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THE INTERFERON SYSTEM IN THE DEVELOPING MOUSE EMBRYO AND IN DIFFERENTIATING TERATOCARCINOMA CELLS.

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DECLARATION

The work presented in this thesis has not been accepted in any previous form for a degree, and has been conducted by myself except where specifically acknowledged. The work presented in Section three was conducted jointly between myself and Beverly Randle of the Department of Zoology, Oxford University.

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SUMMARY

A modified assay to detect interferon production from individual cells has been designed which is more accurate than those already described. Use of this modified assay has demonstrated that the difference between cell lines that can be induced to produce high yields of interferon, and those which are only capable of producing low yields of interferon, resides in the number of individual cells able to produce interferon in that culture. Thus the apparent homogeneous response of a cell culture to an interferon inducing agent, masks the heterogeneous response of the individual cells which make up that culture. This modified assay is probably sensitive enough to detect all cells capable of producing interferon within a given cell population; and the data presented in section one suggests that this assay can be used with confidence to assay interferon production in cell systems which only produce small amounts of interferon.

Cloned 'nullipotent' embryonal carcinoma (ec) cells, like the pluripotent ec cells described by Burke et al (1978), do not possess an active interferon system, and it is proposed that such cells lack the ability to produce interferon mRNA in response to an interferon-inducing agent. When these 'nullipotent' ec cells are treated with retinoic acid they show an activation of the interferon system which extends for approximately ten to fifteen days. The extent of activation seen varied between different embryonal carcinoma cell lines. In these differentiating cultures there is a parallel increase, both in the percentage of individual cells able to produce interferon, and in the yield of interferon per producer cell. The percentage of single cells able to produce interferon always remained small compared to the non-producer cells in the culture. The pattern of development of interferon inducibility and sensitivity does not distinguish between the different types of endoderm-like cell generated by the various differentiating teratocarcinoma cell lines, nor can the amount of interferon produced by different cell lines be used to quantitate the extent of differentiation which has occurred. However, the activation of the interferon system; because it coincides with changes in morphology and in protein synthesis, can be used as an additional positive marker for the production of differentiated cells in this system.

The data presented in section three demonstrates that during the first third of pregnancy the embryo is unable to produce interferon in response to a virus infection, and furthermore suggests that the antiviral action of interferon may be non-specifically inhibited by the tissues of the reproductive system from the adult female mouse. A functional interferon system develops during the seventh day of embryonic development , and the embryonic ectoderm and the visceral extraembryonic endoderm are the last tissues to show a lack of interferon inducibility. Thus, the mouse embryo can be seen to become capable of mounting an interferon-based antiviral response during a period when it is unable to mount a humoral and cell mediated antiviral immune response. This factor may be of importance in the reduced susceptibility to the pathogenic effects of virus infections, which is a feature of the mid to late term mammalian embryo.

ABBREVIATIONS

AFP	Alphafetoprotein
CPE	Cytopathic effect
cAMP	Cyclic adenosine 3'5' monophosphate
diBcAMP	Dibutryl cyclic adenosine 3'5' monophosphate.
DNA	Deoxyribonucleic acid
dsRNA	Double strand ribonucleic acid
ec	Embryonal carcinoma cell
eIF_2	Eukaryotic protein synthesis initiation factor 2
emb.ect.	Embryonic ectoderm cells
emb.meso.	Embryonic mesoderm
EMC	Encephalomyocarditis virus
END	Differentiated endoderm-like teratocarcinoma cell
epc	Ectoplacental cone
ex.emb.ect.	Extra-embryonic ectoderm cells
ex.emb.meso.	Extra-embryonic mesoderm cells
HA (U)	Haemagglutinin units
IFN	Interferon
INAS	Inhibition of viral Nucleic Acid Synthesis
mRNA	Messenger ribonucleic acid
NDV-F	Newcastle disease virus strain F
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PE	Parietal endoderm cells
pfu	Plaque-forming units
Poly rI:rC	Poly ribo-ionsinic and poly ribo-cytidylic acid (a synthetic double strand ribonucleic acid)

RA	Retinoic acid
RNA	Ribonucleic acid
RRL	Rabbit reticulocyte lysate protein synthesis assay
rRNA	Ribosomal ribonucleic acid
SFV	Semliki Forest virus
SV_{40}	Simian virus type 40
TB	Trophoblast cells
TE	Trophectoderm cells
tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer ribonucleic acid
VE	Visceral endoderm cells
v.emb.end	Visceral embryonic endoderm cells
v.ex.emb.end.	Visceral extra embryonic endoderm
VSV	Vesicular Stomatitis virus
VYS	Visceral yolk sac

INTRODUCTION

INTRODUCTION

The role of interferon as an anti-viral agent has been known and accepted for over two decades. However its pleiotropic effects on cell function have only recently been described; and serve to highlight how little is known of the extent and behaviour of the interferon system in vivo. The molecular biology of this system has yielded under a sustained assault over recent years, and it can now be seen that interferon comprises multigene families whose members are functionally heterogeneous and whose mode of action involves both induction of specific enzymes and the alteration of cell surface behaviour. Interferon is by definition an anti-viral agent, but because it is an agent which has a wide range of non anti-viral effects on cells it becomes necessary to have a more complete understanding of the role and status of the interferon system in vivo.

Efforts in this direction have mainly centred on attempts to gain an understanding of the role of interferon in in vivo pathogenesis, with the aim of evaluating interferon as a possible therapeutic agent. This aim, albeit worthy, has resulted in the neglect of the wider potential of the interferon system.

It is generally thought that the interferon system is not restricted to individual tissues or organs but can be expressed throughout the whole of the animal. However very little is known concerning its ontogeny and phylogeny even though such knowledge is necessary if the understanding of the role of interferon is to extend beyond its action as a component of the hosts' anti-viral response.

Interest in the developmental aspects of the interferon system stem from the observation of Burke et al. (1978) that this system

is inactive in undifferentiated teratocarcinoma cells but becomes active when the teratocarcinoma cells are caused to differentiate. The aim of this study is to examine the ontogeny of the interferon system in the developing mouse embryo, and also to examine in more detail the kinetics of the appearance of the interferon system in teratocarcinoma cell cultures undergoing the transition from multi-potent stem cell to a differentiated cell type. This study therefore combines aspects of interest to two disparate fields and thus the aim of this introduction is two-fold; to describe the potential role and use as an experimental tool of the interferon system, and to describe the concepts underlying both mouse embryogenesis and the use of teratocarcinoma cells as *in vitro* analogues of development.

THE INTERFERON SYSTEM

What is interferon ?

Interferon is defined as an anti-viral activity (see reviews in Burke 1977, Baglioni 1979, Marx 1977a,b). It was first described by Isaacs and Lindeman (1957) who demonstrated that cells exposed to a virus released a substance which could be shown to protect other cells from subsequent virus attack. The principle involved was named interferon. In recent years other functions have been attributed to the interferon molecule, e.g.; inhibition of cell growth (see review in Taylor-Papidomitriou 1980) and regulation of the immune response (see review in Bloom 1980). The discovery of these other functions led to the description of interferon as a cellular mediator or regulator (Clemens 1979, Cohen and Bigazzi 1980) or a pleiotropic modifier of cellular function (Stewart 1979a)and even to the proposal that interferon be classified as a hormone. This latter proposal is based on three criteria; these are that interferon interacts with a specific plasma membrane receptor as an initial step in affecting cell function, that it can affect a range of cell functions in a variety of cell types (Taylor-Papidomitriou 1980), and that one class of human interferon strongly resembles the neuroendocrine hormones and is thus capable of mediating some of the effects associated with interferon through interaction with this system (Blalock and Smith 1981). In addition to the proposed functions of interferon, the molecular biology of interferon production and anti-viral sensitivity provides a model system to study the induction and expression of a eukaryotic gene (Havell 1977, Burke 1980).

Interferon has therefore a wide spectrum of effects, it also has

considerable potential as an experimental tool and is thus of interest to workers in several fields which includes virologists, cell and cancer biologists and immunologists. However because interferon is just one of a variety of biologically active molecules which can be elicited under pathological conditions *in vivo* (Cohen and Bigazzi 1980), it is necessary to define precisely what is meant by the term interferon. The acceptance of a working definition will provide a coordinating influence under which the numerous other proposed functions can be examined. The Committee of Interferon Nomenclature (Stewart *et al*. 1980) have accepted the following definition for interferon; "to qualify as an interferon, a factor must be a protein which exerts virus non-specific anti-viral activity at least in homologous cells through cellular metabolic processes involving systhesis of both RNA and protein".

The term 'interferon system' used in this study is used to denote both interferon production and sensitivity to its pleiotropic effects. These are separate and distinct cellular phenomena and occur by separate pathways. Most differentiated cell lines can produce, and are sensitive to the effects of interferon. This is in contrast to undifferentiated teratocarcinoma cells which cannot produce interferon and are insensitive to its anti-viral (Burke *et al*. 1978) and anti-proliferative effects (Wood and Hovanessian 1979). Some differentiated cell lines, e.g.; African green monkey kidney (vero), baby hamster kidney-21, and chinese hamster ovary kl cannot produce interferon but are sensitive to its effects (Desmyter *et al*. 1968, Taylor-Papidomitrious and Stoker 1971, Morgan 1976). Thus although interferon production and sensitivity are properties which can both reside in one cell they can be separated or temporally absent from some cell lines.

Induction and production of interferon

The presence of interferon cannot be detected in the normal state *in vivo* and *in vitro*, but interferon production can be stimulated by exposure of the cell to a specific inducer of interferon. The inducer enters the cell and either directly or indirectly causes the transcription of the interferon gene mRNA and the translation of it into a secretory protein. Interferon production continues for a set period of time which depends on the particular inducer/cell system, and is then switched off (Burke 1980), and this shut-off of interferon synthesis requires post-transcriptional modification of the mRNA (Sehgal *et al*. 1977). Interferon production has been shown to be due to *de novo* transcription and translation and not merely activation of pre-existing molecules by enucleation experiments which demonstrated the necessity of the nucleus (Burke and Veomett 1977), and by the use of inhibitors of transcription and translation, which were shown to inhibit the production of interferon (see review in Burke 1980 and Stewart 1979 p.90-109). There are numerous agents (called inducers) which can stimulate the production of interferon. Most inducers are viruses (Jameson 1977, Stewart 1979 p28-34) but non-viral substances can also induce interferon. These include micro-organisms (Stewart 1979 p39-41) chemical polymers and low molecular weight compounds (Stewart 1979 p47-48).

It is not yet known how the various inducers act within the cell to activate the cellular gene for interferon, or whether this happens directly or indirectly. Because of the known ability of RNA viruses to induce high yields of interferon (Meager and Burke 1971) and the ability of natural and synthetic dsRNA to similarly induce high yields of interferon (Marcus and Sekellick 1977 have shown that one molecule

of dsRNA can induce anti-viral resistance in a human fibroblast cell _in vitro_) it has been proposed that viruses induce interferon via synthesis of a dsRNA intermediate (Morser and Burke 1979).

Thus interferon is not seen _in vivo_ and _in vitro_ unless the cells are first exposed to an inducer, and then its appearance is transient. There are however pathological and other conditions under which interferon is produced by cells which have had no apparent contact with an inducer. This phenomenon may have one of two possible explanations, either the interferon gene has altered and become permanently expressed or an inducer is permanently present in that cell. Since the true nature of the interferon inducer is not clear these two possibilities cannot be distinguished, and the production of interferon in cells not exposed to an inducer will be termed spontaneous interferon production. This phenomenon will be described later in this section.

Classes of interferon

Three main classes of interferon have been identified in all of the animals so far studied. These classes are designated alpha, beta and gamma, and interferons assigned to each on the basis of antigenic similarity.

Class alpha and beta are produced transiently following the exposure to non-mitogen inducers of most differentiated cells, including cells of the immune system. The ratio of alpha to beta interferons produced within a cell depends on the cell type and the inducer, e.g.: human leukocytes produce predominantly alpha, and human fibroblasts predominantly beta. Class gamma is produced by

immunocompetent lymphocytes following exposrue to a mitogenic stimulus, e.g.: specific antigen or plant lectin, and it is thought that T lymphocytes are the main source of gamma interferon (Epstein 1977). Since gamma interferon is produced from lymphocytes following exposure to a mitogen it can also be termed a lymphokine (Cohen and Bigazzi 1980).

Cloned human interferon genes

The concept of the interferon molecule has altered since the availability of cloned interferon genes. So far only human alpha (Nagata *et al*. 1980) and human beta (Derynck *et al*. 1980) have been cloned. The human alpha class has been shown to consist of a family of different but homologous genes situated on the same chromosome, which are capable of cross hybridisation at the nucleic acid level and of cross neutralisation at the protein level. Up to ten alpha genes, including one pseudogene, have so far been identified (Goeddel *et al*. 1981). These show little or no glycosylation (Allen and Fantes 1980), and do not contain introns either in the coding or non-coding genomic sequences (Nagata *et al*. 1980). The alpha multigene family once cloned into *E. coli* can all be translated (except the pseudogene) and therefore have the potential to be expressed within the cell, but it is not yet clear how many members of this family are expressed in cells treated with different inducers, or whether a particular pattern of expression occurs within certain cells treated with certain inducers. However it is thought that the alpha family are functionally heterogeneous and this is supported by the observations of Streuli *et al*. (1981) on the different cross species reactivity amongst the

members of the alpha family. Cloned alpha 1 and 2, which differ in only twenty nucleic acid positions, showed a large difference in inducing anti-viral resistance in mouse cells. It has long been known that some interferons can show reactivity on cell species different to that which produced the interferon (Stewart 1979 p136-42), but interferons were thought in general to show broad species specificity. The cloning studies have clarified this situation and it appears the species restriction of sensitivity resides in specific interferon molecules and is not a general property of interferons.

The human beta class is antigenically different to alpha interferon and also does not cross react at the nucleic acid level (Allen and Fantes 1980). The total number and extent of the beta gene family is not yet clear; Goeddel et al. (1981) found only one beta gene, while Weissenbach et al. (1980) identified two beta interferon genes in cell-free lysate translations of cloned DNA from poly rIrC induced fibroblasts. These two interferons were identified serologically as beta but did not cross-hybridise at the nucleic acid level, and interestingly were not produced in intact cells; suggesting that a sub-class of interferons are modified at a post-transcriptional level. Sehgal et al. (1981) found a similar situation in fibroblast cells induced in the presence of DRB (dichlororibofuranosylbenzimidazole), and has suggested that a second, hetero-disperse, set of interferon genes exist in both the alpha and beta classes. The second set are proposed to contain introns which prevent nucleic acid cross hybridisation.

Interferon was long thought to be a glycosylated protein, and the varied functions and properties of interferon were thought to be related to the degree of glycosylation of each molecule (Stewart et al.

1977, 1979). The amount of glycosylation has still not been fully resolved but Allen and Fantes (1980) and Derynck et al. (1980) have both shown that alpha and beta interferons do not need to be glycosylated to be biologically active, although beta interferon is normally synthesised as a glycosylated protein.

The biological role of the alpha multigene family has not been clarified; the production of functionally heterogenous interferons may explain the ability of interferon to protect cells from a wide variety of viruses, or alternatively it may explain the large variety of target cells which are susceptible to the effects of interferon. R. M. Friedman has suggested (personal communication) that the different interferon species may be effective on different host tissues.

Mouse interferons

Full details of the structure of mouse interferons are not yet available, since the genetic engineering program has for obvious reasons concentrated on human interferon. However it is known that viral induction of non-lymphoid mouse cells, e.g.: Erlich ascites cells and C243, causes two species of interferons to be produced. The molecular weights are approximately 22 and 35 kd. (De Maeyer-Guignard et al. 1978, Kawakita et al.1978, Iwakura et al. 1978). The lower molecular weight molecule shows some sequence homology with human alpha interferon. The number of interferons produced in virus infected mouse cells does vary; Knight (1975) found ten interferon polypeptides were produced in MM virus-infected cells each with anti-viral activity. Heterogeneity of glycosylation was also found by Knight (1975) and by De Maeyer-Guignard et al. (1978).

