Effect of murine γ-interferon on mouse preimplantation development and expression of major histocompatibility complex proteins

by

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TABLE OF CONTENTS

INTRODUCTION

Major Histocompatibility Complex of the Mouse

In 1936, the English immunologist Peter Gorer first described three blood group antigens in mice. He found that one of these antigens, antigen II, also controlled tissue graft rejection (Gorer, 1936). In 1948, on the suggestion of George Snell, this antigen became known as Histocompatibility-2, or H-2 (Snell, 1948). When similar groups of antigens were discovered in other species, they were referred to as the major histocompatibility complex antigens (MHC). In the mouse, MHC and H-2 are synonymous; in man the MHC is known as human leukocyte antigen (HLA).

The H-2 complex resides on chromosome 17 in the mouse. There are six distinct regions within this complex: K, I, S, D, Q, and TL. In these six regions, three classes of proteins are encoded (Fig. 1).

The class I protein products are usually membrane-bound glycoproteins (with a few exceptions, such as Q10 [Devlin et al., 1985a). They are members of the immunoglobulin supergene family, having a great deal of structural homology to the antibody molecules. There are three types of mouse class I proteins: H-2, Q, and Tl. They consist of two chains, a heavy chain of molecular

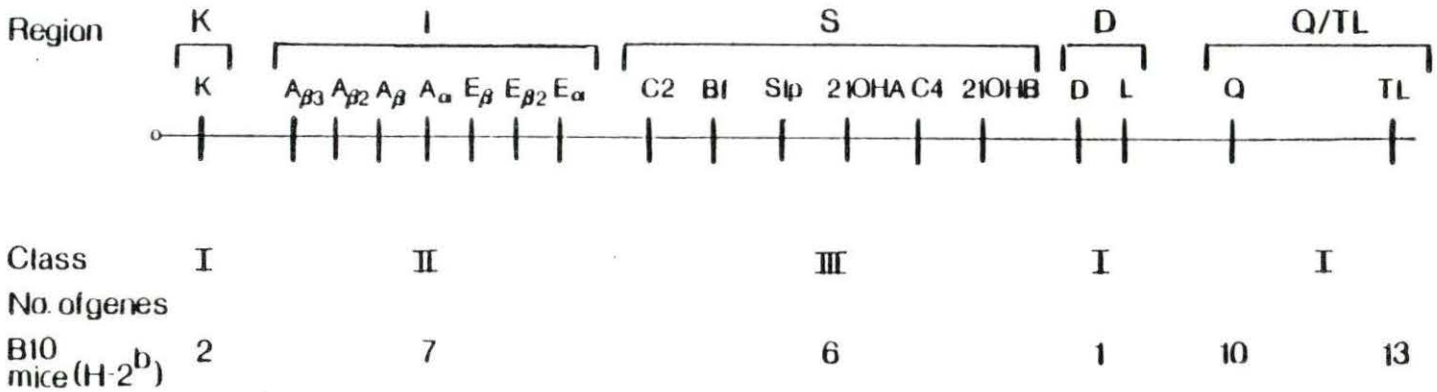

Figure 1. Genetic map of the mouse MHC. The 5 regions encoding the MHC antigens and the class of protein encoded is denoted under the corresponding region. The number of genes in each region of the C57BL/10 mouse strain are shown. This figure was adapted from Mellor (1986)

weight between 40,000 and 45,000 which is non-covalently associated with a light chain protein, β_2-microglobulin, which has a molecular weight of approximately 12,000. The heavy chain consists of three external domains (α_1, α_2, and α_3) of about 90 amino acids each, a transmembrane section of about 40 amino acids in length, and a cytoplasmic tail of about 30 residues (reviewed in Flavell et al., 1986). The 3-dimensional structure of a human class I MHC protein, HLA-A2, has been determined and reveals a binding cleft between the α_1 and α_2 domains large enough to accommodate a small peptide of up to 15 amino acids in length (Bjorkman et al., 1987) (Fig. 2).

The H-2 proteins (H-2K, H-2D, and H-2L) are highly polymorphic and expressed on all somatic tissues with levels of expression varying between tissue types (reviewed in Lew et al., 1986). These are the classic transplantation antigens which are responsible for graft rejection (Gorer, 1938) and restriction of the cellular immune response (Zinkernagel and Doherty, 1974). The Q and Tl proteins are not as polymorphic as the H-2 proteins (Flaherty, 1980). They are expressed on a limited number of cells and have no known function in T cell mediated immunity (Michaelson et al., 1983). There are five known Q/Tl proteins: Qa-1, Qa-2, Qb-1, Q-10, and Tl (Stanton and Hood, 1980; Flaherty, 1981; Devlin et

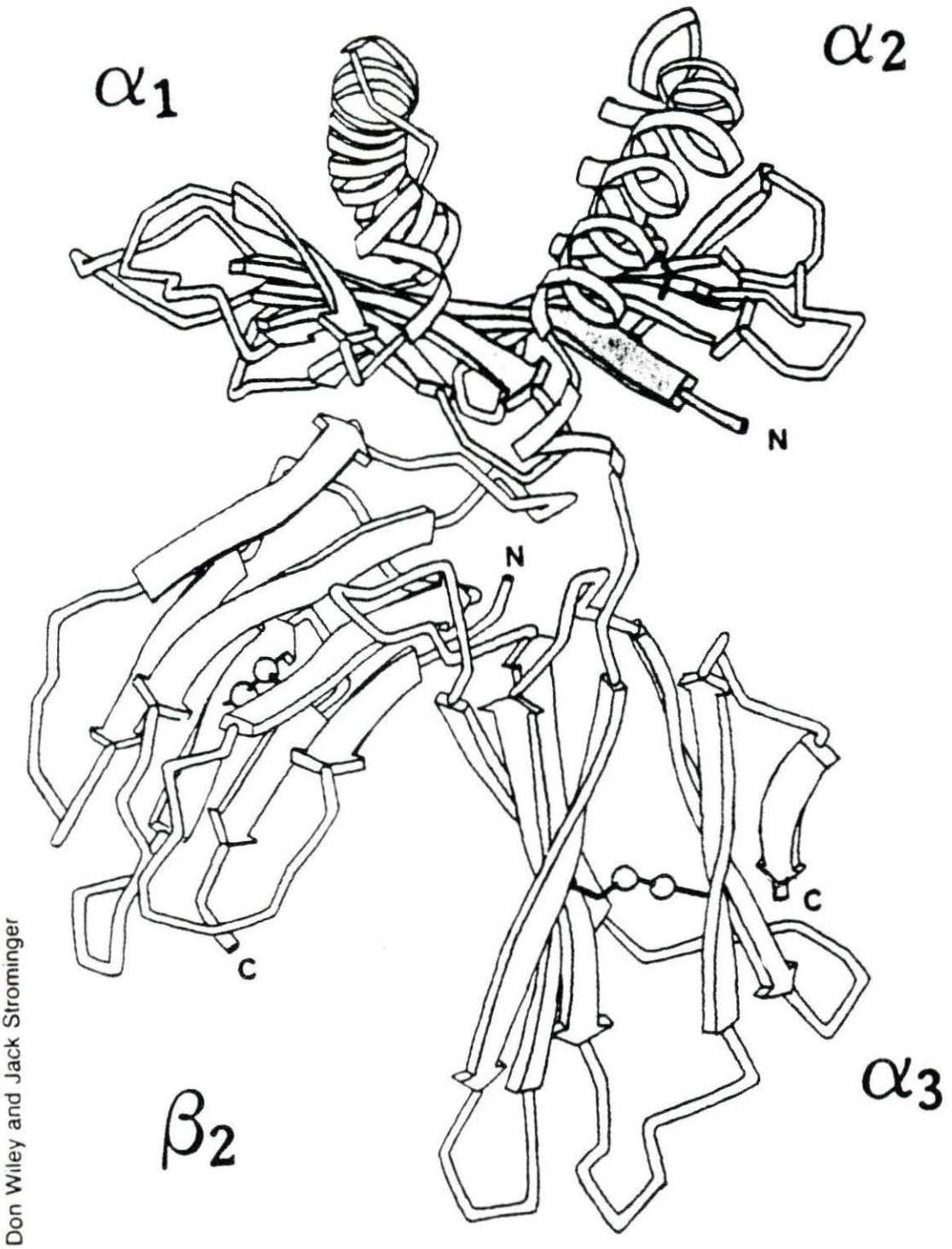

Figure 2. Three-dimensional structure of HLA-A2 (From Bjorkman et al., 1987)

al., 1985; Robinson, 1985). The Qa-2 antigen is the best characterized of the Q/Tl molecules. The heavy chain of this molecule is about 40 kilodaltons in molecular weight and, like the H-2K and H-2D proteins, is associated with β_2-microglobulin. It has a high degree of homology to the H-2K and H-2D protein sequences (Soloski et al., 1982). Recent evidence has shown that the Qa-2 protein is encoded by two genes, Q7 and Q9. (Stroynowski et al., 1987; Soloski et al., 1988). Sequencing has revealed that these two genes have identical exon sequences and only differ in the intron regions (Devlin et al., 1985b).

The murine MHC class II proteins, also called Ia antigens, are membrane-bound glycoproteins encoded in the I region of the MHC complex. Like the class I proteins, they are members of the immunoglobulin supergene family, suggesting a common ancestral gene for these molecules. These molecules are heterodimers formed by the non-covalent association of a 32,000 dalton α-chain with a β-chain of molecular weight approximately 28,000 daltons. Both chains have two extracellular domains, a transmembrane section and a cytoplasmic segment. The 3-dimensional structure of the class II molecule has not yet been elucidated, but is believed to be very similar to that of the class I protein in association with β_2-microglobulin. Class II proteins are highly polymorphic

and are found primarily on B cells, macrophages, and some epithelial cells, although they are not restricted to these cell types. These molecules are involved in presenting foreign antigens to helper T cells, thus initiating the humoral immune response (reviewed in Dorf, 1981).

The class III proteins are encoded in the S region of the MHC. These proteins are 21-hydroxylase (21-OH), Sex-limited protein (Slp) and three components of the complement system, C2, C4, and factor B. There is no homology between these proteins and the class I or class II molecules (reviewed in Hood et al., 1984).

Embryonic Development

For a review of early mammalian development, see Johnson and Everitt (1980). The diploid primordial germ cells in the ovaries, the oogonia, divide mitotically and enlarge into primary oocytes. These cells then undergo two successive meiotic divisions to become a large secondary oocyte and a smaller polar body. At the time of estrous, the eggs are discharged in a clutch surrounded by cumulus cells into the ampullar end of the oviduct.

It is within the oviduct that fertilization occurs. A successful spermatozoan contacts the zona pellucida, an

acellular glycoprotein matrix encircling the ovum. Following this contact the zona pellucida undergoes changes which prevent any subsequent penetration; this is known as the zona reaction (Bleil et al., 1981). The male and female pronuclei fuse together completing the fertilization process by producing a diploid cell, the zygote.

Immediately after fertilization, each zygote undergoes a series of five to six cleavage divisions. In the mouse, the first cleavage occurs about 24 hours after copulation and thereafter once every 12 hours (Snell and Stevens, 1966). These divisions can be asynchronous resulting in embryos with odd numbers of cells. The dividing embryo will pass through six morphologic stages before implanting: fertilized egg, 2 cell, 4 cell, 8 cell, morula, and blastocyst. The first five stages occur in the oviduct; at about the time of the fifth cleavage, the embryo passes from the oviduct into the uterine horn.

The blastocyst, the final stage of development prior to implantation, is composed of two cell types. The trophectoderm is the outer ring of cells which surrounds the inner cavity (blastocoel) and inner cell mass (ICM). Just before implantation the blastocyst sheds the zona pellucida in a process called hatching. At implantation

the trophectoderm begins to form the placental tissues which will act as a barrier between maternal and fetal circulations in the post-implantation stages. The inner cell mass will form the embryo proper.

In mice, the preimplantation phase is approximately 4.5 days long. During this time, the embryo is a free-floating entity, completely separate from, yet surrounded by the maternal environment.

Pregnancy and the Immune Response

Since the discovery of the MHC and its role in tissue transplant rejection, immunologists have been puzzled by the fact that fetal tissue evades this phenomenon. Logically, since the fetus possesses paternal as well as maternal genetic material, it should be recognized as foreign by the mother and rejected. Peter Medawar was one of the first to address this paradox in 1953, when he proposed three mechanisms by which the fetus could protect itself from the maternal immune system (Medawar, 1953).

His first hypothesis suggested that a physical barrier exists between the fetus and the surrounding maternal environment. The zona pellucida appears to be an effective shield against maternal cellular responses. Ewoldsen and coworkers found that cytotoxic T lymphocytes

(CTL) had the ability to kill embryos which had their zona pellucida removed, but lacked this ability when the zona pellucida was left intact (Ewoldsen et al., 1987). It is known that the zona pellucida is permeable to immunoglobulins (Bleil and Wasserman, 1980); however, it is not known if the embryonic alloantigens can initiate an *in vivo* maternal humoral response with the zona pellucida intact. Trophoblast cells have been shown to express paternal Class I MHC antigens in vitro (Zuckerman and Head, 1986; Billington and Burrows, 1986; Chatterjee-Hasrouni and Lala, 1979), and these alloantigens have been shown to induce both humoral (Menge and Beer, 1985) and cellular (Smith et al., 1978; Jacoby et al., 1984) immune responses. These results are controversial and *in vivo* studies using females hyperimmunized against paternal alloantigens revealed no loss or damage to fetal tissue (Zuckermann and Head, 1985; Mitchison, 1953; Wegmann et al., 1979).

