to cells treated with mitogen induced interferon. For example, approximately 1,500 units of interferon activity, which was equivalent to 83 percent of maximum titer, were observed in the RNA induced interferon treated cells. At the same time, only 550 units of antiviral activity, equivalent to 29 percent of the maximum activity, were noted in cells treated with mitogen induced interferon. After 24 hours of interferon-cell interaction, the antiviral levels in cells treated by both types of interferon reached approximately 2,000 units.

Results on antibody neutralization experiments were equivocal. Exposure of bovine interferons to anti-human and mouse interferons resulted

Figure 15. Rates of production of antiviral proteins (AVP). Bovine interferons were incubated with MDBK cells. AVP levels were reflections of the percent of maximum antiviral level. Results expressed as mean values ± S.E. of three experiments.


100
50
PERCENT OF MAXIMUM ANTIVIRAL ACTIVITY
6
12
24
HOURS IN CONTACT WITH CELLS

in a 50 percent reduction of activity. At higher dilution only antibody to human interferon had an effect but this was not consistent or proportional to dilution.

Cross species antiviral activities were found to a limited extend in some heterologous cells (Table 5). Of all the heterologous cells tested, only PK, WI-38, HFF, and MK cells were protected with one or both types of bovine interferon. Other than the homologous MDBK cells, PK cells were found to be protected most effectively by bovine interferon. This was followed by MK cells. HFF responded very little to the RNA induced interferon. Another human cell, WI-38, also responded minimally to the same type of interferon. Responsiveness of cells to bovine interferon depended on the type of interferon. For example, we found that RNA induced bovine interferon consistently protected sensitive heterologous cells greater than the mitogen induced bovine interferon. About 30 percent of the titer of RNA induced interferon was expressed on PK cells but only about 10 percent of the mitogen induced interferon titer was expressed on the same cell type.

TABLE 5

CROSS SPECIES ANTIVIRAL ACTIVITIES OF BOVINE INTERFERONS

Interferon Types (Inducers)	Cell Types[a]										
	MDBK	Rat	PK	HeLa	WI-38	HFF	HEK	L-929	CEC	RK-13	MK
RNA	100[b]	0	31.2	0	0.57	3.2	0	0	0	0	10.6
Mitogen	100	0	9.7	0	0	0	0	0	0	0	4.3

[a]Sources and species of cells can be found on Table 1.

[b]Average percent of activity compared with homologous cells (MDBK). Each number represents an average of two or more experiments.

CHAPTER IV

DISCUSSION

The results of interferon production by bovines *in vivo* in the current studies were similar to the results reported by Rosenquist and Loan (72, 73). They showed that i.v. injections gave rise to the production of interferon within 10 hours, while i.m. and i.n. (intranasal) injections gave rise to circulating interferon 2 or 3 days post injection. With the combined routes (i.m. and i.v.), we observed two peaks of production. The first interferon peak may represent the interferon induced by the i.v. injection of the virus, and the second peak could be the response to the i.m. administration of the vaccine.

These investigators also reported very low levels of serum interferon (72, 73). In our experiments, we observed interferon titers up to 200. These were more than 10 times that reported by Rosenquist. These differences may be due to the use of different virus inducers, doses, and routes, or could relate to the sensitivity of the interferon assay system.

In the current studies, a particularly high antiviral level was found in the prebleeding serum, but it was not determined whether the antiviral activity was due to naturally occurring interferons, antibodies against VSV, or other inhibitors. The nature of these inhibitors was not determined. Prior to the second booster injection, the prebleeding titer was about half of the former.

The abilities of three bovine cell types to produce interferon upon induction by poly I:C were compared. Among the three, MDBK cells did not

produce interferon when induced with poly I:C alone, or poly I:C plus DEAE-Dextran. Polynucleotides have been shown not to induce interferon in most mouse cells except in the presence of DEAE-Dextran (21, 22). The mechanism for the DEAE-Dextran's potentiating ability is that DEAE-Dextran enhances adsorption of RNA by cells (92). Since the MDBK cells did not produce interferon even in the presence of DEAE-Dextran, very likely, the loss of the ability to respond to poly I:C stimulation is due to malfunctioning at a level other than adsoprtion of the inducer.