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Assay of interferon yields

A large area of this study is concerned with the production and assay of interferon yield. The yield of interferon per cell is subject to regulatory controls within the cell (on transcription rate, half-life of mRNA and translation); but it is also regulated through genetic controls not associated with the structural genes. De Maeyer and co-workers (reviewed in De Maeyer and De Maeyer-Guignard 1979) have obtained mice through breeding experiments which have the ability to produce high or low levels of circulating interferon in response to the same virus inducer. This genetic control of interferon yields is only shown by macrophages _in vitro_. A review of the intracellular regulation of interferon can be found in Havell (1977) and Burke (1980). Briefly it is proposed that expression of the interferon gene is inhibited in normal conditions by the synthesis of a repressor molecule and that interferon inducers act to disrupt this equilibrium and allow expression of the interferon gene (Vilcek and Ng 1971). As yet there is no direct confirmation of the existence of the putative interferon repressor, or of any other eukaryotic gene repressor.

The amount of interferon in a preparation is assayed indirectly by measuring the effect that it can exert on cells. Since different mouse cells vary in their sensitivity to interferon the definition of a unit of interferon thus depends on its mode of assay. The effect most commonly measured is that of antiviral resistance. This can be assayed in a variety of ways including, virus RNA synthesis, yield and plaque reduction and cytopathic effect; all of which vary in sensitivity, reproducibility, speed and amount of labour and expense.

This laboratory routinely employs the Inhibition of viral Nucleic Acid (INAS) assay (Atkins *et al.* 1974). Units of interferon are reciprocals of end point dilutions of an interferon preparation and the end point of the INAS assay is the dilution at which virus nucleic acid synthesis is reduced by fifty percent. These assays are all biological assays and variations from many sources will occur; to eliminate these a laboratory standard interferon preparation is included in all assays and results corrected accordingly. To compare results between different laboratories the laboratory standard interferon is calibrated against an international research reference standard made available from the National Institute for Biological Standards, London. Use of a laboratory standard calibrated to the international research reference standard enables interferon titres to be expressed in international reference units.

The recent development of a monoclonal antibody to interferon (Secher and Burke 1980) has enabled non-biological assays to be developed. Secher (1981) has devised an immunoradiometric assay which although it has reduced sensitivity compared to the biological assays is potentially useful since it can measure physical amounts of interferon.

Single cell assay for interferon

The assays described above measure interferon yields from a population of cells and thus represent the average response of many cells. Since the minimum number of cells required to produce an interferon sample which can be used in these assays are approximately ten thousand, they cannot be used in situations in which only a

small number of cells are available, e.g. from early embryo tissues. In addition it is possible that by measuring only the average response, any heterogenous behaviour of either the cells or the inducer is masked. Interferon production from either single cells or from small groups of cells can be examined in a 'single cell assay'. Two types of single cell assay have been developed both based on the biological activity of interferon. Fleischman and Simon (1974) and Rodgers and Merigan (1974) examined interferon production from single cells isolated in microdrops, whilst Osbourn and Walker (1969) and Kronenberg (1977) measured the percentage of cells able to produce interferon in an assay analogous to the infectious centre assay for virus production from single cells. This study employs a modified version of the assay described by Kronenberg (1977).

PLEIOTROPIC ACTIVITIES OF INTERFERON

Interferon is capable of exerting a vast and often bewildering array of effects upon cellular structure and function (Stewart 1979 p224-231). These effects may be due to a single action of interferon or more probably are effected through the concerted action of interferon on many aspects of cell biology. The activities ascribed to interferon mechanisms may be grouped into six major categories:

(a) Alteration in cell surface composition and behaviour.

(b) Antiviral actions against a wide range of viral pathogens.

(c) Alteration of induced and non-induced protein synthesis.

(d) Regulation of humoral and cellular mediated immune responses.

(e) Inhibition of cell replication in normal and tumour cells.

(f) Possible role in the normal control mechanisms of growth and differentiation.

The order of the above groupings is arbitrary and serves to focus attention on the main effects of interferon, whilst not ignoring the fact that the causes of the observed phenomena may involve several of the effects listed above. It is still not clear whether or not all the activities listed above are secondary to the establishment of the anti-viral state within the cell.

The first non anti-viral effects of interferon were described by Isaacs and Burke (1958) who demonstrated the cell multiplication inhibitory effect. These early experiments were performed with impure interferon preparations (probably less than 1% pure) and were open to criticism that these non anti-viral effects were due to impurities in the preparations. These criticisms have now been formally turned over by the use of electrophoretically pure interferon, Gresser *et al.* (1979) used mouse interferon of specific activity 10^9 units per mg., which was resolvable to two bands on polyacrylamide gel electrophoresis, and clearly demonstrated that each band in this preparation could produce all of the effects listed above.

Cell surface receptors?

The interferon molecule is not itself directly the cause of the effects ascribed to interferon, for it is not active within the cell in which it was synthesised but needs first to be externalised (Vengris *et al.* 1975). Interferon does not need to enter the cell but it has not been formally shown that none of the molecule enters

the cell (Burke 1977). This suggests that sensitivity to the pleiotropic effects of interferon is mediated via the interaction between the interferon molecule and the cell surface. Such a step could act to amplify the action of interferon and might account for its high biological activity.

It has been proposed (Stewart 1979 p185, Cupples and Ian 1977, Tan 1976, Revel _et al_. 1976) that the sensitivity of cells to the effects of all the classes of interferon (alpha, beta and gamma) are mediated initially by a common pathway regulated by a gene on chromosome 21 in humans. The evidence for this is mainly based on the increased sensitivity to human interferon shown by cells trisomic for chromosome 21 and the complimentary decreased sensitivity shown by cells monosomic for chromosome 21. Revel _et al_. (1976) proposed that chromosome 21 codes for an interferon cell surface receptor, since they were able to demonstrate that antisera raised to the cell surface of human/mouse hybrids (which contained only chromosome 21 as its human contribution) blocked the action of interferon. Tan (1976) proposed that chromosome 21 directs the production of an intermediate substance (not a receptor) which mediates the action of interferon within the cell. However contrary evidence has cast doubts on the role of chromosome 21 at all. De Clercq _et al_. (1976) examined interferon sensitivity in a wide range of cells both monosomic and trisomic for chromosome 21 and found that the difference in interferon sensitivities was not constant. Moreover they demonstrated that cells monosomic, disomic and trisomic for chromosome 21 had equal ability to remove interferon from culture fluids, suggesting that these cells all bind equal amounts of interferon. However since the experiments of De Clercq _et al_. (1976) were not performed on matched cells, the

general consensus (see review in Stewart 1979 p185-190) still supports a role for human chromosome 21 in mediating the effects of interferon.

The majority of work on interferons has involved the use of the alpha and beta types because in contrast to gamma interferon they are now available in relatively pure preparations; however it is considered that all three classes of interferons possess a similar range of pleiotropic effects.

Alteration in cell surface composition and behaviour

Interaction with or binding to the cell surface is a necessary preliminary to the manifestations of the effects of interferon. This reaction at the cell surface causes chemical, immunological and morphological changes within the membrane before any of the other effects of interferon can be demonstrated (Chang *et al*. 1978, Friedman 1979). These changes include inhibition of the binding of specific low molecular weight molecules; the binding of cholera toxin and thyroid stimulating hormone are inhibited by interferon whilst that of human chorionic gonadotrophin, leuteinising hormone and follicle stimulating hormone are not. This interference with binding is not thought to be due to direct competition for receptors but rather due to conformational changes induced in the cell membrane by interferon. This alteration also changes the expression of some cell surface molecules; expression of cell surface gangliosides is decreased (Friedman 1979) but expression of beta-microglobulin and the major histocompatability antigens is increased (Lindahl *et al*. 1972, 1973; Heron *et al*. 1979). Two general properties of the cell surface have been found to alter: there is an increase in the number of intra-

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membranous granules of diameter 5-10μm (Chang *et al*. 1978), and an increase in the overall net negative charge of the cell (Knight and Korant 1977). This latter change may indicate that the membrane alters in its permeability to certain molecules. The relevance of the membrane changes to the effects of interferon is described later. Friedman (1979) has suggested that changes in the cell surface may be found to account for all of the non anti-viral effects of interferon.

The first detectable intracellular change after interferon interacts with the cell surface is the elevation of the concentration of the cyclic nucleotides adenine and guanosine monophosphate. This elevation has been reported only in those cells sensitive to the effects of interferon (Friedman and Pastan 1969, Weber and Stewart 1975, Meldolesi *et al*. 1977 and Tovey *et al*. 1979). In non-interferon treated cells, the levels of these cyclic nucleotides can be affected by the rate of cell growth and cell density. Tovey *et al*.(1979) developed a system in which L1210 cells cultured in a chemostat produced constant levels of both cyclic nucleotides, and found that after interferon treatment of these cells the levels of CGMP rose within five minutes, but that in contrast, the levels of CAMP did not rise until several hours later. They suggest that CGMP functions as a membrane-nuclear signal. In addition Friedman and Pastan (1969) and Meldolesi *et al*. (1977) have provided further evidence that cyclic nucleotides may be involved in the effects of interferon,by demonstrating that the administration of CAMP added simultaneously with interferon potientiated the degree of anti-viral resistance achieved in the cells, but had no anti-viral action by itself.

ANTI-VIRAL ACTIONS AGAINST A WIDE RANGE OF VIRAL PATHOGENS

All six classes of animal viruses (i.e. enveloped and non-enveloped, single strand and double strand, and DNA and RNA classes - Matthews (1979)) are sensitive to the inhibitory effects of interferon to a lesser or greater extent (Stewart 1979 p202-206). The total anti-viral response of an organism involves cells and other products of the immune system, as well as the production of interferon within infected cells (Roitt 1977 p201). Host humoral and cell-mediated responses are both involved in the immune response to a virus infection; B lymphocytes are involved in antibody production directed towards virus antigens and T lymphocytes are directly involved in the recognition and lysis of virus infected cells. It is of interest that T cell recognition of virus-infected cells is directed through the major histocompatability antigens and that interferon enhances the expression of these antigens.

The production of interferon can be regarded as the first line of defence against virus infection. Gresser *et al.* (1976 a and b) have demonstrated the role of interferon in *in vivo* virus infections. They treated mice infected with a variety of viruses with antiserum to interferon, and found that in most cases, the disease showed a more rapid onset and greatly increased mortality. An interesting exception was that of Influenza type A in which the course of the disease was not affected.

Treatment of cells with interferon will induce anti-viral resistance within the cell, but this resistance is not apparent until the cell is exposed to virus or to dsRNA (Burke 1977, Marx 1979, Revel 1979). This covert antiviral state may be a mechanism to protect host

cell synthesis. The development of this interferon-induced covert anti-viral state requires the presence of the cell nucleus (Radke *et al.* 1974) and requires *de novo* RNA and protein synthesis of molecules previously termed 'anti-viral' proteins (Burke 1977). Although interferon preparations can show broad species specificity the anti-viral proteins can be transferred between, and are active in heterologous cells which are not responsive to the interferon molecule itself (Blalock and Baron 1977).

Interferon is able to inhibit the growth of a wide variety of viruses to varying degrees (Stewart 1979 p202-206), and this may reflect the ability of the interferon-induced anti-viral state to raise many and diverse barriers to virus replication. Interferon has been shown to inhibit uncoating, transcription and translation, and assembly and release of the virion (Revel 1979). The primary effect of the anti-viral state upon virus replication will depend on the particular virus/cell system under study, however effects upon translation are most commonly observed. Revel (1979) has shown that SV40 replication is inhibited at the early and late stages in interferon treated cells affecting uncoating and DNA replication and later assembly and release of the virions. Strachen *et al.* (1977) demonstrated that interferon can prevent expression of mouse mammary tumour virus in steroid induced cells probably acting at both transcription and translation. Joklik and Merigan (1966) have shown that translation is inhibited in interferon treated vaccinia virus infected cells because of the disruption of the association between viral mRNA and host cell ribosomes.

A further example of the multiphasic antiviral state induced by interferon is seen in Murine Leukaemia virus infected cells. The

production of virus RNA and protein is little affected but the yield of infective virus is considerably reduced due to a defect in the budding and release of virions on the host membrane (reviewed in Friedman 1979). Maheshwari and Friedman (1979) and Maheshwari et al. (1980) have demonstrated the same phenomena in interferon treated vesicular stomatitis virus infected cells which are released from the cell by a mechanism similar to that used by murine leukaemia virus.

Specific inhibition of virus synthesis

It has been suggested that virus protein synthesis is preferentially inhibited in interferon treated cells, and whilst this is not certain there may be physical reasons why this is so. Garry and Waite (1979) have shown that lytic viruses induce alterations in the Na^+ and K^+ concentrations within the cell, and that under these new conditions host cell synthesis is inhibited but virus synthesis is not affected and neither is the synthesis of interferon itself. In addition, Garry and Waite have demonstrated that experimental alteraton of the intracellular concentration of these ions prolongs the synthesis of interferon, probably by inhibiting the synthesis of the host protein responsible for shut-off. In such cells virus synthesis will constitute the bulk of the cells synthetic effort and any inhibitors of protein synthesis will have a proportionally greater effect on virus synthesis. In addition Baglioni (1979) suggests that since the antiviral state in an interferon treated cell is not activated until the cell is exposed to virus the inhibitory effect may be localised to the immediate area of the virus. An alternative to these views is the suggestion that exposure of an interferon treated cell to a virus may

cause the destruction of that cell and thus destruction of the virus (Burke 1977).

Mechanism of antiviral action

As mentioned above, interferon induces *de novo* synthesis of anti-viral proteins. The nature of these proteins was elucidated by examining the antiviral action of lysates from interferon-treated and control cells. Kerr *et al* (1977), Kerr and Brown (1978) and Farrell *et al.* (1978) demonstrated that lysates from interferon treated cells showed enhanced sensitivity to the protein synthesis inhibitory effects of dsRNA. This was shown to be due to two dsRNA dependent enzymes; elevated levels of inactive forms of these enzymes formed in interferon-treated cells being converted to an active form by exposure of the cell to dsRNA (or to virus). These enzymes were a cAMP dependent protein kinase and an oligonucleotide synthetase, termed 2,5 oligo A synthetase by Kerr *et al* (1977).

(a) cAMP dependant protein kinase

In view of the reported elevation of cAMP by interferon (Tovey *et al.* 1979) it is of interest that this enzyme is cAMP dependent. The protein kinase phosphorylates two cell proteins of molecular weights approximately 35Kd and 67Kd (Farrell *et al.* 1978). The smaller molecular weight protein is the alpha sub unit of eIF2 (eukaryotic initiation factor-2) (Hovanessian *et al.* 1980). The nature of the larger protein is not clear; Ohtsuki *et al.* (1980) consider that it is a ribosomal protein that may act as an intermediate between the protein kinase and phosphorylation of eIF2. The phosphorylation of eIF2 prevents the initiation of protein synthesis (Lenz and Baglioni 1978 and Baglioni 1979).

(b) 2,5 Oligo A synthetase

This catalyses the formation of a low molecular weight oligonucleotide of the general formula pppA(2'p5'A)n where 'n' could be from 1-10 but most frequently the trinucleotide was observed (Kerr and Brown 1978). The trinucleotide consists of three adenosine residues linked by two phosphodiester bonds at the 2' 5' positions and linked to three phosphate groups; i.e.

A A A OH (2')
P P OH
P P P (5')

The trinucleotide activates an endonuclease which can degrade viral and cellular mRNA, and thus acts to inhibit virus replication at the level of transcription. Further actions of 2'5' oligo A synthetase and the trinucleotide have been proposed; Thang *et al.* (1981) demonstrated that 2'5' oligo A synthetase catalyses the adenylation of tRNA and NAD^+. The function of this is not clear but this process does limit the size of oligo A nucleotide formed, in the absence of tRNA and NAD^+ long chain oligo A polymers are synthesised. Wallach and Revel (1980) have suggested that the trinucleotide as well as activating the endonuclease, may have a more direct anti-viral role, since they detected the 2'5' A trinucleotide bound to the ribonuclear-protein core of VSV and MLV virions released from interferon-treated cells.

The 2'5' A trinucleotide is itself degraded by an interferon enhanced phosphodiesterase and Revel (1979) proposes that this PDE acts as additional pathway to inhibit virus replication by inhibiting directly the amino acylation of tRNA and thus inhibiting peptide chain elongation.