Medawar's second model suggested that the fetal tissue is not recognized by the mother as foreign because the fetus does not express histocompatibility antigens. The expression of functional histocompatibility antigens is now well documented in both pre- and post-implantation stages. H-2 antigens have been detected by electron microscopic examination of immunolabeled blastocyst stage

embryos (Searle et al., 1976; Warner and Spannaus, 1984). The technique of ^{125}I-lactoperoxidase labeling has been used to detect H-2 antigens on the ICM of blastocyst stage embryos (Webb et al., 1977). Eight cell stage embryos have been reported to express H-2 antigens in two studies, both utilizing variations of complement-dependent cytotoxicity (Krco and Goldberg, 1977; Cozad and Warner, 1982). H-2 class I antigens have been detected on mouse embryos as early as the 2 cell stage by immunoprecipitation (Sawicki et al., 1981); however, this is the only report of H-2 antigens at the 2 cell stage.

In addition, the production of H-2 mRNA has been examined by two groups. Warner et al. (1985) have found class I mRNA in preimplantation embryos. Ozato and her colleagues were unable to detect any class I mRNA until day 9 of gestation (Ozato et al., 1985); however, no embryos prior to day 8 of gestation were observed.

In his third hypothesis, Medawar proposed that the maternal immune system is incapable of mounting a response to an allogeneic pregnancy. Much data has accumulated to suggest that this is not the case. Alloantigens are indeed recognized by the maternal immune system during pregnancy. The pregnant female produces antibodies to paternal MHC antigens, has draining lymph nodes in allogeneic pregnancies, and produces suppressor

cells, both in the decidua and other tissues (reviewed in Hunziker and Wegmann, 1986). In spite of this immune response, the allogeneic pregnancy survives. Recent evidence suggests that this maternal recognition of fetal alloantigens is not deleterious, but in fact directly contributes to improved placental function and fetal survival. The suggestion that a maternal immune response may be necessary for pregnancy survival is supported by studies in three different species which demonstrated that immunizing against paternal alloantigens prevents abortions, (Gill and Wegmann, 1987) and others which demonstrated the removal of T suppressor cells or their activity may compromise a pregnancy (Athanassakis et al., 1987; Beaman and Hoversland, 1988).

The *Ped* Gene

The preimplantation period is a critical point in embryonic development. The timing of development at this stage is important for successful implantation and subsequent fetal development. Mouse embryos transferred to a uterus at an earlier stage of pregnancy will wait for the uterus to match their developmental stage before implanting; however, embryos transferred to a uterus at a stage of pregnancy later than their own do not implant and consequently die (McLaren and Michie, 1956; Doyle et

al., 1963). These data demonstrate that embryos which are not at the appropriate stage of development at the time of uterine receptivity do not survive. Thus, embryonic cleavage rate is crucial with respect to embryonic mortality.

Relatively little work has been undertaken in the area of the genetic timing of early embryonic development. One of the first studies performed in this area demonstrated differences in the cell numbers of embryos from the BALB/c and 129/RV inbred strains of mice at 65 and 89 hours post-HCG (Whitten and Dagg, 1972). McLaren and Bowman showed that C57BL/6 strain mice had a greater number of cells per embryo than mice of the C3H strain throughout the preimplantation period (McLaren and Bowman, 1973). Titenko observed that embryos from inbred mice of the CBA strain had fewer cells than embryos from either the C57BL/6 or BALB/c strains (Titenko, 1977). In 1980, two groups suggested that the timing of embryonic cleavage is genetically controlled (Niwa et al., 1980; Shire and Whitten, 1980).

The slow-developing strains in both the McLaren and Bowman (1973) and Titenko (1977) studies were of the $H\text{-}2^{k}$ haplotype. This led Warner and her coworkers to hypothesize that the rate of embryonic cleavage was a trait linked to the major histocompatibility complex.

Verbanac and Warner demonstrated that an MHC-linked gene, Ped (preimplantation embryo development), exists which influences the rate of cleavage of preimplantation embryos (Verbanac and Warner, 1981). The known properties of the Ped gene have been reviewed by Warner (1986). There are two functional alleles of the Ped gene: fast and slow.

Observations in the congenic strains B6.K1 (Qa^a) and B6.K2 (Qa^b) have further linked this gene to the Q region of the MHC (Brownell and Warner, 1988). B6.K1 mice, which lack Q region genes, are Ped slow while B6.K2 mice, which have the full battery of Q genes, are Ped fast. The phenotypic expression of the Ped fast allele has been shown to be concordant with expression of the Qa-2 antigen, as has the expression of the Ped slow phenotype with the absence of Qa-2 (Warner et al., 1987). The current hypothesis asserts that the Ped gene product may be the Qa-2 antigen.

Interferon

For many years, virologists have observed that an individual which has a viral disease almost never develops another viral illness at the same time. This phenomenon is called viral interference. Isaccs and Lindenmann (1957) found that when supernatant from

virally-infected chicken cell cultures was added to uninfected cells which were subsequently challenged with a virus, no infection would occur. They concluded that the virally-infected cells were producing a soluble substance which was preventing further infection, and they called this substance interferon.

There are two types of interferons. The first type are the α-interferons (α-IFN) and β-interferons (β-IFN). These interferons are produced by cells infected with a virus. Although α-IFN and β-IFN are sufficiently similar in structure to use the same receptor, they are encoded by different genes. Both are proteins of approximately 20,000 daltons in molecular weight (Klein, 1982).

The second type of interferon is γ-interferon (γ-IFN), also known as immune interferon. γ-IFN is a true lymphokine; it is produced by mitogen- or antigen-stimulated T lymphocytes. γ-IFN has a more pronounced antiviral effect than either α-IFN or β-IFN (Klein, 1982).

The two main effects of interferon are the prevention of viral replication and inhibition of cell growth. Among the other effects on cells of these molecules are inhibition of antibody formation, enhancement of lymphocyte cytotoxicity, and enhancement of macrophage function (Vilcek and DeMaeyer, 1984).

Modulation of MHC Protein Expression by Interferons

Interferons incite a variety of changes in the cell membrane (Friedman, 1979). Among these are the inhibition of virally-induced cytolysis and cell fusion (Sato et al., 1980; Tomita and Kuwata, 1981), increased binding of proteins (Pfeffer and Tamm, 1982; Lonai and Steinman, 1977), increased expression of Fc receptors (Fridman et al., 1980), and increased expression of MHC molecules. Of these effects, the modulation of MHC protein expression has been extensively studied.

Lindahl, Gresser, and their collaborators were the first to observe the induction of MHC proteins by interferons (Lindahl et al., 1973). The alloantibody-absorbing capacity of L1210 cells was measured following exposure to mouse α and β interferons. They found a dose-dependent increase in alloantibody-absorbing capacity in interferon-sensitive L1210 cells, but no increase in interferon-resistant L1210 cells.

Since interferons are known to be powerful anti-proliferative agents, it was hypothesized that the increased absorbance of alloantisera could be attributed to a "piling up" of cells in one phase of the cell cycle. Killander et al. determined the cell cycle position of individual control of interferon-treated mouse leukemia cells by cytophotometric and autoradiographic techniques

and simultaneously determined the amount of H-2 antigens expressed on the surface of individual cells by quantitative immunofluorescence (Killander et al., 1976). They found that the distribution of interferon-treated cells and control cells in the various stages of the cell cycle were similar, and that the expression of H-2 antigens was enhanced in all phases of the cell cycle. Thus, the enhanced expression of cell surface antigens was not due to a concentration of cells in one phase of the cell cycle.

Both the class I and class II proteins can be modulated by interferon. Class I expression is increased by IFN treatment on all cell types investigated thus far. Class II expression is increased with exposure to interferon in cells which constitutively express class II; additionally, cells which do not express class II antigens (or express them at levels too low to detect) can be induced to express class II upon contact with interferon. These effects have been demonstrated with both natural and recombinant DNA-derived interferons. Both type I and type II interferons have the ability to produce these effects. While α and β IFN have varying ability to induce class II expression, γ-IFN induces both class I and class II very effectively. Some studies have demonstrated 30-fold increases in class I expression and

8-fold enhancement of class II (McNicholas et al., 1983).

The enhancement of MHC proteins by interferons has been found on virtually every cell type, both marrow-derived and non-marrow-derived. Marrow derived cells which respond to interferons in this manner include T cells, B cells, macrophages, monocytes, Langerhans cells, and mast cells. Non-marrow derived cells which have been shown to increase expression of MHC proteins in response to interferons in culture include those derived from heart, kidney, colon, pancreas, bladder, lung, ovary, brain, thyroid, and many melanomas (reviewed by Halloran et al., 1986).

Several species have now been studied and all have revealed similar results. Interestingly, interferons do not appear to work across species in this effect. This appears to be a receptor-oriented function, rather than one of gene regulation. In transformation studies, the ability of an interferon to enhance or induce MHC protein expression is restricted by the host cell species origin rather than by the origin of the transforming gene (Yoshie et al., 1982; Rosa et al., 1983).

Friedman and Stark (1985) reported an interferon response sequence (IRS), a common sequence of about 30 base pairs found in the promoter region of several human genes responsive to α-IFN. Sequences highly homologous

to this human IRS have been found in the promoter regions of several H-2 class I genes including $H\text{-}2K^b$, and $H\text{-}2L^d$ (Israel et al., 1986). Singer and her coworkers have recently found a sequence in the 5' region of a porcine class I gene which has 69% homology to the human IRS (Singer et al., 1988). In comparing the sequence of the Q7 gene (Devlin et al., 1985b), a sequence beginning 139 base pairs upstream of the TATA box can be found which has 82% homology to the human IRS.

Effects of Interferon on Embryos

Little is known regarding the effects of lymphokines on embryo development. Only a few studies have been performed and these have yielded contradictory results. Lindahl-Magnusson and her co-workers (1971) found that mouse interferon preparations inhibited proliferation of mouse embryo cells in a primary monolayer cell culture as determined by ^{3}H-thymidine uptake and cell counts after trypsinization. This study also showed that human interferon had no effect on mouse cell proliferation. IFN has also been shown to have anti-proliferative effects on an embryonic cell line (Drasner et al., 1979); however, in the same study, no effects on development or ^{3}H-thymidine uptake by 2 cell and 8 cell embryos maintained *in vitro* in the presence of 3.4×10^3 U/ml of

interferon. An examination of this data suggests that in some experiments values for development and ^{3}H-thymidine uptake might be slightly higher in the interferon samples. Carthew et al. (1986) found no significant difference in cell number per embryo between cultures of 2 cell embryos maintained *in vitro* 72 hours with 666 U/ml interferon and similar cultures with no interferon. These findings confirm those of Drasner (1979); however, each investigator utilized only one concentration of IFN, leaving the possibility that larger or smaller concentrations might elicit effects not observable at these two concentrations.

Most recently, Hill et al. (1987) studied the effects of various lymphokines on mouse embryo development determined by morphologic scoring. Several interferons including human recombinant γ-interferon (hu r-γIFN) and rat recombinant γ-interferon (rat r-γIFN) were examined at concentrations as high as 10^{6} U/ml in this study, however, no mouse interferons were tested. Dramatic increases in embryotoxicity with both human and rat r-γ interferons were observed at concentrations greater than 10^{5} U/ml, but no significant effect was seen at lower concentrations.

Significant discrepancies exist between these experiments in the method and duration of *in vitro*

culturing. Hill and coworkers cultured 2 cell embryos for 4 days and found that 80% of control embryos developed to the blastocyst stage. In contrast, Carthew and associates and Drasner both cultured 2 cell embryos for 3 days and observed 79% and 78% blastocysts in control cultures, respectively. It is of interest that many researchers have had success culturing 2-cell embryos to the blastocyst stage in 48 hours (Rafferty, 1970).

Even less work has been done in the area of embryonic MHC modulation by interferons. Both H-2K and H-2D expression is enhanced by interferon, while Ia expression is affected little on mouse embryonic fibroblasts (Vignaux and Gresser). Zuckermann and Head (1986) have shown that α/β-IFN not only enhances expression of paternal class I antigens on trophoblast cells, but also increases the percentage of cells which are positive for paternal class I. Ozato and coworkers (1985) were unable to detect class I antigens in day 8 embryonic tissues; however, treatment with either α/β-IFN or γ-IFN resulted in the detection of high levels of H-2.

The effects of mouse r-γIFN on preimplantation embryos have not yet been fully examined. The intent of this thesis research is to study the effects of mouse r-

γIFN on proliferation and DNA synthesis in preimplantation embryos, and to determine whether mouse r-γIFN influences cell surface MHC protein expression on preimplantation mouse embryos.

MATERIALS AND METHODS

Mice

Supply and housing

The CFI outbred strain was obtained from the Portage, Michigan facility of the Charles River Breeding Laboratory. The inbred strain C57BL/6J was obtained from Jackson Laboratories, Bar Harbor, Maine. Mice were maintained on a 14-hour light period (5 a.m. to 7 p.m., Central Daylight Time), and were given food and water *ad libitum*.