BEK cells were chosen as interferon producing cells in our interferon studies since they developed the highest responses to poly I:C induction. The dosage of poly I:C used and the length of incubation in the preliminary experiments were arbitrarily chosen. In these studies, 10 to 40 µg poly I:C per ml were utilized. It was found that 10 µg/ml was the optimal dosage for interferon induction in BEK cells. The number of BEK cells per plate was approximately $4x10^6$. Two mls of media containing 10 µg/ml of poly I:C per plate was equivalent to 5 µg/10^6 cells.

The optimal amount of poly I:C used in lymphocyte cultures was found to be 20 µg/ml or 60 µg per culture. Since we had 3 ml of cell suspension per culture and there were $5x10^6$ lymphocytes per ml of cell suspension, the dose of poly I:C employed was equivalent to 4 µg/10^6 cells. In terms of dosage per cell, 10 µg poly I:C/ml in BEK cultures was similar to 20 µg/ml in lymphoid cultures.

Previous reports have shown that in rabbit and mouse cells, poly I:C induced type I interferons would accumulate in the culture media and remain stable there for as long as 24 hours (63, 96). The persistence of interferon was not observed in both the BEK-poly I:C and lymphocyte-poly I:C

systems when the inducers were removed from the culture media. Instead of plateauing after reaching maximum level, the level of interferon dropped drastically. This drop in titer may be the result of either one or more of the following: (a) inactivation of interferon at 37°C, (b) readsoprtion of interferon to cells, and (c) enzymatic degradation of interferon.

Readsorption of interferon by cells seems to be the least likely because the reduction in titer of detectable interferon between the 8- and 24-hour period of the 10 µg poly I:C/ml induced culture was approximately 15,000 units. Since we had a 3 ml total volume in each culture and used 0.025 ml for assay, the total reduction in the amount of interferon in one culture is about 1.8×10^6 units. This is about 90 percent of the interferon in the culture at the time of maximum titer. It has been shown that only 0.6 to 1.2 percent of the total interferon applied to cultures was bound to cells (24). Furthermore, these investigators also showed that the binding of both types of mouse interferon to mouse cells was rapid and essentially complete after 5 minutes of contact with the cells at 37°C and that the amounts of bound interferon remained unchanged thereafter.

Incubation of cell-free type I (RNA induced) bovine interferon without cells at 37°C overnight had the effect of reducing the interferon titer as much as 70 percent (unpublished data). Therefore, the loss in interferon titer in our cultures may be partially because of its instability at 37°C.

In a recent paper by Halback (44), mouse interferon was found to enhance the fragility of lysosomes in L-929 mouse fibroblasts. If this was actually occurring in our cultures, we would expect a release of lysosomal enzymes into the media following lysis of the cells. The various proteases

in the lysosomes could be responsible for the degradation of interferon in the media.

Cultures of BEK cells induced by 10 µg poly I:C/ml produced the highest titers of interferon and also had the greatest interferon degradation when compared to cultures induced by 5 and 20 µg poly I:C/ml of medium. This could suggest that a threshold level of interferon may be needed to induce a mechanism responsible for the degradation of interferon.

When poly I:C and triple vaccine virus were used to induce interferon in cultures of both splenic and blood lymphocytes, low titers of interferon were obtained. In most cases, splenic lymphocytes did not produce interferon in amounts detectable by our assay system. In comparison, blood lymphocytes responded better than splenic lymphocytes to both poly I:C and virus induction. This is in contrast to Fulton, et al. (38) who found that when splenic and blood lymphocytes were induced by inactivated IBR virus, the former produced more interferon than the latter cell type. This could be explained by different experimental conditions used in the two laboratories, including nature of the inducer, lymphocyte concentration, and age of the animal, etc.

In vitro blastogenesis of bovine peripheral blood lymphocytes by mitogens was reported by Lazary (64). He showed that 10 to 30 µg con A per 10^6 cells induced blastogenesis in bovine lymphocytes to an extent comparable with PHA induction. In the present study, we tried to compare the effectiveness of these two mitogens in interferon induction. It was found that 10 µg con A/10^6 lymphocytes induced interferon at a faster rate and at higher levels than 20 µg PHA/10^6. About 170 units/ml of interferon

were detected in cultures stimulated by PHA after 24 hours, but in cultures stimulated by con A, three times that level of interferon were recovered during the same period of incubation.