The role of 2'5' oligo A synthetase and the cAMP dependant protein kinase in mediating the antiviral action of interferon has been confirmed by many experiments which have correlated levels of these enzymes with both the degree of antiviral resistamce and the level of interferon. Williams and Reed (1981), Krishnan and Baglioni (1980) and Hovanessian *et al* (1981) have demonstrated this correlation in *in vivo* pathogenesis, and Thang *et al* (1981) have found this to be true in interferon treated cells infected with MLV *in vitro*. In addition Hovanessian *et al* (1981) have detected elevated levels of 2'5' oligo A synthetase in the sera of human volunteers treated with poly rA:rU, and also in the mononuclear cells of humans suffering from virus infection.

Role of 'antiviral' enzymes in mediating the non-antiviral effects of interferon

The interaction between the interferon molecule and the establishment of the covert antiviral state within the cell reflects a profound effect on the cells' biology, and because of this it may be considered that the other non-antiviral effects attributed to interferon are secondary events and do not represent a series of distinct non-antiviral functions of interferon. Despite intensive searches a further series of interferon-induced enzymes specific to other effects have not been found, and it is therefore possible that the induction of antiviral enzymes and changes at the cell surface mediate the other effects of inter feron.

If this is so, it should be possible to demonstrate that the interferon-induced antiviral enzymes can produce the non-antiviral effects of interferon.Xinyan *et al* (1981) in addition to demonstrating

that 2'5' oligo A trinucleotide can confer anti-viral resistance against a wide variety of viruses, has shown that this trinucleotide can inhibit DNA synthesis in a variety of human and mouse cells and enhance the activity of macrophages and natural killer cells; and thus can have anti-proliferative and immunoregulatory effects. The anti-proliferative effects of 2'5' oligo A trinucleotide have also been demonstrated by Baglioni (1979) and Kimchi *et al.* (1981) who used this trinucleotide to inhibit the mitogen-induced proliferation of both serum starved 3T3 mouse fibroblasts and mouse lymphocytes.

An interesting adjunct to the role of 2'5' oligo A has come from studies on cells insensitive to the action of interferon. Wood and Hovanessian (1980) have shown that in stem cell teratocarcinoma cells which are insensitive to the actions of interferon, that levels of 2'5' oligo A synthetase are elevated by exposure to interferon but those of the cAMP dependent protein kinase are not. Epstein *et al.* (1981) have shown that in a mouse fibroblast line insensitive to interferon the levels of both the 2'5' oligo A synthetase and the protein kinase are enhanced by interferon but no endonuclease activity can be detected. These experiments suggest that other factors are necessary for the functioning of 2'5' oligo A and also that it may be too simplistic an interpretation to propose this molecule as the common pathway through which interferon exerts its effects.

Alteration of induced and non-induced protein synthesis

Interferon is known to influence the synthesis of specific induced and non-induced proteins as well as changing the overall biosynthetic activity of the cell. These changes may represent a specific

action of interferon or alternatively they may represent the differential stability of cell mRNAs to the intracellular changes induced by interferon, or even the alteration in uptake of component molecules caused by changes in the cell membrane.

Treatment of cells with high or low concentrations of interferon can respectively depress or enhance the production of interferon itself, phenomena celled blocking and priming (Stewart 1979 p233-238).

Interferon is also known to enhance the synthesis of factors associated with the immune response, e.g. IgE-induced histamine release is enhanced (Bloom 1980) and so is the synthesis of prostaglandin E (PG-E) (Yarron _et al_. 1977), PG-E is known to be involved in the inflammatory process associated with infection. The release of molecules such as histamine and PG-E may contribute to the febrile illness often associated with interferon therapy (Gresser 1981).

Levels of DNA, RNA and protein synthesis are generally depressed in interferon-treated cells but in some cell systems the synthesis of specific proteins is depressed more than this background level, e.g. globin synthesis in interferon treated differentiating Friend leukaemia cells (Rossi _et al_. 1977a,b), and the enzyme ornithine decarboxylase in mitogen treated cells (Taylor-Papidomitriou 1980).

Regulation of humoral and cellular mediated immune responses

Interferon has effects on all aspects of the immune response including cell mediated and humoral reactions and the production of soluble factors, e.g. complement. Many of the recent papers (see reviews in Bloom 1980, Ortaldo _et al_. 1981 and De Maeyer 1981) describe the paradoxical effects of interferon upon immune responses,

and by this is meant that an opposite effect can be seen if the dosage or timing of administration is altered. It is not clear why these effects occur but they may represent a combination of direct effects upon the cell and the induced release of other mediators of the immune response. The influence of interferon upon the immune response will first be described and then its mechanism of action considered.

Cell mediated immunity

These responses are carried out via T lymphocyts, and they include the lysis of virus infected cells, delayed hypersensitivity (DHS) and allograft rejection. Interferon has been shown to inhibit mitogen-stimulated T cell proliferation (Matheson *et al*. 1981), and to depress DHS as measured by its *in vitro* counterpart, leukocyte migration inhibition (Sziegeti *et al*. 1980). However it has also been shown that if the timing of interferon treatment is altered to after the administration of the antigen stimulus, then the DHS response can be enhanced *in vivo* (reviewed in Gresser 1981). A similar dual response is seen in allograft rejection, low concentrations of interferon accelerate allograft rejection (Lindahl 1973) and high concentrations prolong survival.

Humoral immunity

Production of antibody from antigen stimulated B lymphocytes is altered by treatment with interferon but the direction depends on the timing of treatment; cells treated at the same time as the antigen stimulus show decreased production (reviewed in Stewart 1979 p254-256). It has been thought that interferon depressed mitogen-induced proliferation in all lymphocytes but recently Matheson *et al*. (1981) have shown that human interferon produced an enhanced proliferation of

of mitogen-stimulated B cells. Whilst this is contrary to the known cell multiplication inhibition of interferon, it may represent elicitation of other factors which cause mitogenesis; Blomgren and Einhorn (1981) have shown that interferon enhances the release of lymphokines which contain mitogen factors.

Antibody-dependent cell-mediated cytotoxicity is enhanced by interferon. This response does not primarily involve B cells, but requires circulating antibody, complement and macrophages (Heberman et al. 1979).

Macrophage and monocyte response

Interferon can enhance or restore phagocytic ability to both monocytes and macrophages, and this effect can be seen in both in vivo and in vitro (Gresser 1981). However Degree et al. (1981) report that this enhancement is only seen if low levels of interferon are used; at high levels the response is depressed. Hovi et al. (1981) have also shown that interferon at low to moderate concentrations can inhibit the differentiation of monocytes to macrophages, whilst at the same time increasing the phagocytic ability of the monocytes.

Natural killer cells (NK cells)

These are non-T and non B-lymphocytes, which although cytotoxic for all cells show enhanced cytotoxicity for neoplastically transformed cells (Bloom 1980). There is a positive correlation between resistance to transplanted tumours and the level of NK activity (Gidlund et al. 1978). Many workers have shown that interferon increases the level of activity of NK cells in vivo and in vitro (Wigzell 1981, Gidlund et al. 1978, Zarling et al. 1979, Heberman et al. 1979 and Huddlestone

et al. 1979). The interferon-enhanced activity of NK cells has two causes; there is an increase in the number of functional NK cells by promotion of differentiation of precursor NK cells (Bloom 1980, Ortaldo *et al.* 1981), and an increase in the lytic activity of pre-existing NK cells (Ortaldo 1981).

From the involvement of interferon in the immune responses described above, it is evident that interferon can influence the overall immune response in a variety of ways. Friedman *et al.* (1981) has suggested that since the majority of the immunoregulatory effects of interferon only require brief treatment, and do not require DNA synthesis or differentiation (NK cells are an exception), then such effects could be mediated via changes in cell surface antigens. It is known that interferon enhances expression of the major histocompatibility antigens on all cells including lymphocytes (Gresser 1981). Fridman *et al.* (1981) have demonstrated that expression of Fc-gamma receptors, which mediate many immune functions, are enhanced by interferon.

It should be appreciated that although application of exogenous interferon can have profound effects upon the immune system, the production of interferon itself by mitogen or antigen stimulated lymphocytes is part of the immune response itself (Roitt 1977, Cohen and Bigazzi 1980). This close involvement of interferon in the immune response and the fact that not only can interferon promote differentiation of NK cells but may be essential to their normal differentiation *in vivo* (Heberman *et al.* 1979) does strongly suggest that interferon has a natural role in the regulatory machinery of the immune response of the host to virus infection and to tumourigenesis.

Inhibition of cell replication in normal and tumour cells

The anti-proliferative effects of interferon were initially demonstrated by Paucker et al. (1962) and these effects were later confirmed by several groups using pure interferon (Gresser 1979, De Maeyer-Guignard et al. 1978, Iwakura et al. 1978, Knight 1976, reviewed in Stewart 1979 and in Taylor-Papidomitrious 1980).

In multicellular organisms in the animal kingdom, cells grow at different rates to reach a finite cell number and therefore an inherent ability to control cell and tissue growth must be present in all cells. In addition it may be that organisms which contain cells growing at different rates and with different regenerative capacities employ various methods to regulate growth applicable to different cell types. Malignant transformed cells can be considered as cells which have escaped the influence of these normal growth control mechanisms; and it has been proposed (Gresser 1981, Hicks et al. 1981) that the mechanism underlying the antiproliferative effects of interferon may be one which increases the sensitivity of normal and tumour cells to these various normal growth control mechanisms. This increase in sensitivity would be analogous to that seen in interferon-treated cells exposed to dsRNA which results in increased sensitivity to the inhibition of protein synthesis.

Interferon can inhibit cell replication in both normal and tumour cells *in vivo* and *in vitro*, but it does not have a significantly greater activity or bind preferentially to tumour cells (Taylor-Papidomitriou 1980). The majority of normal and transformed cells are sensitive to the anti-proliferative effects of interferon to varying degrees, but some exceptions have been identified. Some of these cells

(Wood and Hovanessian 1979, and Epstein _et al_. 1981) have been shown to lack components of the interferon system; however there is no evidence to suggest that similar abnormalities of the interferon system are involved in _in vivo_ tumourigenesis.

The anti-proliferative action of interferon causes many changes in cell phenotype and behaviour which vary according to the cell system under study; these changes can be seen to have two main effects. These are inhibition of cell replication and in some tumour cells, a reversion towards a more normal phenotype.

Gresser _et al_ (1975) demonstrated that replication of transplanted normal bone marrow and spleen cells is inhibited by interferon in immune-suppressed hosts, and that interferon could also inhibit regeneration in partially hepatectomised mice. Pfeffer _et al_. (1979) has shown that cell replication _in vitro_ in human fibroblasts is inhibited by interferon but the rate of cell growth, as determined by increase in cell volume and area, was not altered. Similar inhibitory effects on tumour cell replication have also been observed (DeMeyer-Eichberger _et al_. 1981). The cell replication inhibitory effect of interferon acts to progressively lengthen the cell cycle at each mitotic division thus reducing the overall rate of replication; the period of G1 is seen to be extended by several hours (Taylor-Papidomitriou 1980). This effect is not often directly lethal but it can be to some cells that seem to require a minimum replication rate to be viable, e.g. human Burkitt Daudi cells (Gresser 1981).

It has been reported that interferon treatment of some transformed cell lines produces a reversion towards a more normal phenotype and this phenomenon may be the cause or effect of interferon on growth. De Maeyer-Guignard _et al_. (1978) and Hicks _et al_. (1981) both report changes in

the cytoskeleton of interferon treated transformed cells cultured *in vitro*. They observed a partial restoration of the microfilament bundles associated with increased cell spreading. In additon Hicks *et al*. have shown that the *in vitro* transformation-specific phenomena of focus formation, and colony formation in agar are similarly inhibited by interferon.

Other reports have noted that interferon-treated transformed cells show a reduction in cell saturation density (Taylor-Papidomitriou and Stoker 1971, Brouty-Boye *et al*. 1980).

Mechanism of action.

The mechanism of action of the growth inhibitory effects of interferon are not as fully understood as are the mechanisms controlling the anti-viral state. As mentioned above these anti-proliferative actions may be secondary to the establishment of a covert anti-viral state; this idea is supported by the experiments which demonstrate the anti-proliferative actions of the induced anti-viral enzymes, and also by the fact that a specific factor is not induced by interferon to mediate its effects on growth. Interferon may act to inhibit cell replication by general inhibiton of cell biosynthetic machinery, since there are many reports citing the anti-proliferative effects of interferon which have also reported a depression of DNA, RNA and protein synthesis (reviewed in Taylor-Papadomitriou 1980). However the reports on the inhibition of DNA synthesis do vary in the extent to which DNA synthesis is affected (Stewart 1979 p242). This variation may be related to the stage of the cell cycle when the cells were treated.

The role of interferon during *in vivo* tumourigenesis is not

limited to its direct effect on the tumour cell because it is also involved in mediating the immune response to the tumour (see above); and possibly also in increasing the antigenicity of tumour cells by alteration of cell surface antigens. There has been one report by Attollah *et al* (1979) which has shown that interferon increased the expression of a tumour specific antigen (Carcinoembryonic antigen) on the surface of human colon carcinoma cell lines.

Possible role in the normal control mechanisms of growth and differentiation

It has been proposed that since effects on cell replication and differentiation have been observed with exogenous applications of interferon that this might suggest that interferon has a natural function in regulating these phenomena (Taylor-Papidomitrious 1980, Stark *et al*. 1979). If interferon has such a function then it would be expected that either interferon or enzymes induced by interferon could be detected in cells growing and undergoing differentiation.

There have been many reports of cells not previously treated with interferon, but containing high levels of the anti-viral enzymes known to be induced by interferon. Rabbit reticulolysate (RRL) has been shown by Petryshyn *et al*. (1979), Ernst *et al*. (1979), Datta *et al*. (1977) to have a high degree of sensitivity to the protein synthetic inhibitory effects of dsRNA; similar to that found in extracts of interferon-treated cells. These RRL samples are found to contain a cAMP dependent protein kinase, activated in response to dsRNA which, like the interferon-induced cAMP dependent protein kinase, can phosphorylate the alpha sub-unit of eIF2. RRL samples contain an

additional cAMP independent protein kinase, antigenically distinct from the dsRNA activated cAMP dependent protein kinase, which is activated by the absence of haemin. A 2'5' oligo A synthetase has also been shown to be activated in RRL samples by dsRNA (Hovanessian and Kerr 1979). This has led to the suggestion by Hunt (1978) that either interferon or the anti-viral enzymes themselves have a natural role in the growth and differentiation of reticulocytes.

Stark et al. (1979) have identified inactive 2'5' oligo A synthetase in a wide range of mammalian and chick tissues. The levels were found to vary considerably and all could be elevated by treatment of the cell with interferon. This led to their intriguing suggestion that the interferon-induced elevation of 2'5' oligo A synthetase during the anti-viral response is only one function of this synthetase. These workers propose that 2'5' oligo A synthetase could function as a general agent to regulate cell translation in normal conditions in the cell.

Shimizu and Sokawa (1979) have shown that quiescent lymphocytes have high levels of 2'5' oligo A synthetase, similar to those seen in interferon-treated cells, and propose this may be to prevent replication until the cell is exposed to a specific mitogen stimulus. In addition Kimchi et al. (1979, 1981) found that the levels of 2'5' oligo A synthetase rise in cultures of Friend leukaemic cells during the transition from growth to quiescence. In support of this finding it is interesting to note that interferon can inhibit the growth of these cells (Rossi et al. 1977a,b).

Kimchi et al. (1981) have proposed a model whereby levels of 2'5' oligo A synthetase within the cell regulate the ability of that cell to replicate; mitogen stimulated lymphocytes have decreased levels

of 2'5' oligo A synthetase and high levels of phosphodiesterase (which degrades the trinucleotide), interferon increases the levels of 2'5' oligo A synthetase (and to a lesser extent the phosphodiesterase). Thus mitogens act to remove the 2'5' oligo A trinucleotide block on translation and interferon acts to reimpose it.