Superovulation

Embryos were collected from hormonally superovulated mice. Female mice of age 6 weeks or older were given 5 I.U. Pregnant Mare Serum (PMS) (Sigma, St. Louis, MO) intraperitoneally in the 11th hour of the light cycle, followed 45 hours later by an injection of 10 I.U. human chorionic gonadotropin (hCG) (ICN Nutritional Biochemicals, Cleveland, OH) for the inbred strain, or 5 I.U. hCG for the outbred strain. Prepubertal mice (3 to 4 weeks of age) received half dosages. Three hours later mice were monogamously paired with a male. Females were checked the following morning for a vaginal plug to identify a successful mating.

Collection of embryos

Embryo collection was performed as described in Rafferty (1970). Briefly, successfully mated females were sacrificed by cervical dislocation and their uterine horns removed. Embryos were collected by flushing through the oviduct with 0.5 ml of Whitten and Biggers medium (Whitten and Biggers, 1968). Collection was performed at 47 hours post-hCG to recover 2 cell embryos and at 69 hours post hCG to recover 8 cell embryos.

Microdrop cultures of embryos

Embryos were cultured to the blastocyst stage from the 2-cell or 8-cell stage by placing them in microdrops under oil as described in Rafferty (1970). Paraffin oil (Fisher, Pittsburgh, PA) is equilibrated with Whitten and Biggers medium which does not contain BSA and is placed in a 60 mm plastic petri dish (Becton Dickinson, Rutherford, NJ) to a depth of approximately 5 mm. Drops of medium were placed under this oil (approximate volume 10 µl). Cultures were incubated at 37° C in 5% CO_2 in air.

Solutions, Buffers, and Culture Media

Table 1 lists the components of the solutions, buffers and culture media used for the experiments in this thesis.

Table 1. Components of solutions, buffers, and media

Solution	Component	Concentration
Phosphate Buffered Saline (PBS)	$NaH_2PO_4 \cdot H_2O$	3.26 mM
	Na_2HPO_4	6.69 mM
	NaCl	0.127 M
ELISA PBS	Fetal Calf Serum (IgG free)	10%
	NaN_3 in PBS	1%
PT	Tween 20 in PBS	0.05%
ELISA Assay Buffer	Tris-HCL	10 mM
	NaCl	10 mM
	$MgCl_2$	1 mM
Stop Solution	Na_2CO_3	1 M
	NaN_3	0.2%
0.85% Na Citrate	Na Citrate	0.85%
Whitten and Biggers Medium (1968)	NaCl	87.95 mM
	KCl	4.77 mM
	KH_2PO_4	1.19 mM
	$MgSO_4 \cdot 7H_2O$	1.19 mM
	$NaHCO_3$	25.07 mM
	Glucose	0.1%
	Ca lactate	17.09 mM
	Na pyruvate	0.0036%
	Penicillin G, K salt	0.0075%
	Streptomycin SO_4	0.005%
	D,L lactic acid	0.37%
	Phenol red	0.05%
	Bovine serum albumin	0.3%

Table 1 (continued).

Solution	Component	Concentration
Ringer's Solution	$CaCl_2$	2.25 mM
	KCl	5.63 mM
	NaCl	0.15 M

Cell Lines and Antibodies

The WEHI-3 cell line (ATCC, Rockville, MD) is a macrophage-like cell line of H-2^d haplotype. Monoclonal antibody-producing cell lines include, 27-11-13 which produces an antibody which is specific for H-$2D^{b/d}L^d$ molecules and was kindly provided by Dr. David Sachs; 11.4-1, specific for H-$2K^k$, which was obtained from the Salk Institute (San Diego, CA); and TIB 108 (ATCC) which produces N-S.8.1 antibody which is specific for sheep red blood cells and was used as a negative control. A monoclonal antibody specific for Qa-2 antigens, 141.16.6, was the kind gift of Dr. Ian McKenzie, and was used as ascites fluid. The hybridoma cell lines were maintained in either DMEM or RPMI media (GIBCO, Grand Island, NY) at 37°C in 5% CO_2 in air.

Interferons

Mouse recombinant γ-IFN was the kind gift of Dr. Michael Shepherd (Genentech, Inc., San Francisco, CA). It was at a concentration of 2.3×10^7 U/ml in a buffer of 20 mM TRIS, 5 M NaCl. Rat recombinant γ-IFN (AMGen, Thousand Oaks, CA) was at a concentration of 4×10^6 U/ml in a solution of PBS, 1% fetal calf serum, pH 8.5. Human recombinant γ-IFN (Collaborative Research, Inc., Bedford, MA) was at a concentration of 5×10^6 U/ml in 82.3 mM Na_2HPO_4, 51.7 mM NaH_2PO_4, 79 mM sucrose, 2.7% human serum albumin.

Fluorescence Assay

WEHI-3 cells were analyzed for expression of Class I MHC molecules by a procedure modified from that described by McNicholas et al. (1983). Cells were cultured in the absence or presence of 55 U/ml of murine γ-IFN. Cells were washed 3 times in RPMI medium (GIBCO) supplemented with 0.1% sodium azide (Fisher Scientific Company, Fair Lawn, NJ), then placed in a 96-well v-bottom microtiter plate at a concentration of 2.5×10^5 cells/well in a volume of 50 µl. Appropriate monoclonal antibodies diluted in RPMI were placed in each well at saturating levels in a 50 µl volume and the plate incubated for 30 minutes at room temperature. The plate was then washed 3

times in a 100 µl volume of fresh RPMI medium per well. Following the final wash, cells were resuspended in a 50 µl volume of fresh medium. A fluorescein isothyocyanate (FITC)-conjugated goat anti-mouse IgG antiserum (ICN, Plainview, NY) was added at the appropriate concentration and the plate incubated at room temperature for 30 minutes. The plate was washed as before. The cells were analyzed on an EPICS model 752 flow cytometer (Coulter Electronics, Hialeah, FL) utilizing a logarithmic scale. A standard curve was created by analyzing FITC-conjugated beads of known fluorescence intensity on a logarithmic scale. On this scale, a difference of 10 fluorescence channels represents a two-fold difference in fluorescence intensity.

Determination of Embryonic Cell Number

Cell numbers were determined by microscopic examination of embryos fixed on slides by the method of Tarkowski (1966). Embryos were washed 3 times in Ringer's solution followed by 3 washes in 0.8% sodium citrate. The embryos were allowed to remain in the final sodium citrate wash for up to 20 minutes before being transferred to a clean microscope slide. Embryos were fixed with an application of a small volume of freshly prepared 20% glacial acetic acid in methanol. Slides

were stained overnight in Giemsa stain (GIBCO) and rinsed by dipping in distilled water. Samples were examined under a phase contrast microscope (Zeiss, Cincinnati, OH) at 400x magnification and the number of nuclei per embryo were counted.

Tritiated Thymidine (^{3}H-Tdr) Assay for DNA Synthesis

The incorporation of ^{3}H-Tdr was used as a measure of DNA synthesis and was performed as described by Cozad et al. (1981). A working stock of ^{3}H-Tdr was made by diluting Methyl-labeled ^{3}H-Thymidine in an aqueous solution (20 Ci/mmole, NEN) with an equal volume of double-strength Whitten and Biggers medium. Fifty µl of Whitten and Biggers medium was added to the wells of a 96-well flat-bottomed microtiter plate (GIBCO), followed by 5 to 20 embryos in as small a volume of medium as possible. A working stock of ^{3}H-Tdr in Whitten and Biggers medium was made 8 µCi/ml, and 50 µl of this solution were added to each well (final concentration of 4 µCi/ml). The plates were incubated at 37° C in 7% CO_2 in air for 3 to 5 hours. The reaction was stopped by placing the plates on ice and the contents of the wells were harvested onto filter paper using a Titertek cell harvester (Flow Laboratories, Ltd., McLean, VA). The filters were dried in a 65°C drying oven. The filter

disks were placed in vials containing toluene fluor and counted in a liquid scintillation counter (Packard Instruments Co., Downer's Grove, IL). DNA synthesis is expressed as CPM/embryo number in sample/hour of incubation.

Embryo ELISA

Expression of cell-surface proteins on embryos was measured by an enzyme-linked immunosorbent assay (ELISA) as described by Goldbard et al. (1984) and Brownell and Warner (1988). Blastocyst-stage embryos were washed 3 times in ELISA PBS solution (10% heat inactivated FCS and 0.1% NaN_3) and then placed in a 200 µl volume of a primary monoclonal antibody diluted 1:8 in ELISA PBS solution. The embryos were incubated in humidified chambers at 37° C in 7% CO_2 in air for 2 hours, then washed 3 times in ELISA PBS, one time in PT, and once again in ELISA PBS. Embryos were then incubated with sheep anti-mouse IgG $F(ab')_2$ fragments which are conjugated to β-galactosidase (Bethesda Research Laboratories, Gaithersburg, MD) diluted 1:25 in ELISA PBS solution and incubated for one hour and washed as described above. The embryos were then placed in 100 µl of freshly prepared substrate solution (4 mg/ml p-nitrophenyl β-D-galactopyranoside [Sigma], 7 µl/ml β-mercaptoethanol [BioRad, Richmond, CA], in assay buffer),

15 per well, in a 96 well Immulon 1 microtitration plate (Dynatech Laboratories, Inc., Alexandria, VA). The plates were incubated 3-4 hours at 37°C. The reaction was stopped by the addition of 1.0 M Na_2CO_3. The absorbance at 410 nm of each well was read on an ELISA plate reader (Dynatech).

Statistical Analysis of Data

The student's t-test was used to compare the means of different sample populations. The test assumes all populations are normally distributed with the same variance. Applications in this work compared the means of experimental points to the means of control samples. For the ELISA and ^{3}H-Tdr incorporation studies, the degrees of freedom were based on the number of replicate samples. For the cell number assays, the degrees of freedom were based on the number of embryos counted. Mean cell number data were calculated using n = number of embryos, with n being greater than or equal to 15 for all samples.

RESULTS

Fluorescence Analysis of Class I Expression

The expression of H-2D^d and H-2L^d molecules on WEHI-3 cells cultured with or without mouse r-γIFN is presented in Figure 3. At a 1:10 concentration of primary antibody there is a difference of 94.34 fluorescence channels between samples cultured with 50-60 U/ml γ-IFN and control cells. This difference correlates to a 20-fold difference in fluorescence intensity. This experiment successfully repeated the results of McNichols et al. (1983), thus demonstrating the ability of the mouse r-γIFN to enhance MHC expression.

Effects of γ-interferon on Embryonic Cell Number

The determinations of embryo cell numbers in CF1 embryos cultured from the 2 cell stage for 48 hours in the presence of human (hu) r-γIFN or rat r-γIFN are shown in Figures 4 and 5, respectively, and are summarized in Table 2. At least 15 embryos were counted for each sample in each experiment. Error bars represent the standard error of the mean (SEM). There were no significant differences in mean cell number per embryo between control embryos and embryos treated with either hu r-γIFN or rat r-γIFN in concentrations as high as 10^5

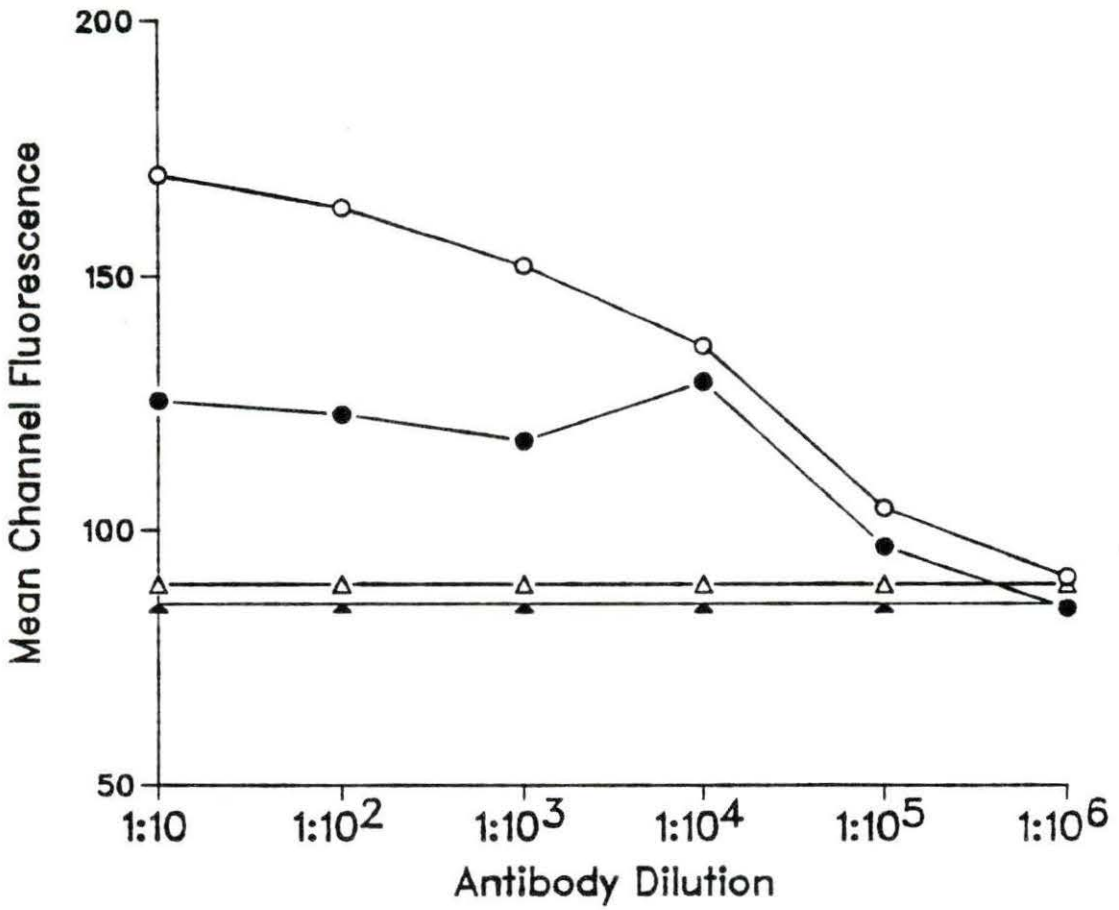

Figure 3. Analysis of H-2D^{d}L^{d} expression on WEHI-3 cells. Circles represent values for 27-11-13 antibody. Triangles represent values for samples with 2nd antibody only. Solid symbols represent control cells, open symbols represent IFN-treated cells