In addition to comparisions between mitogens, we also looked at the levels of con A which would produce maximum amounts of interferon. Ten and 30 μg con A/10^6 lymphocytes induced almost equivalent amounts of interferon during the first 48 hours of stimulation. From 48 hours onwards, cultures stimulated by the lesser amount of con A continued to produce interferon while those induced by the larger amount of con A showed a decrease in interferon levels. Although 30 μg con A/10^6 cells did induce high levels of interferon, this concentration was found to be more toxic to lymphocytes as evidenced by increased cell death (trypan blue uptake). Poor viability of lymphocytes has been reported by other investigators (82). Stewart, et al. showed that even without mitogen stimulation, there was a loss of 30 to 50 percent of human lymphocytes during incubation and that the rate was higher in cultures with greater cell densities. Since the cell concentrations in our studies were approximately 5 times that of Stewart, et al., we would anticipate a lower cell viability.

We also tested splenic lymphocytes for their responses to con A. When con A at 10 μg/10^6 cells was added to splenic cultures, we noted a slow rate of interferon production. The highest titers of approximately 200 units were found after 3 days of cultivation. This suggests that splenic lymphocytes were less efficient producers of interferon when compared to peripheral blood lymphocytes. These results could also reflect differences

in experimental conditions as well as cell populations. Similar effects were also noted with poly I:C previously.

The continuous presence of con A during cultivation of blood lymphocytes resulted in a biphasic production curve. These observations suggest two possibilities: (1.) two distinct classes of interferon--one class could have been a less stable interferon which was inactivated during the first few hours of incubation; and/or (2.) different lymphocyte subpopulations were activated by the mitogen at different times. Some produced interferon early and others after a lag period. Before definite conclusions can be drawn, further experiments designed to separate cell populations as well as methods to test the heterogeneity of the interferons from the early and late phases must be performed.

Friedman (36) found that the production of interferon in human lymphoid cultures ceased once PHA was removed from the cultures. If the mitogen was readded to the cultures, production of interferon resumed and continued throughout the subsequent days of cultivation. Based on these previous findings, we tested the effects of pulse induction and continuous induction on interferon production. We found that for the continuous production of high titered mitogen type interferon, the continuous presence of the inducer during cultivation period was required.

The maximum amounts of interferon found in cultures stimulated by a pulse induction of con A were observed at a post induction time equivalent to that of the first peak observed in the biphasic production of interferon, i.e., approximately 10-hours post induction. It is likely that the lymphocyte-mitogen contact during the first few hours triggers the manufacture of interferon. Since this early interferon was found to be readily

inactivated in the media, which was also true for poly I:C induced interferon in BEK cells and lymphocytes, there are indications that the interferon produced undergoes a similar inactivation process irrespective of the inducer.

Trypsin inactivation of antiviral activity in samples containing "interferon activities" suggested that we were dealing with a protein. The inability of MDBK cells to respond to poly I:C induction plus the fact that ribonuclease treated samples did not lose their antiviral activity confirmed that the antiviral state expressed in our interferon assays was the result of interferon action alone and not caused by residual poly I:C in the culture media.

It is generally believed that interferons from all species of animals can be categorized into two types relative to low pH stability; type I being acid stable and type II acid labile. This generalization has not held with bovine interferons. There have been reports indicating that bovine type I interferon induced by viruses was unstable at pH 2 (62, 38). These observations were also recorded in our laboratory. The possible explanations could be that both types of bovine interferon are acid labile, or perhaps only a single type of interferon is produced by bovine cells irrespective of the kinds of inducers used, and that this interferon produced is similar to type II in nature, i.e., labile at pH 2.

It is unlikely that only one class of interferon is produced, because at pH 4 and pH 5 only the interferon preparations obtained from cultures of lymphocytes induced by con A were inactivated and not those from poly I:C exposed BEK cells. Both types of interferon are inactivated at pH 2 and 3. These results suggest that at least two populations of bovine

interferon are produced by bovine tissues and lymphoid cells upon stimulation with "type I or type II" interferon inducers.