Control of differentiation

There has been some support for the suggestion that either interferon or 2'5' oligo A synthetase are involved in the regulation of some cell differentiation events. Some differentiation events in the immune system have been shown to be influenced by interferon. Natural killer cell differentiation is enhanced whilst the differentiation of monocytes into macrophages is inhibited (see above). Kimchi et al. (1981) have found that levels of 2'5' oligo A synthetase increase (fifty times beyond control cultures) in Friend cells induced to differentiate by addition of dimethylsulphoxide. Moreover it was shown that addition of antisera to mouse interferon could abolish this rise.

There is also some evidence that interferon or 2'5' oligo A synthetase are involved in differentiative events outside of the bone marrow stem cells. Chick oviducts if treated with oestrogen synthesise specific proteins, and withdrawal of the steroid results in inhibition of synthesis of these specific proteins. The kinetics of this inhibition are paralled by a rise in 2'5' oligo A synthetase. Stark et al. (1979) propose that the role of interferon is to cause the degradation of the mRNA and thus to rapidly terminate protein synthesis.

The report of Kimchi et al (1981) appeared initially to contradict an earlier finding by Rossi et al (1977) who demonstrated that the differentiation of Friend cells could be inhibited by interferon.However this laboratory

has since reported (Dolei et al. 1980) that although high doses of interferon do inhibit differentiation in DMSO induced Friend cells, low doses enhance differentiation and will even promote differentiation in Friend cells not exposed to DMSO. The inhibitory effect seen at high doses might reflect a need for cell division to occur during differentiation.

The work of Kimchi et al. (1981), Stark et al. (1979) and Dolei et al. (1980) might be seen to indicate that interferon acts to promote differentiation, but the work of Hovi et al. (1981) on monocyte differentiation, and a recent paper bu Grossberg et al. (1981) suggest that this might be a too simplistic interpretation. Grossberg et al. have observed that very low amounts of interferon (approximately one unit per ml) inhibit the differentiation of 3T3L1 mouse fibroblasts to adipocytes when the former are exposed to insulin. The transition to adipocytes involves loss of cytoskeleton, collagen synthesis and the accumulation of lipid, and all these are prevented by interferon. This report is interesting in that although it suggests that interferon is inhibiting differentiation it also indirectly suggests that interferon is promoting a phenotype more commonly associated with the normal cell, i.e. well defined cytoskeleton and synthesis of collagen.

These reports on the influence of interferon in growth and differentiative events are interesting, and if nothing else demonstrate that the area deserves further attention. These reports all involved cells which were partially differentiated; the reports that exist concerning undifferentiated cells show that these cells are not sensitive to the effects of interferon. Thus interferon cannot be proposed as a universal regulator of cell differentiation and growth per se. The

reports do however suggest that interferon can interfere with terminal cell differentiation in some cell systems; in accordance with the other known effects of interferon it may "interfere" by establishing an interferon-specific pattern of cell growth, morphology and replication; which acts as the trigger to promote synthesis of certain differentiative products.

MOUSE EMBRYOGENESIS

Mammalian development consists of many processes, which interact continuously, whereby a single fertilised egg gives rise to a complex differentiated organism. These processes include determination and differentiation; which leads to the acquisition of phenotypic variation amongst cells of an identical genotype, morphogenesis; which is the attainment of correct spatial and temporal relationships between phenotypically different cells, and growth; the increase in size of the organism.

These processes must also include the interaction between the embryo and its maternal host, defined by Johnson (1977) as "a parabiotic liaison with a genetically alien individual". It has been suggested (Billington and Wild 1979) that the maternal response to embryonic antigens may influence some differentiative events. However this has been shown not to be true for early developmental events, mainly because it can be seen that transplantation of early cleavage embryo (two to four cell stage) to ectopic sites does not impair development to egg cylinder or neural plate formation (Herbert and Graham 1974). The maternal environment can however negatively effect normal growth and development of the later foetus, and pathological conditions in the maternal host can induce stunted growth and foetal malformations (Cohen 1979, Morris 1979, reviewed by Newth and Balls 1979).

Observation of embryogenesis has shown that there exists a synchrony of development amidst animals of one species, such that the timing of crucial events can be expressed in hours and even minutes

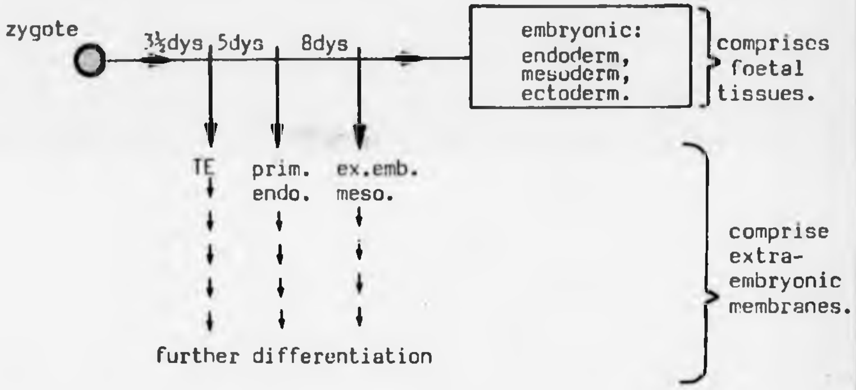
since the initiation of development. It is therefore conceivable that a timing mechanism may operate to control the emergence of developmental events; for example, Tarkowski and Wroblewska (1967) demonstrated that the accumulation of fluid in individual cells of the eight cell embryo was a process which occurred after a definite period of time, irrespective of the number of nuclear cycles or of cleavage inhibition. However, most agree that later embryological development is not timed by a biological clock set in motion at the time of fertilisation (reviewed in Snow and Tam 1980).

Observation has also shown that an obvious feature of normal embryological development is that cell types appear in a fixed sequence in which differentiated cells are successively thrown off from a multipotent stem cell population (Graham 1977). An example of what is meant by this is shown in figure 1, which illustrates early events in mouse embryogenesis . The initial differentiation event in mouse embryogenesis is the production of the trophectoderm from the outer cells of the morula ; the remaining multipotent stem cells even though they generate the embryonic ectoderm, endoderm and mesoderm, are not completely totipotent since they are unable to produce trophectoderm (Rossant 1977, Johnson et al. 1977).

It is not yet clear if subsequent foetal development depends upon an initial orderly sequence of developmental events. Little information is available from embryonic material itself but observation of teratocarcinoma cell differentiation has shown that differentiated cells representative of the three embryo germ layers can arise without an initial orderly developmental sequence. For example, testicular teratocarcinoma which are composed of stem and differentiated cells can be induced to form directly from primordial germ cells (see review in Rossant 1977).

FIGURE 1. The early events in mouse embryogenesis.

(taken from Hogan 1977, Rossant and Papiaoanou 1977).



The single fertilised egg, the zygote, undergoes cleavage to become an aggregate of pluripotent stem cells; the morula . This produces in turn, the trophectoderm (TE), the primitive endoderm (prim.endo.), and the extra-embryonic mesoderm (ex. emb.meso.); the remaining cells contributing to the foetus itself.

Differentiation of phenotypically altered cell types is marked by changes in cell morphology, synthesis of specific proteins and changes in cell behaviour; all of which can be used as markers of cell differentiation. However before these changes are manifest the cell is thought to be covertly committed to its subsequent development fate cells in this condition are said to be determined (Rossant and Papiaoannou 1977). Determination can be seen as a stage during which the developmental potential of a multipotent cell becomes restricted with no overt change in phenotype. It can be envisaged as a single cellular event (Levenson and Houseman 1981), or as a labile determinative period in which cells are gradually biased towards a particular fate (Johnson et al. 1977). Determination may or may not be detectable in all differentiative processes, and it may well be that with increasing sensitivity in assay techniques, that determination and differentiation may be seen to arise together. However within the confines of the present state of knowledge, a state of determination defined as above can be detected during some stages of embryogenesis.

The study of differentiation has been advanced with the discovery of cell specific biochemical markers and of sensitive assay techniques for them (Bode and Dziadek 1979, Dziadek and Adamson 1978, Adamson 1976, Strickland and Sherman 1976 and Burke et al. 1978); and also by the development of antibodies, including monoclonal antibodies, to specific stages of embryogenesis (Gooi et al 1981, Kemler et al. 1979, Solter and Knowles, 1978, Brulet et al (1980).

Activation of the interferon system appears to be associated with the establishment of the differentiated cell type in teratocarcinoma cells (Burke et al. 1978); and it is one of the aims of this study to see if this observation can be confirmed in the mouse embryo.

The gestation period in mice is complete in approximately 19 - 21 days, copulation results in a visible plug which marks day one of embryogenesis. The developmental age of the embryo is taken to begin on midnight, of the night before the day, on which the plug was found. The main stages of mouse embryogenesis are complete by the first half of gestation; these stages involve the establishment of the extra-embryonic membranes, the embryonic germ layers and the organ rudiments. Embryogenesis in the mouse (and in other related animals, the rat, guinea pig and rabbit) differs from that of other mammals, in that the initial arrangement of the three embryonic germ layers is reversed. The embryonic endoderm forms outside the embryonic ectoderm initially, but this inversion of germ layers is corrected when the embryo undergoes a complex rotation at approximately the tenth day of development (Snell 1941, Balinski 1975, Hogan 1977 and Green 1960).

Cleavage stage embryos: determination and differentiation

For the first four days of development the embryo is free in the reproductive tract and isolated by a jelly coat, called the Zona Pellucida. The single fertilised cell cleaves to two four and eight cells which initially aggregate loosely; however at the eight to sixteen cell stage, compaction occurs and cell boundaries become less obvious. The embryonic genome is active early (Johnson *et al* 1977), the four cell embryo shows rRNA, tRNA and mRNA synthesis as well as qualitative changes in protein synthesis (Van-Blerkom and Brockway 1975, Izquierdo 1977 and Barlow *et al* 1972).

It has recently been shown by Johnson and Ziomek (1981) that the sixteen cell embryo contains two morphologically distinct cell populations, which differ with respect to the distribution of surface microvilli. At the eight cell stage all cells show a polar distribution of these microvilli, but by the sixteen cell stage the cells have either a polar or even distribution of microvilli. This latter stage coincides with the enclosure of some cells within the aggregate which are thus no longer exposed to the external milieu. Johnson and Ziomek (1981), propose that the 'decision' of cells to be enclosed may depend on the absence of a polar distribution of surface microvilli. Thus it is suggested that the cells of the eight cell cleavage embryo may possibly be committed to produce either enclosed or exterior cells, whose subsequent fate is restricted by assuming these positions.

However experiments involving the reaggregation of cells from four or eight cell cleavage embryos have shown that the developmental fate of these cells is not restricted at this stage. Each of the cells from these early embryos is able to colonise either the trophectoderm or the inner cell mass of the later blastocyst. (Wilson and Stern 1975, Rossant 1977, reviewed in Graham 1977 and Rossant and Papiaoannou 1977); and therefore the cells from the four or eight cell embryo cannot be regarded as determined as defined above.

Blastocyst: determination and differentiation

Cleavage occurs until the cell aggregate, now called the morula,

reaches approximately 32 - 64 cells, and by this stage the external cells of the morula have become morphologically differentiated. These altered external cells begin to pump fluid from the external environment into the centre of the morula. This action mechanically displaces the central cells to one pole of the embryo, producing a cavity called the blastocoele. This stage is now called the blastocyst, the outer cells are termed the trophectoderm and the enclosed cells are termed the inner cell mass (ICM)(see figure 2a).

The behaviour of these two cell populations are different, the cells of the ICM synthesise DNA more rapidly and have a faster doubling time than those of the trophectoderm (Barlow *et al* 1972). Trophectoderm vesicles can invoke a decidual response in the uterus and, in contrast to the cells in the ICM, cannot replicate *in vitro* (Gardner 1972).

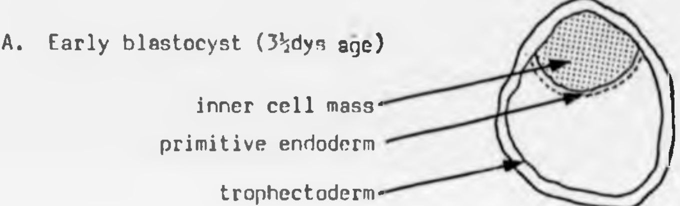
The ability of the cells of the early morula to form either trophectoderm or ICM cells has been shown to be dependent on the position of these cells in the morula. The positional hypothesis considers that the blastomeres enclosed in the morula become ICM, whilst those on the outside, in contact with the uterine secretions become trophectoderm. This was originally proposed by Tarkowski and Wroblewska (1967), and has been confirmed through reaggregation experiments using four or eight cell embryos by the work of Hillman *et al* (1972), and is also supported by the work of Johnson *et al* (1977), Herbert and Graham (1974), Wilson and Stern (1975).

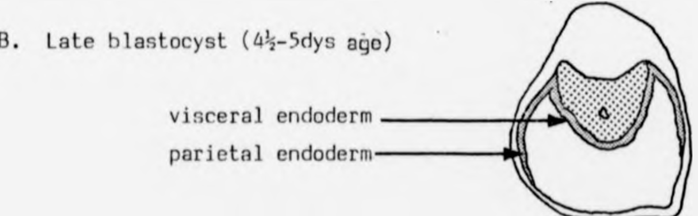
Development of the extra-embryonic membranes and the placenta

At the late blastocyst stage, approximately 4 - 4½ days development, a third morphologically different tissue forms

FIGURE 2. STAGES OF EARLY MOUSE EMBRYOGENESIS.

(Taken from Bode and Dziadek 1979, Snell 1941, Herbert and Graham 1974 and Martin 1978.)





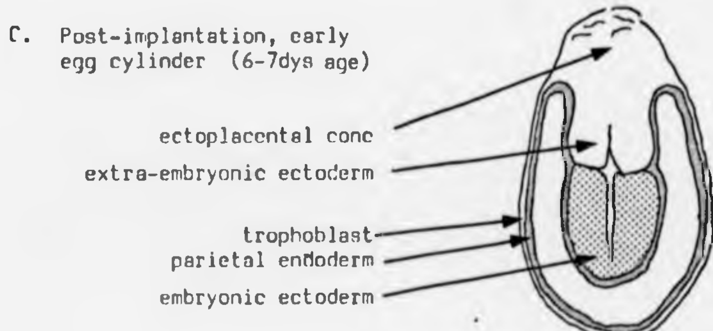
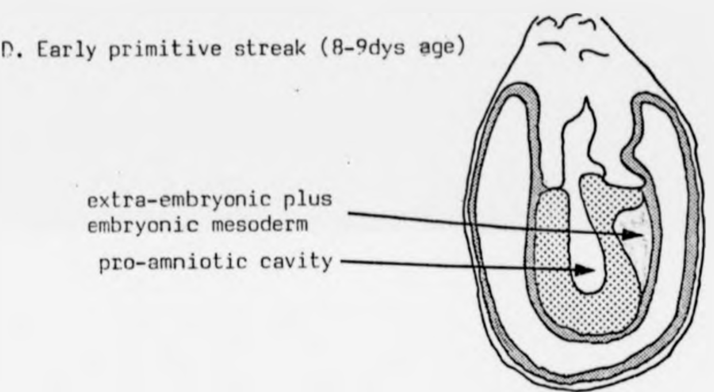


FIGURE 2. (continued) STAGES OF EARLY MOUSE EMBRYOGENESIS.