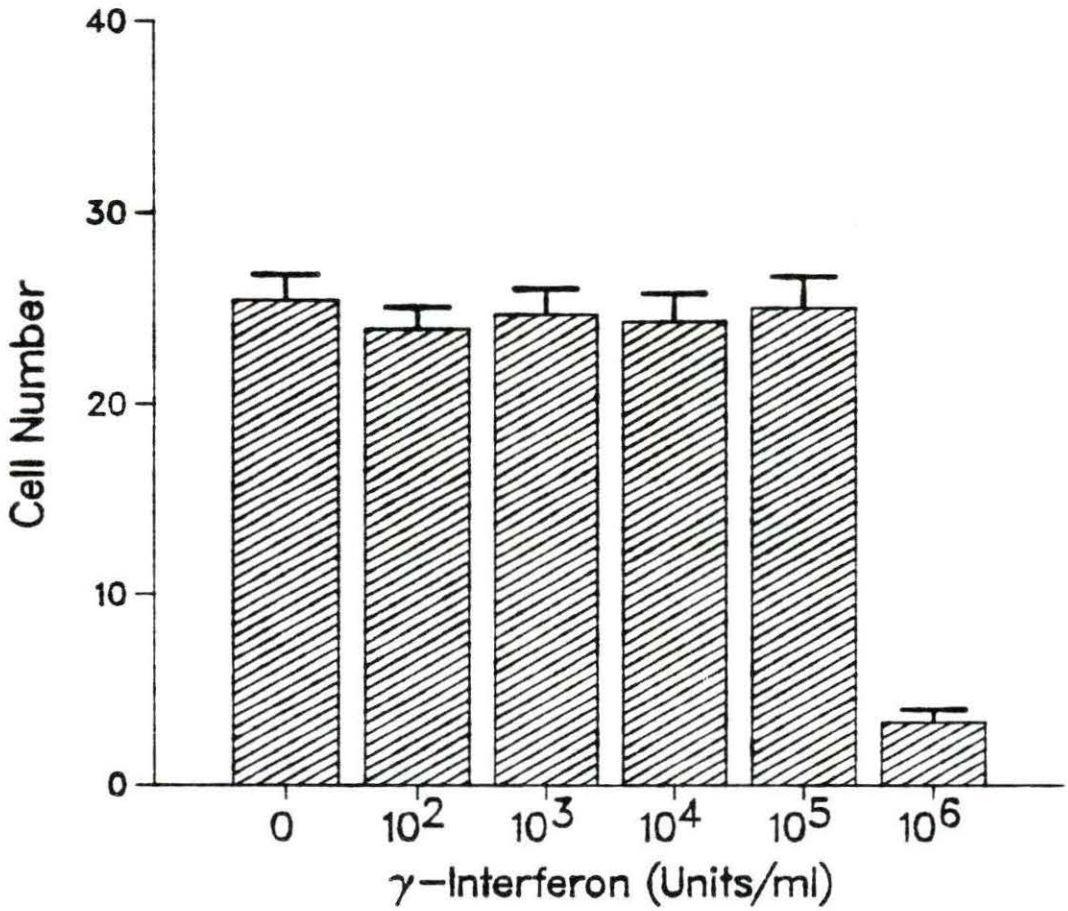

Figure 4. Effect of human γ-IFN on CF1 embryo cell number. Embryos were cultured from the 2 cell stage for 48 hours

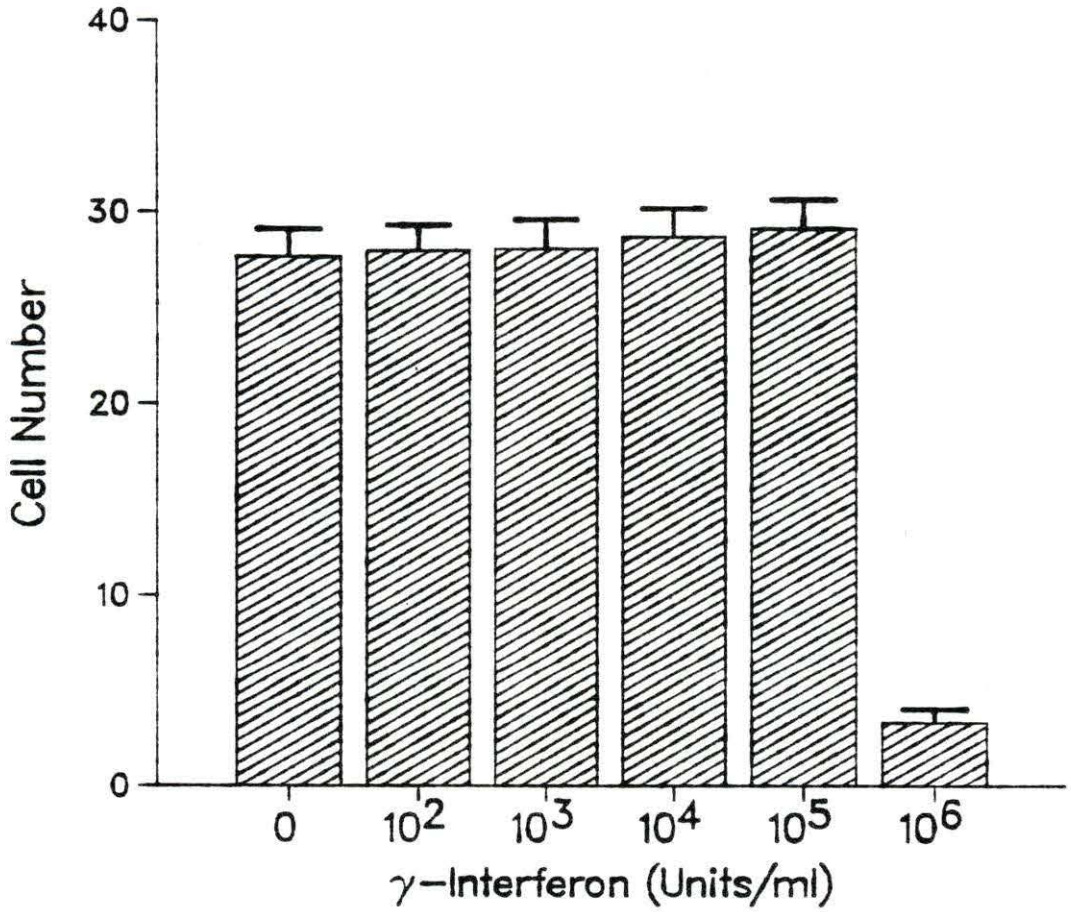

Figure 5. Effect of rat γ-IFN on CF1 embryo cell number. Embryos were cultured from the 2 cell stage for 48 hours

U/ml. Both hu r-γIFN and rat r-γIFN had significant embryotoxic effects at concentrations of 10^6 U/ml ($p \leq 0.001$).

Table 2. Effects of xenogeneic γ-IFN on CF1 embryonic cell number

Interferon	Concentration of IFN (U/ml)	Number of Embryos Scored	Mean Cell Number/Embryo (SEM)	$p \leq$
Rat r-γIFN	0 (Control)	16	27.69 (1.36)	
	10^2	18	28.00 (1.26)	NS[a]
	10^3	17	28.06 (1.47)	NS
	10^4	18	28.67 (1.44)	NS
	10^5	15	29.07 (1.49)	NS
	10^6	15	3.27 (0.64)	0.001
Human r-γIFN	0 (Control)	17	25.41 (1.29)	
	10^2	19	23.89 (1.13)	NS
	10^3	18	24.67 (1.27)	NS
	10^4	16	24.25 (1.42)	NS
	10^5	15	25.00 (1.58)	NS
	10^6	15	3.27 (0.82)	0.001

[a]NS = Not Significant.

The effects of mouse r-γIFN on mean cell number per embryo for 2 cell stage embryos cultured 48 hours for the CF1 and C57BL/6 strains are presented in Figures 6 and 7. Mouse r-γIFN showed marked effects on cell number per embryo in CF1 mice; a steadily increasing trend can be seen, the results at 10^4, 10^5, and 10^6 U/ml were significantly different from values for control embryos ($p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively). In contrast, C57BL/6 embryos demonstrated no significant change in cell number at 10^4 U/ml, but values were higher than those for controls at 10^5 and 10^6 U/ml ($p < 0.05$ and $p \leq 0.001$, respectively). Results of these experiments are summarized in Table 3.

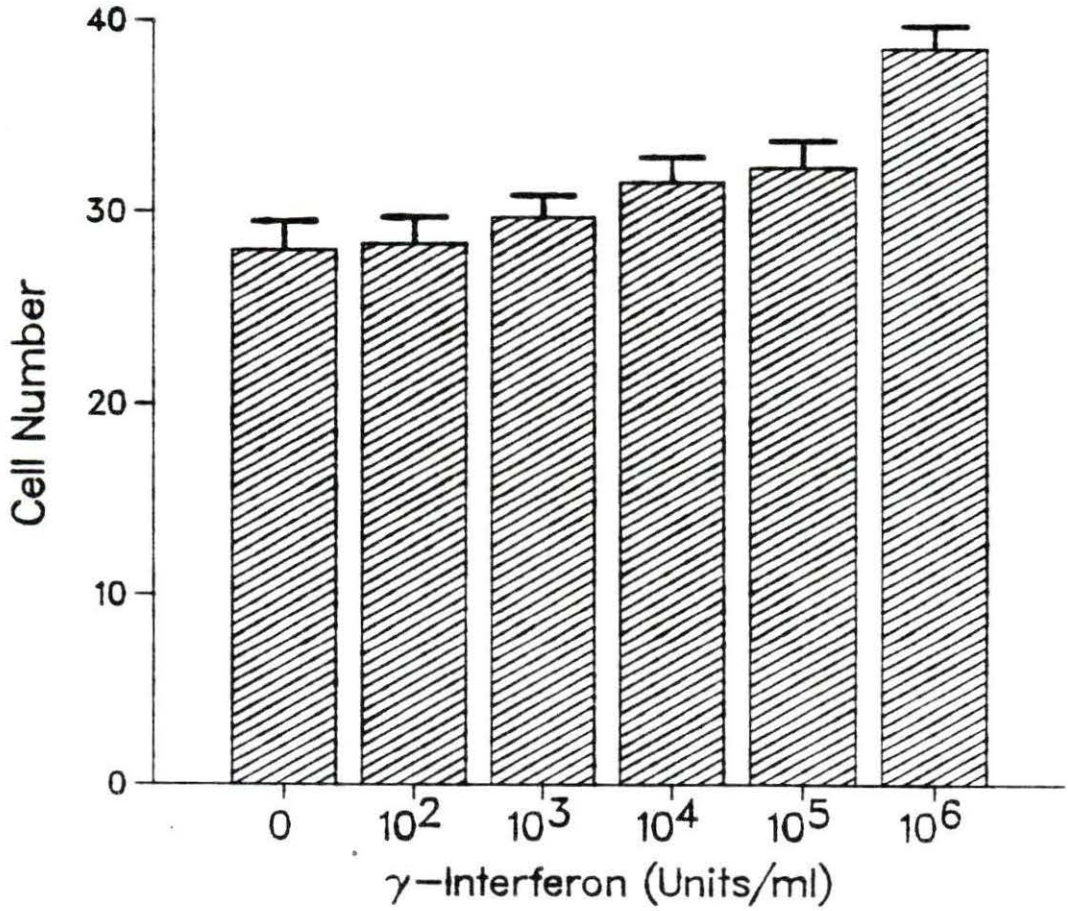

Figure 6. Effect of mouse γ-IFN CF1 embryo cell number. Embryos were cultured from the 2 cell stage for 48 hours