In experiments on kinetics of production, we noted a biphasic interferon production pattern in lymphocyte cultures stimulated by con A. The heterogeneity of the interferon produced in the two phases was questioned. Attempts to elucidate the nature of the interferons in these two phases were devised. Since pH 4 may serve as a parameter to distinquish the two types of bovine interferon, it may offer a means to reveal the heterogeneity of the interferon produced in the two phases of mitogen induction.

Both the EMIF and LMIF were subjected to pH 4 dialysis. The same procedure was repeated with ERIF and LRIF. Differences in amounts of interferon inactivation could suggest that EMIF and LMIF contain different molecular populations of interferon while ERIF and LRIF may be just one homogeneous type. The average percent reduction in titers of EMIF interferon after pH 4 dialysis was about 30 percent less than the average reduction in titer of the LMIF, since there could be a 20 to 50 percent variation in titers in interferon assay. Therefore, the data reported here do not support in a definitive manner the existance of subpopulations of interferons.

The lack of stability of bovine interferons was again demonstrated by their rapid inactivation by high temperature. Both types of bovine interferons were inactivated almost completely after 15 minutes of incubation at 56°C. Since pH 4 but not pH 2 distinquishes the different biophysical properties of the types of bovine interferon, it was thought that a temperature lower than 56°C could reveal different thermal stabilities of the two types of bovine interferon. A temperature of 45°C was found to

inactivate both types of bovine interferon at different rates. After 30 minutes at 45°C, bovine mitogen type interferon activity was reduced about 2 percent, while type I activity was cut about 75 percent compared to control samples at 4°C. Further incubation at this temperature did not have significant additive effects on reduction.

The literature is somewhat confused regarding thermal inactivation of interferon. For example, endotoxin induced interferon in rabbits (49) and in rabbit macrophage cultures (81) was reported to be inactivated by 56°C, although this was not true in mice (87). Human type II interferon induced by PHA was labile at 56°C (98), while bovine type I interferon induced by virus was also labile at this temperature (72, 73). Mouse immune specific interferon was reported to be stable at 56°C (101). Therefore, it would appear that thermal stabilities of interferon depend on the species or the cell type that produced them as well as the type of inducer.

Since the two types of bovine interferon have different biophysical properties such as pH and temperature sensitivity, it may be expected that these types of interferon would be different in their biological properties such as cellular activation, etc. Comparative studies on the rates of cellular activation leading to antiviral state were conducted. Data obtained agreed with previous findings of Dianzani (24) in which type I interferon activated cells earlier than type II did. This was unlike human and murine type I interferons which impart the complete antiviral state to homologous cells within four hours of cell-interferon contact (24). Bovine RNA type interferon takes at least 12 hours to induce 80 percent of the full antiviral state in MDBK cells. Dianzani showed that mouse type II interferon induced antiviral state on mouse cells faster than human type II

did on human cells. We found that bovine mitogen type interferon took longer to induce the antiviral state in MDBK cells than both human and mouse interferons did in their homologous cells, suggesting that interferons from different species interact with cells at different rates. Nevertheless, irrespective of species of origin, type I interferon activates cells more rapidly than type II interferon.

The rate of activation of bovine interferon, when compared to human and mouse interferons, may be the result of several possibilities. First, Dianzani, et al. used purified interferons as opposed to crude bovine interferons. There may be present in the crude bovine interferon preparations substances capable of competing with interferon for receptor sites on the cell surface. Secondly, there may be a difference in availability of cellular receptors between bovine, human, and mouse cells; and thirdly, there may exist a difference in mechanism of activation of the AVP among the three cell types.