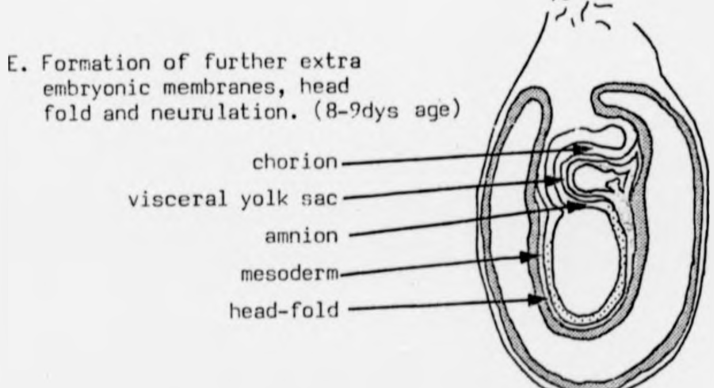
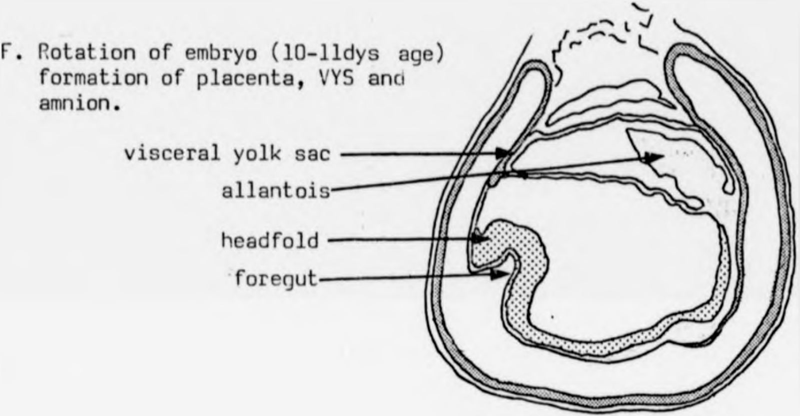
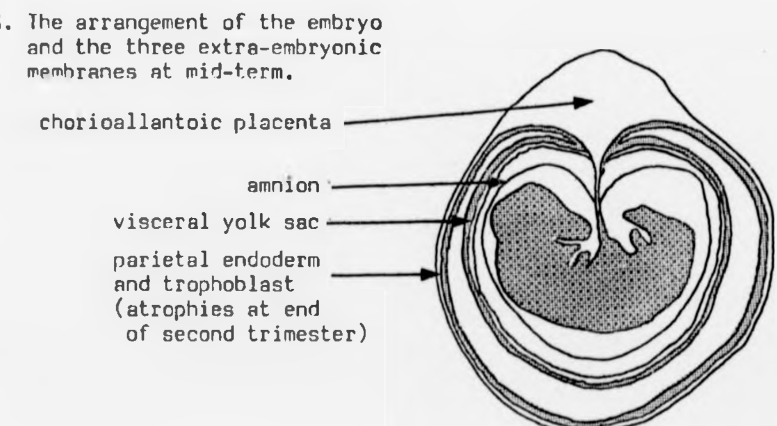


FIGURE 2. (continued) STAGES OF EARLY MOUSE EMBRYOGENESIS.



F. Rotation of embryo (10-11dys age) formation of placenta, VYS and amnion.



G. The arrangement of the embryo and the three extra-embryonic membranes at mid-term.

from the cells of the ICM exposed to the blastocoele fluid. This single layer of cells is called the primitive endoderm and the ultrastructure of these cells differs from that of the remaining ICM cells (which are now called primitive embryonic ectoderm). The cells of the primitive endoderm are marked by prominent rough endoplasmic reticulum, intracellular vesicles and surface microvilli (Hogan 1977). Formation of primitive endoderm is not thought to arise from predetermined cells in the ICM because isolated ICMs have the ability to form a single layer of endoderm over the entire surface (Rossant and Papiaoannou 1977).

The primitive endoderm is not thought to contribute substantially to the embryo itself, instead it produces two types of extra-embryonic endoderm during the next few days of development. These are the parietal endoderm which grows adjacent to the trophectoderm and the visceral endoderm which grows adjacent to the primitive ectoderm (Hogan 1977), this stage is illustrated in figure 2b.

At approximately 4½-5½ days the zona pellucida is lost and the embryo implants into the wall of the uterus, the presence of the embryo inducing a decidual swelling in the uterine stroma.

After the development of the primitive endoderm several changes occur both in this tissue and in the trophectoderm. The trophectoderm can be divided into two populations of cells which are morphologically distinct, these are termed because of their positions, mural and polar trophectoderm (see figure 2b). The inner surface of the mural trophectoderm is initially in contact with the blastocoele fluid, these cells soon cease cell division but continue DNA replication eventually producing primary giant trophoblast cells. The polar trophectoderm overlies the primitive ectoderm, these cells replicate normally and are the source of the remaining trophoblast tissue in the embryo (Hogan 1977).

The further differentiation of the polar trophectoderm is regulated by contact with the primitive ectoderm (Gardner and Papiaoannou 1975, Johnson 1972 appendix, Gardner 1972).

The polar trophectoderm at the exterior surface differentiates into cells of the ectoplacental cone (EPC), which are capable of producing secondary giant trophoblast cells. The interior surface differentiates into the extra-embryonic ectoderm (ex.emb.ect.). The formation of these new tissues displaces the primitive embryonic ectoderm producing an elongation of the embryo, the whole embryo because of this elongation is now called the embryonic egg cylinder (see figure 2c).

Confirmation that the EPC, the secondary giant trophoblast cells and the ex.emb.ect. do arise from the polar trophectoderm has been obtained from the work of Gardner and Papiaoannou (1975). These workers produced chimaeric embryos via injections of the ICMs' of one mouse strain into the blastocyst of another strain or species. Chimaerism in the tissues was examined by karyotype analysis or isozymal variants, and showed that the EPC, secondary giant trophoblast cells and the ex.emb.ect. was never of ICM origin. In addition Johnson and Rossant (1981) demonstrated that these three tissues could produce similar two-dimensional gel profiles.

The embryo at this stage (see figure 2c) is thus enclosed by one extra embryonic membrane, which consists of an outer layer of trophoblast cells and an inner layer of parietal endoderm cells, separated from each other by a layer of inert basement membrane material termed Reicherts' membrane (the latter is specific to rodents). This outer membrane is only a temporary structure and begins to atrophy at approximately days 14 - 15 of development.

Formation of the three embryonic germ layers

The next stage of development involves the production of the embryonic mesoderm and endoderm from the primitive embryonic ectoderm. The ability of the primitive embryonic ectoderm to produce all three embryonic germ layers has been demonstrated by Diwan and Stevens (1976). These workers transplanted primitive embryonic ectoderm from early egg cylinder stages, to ectopic sites (e.g. under the kidney capsule) and showed that these cells produced derivatives of all three germ layers.

Primitive streak formation occurs at approximately 8½-9 days. and results initially in the formation of the extra-embryonic mesoderm (ex.emb.meso) and the embryonic mesoderm (emb.meso), (see figure 2d). The primitive streak defines the posterior axis of the embryo, since the head fold develops opposite to this area, while the embryonic endoderm (emb.end) is thought to originate from the head fold region (Hogan 1977, Gardner and Papiaoannou 1975, Rossant 1977).

The body cavity and the organ rudiments are then established; neurulation and rotation of the embryo occurring at approximately day 10-11 . This rotation of the embryo, and other differentiative events occurring at this time leads to the establishment of two further extra-embryonic membranes, this process is illustrated in figures 2e to 2g. The innermost membrane adjacent to the embryo is of ex.emb.mesoderm plus emb.ect. origin and is termed the amnion; the layer in between the amnion and the outer trophoblast and parietal endoderm is called the visceral yolk sac. This latter membrane is composed of visceral extra-embryonic endoderm and ex.emb.meso and has many functions; it forms the outermost embryonic membrane during late development and

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is then able to absorb material from the maternal circulation for foetal use, in addition the mesodermal layer generates foetal haemopoetic stem cells in areas called blood islands, and at least some of these cells later go on to populate the foetal liver and bone marrow.

Thus the embryo at mid-gestation is surrounded by three membranes. These membranes function as mechanical barriers and isolate the embryo from potential pathogens. A further aim of this study was to determine whether or not these membranes could function as a means to protect the embryo from virus attack, this protection would occur if the membranes were able to produce interferon and be sensitive to its effects before the interferon system was operative in the embryo itself.

Ontogeny of the immune response

Since it is proposed to assess the relative role of the interferon system in conferring protection to the foetus from viral pathogens, it is necessary to briefly consider the ontogeny of the immune system. (A review of the immune system can be found in Roitt 1977 and Wood, Weissman and Hood 1978.)

There is a known association between some virus infections which occur during the early gestation period and foetal deaths and malformations; a classical example of this is Rubella virus, infection with which can cause malformations and even abortions and stillbirths (Mims 1977, 1968). It therefore becomes important to discover whether the susceptibility of the foetus to some virus infections is due to an immaturity of the interferon system, similar to that seen in undifferentiated teratocarcinoma cells.

The development of the immune response (i.e. cell mediated and humoral actions of lymphocytes) parallels the development of the lymphoreticular system in the foetus (Ritter 1975). The elements of this system comprise the haemopoietic stem cells, liver, bone marrow and lymphoid tissue. The immune response is mediated via lymphocytes; either T lymphocytes (T represents thymus derived lymphocytes which are involved in cell mediated immunity (CMI)),or B lymphocytes (Bursa equivalent derived lymphocytes involved in humoral mediated immunity).

The foetus initially produces haemopoietic stem cells in the blood islands of the viseral yolk sac, (at approximately 10-11 days). These stem cells migrate primarily to the foetal liver which takes over the role of haemopoiesis, and then secondarily during later foetal development to the bone marrow cells. Only the cells of the bone marrow retain this function during the final stages of foetal development (Ritter 1975, Tyler 1971). The foetal lymphocytes do not become immunocompetent until they have undergone a period of differentiation in either the liver or the thymus; lymphocytes can be detected in the thymus from approximately day 15 . (Roitt 1977, Ritter 1975) and the presence of IgG and IgM bearing lymphocytes can be detected in *in vitro* cultures of liver from approximately day 14. (Owen *et al*. 1974). Wolfe *et al* (1957) and Silverstein (1963) similarly failed to elicit significant antibody titres in early chick embryos and foetal lambs.

The lack of immunocompetant cells, and the lack of demonstrable immune function both suggest that the foetus is unable to mount an immune response for the first half of the gestation period. In addition the immune response is thought to be depressed in most species in the neonate, in humans for example, the production of antibody titres does not reach adult levels until the child is approximately five years of age (Mims 1977).

Status of the foetal interferon system

Early experiments by Isaacs and Baron (1960) demonstrated that interferon production and anti-viral sensitivity was reduced in early mouse and chick embryos. These workers were the first to propose that this depression of the interferon system in early embryogenesis might be responsible for the susceptibility of the foetus to agents such as Rubella virus. These results have been supported by Mendelson et al (1970) who demonstrated that interferon production was absent in 12 day rat embryos in utero but was present in 19 day embryos. Drasner et al (1979) in addition, demonstrated that the growth inhibitory effect of interferon could only be seen in in vitro cultures taken from post-implantation mouse embryos, and not in in vitro cultures of pre-implantation stages.

Some interesting work has been done on the role of interferon during Rubella virus infections in utero, which suggest that the early embryo does not produce interferon during infection, despite the fact that Rubella virus is capable of inducing interferon production in in vitro cultures of amnion cells (Neva and Weller 1964). Desmyter et al (1967) and Rawls and Melnick (1966) both failed to detect interferon production either in the serum and urine from an infected foetus or from cultured explants taken from an infected foetus.

The literature concerning the ontogeny of both the immune response and the interferon system in mammals suggest that these phenomena are absent or severely depressed in early embryogenesis; thus the embryo appears to be unprotected from pathogens during a crucial period of development. However, in reality this is not so, not only does the foetus passively receive maternal immunoglobulins via the placenta and the visceral yolk sac, but the maternal immune response would be activated in the advent of a foetal infection. In addition the anatomy

of the sites of foetal-maternal interaction (at which foetal and maternal circulations are not in direct contact) make it unlikely that the majority of maternal born pathogens will reach the foetus.

There have been some reports that the maternal immune response is slightly depressed during gestation (Billington and Wild 1979, Mims 1977), and this may increase the potential hazard to the foetus. The maternal interferon system has not yet been investigated and reports vary as to whether or not maternal interferon can cross the placenta. Overall and Glasgow (1970) and Fowler _et al_. (1980) have presented evidence that interferon cannot cross the placental barrier, but Williams and Reed (1981) found levels of interferon induced 2'5' oligo A synthetase in mononuclear cells from cord blood samples in proportion to circulating maternal levels (the former experiments were performed on sheep and mice, and the latter on human term placentas, and may reflect a species variation).

Because of the known lack and inhibition of the classical immune response during early embryogenesis, it becomes interesting and relevant to define the role and relative importance of the foetal and maternal interferon systems during viral infection of the foetus, this will be discussed in section three.

TERATOCARCINOMA, AND THEIR USE AS *IN VITRO* ANALOGUES OF DEVLOPEMENT

The term teratocarcinoma describes a tumour containing undifferentiated multipotent stem cells and a variety of differentiated cell types.

The undifferentiated multipotent stem cells are called embryonal carcinoma or ec cells and are malignant *in vivo* and can be established as cell lines *in vitro*; the differentiated cell types are generally benign *in vivo* and since they divide relatively little *in vitro*, cannot be established as cell lines (Adamson and Graham 1979). The ec cell gives rise to the variety of differentiated cell types seen in a teratocarcinoma This was definitively shown by Kleinsmith and Pierce (1964), who transplanted a single ec cell under the skin of histocompatible mice and obtained tumours containing ec cells plus a variety of differentiated cell types; these latter were representative of tissues derived from the three embryonic germ layers, ectoderm, endoderm and mesoderm.

The ability of the ec cell to differentiate into the full range of differentiated cell types does vary between different teratocarcinoma cell lines. The ec cell component of a teratocarcinoma can be pluripotent; that is, capable of giving rise to a variety of cell types, or they can show a more restricted differentiation or even show no spontaneous differentiation at all, the latter are termed "nullipotent".

Teratocarcinoma were originally observed and studied as solid tumour, a typical teratocarcinoma is illustrated in the review by Illmensee and Stevens (1979), and can consist of ec cells plus ectodermal tissue (e.g. neural cell), endodermal tissue (e.g. glandular epithelium) and mesodermal tissue (e.g. bone and cartilage). It is

a characteristic of such teratocarcinoma that these differentiated cell types are found in small groups distributed throughout the tumour in a manner which might at first seem chaotic. However, particular cell types can be seen to grow in locally organised arrangements such that nerves can be attached to muscle, cartilage is characteristically next to bone and bone tissue may contain nucleated erythrocytes within it (Graham 1977).

The name teratoma can also be applied to these tumours which has led to a slight confusion over terminology ; a teratoma is defined (Sherman and Solter 1975) as a benign tumour containing one or more differentiated cell types but not containing ec cells, it is not usually. transplantable nor can it be passaged in vitro.

Reviews concerning the behaviour, and the use of teratocarcinoma as experimental models can be found in, Illmensee and Stevens (1979), Martin (1975, 1978), Graham (1977), Hogan (1977) and in Sherman and Solter (1975).

Teratocarcinoma are used as experimental models in both tumourigenesis and developmental studies. The ec cells of a teratocarcinoma are malignant and can be thought of as normal embryonic cells which behave abnormally because they have escaped the usual maternal, or other influences, which induce appropriate differentiation. The use of teratocarcinoma as a model system for tumourigenesis is reviewed in Martin (1975), Strickland (1981), Manes (and others 1976) and Pierce (1967). The ec cell, although malignant, resembles certain multipotent stem cells of the early embryo, and their behaviour _in vitro_ can provide an analogue for some developmental events which occur in embryogenesis. The use of teratocarcinoma have an advantage over the use of the mouse (or other mammalian embryo) in the provision of larger amounts of

material and the ease with which certain differentiation events can be both controlled and observed *in vitro*.

Factors influencing the formation of spontaneous or experimentally induced teratocarcinoma and teratomas

Spontaneous teratocarcinoma are very rare in most strains of mouse and appear to be a species limited phenomenon unique to inbred strains. This suggests that the development of these tumours is under genetic control. However the incidence of spontaneous teratocarcinoma does not appear to be correlated with the tendency to develop other spontaneous tumours, e.g. hepatomas, mammary gland tumours or even testicular (non-teratocarcinoma) tumours; in addition although this appears to be influenced by the genetic constitution of the animal, there is no correlation with particular major histocompatibility antigens (Solter *et al*. 1979).

Some strains of mouse have been observed which have an abnormally high incidence of spontaneous teratocarcinoma; a sub-line of strain 129 has been bred which have an incidence of up to thirty percent of testicular teratocarcinoma, and strain LT has a reduced but still high incidence of ovarian teratocarcinoma (reviewed by Stevens 1979).