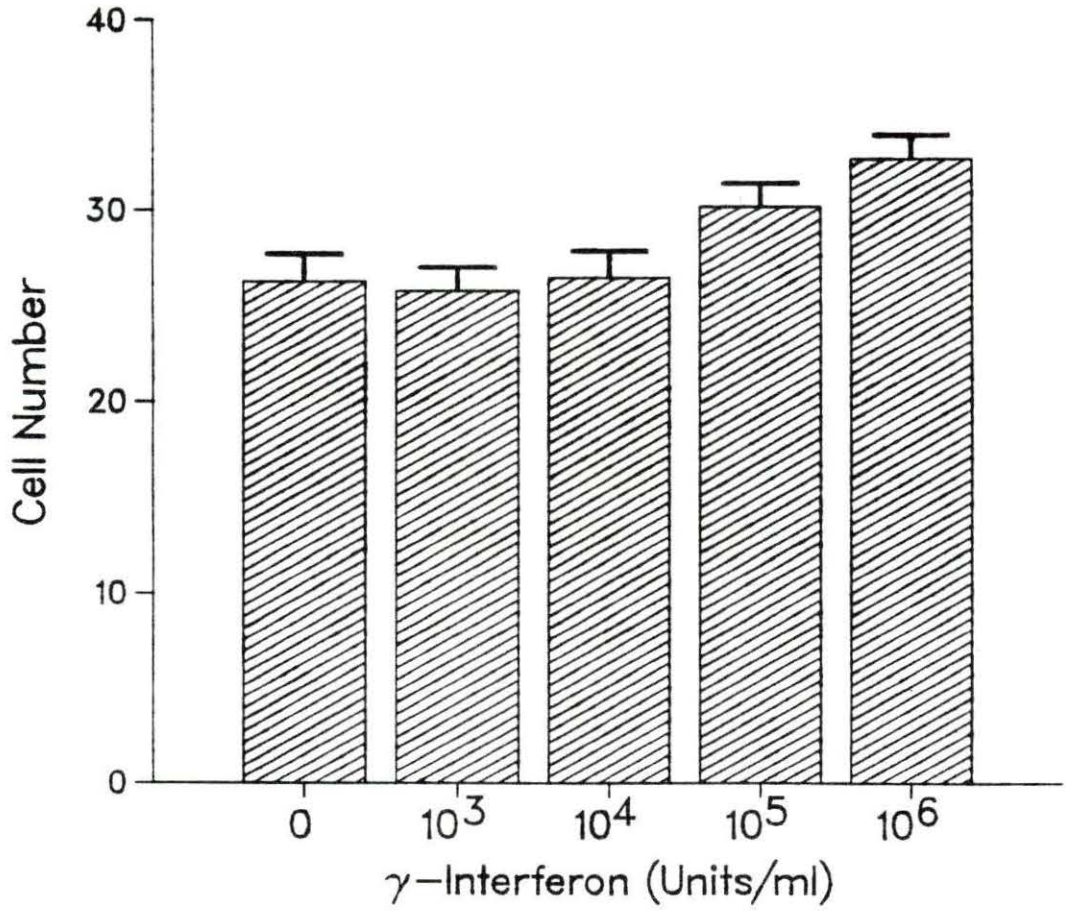

Figure 7. Effect of mouse γ-IFN on C57BL/6 embryo cell number. Embryos were cultured from the 2 cell stage for 48 hours

Table 3. Effects of mouse γ-iFN on embryonic cell number following 48 hours in culture

Strain	Concentration of γ-iFN (U/ml)	Number of Embryos Scored	Mean Cell Number/Embryo (SEM)	p≤
CF1	0 (Control)	18	28.00 (1.43)	
	10^2	16	28.31 (1.33)	NS[a]
	10^3	19	29.68 (1.13)	NS
	10^4	15	31.53 (1.29)	0.05
	10^5	17	32.29 (1.36)	0.01
	10^6	16	38.50 (1.12)	0.001
C57BL/6	0 (Control)	17	26.25 (1.42)	
	10^3	18	25.76 (1.21)	NS
	10^4	16	26.43 (1.38)	NS
	10^5	15	30.19 (1.15)	0.05
	10^6	17	32.65 (1.19)	0.001

Results for the determination of mean cell number per embryo for CF1 and C57BL/6 8 cell stage embryos cultured 24 hours in the presence of mouse r-γIFN are shown in Figures 8 and 9 and are summarized in Table 4. As in the experiments with CF1 2 cell embryos cultured 48 hours, the CF1 8 cell embryos cultured 24 hours in the presence of γIFN displayed a dose-dependent increase in mean cell number. This increase was significant. Values for samples cultured in 10^4 U/ml γ-iFN had differences

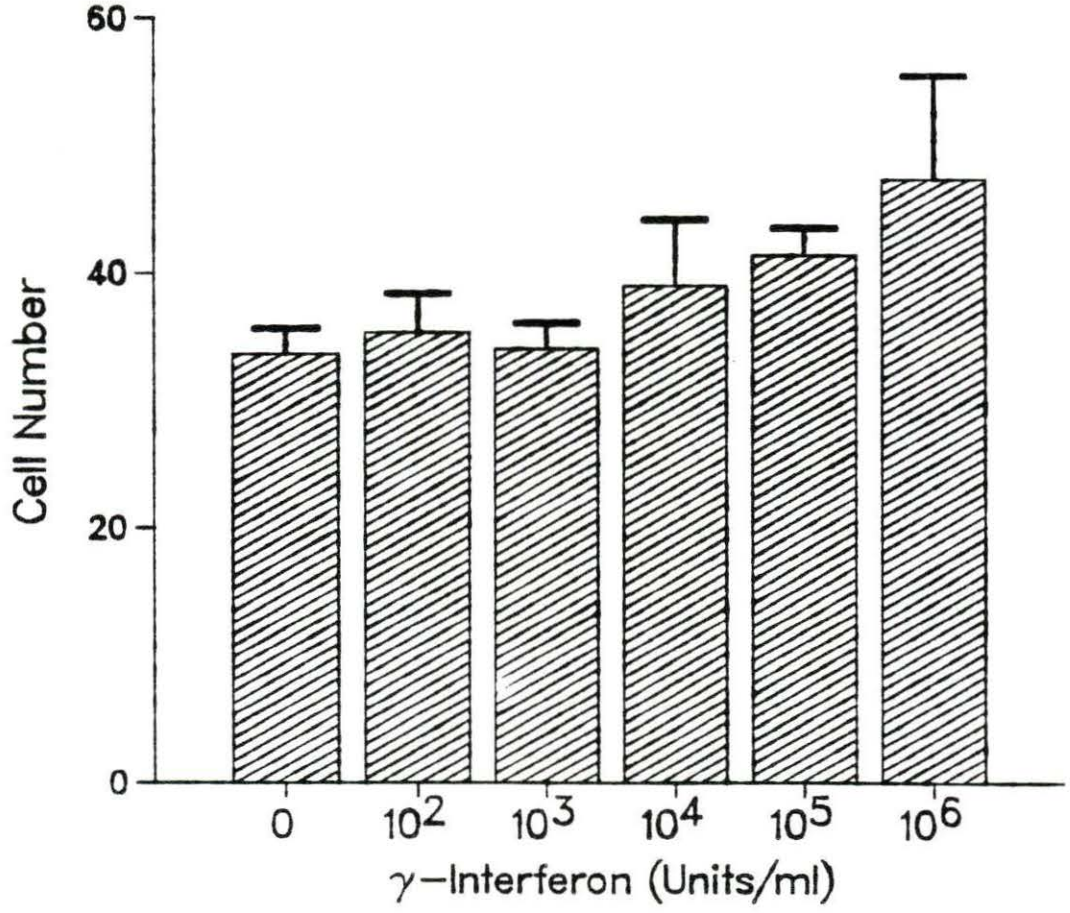

Figure 8. Effect of mouse γ-IFN on CF1 embryo cell number. Embryos were cultured from the 8 cell stage for 24 hours

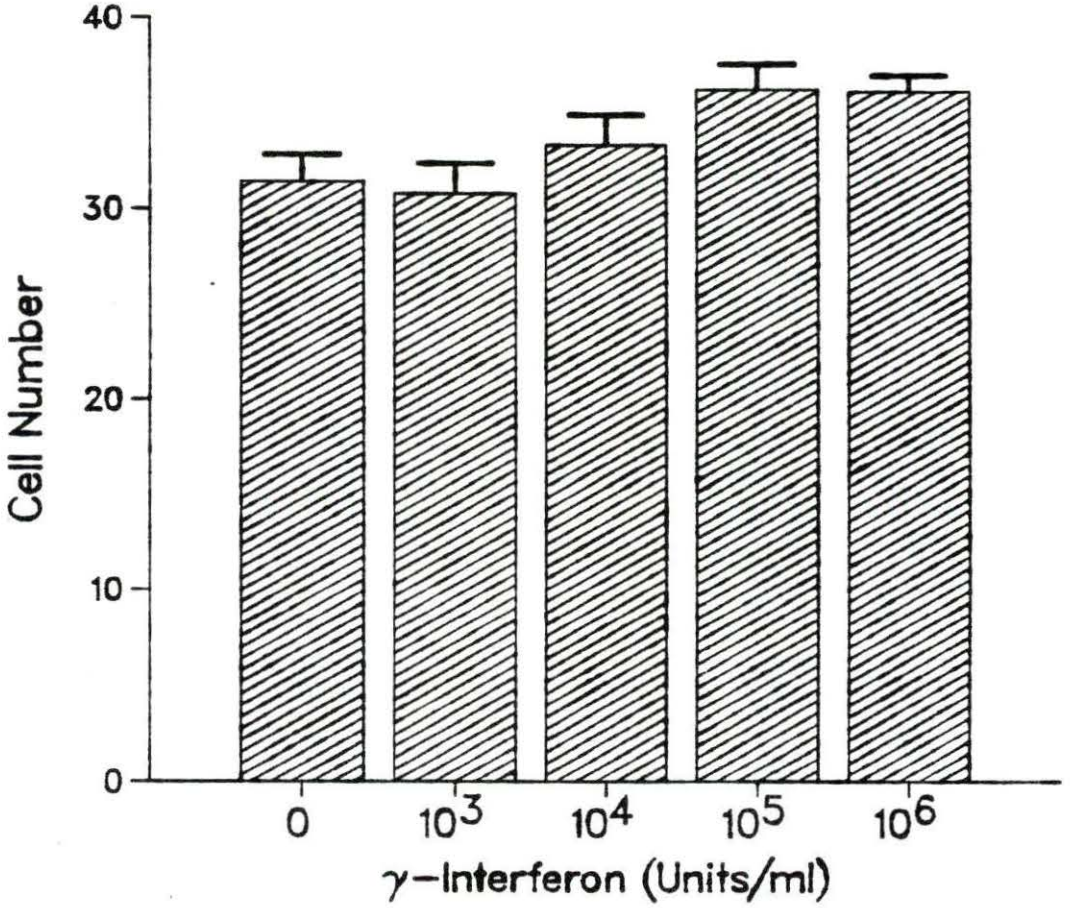

Figure 9. Effect of mouse γ-IFN on C57BL/6 embryo cell number. Embryos were cultured from the 8 cell stage for 24 hours

significant at $p \leq 0.05$, and those at 10^5 U/ml and 10^6 U/ml γ-IFN showed significance at $p \leq 0.001$ when compared to control values. In contrast to the CF1 results, the C57BL/6 embryos showed no significant differences in mean cell number between experimental and control embryos at concentrations of γ-IFN less than 10^5 U/ml. Embryos cultured in 10^5 and 10^6 U/ml γ-IFN revealed cell numbers significantly different from controls ($p \leq 0.005$).

Table 4. Effects of mouse γ-IFN on embryonic cell number following 24 hours in culture

Strain	Concentration of γ-IFN (U/ml)	Number of Embryos Scored	Mean Cell Number/Embryo (SEM)	$p \leq$
CF1	0 (Control)	20	33.7 (1.9)	
	10^2	20	35.4 (2.9)	NS[a]
	10^3	20	34.1 (1.9)	NS
	10^4	19	39.0 (5.0)	0.05
	10^5	20	41.3 (2.1)	0.001
	10^6	20	47.3 (3.4)	0.001
C57BL/6	0 (Control)	17	31.41 (1.38)	
	10^3	16	30.75 (1.57)	NS
	10^4	15	33.33 (1.53)	NS
	10^5	15	36.20 (1.30)	0.005
	10^6	15	36.07 (0.82)	0.005

[a]NS = Not Significant.

Effects of γ-interferon on Embryonic DNA Synthesis

Figure 10 demonstrates the relationship between ^{3}H-Tdr uptake and cell number. As can be seen, this relationship is linear and a linear regression analysis of this data reveals a line with a slope of 2.4.

Figure 11 shows the analysis of ^{3}H-Tdr uptake by CF1 embryos cultured from the 2 cell stage for 48 hours in the presence of mouse r-γIFN. There is an apparent increase in DNA synthesis in all of the samples which were cultured with γ-IFN, and this increase is dose-dependent. Samples of embryos cultured in 10^{2} U/ml γ-IFN displayed values different from those of control embryos at a significance level of $p \leq 0.01$. A significance level of $p \leq 0.005$ can be assigned to samples of embryos cultured in 10^{3} and 10^{4} U/ml γ-IFN. At concentrations of 10^{5} and 10^{6} U/ml γ-IFN, significance increases to a level where $p \leq 0.001$. A small but significant increase in ^{3}H-Tdr uptake is seen in embryos of the C57BL/6 strain after 48 hours in culture with 10^{6} U/ml γ-IFN (Fig. 12). Results of these experiments are summarized in Table 5.