The idea of using anti-human interferon to neutralize bovine interferon came from the speculation that the two species of interferons might bear antigenic similarities since bovine and human interferons have been shown to be cross-reactive (4, 43). Levy-Koenig (65) had reported a marginal degree of neutralization of human interferons by antibodies against heterologous interferons from other species which cells were protected by human interferons. This marginal degree of cross neutralization was suggested to be non significant by Paucker, et al. (70). Using improved experimental techniques, they have shown that human and rabbit interferons do not contain similar antigenic determinants even though the former protects rabbit cells to some extent. Our data suggest that in some instances

crude bovine interferons may be neutralized by anti-human leukocyte interferon globulins. We could not draw definite conclusions as to whether or not this degree of neutralization was specific because sera of animals from which the antibodies were isolated could also contain inhibitors to bovine interferons. In addition, proper control sera were not available.

The poly I:C and mitogen induced bovine interferons were tested for their ability to protect heterologous cells. Crude poly I:C induced interferons were preincubated with ribonuclease as described in order to eliminate any residual poly I:C. The levels of cross species antiviral activities by the two types of bovine interferons were somewhat different from previous authors. For example, 30 percent of homologous activity of our RNA induced interferon was expressed in PK cells as compared to 7 percent reported by Babiuk (4). On the other hand, Babiuk reported 218 percent of homologous activity of their immune-type bovine interferon on porcine cells. Using mitogen induced bovine interferon only 10 percent of homologous activity was noted in porcine cells. Since the PK cells used are not the same cell type that Babiuk, et al. employed in their experiments, it is conceivable that differences in cell types as well as assays and other experimental conditions may have contributed to the differences in cross-species antiviral states observed between the two laboratories. Mitogen induced interferon which we used might be biologically different from immune-specific interferon, although this does not seem to be the case.

In these present studies, bovine interferons protected human cells to a minimal extent with some human cells being insensitive to bovine interferons. This difference in sensitivity to bovine interferon between human

cells was also reported by Sutton (90) who found that only human adult thyroid cells but not human amnion cells were protected by bovine type I interferon.

The low degree of protection on human WI-38 cells by RNA induced bovine interferon was in agreement with Gresser's findings (43). Also, the observation that both types of bovine interferon protected monkey cells more than human cells agreed with the reports of Babiuk (4).

It should be emphasized that those cells which are not sensitive to bovine interferon may not necessarily be refractory to the action of bovine interferon. Studies have shown that if different challenging viruses were used in the assay system, very different titers for the same interferon sample were observed (1). This is due to the difference in sensitivity of different viruses to interferon. Therefore, the lack of protection by bovine interferon on certain cell types we tested might possibly be the result of insensitivity of the challenging virus to bovine interferon in that particular cell type.

Among the heterologous cells sensitive to bovine interferon, a consistent trend showed that RNA induced bovine interferon cross-protected heterologous cells more than mitogen induced bovine interferon. Since the differences were not great, except for PK cells, and because the challenging viruses and cell types used in the assay may influence the outcome of the assay, it was difficult to assess the significance of these findings.

CHAPTER V

SUMMARY AND CONCLUSION

Significant amounts of RNA type and mitogen type bovine interferons were induced in bovine embryonic cells and bovine peripheral lymphocytes respectively. Interferon production in bovine lymphoid cultures induced by con A followed a biphasic pattern with the first peak at approximately 10 hours post induction. The second phase of production lasted from 12 to 72 hours.

Various properties of these interferons were studied. It was found that both types of interferon were unstable at pH 2. Mitogen induced interferon was also unstable at pH's 4 and 5. Approximately 90 percent of its activity was lost at this pH whereas less than 10 percent of the poly I:C induced interferon was inactivated. Differences in temperature stability at 45°C were also noted. RNA induced interferon was inactivated to a greater extent than mitogen induced interferon at this temperature.

Mitogen type interferon activates cells to produce antiviral proteins at a much slower rate than the RNA induced interferon. It was found that MDBK cells expressed about 80 percent of the maximum antiviral activity of the RNA induced interferon after 12 hours of cell-interferon contact. Approximately 20 percent of the activity of the mitogen induced interferon was expressed on MDKB cells after the same incubation period.

In terms of cross species antiviral activity, both the RNA and mitogen induced bovine interferons have a low level of activity on the human cell types. However, porcine cells and monkey cells were more sensitive to both

types of bovine interferon. Porcine cells were protected about 3x better by RNA type bovine interferon than by the mitogen induced type.

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