The susceptibility of mice to experimentally induced teratocarcinoma is dependent on strain and in a few cases on the sex of the animal host. Solter *et al*. (1979) observed the ratio of malignant teratocarcinoma to that of benign teratoma formation after transplantation of early embryos to ectopic sites, using a wide range of host species. These workers found that some mouse strains, e.g. A/J and BalbC/J had a high incidence of teratocarcinoma, whilst others, e.g. C57Bl/CJ had a low incidence of the malignant form. In addition in some strains the

incidence of teratocarcinoma was sex controlled, indicating some hormonal influence on the development of these tumours, e.g. 129J males had a high incidence compared to 129J females. In addition to all this, it should be noted that mice in general are more susceptible to experimentally induced teratocarcinoma than are other species, e.g. only benign teratoma can be experimentally induced in rats and hamsters.

Cellular origins of teratocarcinoma and teratoma

Teratocarcinomas and teratomas can either arise spontaneously in the male or female gonads; or be experimentally induced in mice following transplantation to an ectopic site; of early embryos (3-9 days old) or of genital ridges taken from later embryos (12-16 days old). Analysis of the origin of these various tumours has suggested that *in vivo* teratocarcinogenesis has more than one origin. In addition to the above, Evans and Kaufman (1981) have demonstrated that undifferentiated, but pluripotent cells can be established *in vitro* directly from the embryo.

Spontaneous testicular teratocarcinoma are thought to arise from the primordial germ cells. Stevens (1962) observed the development of this form of the tumour in embryos of strain 129J, and found that they were clearly detectable within the seminiferous tubules of the 15 day embryo; these tubules contain only Sertoli cells and the primordial germ cells, and the ec cells of the tumour were thought to more closely resemble the primordial germ cells. Stevens has provided further evidence for the direct involvement of primordial germ cells (Stevens 1967), by demonstrating that genital ridges taken from mice homozygous for the mutation "steel" (∴ lack most of their primordial germ cells), could not induce teratocarcinoma formation upon transplanation to an

ectopic site. This led to the suggestion that the primordial germ cells were the precursor of teratocarcinoma, and it was proposed that teratocarcinoma derived from transplantations of the early embryo generated primordial germ cells before producing the tumour.

Female primordial germ cells from genital ridges of similar aged embryos (12-16 days old) do not form teratocarcinoma or teratoma after similar procedures. One reason for this difference may be that female primordial germ cells begin meiosis at approximately day 12-13, whilst the male cells do not begin meiosis until after birth of the foetus.

Spontaneous teratocarcinoma of ovarian origin does not arise from the primoridal germ cells; a histological examination of these tumours shows numerous embryos at various stages of development (e.g. morulae, blastocysts and egg cylinders can be seen) and it is proposed that ovarian tumours arise from parthenogenetic activation of oocytes (Stevens 1979).

Teratocarcinoma and teratoma can be induced to form by transplantation of early embryos to extra-uterine sites. The incidence of tumour formation by this method reaches a maximum at eight days post-gestation (reviewed in Graham 1977 and in Illmensee and Stevens 1979). It is not yet clear why the incidence of tumour formation from transplantation follows this pattern although this may be related to the ability of the multipotent cells of the embryo to survive the necessary manipulation procedures (Graham 1977). The formation of teratocarcinoma have been correlated with the presence of the embryonic ectoderm in the egg cylinder stages (Diwan and Stevens 1976), and thus may be related to the presence of multipotent stem cells. The suggestion that embryo derived tumours initially generate primordial germ cells is not

generally supported mainly because of the differences which exist between teratocarcinoma of primordial germ cell and embryo origin. The former only have a male karyotype whilst the latter can have either male or female karyotype, thus the former differ in that they arise from cells which have not undergone meiosis. In addition many mouse strains do not produce tumours of primordial germ cell origin, but will form tumours from transplantation of early mouse embryo, thus the tumours have different survival ability in the same host (Graham 1977).

Further support for a non primordial germ cell origin of teratocarcinoma derived from early embryo comes from the work of Mintz *et al*. (1978). These workers showed embryo derived teratocarcinoma could be obtained from transplantation of early embryos which were homozygous for "steel" (see above),and also from embryos of the W/W sterile genotype, neither of which can produce primordiol germ cells.

There are thus considered to be four sources of teratocarcinoma.

(1) Primordial germ cell origin, spontaneously arising in the testis or via transplantation of the male genital ridge of 12-16 day post-gestation embryos, to extra-uterine sites.

(2) Parthenogenetic activation and development of oocytes,which occurs spontaneously in the ovary.

(3) Embryonic somatic cell origin, via transplants to extra-uterine sites, of embryos of 2-9 days development.

(4) *In vitro* cultivation of early embryos.

Maintenance of teratocarcinoma

Both spontaneous and experimentally induced tumours can be maintained by transplantation into an histocompitable host, providing

that embryonal carcinoma cells are present. Teratocarcinoma are passaged *in vivo* in two ways:

1. By subdermal injection of ec cells, this develops into a solid tumour of the type described above.
2. By injection of a suspension of ec cells into the peritoneal cavity of mice. These cells stimulate the secretion of ascites fluid from the peritoneal cells and grow in suspension in this fluid forming aggregates of cells which are called embryoid bodies.

Two types of embryoid body can form, the type and size being influenced by both the strain of the tumour and of the host (Stevens 1979). Simple embryoid bodies consist of an inner core of ec cells surrounded by a layer of endoderm like cells (Pierce and Dixon 1959); the teratocarcinoma can be passaged indefinitely as simple embryoid bodies, and yet the ec cells will still retain their full pluripotentcy when placed in a suitable environment. Illmensee and Mintz (1976) used ec cells from these forms, which had been in passage for eight years, to produce chimaeric mice. A single ec cell was injected amongst the inner cell mass cells of a preimplantation mouse blastocyst which was then grown to term in a pseudopregnant host mouse; examination of the chimaeric offspring showed that the ec cell had contributed to a wide range of tissues, even to the germ line. The second type of embryoid body which can form is termed a cystic embryoid body, this comprises ec cells, a fluid filled cyst and a variety of differentiated cell types, and more closely resembles the solid tumour form.

Teratocarcinoma can also be maintained in tissue culture, but generally only after several passages have been undertaken *in vivo*. This adaption to tissue culture can be done by *in vitro*

cultivation of embryoid bodies using adherent dishes; or via "nursing" ec cells from solid tumours on a layer of non-dividing fibroblast cells. The fact that *in vitro* cultures cannot usually be established directly from *in vivo* tumours and also the fact that cell lines can only be established from ec cells and not from differentiated cells; suggests that the process of establishing a teratocarcinoma cell line is selective; first for cells which can withstand *in vivo* passage and second for cells which do not differentiate readily in culture (Graham 1977).

Teratocarcinoma cells can be maintained *in vitro* as simple embryoid bodies, by culturing a suspension of ec cells on non adherent dishes, or maintained as monolayer culture on normal tissue culture plates. Pluripotent teratocarcinoma cell lines which differentiate spontaneously, can be maintained as relatively homogeneous cultures of ec cells by appropriate culture techniques, usually involving frequent subculture (Burke *et al*. 1978). Teratocarcinoma cell lines which undergo little or no spontaneous differentiation (called nullipotent cells) are thus more easily maintained, and have the advantage in that they can be cloned to genetic homogeneity (Berstine *et al*.1973, Evans and Martin 1975, Adamson *et al*. 1977, Lehman *et al*. 1975 and Nicolas *et al*. 1976).

Ec cells from cloned tissue culture cell lines which have been maintained as a homogeneous ec culture, have also been shown to retain full pluripotentcy. McBurney (1976) and Martin and Evans (1975) have both demonstrated that single ec cells from such cultures, can produce a well differentiated tumour upon sub-dermal injection into a histo-compatible host. In addition Papiaoannou *et al*. (1975) have shown that ec cells from such lines retain full genetic totipotency , by being able to contribute to a wide variety of tissues in a chimaeric mouse, created by the technique described above (Illmensee and Mintz 1976).

Comparison of the properties of embryonic stem cells and of ec cells

The process of differentiation occurring in teratocarcinoma, in which varied differentiated cell types are generated from a single stem cell, has been proposed as a model system to study embryogenic differentiation. Ec cells do have many features in common with embryogenic multipotent cells (i.e. primitive embryonic ectoderm of pre, and post-implantation embryo). These include:

(1) Ultrastructure: in common with primordial germ cells, ec and embryonic stem cells, have little condensed chromatin apart from nucleoli, the cytoplasm is rich in ribosomes, but lacks prominent golgi or rough endoplasmic reticulum (Pierce and Beale 1964, Damjanov and Solter 1975).

(2) X chromosome inactivation: female mouse embryos show condensation of sex chromatin beginning at approximately six days development, similar X chromatin is seen in some but not all ec cell lines which are XX (McBurney and Adamson 1976). It has been proposed that this suggests that different ec cell lines represent different embryological development stages (McBurney and Strutt 1980).

(3) Cell surface antigens: many workers have raised antibodies to the cell surface of ec cells and demonstrated that these are present at specific stages of mouse embryogenesis (reviewed by Kemler *et al.* 1979, and see later). Antibodies have been produced from different ec cell lines which react to different stages of embryogenesis, which supports the view that different ec cell lines represent different embryological development stages.

(4) Biochemical markers: ec and embryonic stem cells can be identified by the presence and behaviour upon differentiation, of many biochemical

markers; such as the production of enzymes, and secretory and matrix proteins; all of which can be used to qualitate and quantify the process of differentiation (see later).

The homology in structure and behaviour between the stem cell population of the mouse embryo and of teratocarcinoma, should not mask their obvious differences. Ec and embryonic stem cells are not the same, ec cells are malignant and kill their host, embryo cells do not (Graham 1977); the two stem cell populations are also temporally disparate, in that ec cells are obtained from tumours which can have been in passage for up to ten years whilst embryonic stem cells result from events five to seven days old, and occurring in very different culture conditions. Thus although homologies exist between the two cell populations sufficient for ec cells to provide an in vitro model for some stages of development, differences between these cell types must also be expected.

Differentiation of teratocarcinoma cells

Cultured ec cells, which are induced to differentiate, can provide a system to study gene expression under defined conditions, away from maternal and other influences. All teratocarcinoma cell lines so far studied have been shown to differentiate in culture (this includes those teratocarcinoma lines previously classed as nullipotent), the range and extent of differentiation may however vary with the strain of the tumour and with culture conditons. The full extent of teratocarcinoma differentiation is at present only seen when ec cells interact with normal embryo cells. Such experiments have shown that ec cells can become the stem cells of the embryo. The ec cells and their

differentiated progeny participate fully in developmental interactions with normal embryo cells demonstrating that ec cells possess full genetic totipotency (Illmensee and Mintz 1976, Papisoannou _et al_. 1975, 1978).

Methods have been developed to synchronise differentiation of teratocarcinoma lines in culture. These methods vary with different cell lines. Pluripotent cell lines which will undergo spontaneous differentiation can be synchronised by being plated as a monolayer culture at low density (Burke _et al_. 1978, Adamson _et al_. 1979), other lines can be caused to differentiate via aggregation of the cells either by culture at high denisty or by allowing the formation of embryoid bodies by culture in non adherent dishes (Martin 1975, Hyafil _et al_. 1980). Cells previously thought to be nullipotent _in vivo_ and _in vitro_ have now been shown to undergo limited differentiation by culture in physiological amounts of retinoic acid (Strickland and Mahdavi 1978).

"nullipotent" teratocarcinoma cell lines: differentiation in retinoic acid

The cell lines used in this study are those which were previously thought to be nullipotent, i.e. to undergo no differentiation. These have now been shown to undergo limited differentiation when treated with retinoic acid, to an endoderm like cell.

Retinoic acid is the acid of Retinol, known as vitamin A, the structures of these two molecules is shown below (Pawson 1981):

Retinoic acid

COOH (replaced by CH_2OH in vitamin A)

Most, but not all of the acid analogues of vitamin A have biological activity (Jetten and Jetten 1979), and the biological activity of the molecule appears to reside within the carboxyl group, since modification of the cyclohexenyl ring does not affect activity (Strickland and Sawey 1980).

The mechanism of action of retinoic acid in inducing differentiatin in ec cells is not yet clear. It has long been known, from observation of vitamin A deficiency diseases, that this vitamin has many important biological roles. These affect visual processes and the normal growth and differentiation of many, but particularly epithelial tissues (reviewed in Pawson 1981). In addition to its effect on nullipotent teratocarcinoma cell lines, retinoic acid will accelerate the differentiation of pluripotent lines (Jetten and Jetten 1979), and has also been shown to alter the growth and morphology of "normal" mouse fibroblast cell lines (Jetten et al. 1979).

It has also been suggested that retinoic acid exerts its effects in a manner analogous to that of the steroid hormones. Jetten and Jetten (1979) and Schindler et al. (1981) have both identified a retinoic acid binding protein (RBP) in the cytosol of cells which are sensitive to the effects of retinoic acid. This RBP interacts with retinoic acid in the cytosol, and after this retinoic acid can be found in the nucleus of the cell; it is therefore suggested by the above authors that retinoic acid may act to directly modify gene transcription.

There have been some reports of cells which are insensitive to the effects of retinoic acid, and which have also been shown to lack RBP activity. Schindler et al. (1981) have isolated ec cells from mutagenised stocks which are resistant to the differentiative effects of retinoic acid and which lack RBP. Jetten et al. (1979) have shown that SV40 virus transformed fibroblasts are insensitive to retinoic acid and

possess the RBP in the absence of the transforming virus.

There is some evidence that retinoic acid as well as influencing transcription events, can also have direct effects upon the cells biochemistry and behaviour. J. D. Pitts (personal communication) has shown that Retinoic acid at high concentrations can rapidly block junctional communications between cells, and Jetten et al. (1981) have shown that similar levels of retinoic acid can increase the fluidity of cell membranes irrespective of the biological activity of the molecule. Retinoic acid is also known to be directly involved in glycosylation events within the cell probably forming an intermediate retinyl-phosphate carrier molecule (Prehm 1980, Shidaji et al 1981)

Thus retinoic acid may act to influence cell differentiation by affecting both transcription, biochemical and cell interaction events. However, since many pluripotent stem cells are able to differentiate spontaneously simply by manipulation of culture conditions, it is difficult at present, to place retinoic acid as a general differentiative agent; although it is possible that cell lines which spontaneously differentiate have enhanced sensitivity to trace amounts of retinoic acid which are present in normal growth media.

The production of endoderm-like cells from ec cells

Endoderm-like cells are commonly the first cell type to appear as teratocarcinoma cells differentiate. However they are not always observed in the differentiated cell types from all pluripotent strains of teratocarcinoma, and therefore may not be a necessary preliminary to further differentiation (Graham 1977). In some differentiating systems, e.g. simple eb bodies cultured *in vivo* or *in vitro*, and in

"nullipotent" ec cells exposed to retinoic acid, endoderm-like cells are the only differentiated cell type to appear. These differentiating systems will produce endoderm-like cells which can have characteristics of either of the types of extra embryonic endoderm (ex.emb.endo) produced during mouse embryogenesis (i.e. primitive, parietal and visceral). Pluripotent cell lines can produce more than one form of endoderm upon differentiation as well as mesoderm and ectoderm tissue.

The differentiation of teratocarcinoma ec cells into endoderm-like cells is thought to represent the similar transition of multipotent embryonic stem cells (the primitive ectoderm of the pre, and post implantation embryo) to endoderm which occurs in early embryogenesis.

The transition of ec to endoderm-like cell (and also of embryonic ectoderm to endoderm), is marked by characteristic gains and losses in the production of specific products, e.g. matrix and other secretory proteins, enzymes and cell surface antigens and receptors. In vitro culture conditions have been shown not to inhibit the production of biochemical products characteristic of endoderm (Graham 1977). Some of these products can be used to distinguish between the viseral and parietal forms of ex.emb.endo, others are found to be equally present in both forms of endoderm.

Biochemical products characteristic of endoderm and which are also seen in the endoderm-like cells produced in differentiating teratocarcinoma cultures are listed below.