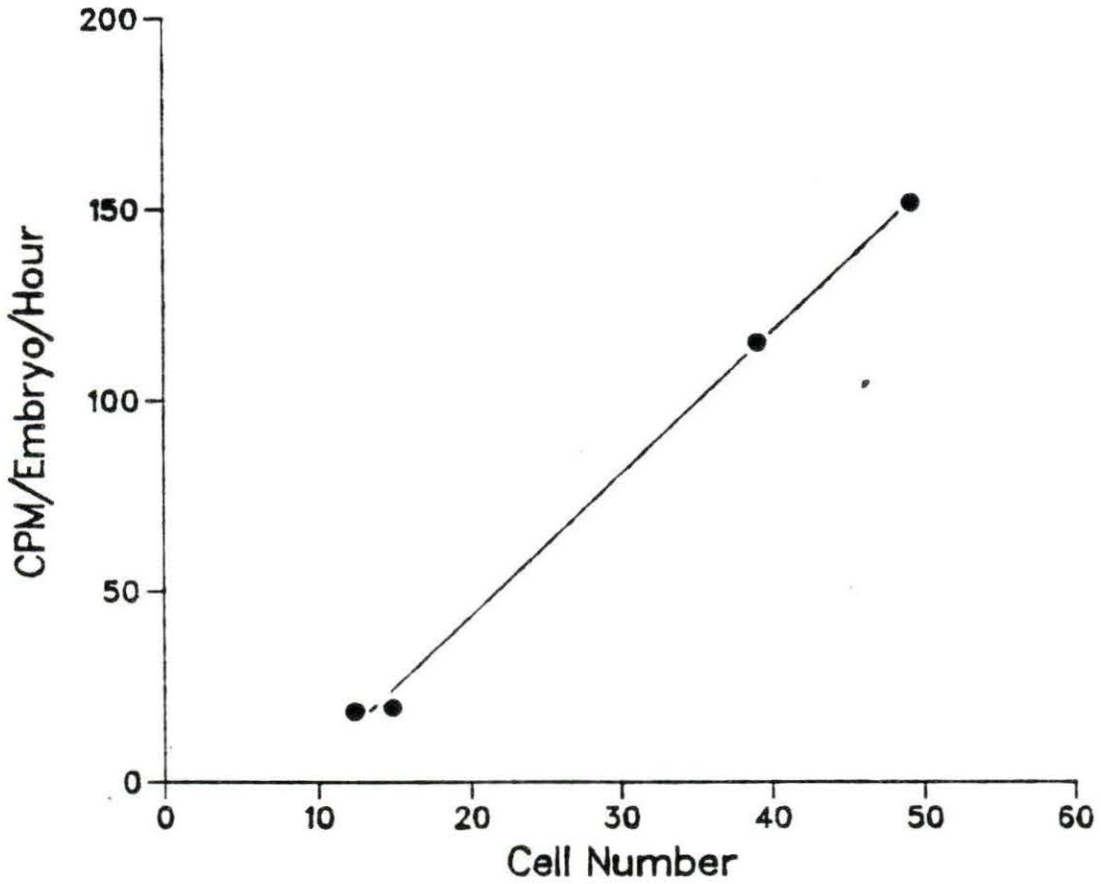

Figure 10. Relationship between uptake of ^{3}H-thymidine and mean cell number per embryo

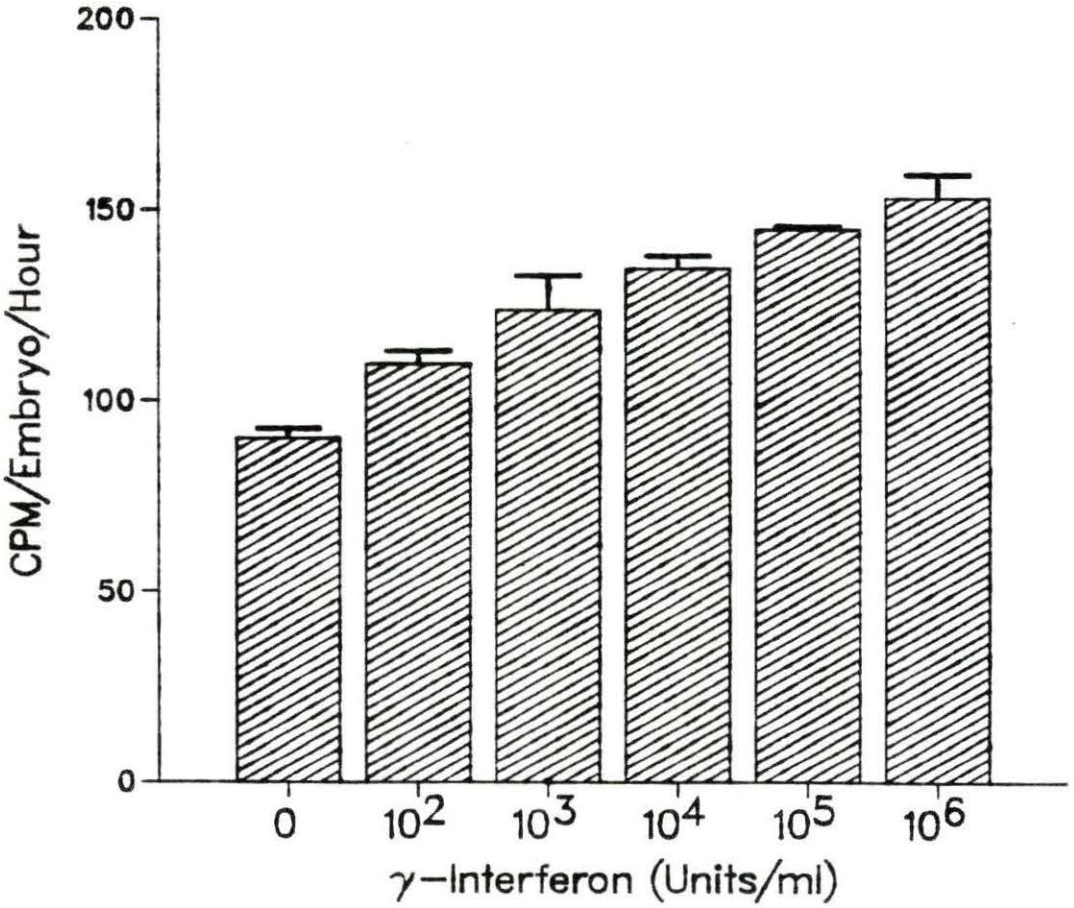

Figure 11. Effect of mouse γ-IFN on uptake of ^{3}H-thymidine by CF1 embryos. Embryos were cultured from the 2 cell stage for 48 hours

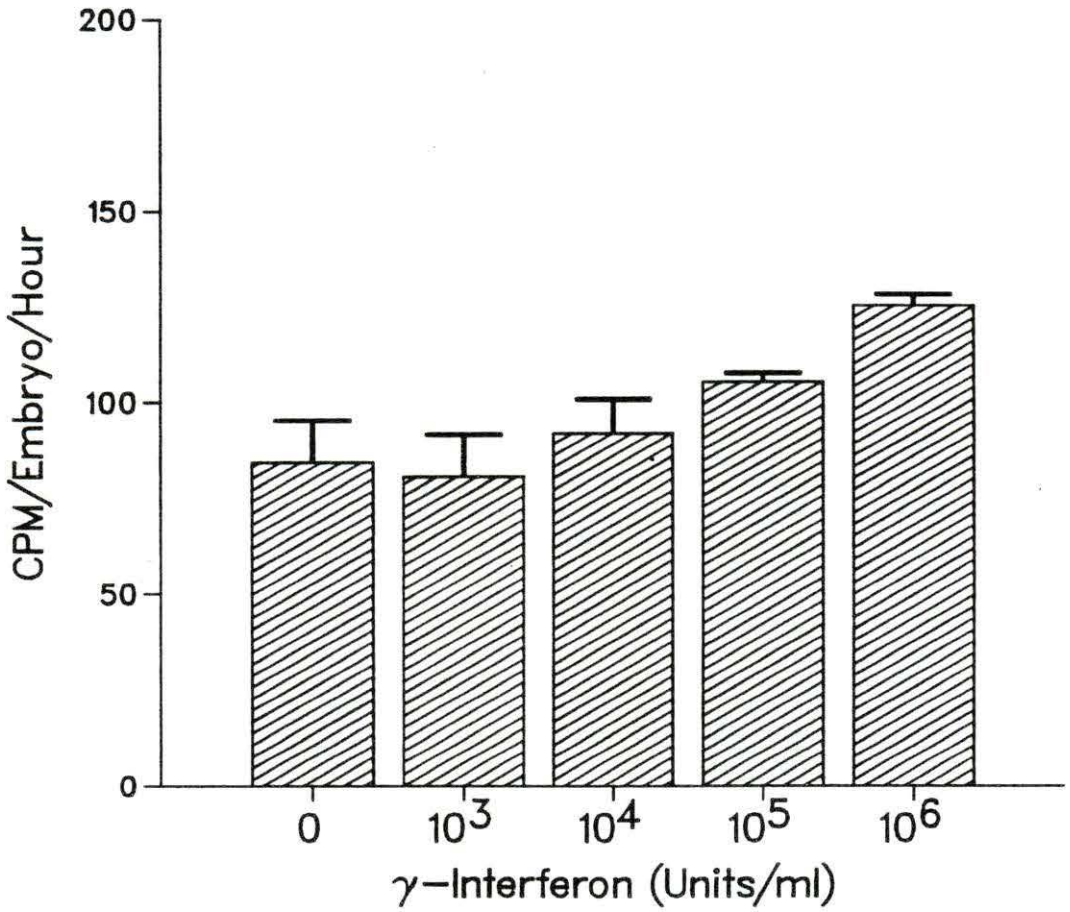

Figure 12. Effect of mouse γ-IFN on uptake of ^{3}H-thymidine by C57BL/6 embryos. Embryos were cultured from the 2 cell stage for 48 hours

Table 5. Effects of mouse γ-IFN on embryonic DNA synthesis following 48 hours in culture

Strain	Concentration of γ-IFN (U/ml)	Number of Embryos Tested	CPM/Embryo/ Hour	STD	p≤
CF1	0 (Control)	15	90.1	2.3	
	10^2	15	109.4	3.3	0.01
	10^3	15	123.4	9.0	0.005
	10^4	15	134.4	3.3	0.005
	10^5	15	144.7	0.7	0.001
	10^6	15	152.9	5.8	0.001
C57BL/6	0 (Control)	15	84.2	10.8	
	10^3	15	80.3	11.0	NS[a]
	10^4	15	91.8	8.7	NS
	10^5	15	105.3	2.0	NS
	10^6	15	125.2	2.7	0.05

[a]NS = Not Significant.

The effects of mouse r-γIFN on ^{3}H-Tdr uptake by CF1 and C57BL/6 8 cell stage embryos cultured for 24 hours are presented in Figures 13 and 14. As in the experiment in which CF1 2 cell were cultured for 48 hours, the CF1 embryos showed a dose-dependent increase in DNA synthesis. This effect is observed up to a concentration of 10^5 U/ml of γ-IFN ($p \leq 0.005$); at 10^6 U/ml values fall

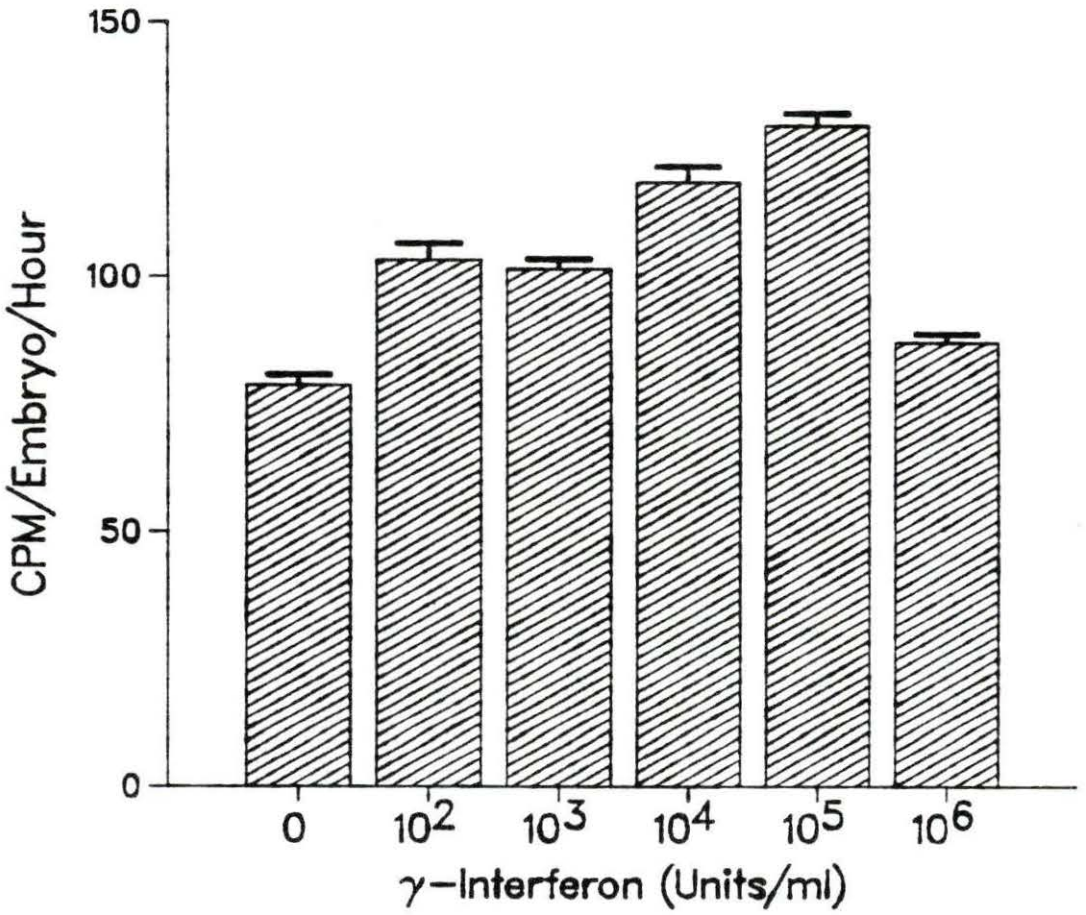

Figure 13. Effect of mouse γ-IFN on uptake of ^3H-thymidine by CF1 embryos. Embryos cultured from the 8 cell stage for 24 hours

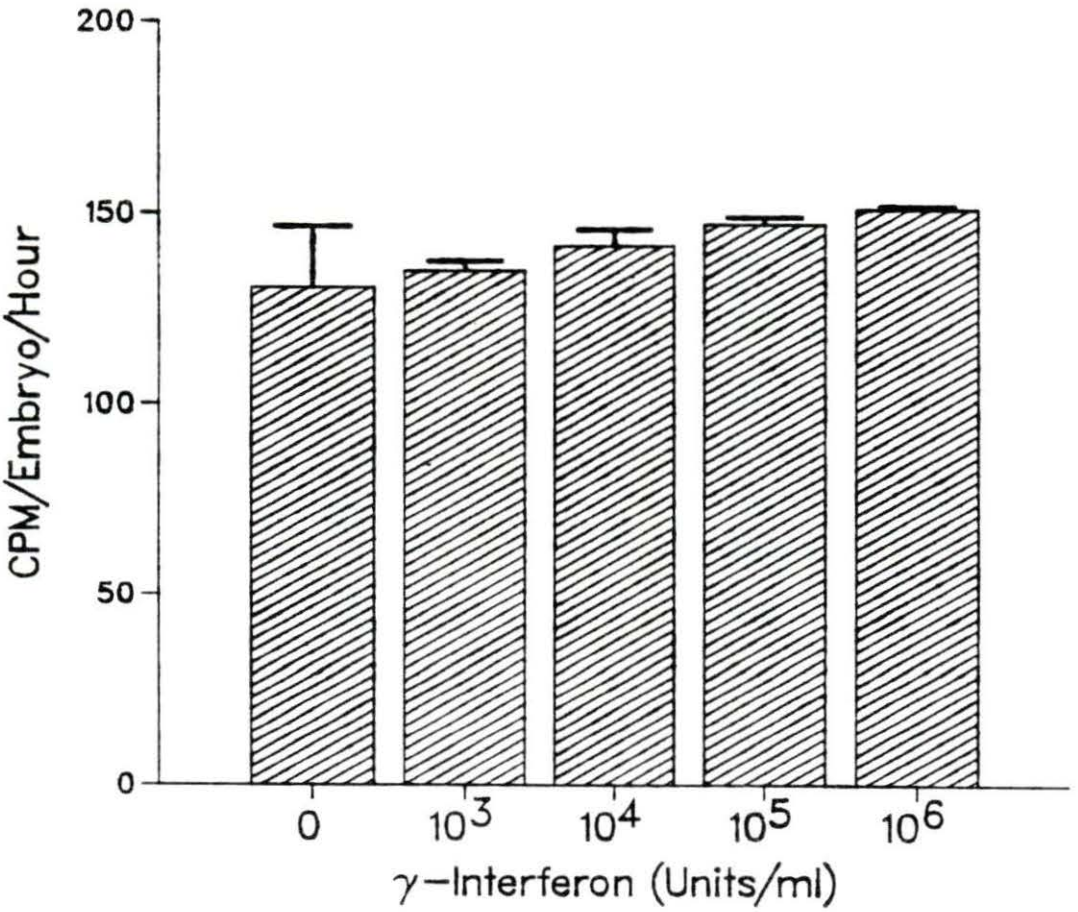

Figure 14. Effect of mouse γ-IFN on uptake of ^{3}H-thymidine by C57BL/6 embryos. Embryos were cultured from the 8 cell stage for 24 hours

to levels closer to those of control samples, but remain significantly different ($p \leq 0.05$). C57BL/6 embryos showed no significant response to mouse γ-IFN in ^{3}H-Tdr uptake. Results of these experiments are summarized in Table 6.

Table 6. Effects of mouse γ-IFN on embryonic DNA synthesis following 24 hours in culture

Strain	Concentration of γ-IFN (U/ml)	Number of Embryos Tested	CPM/Embryo/ Hour	STD	$p \leq$
CFi	0 (Control)	15	78.8	1.8	
	10^2	15	103.3	3.0	0.005
	10^3	15	101.4	1.8	0.005
	10^4	15	118.5	2.9	0.005
	10^5	15	129.5	2.3	0.005
	10^6	15	87.1	1.4	0.05
C57BL/6	0 (Control)	15	130.5	5.8	
	10^3	15	134.8	2.3	NS[a]
	10^4	15	141.3	4.0	NS
	10^5	15	146.9	1.7	NS
	10^6	15	150.8	0.6	NS

[a]NS = Not Significant.

Effects of γ-interferon on Embryonic Class I Expression

The effect of γ-IFN on H-2 expression in C57BL/6 embryos was determined by the embryo ELISA using 27-11-13 antibody for detection of the H-$2D^b$ molecules and N-S.8.1 antibody as a negative control. C57BL/6 embryos were collected at the 8 cell stage and cultured 24 hours with or without 10^5 U/ml γ-IFN (Fig. 15). The averages of three samples for each experimental point were used; error bars represent the standard deviation. An approximate doubling of class I antigen expression can be seen in the IFN-treated sample. Values for this experiment are found in Table 7.

Table 7. Effects of mouse γ-IFN on H-2^b expression on C57BL/6 embryos

Sample	Antibody	Total number of Embryos Tested	Mean A_{410}	STD	$p \leq$
Control	N-S.8.1	45	0.11	0.014	
	27-11-13	45	0.21	0.008	
10^5 U/ml γ-IFN	N-S.8.1	45	0.10	0.014	
	27-11-13	45	0.37	0.017	0.05

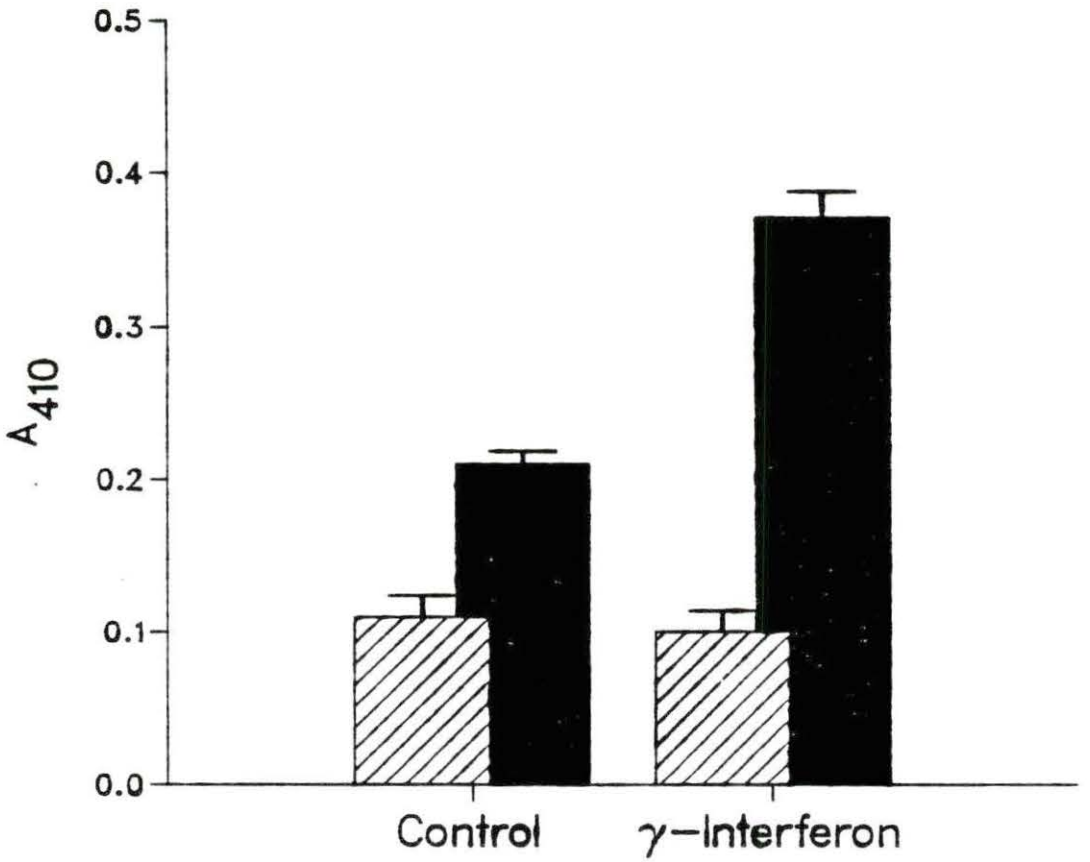

Figure 15. Effect of mouse γ-IFN on H-2D^{d}L^d expression by C57BL/6 embryos. Embryos cultured from the 8 cell stage for 24 hours in the presence of 10^5 U/ml γ-IFN. Hatched bars represent α-SRBC negative controls. Solid bars represent H-2D^{d}L^d-specific antibody, 27-11-13. Primary antibodies were used at a 1:8 dilution

Analysis of Qa-2 expression on CFI and C57BL/6 embryos cultured with γ-IFN was determined by an embryo ELISA using 141.16-6 antibody for detection of Qa-2 and N-S.8.1 as a negative control (Fig. 16, Fig. 17). CFI embryos cultured with γ-IFN displayed an approximate two-fold increase in of Qa-2 expression over levels expressed by embryos from control cultures. A three-fold increase in Qa-2 expression was found in C57BL/6 embryos from IFN-treated cultures when compared to samples of embryos from control cultures. Results of these experiments are summarized in Table 8.

Table 8. Effects of mouse γ-IFN on embryonic Qa-2 expression

Strain	Sample	Antibody	Total No. Embryos Tested	Mean A_{410}	STD	$p \leq$
CFI	Control	N-S.8.1	45	0.05	0.012	
		141.16.6	45	0.14	0.017	
	γ-IFN	N-S.8.1	45	0.04	0.008	
		141.16.6	45	0.27	0.029	0.05
C57BL/6	Control	N-S.8.1	30	0.04	0.005	
		141.16.6	30	0.54	0.040	
	γ-IFN	N-S.8.1	30	0.06	0.020	
		141.16.6	30	1.55	0.100	0.005

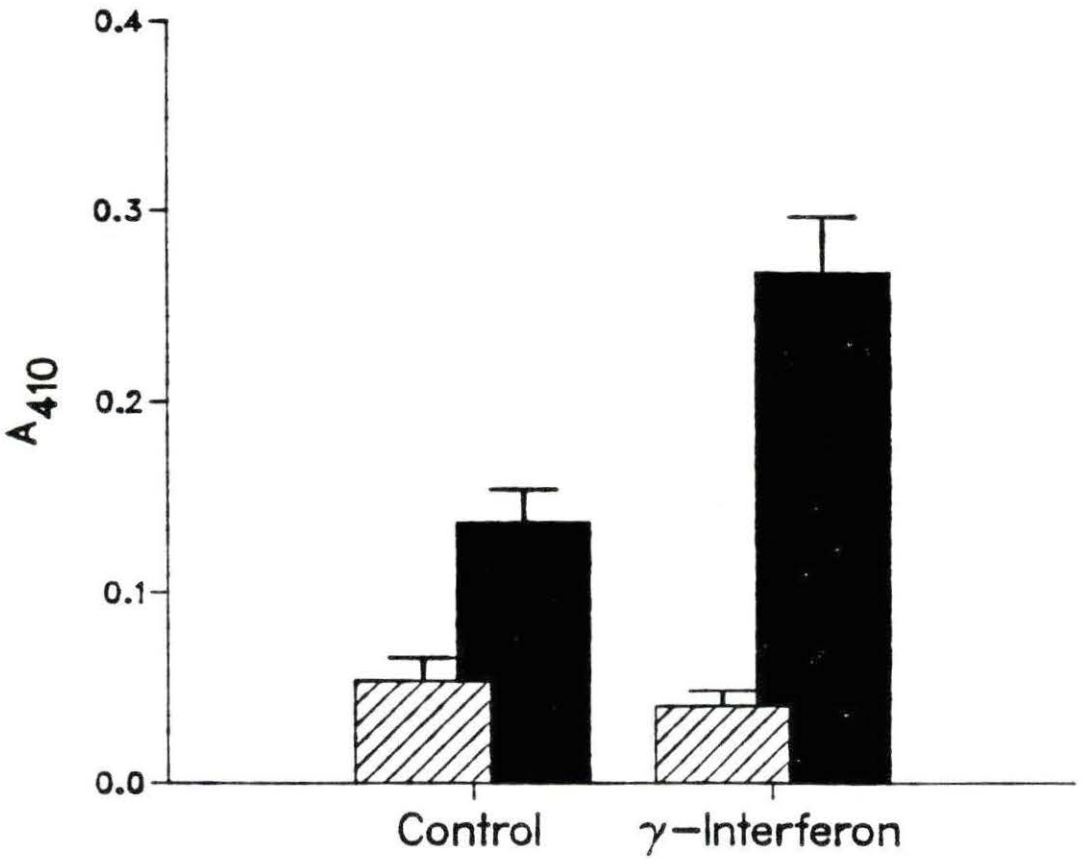

Figure 16. Effect of mouse γ-IFN on Qa-2 expression by CF1 embryos. Embryos were cultured from the 8 cell stage for 24 hours in the presence of 10^5 U/ml γ-IFN. Hatched bars represent α-SRBC negative controls. Solid bars represent Qa-2$^-$specific antibody, 141.16.6. Primary antibodies were used at a 1:8 dilution