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	MOLECULE (Mwt)	PARIETAL ENDODERM	VISCERAL ENDODERM
Secretory and other proteins	Interferon (23-35K)	?	?
	Plasminogen Activator (79K)	+	+
	Alphafetoprotein (70K)	-	+
	Alkaline Phosphatase (80K units)	+	+
extracellular matrix proteins	Fibronectin (265K)	+	+
	Glycoprotein (350K)	+	-
	(250K)	+	-
	Laminin (150-450K)	+	ND
	Collagen	Type IV (160K)	type I (95K)
Cell surface antigens and receptors	EGF receptors (185K)	ND	+
	Cell surface antigens	+	+

Secretory and other proteins

INTERFERON: The interferon system (which has been described above) has been shown to be inhibited in the ec cell of pluripotent cell lines, and then to become active as these cells differentiate by culture at low density (Burke *et al*. 1978). The production of induced gene products is necessary for the operation of the interferon system and the transcription of these genes is inhibited in the ec cell. It is not clear if this inhibition is specific to the interferon system or, if it is true of all inducible genes or, merely representative of the region of the chromosomes which contains the interferon genes. The interferon system, because it is an inducible gene system, provides a model to examine control of gene expression; the elucidation of the block on interferon production and sensitivity in ec cells will provide an insight into the mechanisms which regulate gene expression in multipotent stem cells. This study examines the appearance and kinetics of the interferon system in "nullipotent" ec cells caused to differentiate to endoderm-like cells by culture in retinoic acid, with

the aim of examining its role as a marker of differentiation in this system.

PLASMINOGEN ACTIVATOR: This molecule is thought to have a biological role in tissue remodelling and cell migration during embryogenesis. An increase in plasminogen activator synthesis can be detected as ec cells differentiate to endoderm-like cells (Linney and Levinson 1977), and also during endoderm formation during embryogenesis (Strickland _et al_. 1976). It has been found to have equal activity in both the visceral and parietal endoderm of the foetus (Bode and Dziadek 1979). The increase in plasminogen activator activity seen during endoderm formation can be greatly enhanced by treatment of the cells with cAMP (Strickland and Mahdavi 1978), unlike the retinoic acid elevated plasminogen activator activity seen in normal chick fibroblast cells (Wilson and Reich 1978).

ALPHAFETOPROTEIN: This is an alpha globulin synthesised by the foetus (and also by the adult, but only under certain pathological conditions and in some tissue culture cells (Graham 1977)) The biological role is unclear, it has a high affinity for oestrogen and may function as a specific or general carrier protein. Its synthesis is restricted in the early embryo to the visceral endoderm, but in the later embryo synthesis is seen in the foetal liver (Dziadek and Adamson 1978). Alphafetoprotein synthesis can thus provide a marker which can distinguish between visceral and parietal endoderm, but because it can be found in other differentiated tissues, it cannot be used as a marker indicative of visceral endoderm itself.

ALKALINE PHOSPHATASE: This enzyme is present in high levels in both ec and in embryo stem cells (Damjonov and Solter 1975), differentiation

of both these stem cells results in a fall in enzyme activity (Bernstine et al. 1973). Ec cells contain many isoenzyme variants of alkaline phosphotase including one unique to ec cells (Wada et al. 1976).

Extra cellular matrix proteins

GLYCOPROTEINS: Parietal endoderm synthesises and secretes two glycoproteins of Mwt 350 and 250Kd (Hogan 1980). Both are secreted by a differentiated parietal endoderm cell line (PYS) and by the parietal endoderm of the mid-term mouse embryo. Only trace amounts of one of these glycoproteins (350Kd) is secreted by a differentiated visceral endoderm cell line (PSA 5E), and neither can be detected in visceral endoderm of the mid-term embryo. In addition, they are not synthesised in the endoderm-like cells produced by treatment of the teratocarcinoma cell line PC13 clone 5 with Retinoic acid which is considered to produce cells characteristic of visceral endoderm (Hogan 1980).

LAMININ: This protein has been shown to be secreted by the treatment of the teratocarcinoma cell line F9 with retinoic acid, which is considered to produce cells characteristic of parietal endoderm (Cooper et al. 1981).

COLLAGEN: Adamson et al. (1979) has shown that in differentiating ec cells which produce cells characteristic of visceral endoderm (e.g. PC13 clone 5) that collagen synthesis undergoes a shift from predominantly type IV to predominantly type I; this does not happen in cultures of ec cells which produce cells characteristic of parietal endoderm (e.g. F9) Adamson and Ayers (1979) have shown this phenomenon

also occurs in post implantation mouse embryo; parietal endoderm produces predominantly type IV, and visceral endoderm produces predominantly type I collagen.

FIBRONECTIN: Fibronectin is thought to have a biological role in morphogenesis and is found in extracellular matrix material of both the foetus and the adult. Ec cells have little intra or extra cellular fibronectin whereas endoderm-like cells of a pluripotent teratocarcinoma cell line are found to have increased surface fibronectin (Wartiovaara et al. 1978a,b).

CELL SURFACE ANTIGENS AND RECEPTORS

Recent advances in techniques have allowed the development of monoclonal antibodies which can identify antigens present on the surface of both ec cells and embryonic cells. Solter and Knowles (1978) describe such an antibody, raised to the F9 ec cell which can identify surface antigens on the eight cell stage embryo. This antigen is transiently expressed by trophectoderm cells and is finally confined to cells of the inner cell mass of the embryo. This antigen, termed SSEA-1 is present on both human and mouse embryonic cells, and is shown to dissapear from the cell surface as F9 ec cells are caused to differentiate into endoderm like cells.The SSEA-1 antigen is one of the few antigens shown to undergo stage-specific expression which have also been identified. Gooi et al (1981) have identified the antigenic determinant recognised by the monoclonal antibody raised to the SSEA-1 antigen, as being formed by the fucosylation of the blood group antigen Ia.

Rees et al (1979) have demonstrated an increase in the number of

EGF (epidermal growth factor) receptors during the differentiation of the cell line PC13 clone 5 (which produces cells characteristic of visceral endoderm), the differentiated cells are also able to respond to the mitogenic action of EGF. Retinoic acid can also enhance the number of EGF receptors on cells not undergoing differentiation, e.g. fibroblast and epithelial cells treated with retinoic acid show an increased number of EGF receptors (Jetten 1980).

The variety of changes associated with the differentiation of both ec and embryonic stem cells into endoderm includes many which appear to mark the differentiation of cells in general, and although some can be used to distinguish between different forms of endoderm (e.g. glycoprotein synthesis and alphafetoprotein production) none are unique to endoderm itself. Interferon production and sensitivity is thought to be a characteristic of all differentiated cell types (see above) and thus, activation of the interferon system will not be expected to be specific to one form of endoderm or to endoderm itself (although it may show varied kinetics of activiation in different cell types). The activation of the interferon system might be found to coincide with endoderm production but only because this is one of the earliest differentiated cell types to form in both embryogenesis and teratocarcinoma cell differentiation.

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AIMS OF THIS STUDY

The aims of this study are twofold:

1. The examination of the appearance and kinetics of the interferon system in "nullipotent" embryonal carcinoma cells undergoing the transition from multipotent stem cell to a differentiated cell type; with the aim of evaluating the use of the interferon system as a marker for the differentiation of endoderm in both teratocarcinoma cells and mouse embryos.

2. The examination of the ontogeny of the interferon system in the developing mouse embryo with the aims of relating the onset of interferon production to the appearance of differentiated cell types in the embryo; and also of determining the relative importance of the interferon system compared to the other foetal anti-viral defences.

The results and discussion resulting from the above investigations are in fact divided into three sections. This is because much of the work relied upon a single cell assay technique for measuring interferon production from single or small groups of cells; it is therefore necessary to describe and characterise the features of this assay.

Thus the next three sections will contain results and discussions from:

1. The characterisation of a single cell assay for the production of interferon.

2. The interferon system in differentiating teratocarcinoma cells.

3. The interferon system in the developing mouse embryo.

MATERIALS AND METHODS

MOUSE EMBRYOS

These were obtained from the progeny of a mating between strain 129J females and strain F_1C57Bl/CBA males, maintained in a breeding colony in the animal house at the Department of Zoology, Oxford University, by B. Randle of that department.

MOUSE CELL LINES

L929 -- A clone derived from Strain L, established from the connective tissue of C3H/An mouse, and maintained in this laboratory in continious culture for approximately fourteen years. The original clone is available from the American Type Culture Collection through Gibco Bio-cult Ltd, Paisley, Scotland. The cell line maintained in this laboratory was cultured in medium different to that recommended by the ATCC and is therefore considered a derivative or subline of L929, the karyotype has not recently been observed.

C3H/10T½ -- A continious line of mouse embryo fibroblasts, which are aneuploid and capable of indefinite growth _in vitro_, but are maintained in culture for a limited number of passages only because their growth properties and ability to support virus growth is known to change with increasing time in culture. This cell line was originaly described by Reznikoff CA, Brandow DW and Heidelberger C, Cancer Research _33_ , pp 3231-3230. 1973.

PSA 5E -- A differentiated teratocarcinoma cell line which has visceral endoderm-like characteristics, derived from a pluripotent teratocarcinoma cell line PSA 5 (Adamson _et al_ 1977).

PYS -- A parietal yolk sac carcinoma cell line isolated from a teratocarcinoma which has parietal endoderm-like characteristics (Lehman _et al_ 1974).

F9 Cl 9 -- A differentiated teratocarcinoma cell line which has parietal endoderm-like characteristics, cloned from a culture of F9 embryonal carcinoma cells which had been caused to differentiate *in vitro*. (Solter *et al* 1979b)

PC13 Cl 5 -- A "nullipotent" teratocarcinoma cell line, passaged as embryonal carcinoma cells which do not undergo spontaneous differentiation *in vitro* but will differentiate into a variety of cell types *in vivo* (Bernstine *et al* 1973 and Hooper and Slack 1977), and into a visceral endoderm-like cell after treatment with retinoic acid (Rees *et al* 1979). PC13 was originally established from outgrowths of cultured embryoid bodies from the tumour OTT6050B (the latter established by Dr. L. Stevens) by Bernstine *et al* (1973). PC13 Cl 5 was isolated from PC13 by Dr. S. Gaunt (Zoology Dept, Oxford University). This clone was recieved at passage five and was not used beyond a total of twenty passages.

F9 -- A "nullipotent" teratocarcinoma cell line, passaged as embryonal carcinoma cells which undergo little or no spontaneous differentiation *in vitro*. F9 was originally established from outgrowths of embryoid bodies of the tumour OTT6050970 (the latter established by Dr. L. Stevens) which were cultured on an agar substratum.by Bernstine *et al* (1973). F9 was able to produce well differentiated tumours *in vivo* at the time of isolation but the range of tissues generated was reduced with increasing passage (Bernstine *et al* 1973), and the pluripotency of this cell line *in vivo* has not recently been demonstrated. Monolayers of F9embryonal carcinoma cells treated with retinoic acid will differentiate into a parietal endoderm-like cell (Strickland *et al* 1978), and it has been recently shown by Hogan *et al* (1981) that aggregates of F9 ec cells similarly treated will differentiate into

cells resembling visceral endoderm.

NULLI 2A -- A "nullipotent" teratocarcinoma cell line, passaged as embryonal carcinoma cells which undergo little or no spontaneous differentiation *in vitro*, but which will differentiate into an endoderm like cell when treated with retinoic acid. Nulli 2A was established from a spontaneous teratocarcinoma (LS 402C 1684 established by Dr. L. Stevens). Various cloned embryonal cell cultures were established from this line, as described in Martin and Evans (1975), including Nulli 2A. This cell line was obtained from Dr. P. Stern (Zoology Dept, Oxford University),and has not been recently recloned.

All teratocarcinoma cell lines (except Nulli 2A) were gifts from Dr. C.F. Graham of the Zoology Department, Oxford University.
The establishment of the original teratocarcinomas from which the above cell lines were obtained is described in Stevens (1970). All "nullipotent" teratocarcinoma cell lines were not used beyond twenty passages, the karyotype of these cells is thought to be normal for this period.
However Papiaoannou et al (1979) have shown that the G-banding pattern is abnormal in some embryonal carcinoma cell lines which have a normal karyotype, this suggests that these cell lines are in some manner "abnormal".

STORAGE OF CELLS

A large stock of all cell types was prepared at the begining of this study. Confluent cell monolayers were trypsinised to a suspension of single cells, washed once with maintenance media and resuspended at a concentration of 1 x 10^6/ml in media containing 50% foetal calf serum plus 10% dimethylsulphoxide (DMSO). One ml aliquots were placed in plastic screw capped vials and placed at -70°C for five hours contained inside a 2cm thick polystyrene box. The cells were then transferred to liquid nitrogen for permanent storage.

Cells retrieved from liquid nitrogen were thawed rapidly in a 37°C water bath and then spun at 1 000 rpm for five minutes to pellet the cells. The supernatant was removed and the cells gently resuspended in five ml of growth media and placed in a suitable culture flask. All cells were passaged at least twice after removal from liquid nitrogen before being used because it was noticed that the growth of the embryonal cells differed during the period when the culture established itself from frozen storage.

DMSO was obtained from BDH chemicals, Atherstone, Warwickshire.

MYCOPLASMA TESTING

All cell lines used in this study were examined for the presence of mycoplasma using the technique described by Russell *et al* 1975), which visualises these organisms through the use of a stain which identifies the presence of mycoplasma DNA in the cytoplasm of infected cells. Cells were seeded sparsley onto glass coverslips and stained with 4'-6-Diamidino-2-phenylindole (DAPI) and examined under ultra violet illumination using the Reichert Zetopan flourescence microscope.

DAPI was obtained from Flourochem Ltd, Glossop, Derbyshire.

CELL MEDIA

nb: 'growth media ' were supplemented with 10% serum and 'maintenance media ' with 2% serum.

L929 and C3H cells were grown in the Glasgow modification of Eagles' medium containing 10% new born calf serum, and supplemented with glutamine (200mM) and antibiotics (benzylpenicillin 50 units per ml and streptomycin 50 µg per ml). L929 cells were grown routinely in one litre glass culture bottles and C3H cells were grown in 250cm^2 plastic culture bottles.

All teratocarcinoma cells were grown in alpha modification of Eagles' medium containing 10% foetal calf serum, and supplemented with glutamine (200mM) and antibiotics as described above, plus additional nucleosides (adenosine 30µM, cytidine 30µM, guanosine 30µM, thymidine 10µM and uridine 30µM) as described in Rees *et al* (1979). The differentiated teratocarcinoma lines were cultured in plastic dishes and the undifferentiated embryonal carcinoma lines were cultured on gelatinised plastic dishes. Gelatinised dishes were prepared by coating plastic culture dishes with 0.5% solution (w/v) of gelatin for two hours at 4°C. The gelatin was then removed and the dishes inverted and left to dry inside a laminar flow tissue culture hood. The plates were then taped together and could be stored indefinitely in a sealed container.

Alpha MEM, GMEM, glutamine, foetal calf and new born serum were all obtained from Flow Laboratories Ltd, Irvine, Ayrshire.

Penicillin and streptomycin were obtained from Glaxo Laboratories Ltd, Greenford, Middlesex.

Gelatin and nucleosides were obtained from Sigma Chemicals Ltd, Poole, Dorset.

Plastic culture dishes were obtained from Gibco-Biocult Ltd, Paisley, Scotland, and one litre glass culture bottles from Gibco, Europe.

DETERMINATION OF CELL NUMBER

Cell number was determined using the 'improved Neubauer' counting chamber,with cells suitably diluted with maintainance media and mixed with an equal volume of PBS containing 1% trypan blue. Only cells excluding this dye were considered viable and counted (however non-trypan blue excluding cells were not often seen).
Trypan blue was obtained from BDH chemicals, Atherstone, Warwickshire.

PASSAGE OF CELL LINES

All cell lines were passaged using a trypsin/versene mixture. L929, C3H and the differentiated teratocarcinoma cell lines were passaged using trypsin at a concentration of 80.0µg per ml for 3 - 4 minutes at 37°C for 3 - 4 minutes. All embryonal carcinoma cell lines were passaged using Trypsin at a concentration of 8.0µg per ml for 30 - 60 seconds at 37°C. Cell monolayers were washed with PBS then incubated with sufficient trypsin/versene to cover the cell sheet for two to three minutes at 37°C. The trypsin/versene mixture was neutralised with two volumes of growth media and the cell monolayer disrupted to a suspension of single cells by vigorous pipetting All cell lines were passed when confluent and reseeded to reach confluency in approximately seven to ten days.
A stock solution of fifty times concentrated trypsin/versene was prepared (ethylenediaminetetra acetate - 4mgs/ml, NaCl - 7.6mgs /ml, KCl - 0.224mgs/ml, glucose - 0.18mgs/ml, Na_2HPO_4 - o.142mgs /ml, trypsin - 4mgs/ml, adjusted to pH 7.2 with 1M NaOH) and diluted to a one times solution prior to use.
All chemicals were obtained from Sigma Chemicals Ltd, Poole, Dorset.