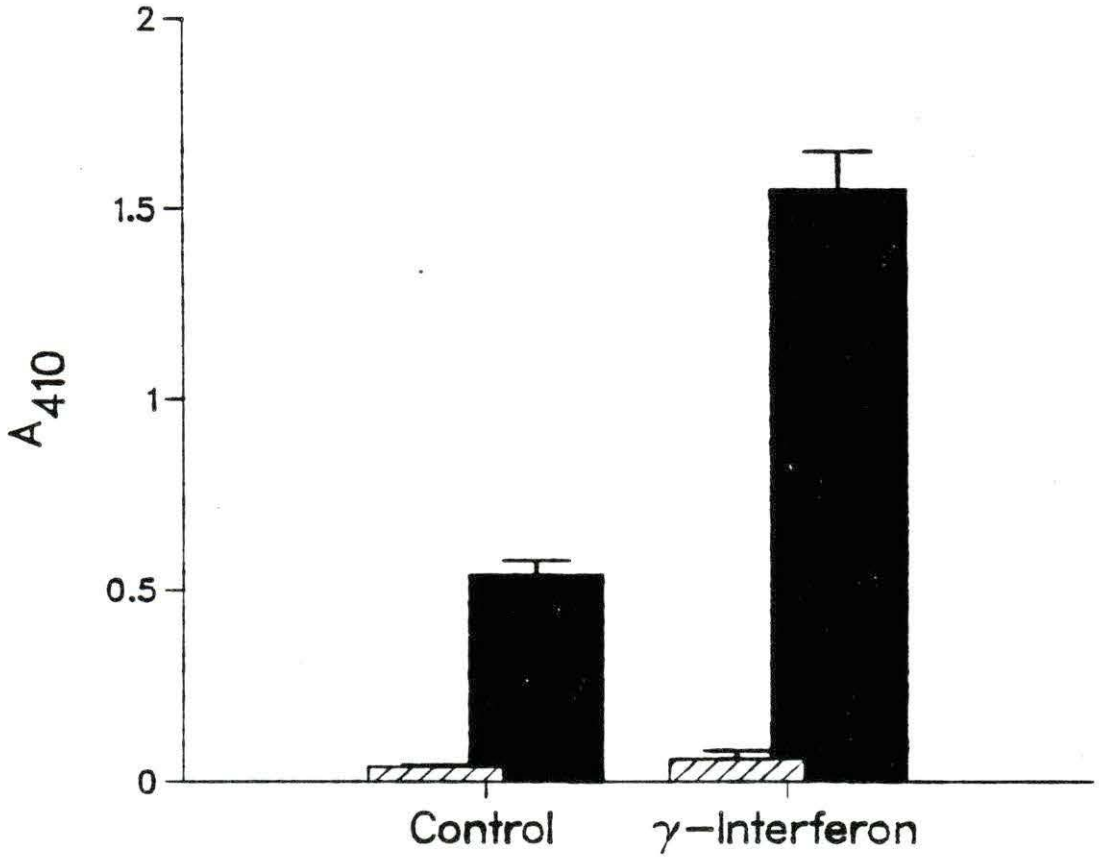

Figure 17. Effect of mouse γ-IFN on Qa-2 expression by C57BL/6 embryos. Embryos were cultured from the 8 cell stage for 24 hours in the presence of 10^5 U/ml γ-IFN. Hatched bars represent α-SRBC negative controls. Solid bars represent Qa-2$^-$specific antibody, 141.16.6. Primary antibodies were used at a 1:8 dilution

DISCUSSION

Embryonic Developmental Responses to γ-Interferon

The work in this thesis shows that the rate of development of preimplantation mouse embryos increases in response to mouse r-γIFN. Although interesting, these findings were quite unexpected. The original intention of the cell number assays was to titer the interferon in order to find a concentration which would not impede development at which further studies in protein expression could be performed. The results clearly show that γ-IFN not only is not deleterious to embryos maintained in culture, but has a definite proliferative effect. This is the first report of an increased rate of embryonic development in response to γ-IFN. Increased cell numbers per embryo were found both in cultures of 2 cell embryos cultured for 48 hours and 8 cell embryos cultured for 24 hours. Embryos from the C57BL/6 strain are unresponsive to γ-IFN at low concentrations of mouse γ-IFN, although some significant increase can be seen in cultures maintained in very high concentrations of γ-IFN.

These results contrast with the findings of Hill et al. (1987) who found dramatic embryotoxic effects of xenogeneic γ-IFNs at similar concentrations. To address this apparent discrepancy, experiments were undertaken in

an attempt to repeat their results in cultures of 2 cell embryos in the presence of human γ-IFN and rat γ-IFN. Hill et al. (1987) found that in cultures of embryos treated with 10^5 U/ml hu r-γIFN for four days, less than 30% of the embryos were scored at the morula or blastocyst stages. An even smaller percentage, less than 20%, of embryos cultured with rat r-γIFN developed to the blastocyst stage.

Experiments described in this thesis demonstrated that no statistical differences existed between control embryos and embryos cultured with 10^5 U/ml human or rat r-γIFNs, and also found >95% of control embryos to be at the blastocyst stage after 48 hours in culture. Hill et al. found only 80% of control embryos to be at the blastocyst stage after four days in culture. It should be noted that the present work revealed an embryotoxic effect with both human and rat r-γIFN at concentrations of 10^6 U/ml; however, this can most likely be attributed to the volume of the stock buffer which was added to the embryo cultures. Embryos are highly sensitive to changes in salt concentration, and to provide such high concentrations of IFN it was necessary to make a 1:4 dilution of IFN stock buffer in Whitten and Biggers medium. A comparison of results from Hill's work and work performed for this thesis is summarized in Table 9.

Table 9. Differences observed in embryonic developmental rates from two research groups

Interferon	Concentration	Percent Reaching Blastocyst Stage	
		Hill et al. (96 hours)	Almquist (48 hours)
Rat r-γIFN	0 (Control)	~ 80%	>95%
	10^2	ND[a]	>95%
	10^3	~ 60%	>95%
	10^4	~ 50%	>95%
	10^5	~ 18%	>95%
	10^6	~ 2%	0%
Human r-γIFN	0 (Control)	~ 80%	>95%
	10^2	ND	>95%
	10^3	ND	>95%
	10^4	~ 82%	>95%
	10^5	~ 26%	>95%
	10^6	~ 5%	0%

[a]ND = Not Determined.

Results of studies of uptake of ^{3}H-thymidine by CF1 embryos displayed similar proliferative effects of γ-IFN. In both 8 cell cultures maintained 24 hours and 2 cell cultures maintained 48 hours dramatic increases in DNA synthesis can be seen. A drop in CPM/embryo is seen in cultures maintained 24 hours in 10^6 U/ml γ-IFN. An

examination of the data for cell number/embryo in these conditions suggests that these embryos have already undergone the last cleavage division in culture, therefore, the lower rate of DNA synthesis is to be expected. This same drop in DNA synthesis is not found in cultures maintained 48 hours at the same concentration. These embryos are most likely dividing slower due to the longer period of time spent *in vitro* and, therefore, have not reached the last cleavage division.

As in the cell number assays, only small increases in developmental rate were seen for C57BL/6 embryos maintained *in vitro* 24 hours. An increase in ^{3}H-Tdr uptake can be seen in cultures of C57BL/6 embryos maintained 48 hours in the presence of 10^{6} U/ml γ-IFN. It can be concluded that both the CF1 outbred strain and the C57BL/6 inbred strain embryos increase their rate of cleavage when cultured with mouse γ-IFN, however, the effect on C57BL/6 embryos is slower and a 48 hour period in culture is necessary to obtain significant increases in cleavage rate.

Modulation of Embryonic Class I by γ-Interferon

Both the CF1 and C57BL/6 strain embryos demonstrated increased expression of class I proteins in response to culture with γ-IFN. H-2D expression was increased two-fold in C57BL/6 embryos. A doubling of Qa-2 expression was observed in CF1 embryos, while Qa-2 levels nearly tripled in the C57BL/6 embryos. These increases were visible after only 24 hours in culture. This observation is especially significant in the C57BL/6 strain where no change in rate of development was observed in this time frame. This would suggest that an increase in H-2D and Qa-2 expression precedes any developmental response to γ-IFN.

Physiologic Role of Interferon in Development

One question prompted by the findings of this study is: what is the role in development of γ-IFN? Certainly the most startling effects in the studies presented here were found at concentrations much higher than reported physiologic concentrations (Booch, 1985); however, it is almost impossible to measure the local concentration of IFN around the embryo <u>in vivo</u>, and the local concentration could be much higher than that found in the maternal blood stream.

There has been at least one report of cultured cells

regulating their own class I MHC expression during differentiation by autocrine production of β-IFN (Yarden et al., 1984). This is especially interesting in light of recent findings by Roberts and Imakawa (1988) who have found that oTP-1, the major protein product of sheep preimplantation conceptuses is a member of the α_2 subclass of interferons. This protein has displayed classic IFN activity: namely, viral inhibition and inhibition of cellular proliferation.

A model can then be suggested for the interactions of interferons and Qa-2 molecules. If the Qa-2 molecule is indeed the Ped gene product, then it can logically be assumed that possession of the gene is not enough to initiate the Ped fast phenotype, for an embryo to display fast development it must express the protein product. A potential role for IFN in preimplantation development could be the regulation of Qa-2 expression. These regulating IFNs could be provided by the maternal environment, but the work by Yarden et al. (1984) and by Roberts and Imakawa (1988) have provided evidence that autocrine regulation should not be ruled out.

It can also be hypothesized that there is a certain level of Qa-2 expression at which the embryo reaches its maximum developmental capacity and additional expression above this level will have no further effects on cleavage

rate. Thus while the C57BL/6 embryos, which are inbred and strongly Qa-2 positive, displayed increased expression of Qa-2, they demonstrated less change in development, since they were most likely near their maximum potential before exposure to interferon. All of the embryos of this strain have the genes necessary to produce Qa-2 and a strong positive response to γ-IFN is the result. The CF1 outbred strain is a heterogenous population with respect to Qa-2 and levels of expression among individuals would be expected to vary, as would the rates of preimplantation development. In this strain, Qa-2 expression can be modulated by γ-IFN, but the extent of the response may be limited by the number of individual embryos which possess the genes necessary for Qa-2 expression. The rate of development for CF1 mice can be modulated by γ-IFN to a greater degree than for the C57BL/6 strain because not all of the outbred embryos are performing at their maximum capacity. The culturing of embryos with γ-IFN may be "turning on" expression of the Ped gene.

Future Studies

There is a great deal of work to be done in the study of the interactions of interferons, MHC proteins and development. A first step would be to examine the effects of interferon on embryos at the molecular level.

Changes in levels of mRNA expression in response to interferons could provide insight into the mechanistic action of interferon in MHC expression. Additionally, transgenic mice could be created by inserting the Q7 or Q9 genes into a host which is Qa-2 negative. The Ped gene phenotype of these mice and their developmental responses to interferon would further define the roles of interferon and Qa-2 expression in development.

Inbred strains of mice with lower levels of expression of Qa-2 antigens on embryos should be studied to observe their responses to culture with interferon for a comparison with the C57BL/6 strain. These studies could be used to examine the relationship between the level of inherent Qa-2 expression and ability to respond to interferon with regard to protein expression and development.

A study of embryonic responses to α and β interferons should be performed in light of the findings of Yarden (1984) and Roberts and Imakawa (1988). Gamma-IFN was used in the initial studies presented in this work because it has been shown to produce stronger effects than type I interferons, however, the in vivo exposure of the embryo may be to type I interferons rather than type II. Finally, it would be of interest to discern if mouse embryos, like sheep embryos, produce an

interferon during the preimplantation stages of development. These findings could be invaluable in unlocking the mysteries of the events of this phase of development which is so critical for survival.

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