DIFFERENTIATION OF EMBRYONAL CARCINOMA CELLS

Embryonal carcinoma cells from PC13 Cl 5, F9 and Nulli 2A cell lines were seeded at a density of 3 000 cells per cm^2 onto three cm gelatinised plastic culture dishes, into growth media containing 3×10^{-6}M retinoic acid. The cells were cultured over a period of twenty days and the media changed every two days; retinoic acid containing media was removed and the cell sheet washed with PBS prior to interferon induction.

Retinoic acid (all trans) was dissolved in DMSO at a concentration of 4mgs per ml and 25µl of this stock solution added per 100mls of growth media. Retinoic acid was obtained from Sigma Chemicals Ltd, Poole, Dorset.

ASSAYS FOR INTERFERON

A) INAS ASSAY --- Inhibition of viral Nucleic Acid Synthesis (Atkins et al 1974). L929 cells were routinely used to assay all mouse interferon samples, 2×10^5 cells were seeded into 2" x ½" flat bottomed glass vials in one ml of growth media and left for twenty four hours. The media was then removed and replaced with maintenance media containing different amounts of interferon. After approximately twenty hours the interferon-containing medium was removed and replaced with 0.2ml of maintenance media containing 3µg per ml actinomyocin-D and Semliki Forest virus (1/15 dilution of stock virus). After 2.5 hours incubation at 37^oC an additional 0.2ml of media containing actinomycin-D plus 2uCi/ml ^{3}H-uridine, were added to each vial. After five hours of virus growth the media was removed from each vial and the cell sheets washed twice with ice-cold trichloroacetic acid (TCA) and once with ethanol. The cell sheets were then dried and solubilised in 0.2mls of Soluene

(dissolved 1:2 in toluene). A further 2.0 mls of acidified scintillant was added to each vial and each sample counted in an LKB Rackbeta liquid scintillation counter. The titre of each sample of interferon is the reciprocal of the dilution found to inhibit the synthesis of viral nucleic acid by 50%. This 50% level is obtained from the difference between virus and cell control vials which are always included with each assay. These vials are not exposed to interferon and are either infected with virus or not infected. One unit of interferon in the L929 INAS assay is equivalant to one international reference unit of interferon (see introduction).

Preparation of interferon containing samples: A dilution series was prepared from each interferon containing sample to be placed in the INAS assay. Interferon titres were seldom expected to exceed 10^6 units/ml, therefore a dilution series extending from 0.5 to 4.5 $logs_{10}$ was used routinely. Ten vials were used for each sample, each dilution being placed on duplicate vials.

Preparation of Semliki Forest virus: Primary stocks were prepared from _in vivo_ infections of brains of suckling mice (as described in Walters _et al_ 1967). These primary stocks were used to prepare secondary stocks from infection of either baby hamster kidney or chick embryo fibroblast cells (as described in Kennedy and Burke 1972). The secondary stock was diluted 1/15 for use in the assay and was not used to grow further stocks of virus. The titre of the secondary stock on BHK cells was 10^9 plaque forming units (pfu) per ml.

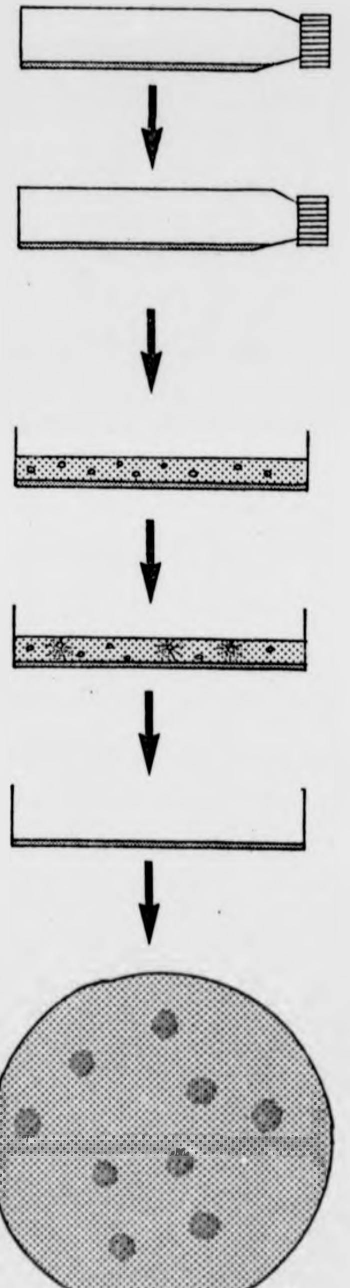
Glass assay vials were obtained from Regina Industries Ltd, Stoke-on-Trent.

^{3}H-uridine (activity, 25 - 30 µCi per ml) was obtained from

Radiochemical Centre, Amersham, Buckinghamshire.
TCA was obtained from BDH Chemicals, Atherstone, Warwickshire.
Soluene was obtained from the Packard Instrument Co, Caversham, Berkshire.
Acidified scintillant was prepared from 5gm 2,5, diphenyloxazole and one ml acetic acid per litre of toluene. (Chemicals obtained from Fisons Ltd, Loughborough, Leicestershire.

B) DETECTION OF INTERFERON PRODUCTION FROM SINGLE CELLS: this assay is a modification of that described by Kronenberg (1977) and is illustrated in figure 3.
The indicator monolayer is prepared by seeding 1 x 10^6 L929 cells onto a gelatinised five cm plastic culture dish for twenty-four hours. The cells under test are induced to produce interferon whilst still in a monolayer. The test cells were infected with 100 HAU of NDV-F for one hour at 37°C, washed three times with PBS and then incubated for a further hour with media containing antiserum raised to NDV-F. The test cells were then trypsinised to a suspension of single cells; the number of trypan-blue excluding cells counted and the cell suspension diluted to contain 100 cells per 0.2mls (this concentration varied with different experiments). The media was removed from the indicator monolayers and o.2mls of the cell suspension added per dish. Immediately 1.5mls of agar-media (warmed to 37°C) was added and the dish agitated to disperse the single cells evenly. The agar was allowed to gel at room temperature before the assay plates were returned to 37°C. After 16 - 20 hrs culture the agar and single cells were removed from the indicator monolayers by tapping the dishes sharply whilst held at an angle of 45°. The monolayers

FIGURE 3. A modified assay to detect interferon production from single cells.



Test cells are infected with virus for one hour

Test cells are washed then incubated with antiserum to the inducing virus for a further hour, and tryps-inised to a suspension of single cells.

A known number of single cells are suspended in agar above an indicator monolayer of similar cells.

Interferon produced by the single cells diffuses across to the indicator monolayer.

The agar and single cells are removed and the mono-layer infected with a cytopathic virus.

Staining of the monolayer reveals foci of interferon protected cells amongst a background of dead cells.

were then washed with PBS to remove particles of agar and then infected with 4 pfu Encephalomyocarditis (EMC) virus per cell. After one hour excess virus was removed and the cells refed with 5ml of maintenance media then incubated at 37°C for 12 hours. The media was then removed from all dishes and the cell sheet fixed by the addition of methanol for one minute and then stained with 0.1% crystal violet for fifteen minutes.
Staining reveals foci of interferon protected cells, of various sizes, in the indicator monolayer amongst a backgroud of dead cells; each foci representing the area underneath an interferon producing single cell. Each single cell assay contained a control plate which differed only in that it did not contain induced single cells. This plate will therefore indicate the presence of any spontaneously occurring foci produced by the indicator monolayer cells; all such control plates were negative throughout use of this assay.
Photographs of the stained assay plates were taken using a yellow filter (Asahi Pentax Y2) on Tri-X Pan 35mm film, with the plates illuminated by a fluorescent light box.

GROWTH OF VIRUSES

A) NEWCASTLE DISEASE VIRUS, STRAIN F (NDV-F), was grown in 11 day embryonated chicken eggs. 0.1ml of a 10^{3} dilution of a stock solution (previously titred at 10^{4} haemagglutinin units (HAU) per ml) was injected into the allantoic cavity of each egg. The eggs were incubated at 37°C for 60 hours then at 4°C for 24 hours, the shell tops were then removed and the allantoic fluids harvested. The virus containing fluids were then spun at 3 000 rpm for 30 minutes to remove the heavier allantoic proteins, and then dispensed into 0.25ml aliquots and snap frozen. The

virus was stored at -70^{0}C and was not refrozen once thawed. The concentration of virus determined by haemagglutinin assay (as described by Borland and Mahy 1968) was 10^{4} HAU per ml.

B) ENCEPHALOMYOCARDITIS VIRUS was grown in L929 cells. The media was removed from one litre culture bottles containing confluent monolayers of L929 cells and replaced with 2ml of low passage virus stock. After one hour at 37^{0}C the surplus virus was removed and the cells refed with twenty ml of maintainance media. At twenty hours post-infection (when cell lysis was evident) the supernatant is harvested and spun at 5 000 rpm for five minutes to remove cell debris. The virus was dispensed into 0.25 aliquots, snap frozen and stored at -70^{0}C, and was not refrozen once thawed. The concentration of virus determined using a standard plaque assay technique (on L929 cells) was 2×10^{8} pfu per ml.

PREPARATION OF ANTISERUM TO NDV-F: A 100ml sample of NDV-F, prepared as above, was purified as described by Avery and Niven (1979). Briefly, the virus was pelleted from the allantoic fluid by centrifugation at 21 000 rpm for three hours and then resuspended in a minimal amount of buffer. The concentrated virus was then banded on a sucrose density gradient and finally pelleted through a sucrose velocity gradient, before being resuspended in a small volume of tris/EDTA/NaCl buffer. The protein content of the purified virus sample was estimated using the technique described by Lowry _et al_ (1951), and the sample diluted to 1.5mg protein per ml. The immunisation programme consisted of an initial injection composed of an emulsion of one ml virus plus one ml Freunds Complete adjuvant. This was followed at two weekly intervals by injections composed of an emulsion between one ml virus plus one ml Freunds Incomplete

adjuvant. Two "half lop" rabbits (obtained from Hylyne Ltd, Marston) were used to produce antibody, and the titre of antibody obtained by means of a haemagglutinin inhibition assay . The titre is expressed as the reciprocal of the dilution of antibody required to prevent agglutination of erythrocytes by 4 HAU of NDV-F. The stock antiserum had a titre of 640 units and was diluted 1 in 10 for all experiments.

Freunds Complete and Incomplete adjuvants were obtained from Gibco Biocult, Paisley, Scotland.

PREPARATION OF AGAR MEDIA: Fifty ml of agar-media (0.3% agar) were prepared thus; 9.7ml of 1.8% agar (Difco Laboratories, East Molesey, Surrey), 5ml of foetal calf serum, 3mls of 0.85M $NaHCO_3$, 0.1ml of antibiotics, 5ml of ten times concentrated GMEM (Flow Laboratories, Irvine,Ayrshire), 0.5ml of 200mM Glutamine and 26.7ml distilled water. All fluids except the agar were mixed and maintained at 37°C, the agar was liquified by boiling and left at 45°C ; when required the agar was added to the other fluids and the whole mixture left to reach 37°C before use.

INDUCTION OF INTERFERON SYNTHESIS BY VIRUS INFECTION

Cells were routinely grown in 3cm culture dishes prior to interferon induction. 100µl of media containing 100 HAU of NDV-F was added to each monolayer for one hour at 37°C.and the cells then washed three times with PBS before being refed with 2ml maintenance medium. After incubation for a total of 20 hours post-infection at 37°C the supernatants were harvested and the cell number counted. The supernatants were dialysed at 4°C for five hours in a pH 2 solution (6.5mls of two molar HCL pus 43.5mls of two molar KCL were made up to one litre with distilled water). Each sample was then dialysed for one day in PBS to restore the pH to 7.4. All samples were stored

at $-20^{o}C$ until assay and results are presented as interferon units per cell in culture.

Purified mouse interferon was a gift from Dr. A.G. Morris (of this laboratory), this interferon had a specific activity in excess of 10^{7} units per mg protein, and was used in section two (figure 30) to examine the antiproliferative effects of interferon upon teratocarcinoma cells.

Interferon antiserum, used in section one (figure 12), was similarly a gift from Dr. A.G. Morris. This antiserum had a titre of $10^{5.5}$ against four units of interferon.

POLYACRYLAMIDE GEL ELECTROPHORESIS OF CELL LYSATES

20 x 20 cm polyacrylamide gradient (10 - 20%) slab gels containing sodium dodecyl sulphate, were prepared using the tris/glycine buffer system (as described in Laemmli 1970). Samples containing an equal number of acid precipitable counts were loaded onto each track; gels were fixed in 45% methanol plus 10% acetic acid and dried under suction onto filter paper, then exposed to Fuji X-ray film (Fuji Photo Co Ltd, London) for approximately one week.

Preparation of cell lysates: Cell cultures were labelled with ^{35}S methionine (activity 740 Ci/mMol) as described in figure 34a , washed three times with ice cold PBS then harvested using a rubber bung into a tris/sodium dodecyl sulphate/mercaptoethanol buffer (pH 9). An equal volume of this lysate was then mixed with an equal volume of "blue buffer" , boiled for three minutes and then spun for four minutes in a microfuge. The number of acid precipitable counts were then counted and an equal number of counts per lysate loaded on to the gel.

"Blue buffer" : 10 mls of this solution was prepared from 1 ml methionine

0.5 ml of 0.1% Bromophenyl blue and 8.5 mls of 'extra strong buffer' (ie: 2.5mls 0.5M tris-HCL pH 6.8, 2g sodium doddecyl sulphate, 2mls mercaptoethanol, 12mls glycerol, 3.3mls distilled H_2O; diluted to two volumes with o.5M tris-HCL pH 6.8)

Counting of cell lysate samples: 5µl of the cell lysate (previously mixed with 'blue buffer' and boiled for three minutes) were spotted onto 1 cm^2 of filter paper and dried under an infra-red lamp. The filter paper squares were then boiled for ten minutes in 5.0% TCA then washed twice in ice-cold TCA, once in ice-cold ethanol and dried under an infra red lamp. The squares were then placed in a scintillation mixture (toluene plus 5.0% 2,5, diphenyloxazole) and counted in a LKB Rackbeta liquid scintillation counter.

EXTRACTION OF CELLULAR RNA AND QUANTITATION OF INTERFERON mRNA PRODUCED IN VIRUS INFECTED CELLS.

Cellular RNA was extracted from virus infected cells using two methods.

A) Total cellular RNA:

Intact cells were harvested at six hours post virus infection and lysed in Guanidine thiocyanate (4M) the total cell RNA was obtained by centrifugation of this lysate (at 40 000 rpm for 16hrs) through 5.7M caesium chloride. The pellet was solubilised and RNA extracted using butanol/chloroform, this method is described in Morser *et al* (1979).

B) Cytoplasmic cellular RNA:

Intact cells were harvested at six hours post-virus infection into ice-cold isotonic buffer and then washed and resuspended into ice-cold hypotonic buffer. After addition of a non-ionic detergent the cells were gently homogenised using a Dounce glass homogeniser, until all cells were shown to be non-trypan blue excluding. Nucleii and other cell debris were removed by centrifugation for ten minutes at 10 000 rpm, RNA was then obtained from the remaining supernatant by phenol/chloroform extraction.

The RNA obtained from both these methods was precipitated three times under ethanol before being used in subsequent experiments.

XENOPUS OOCYTE INJECTIONS: RNA at a concentration of 5mg/ml was injected into both the nucleus and cytoplasm of the Xenopus oocyte by Dr. A Colman (of this department). It has been previously shown (Colman and Morser 1979) that the oocyte will secrete interferon manufactured from injected messenger RNA. Thirty injected oocytes were placed in 200µl medium which was assayed (by INAS assay) at 24 and 48 hours post-injection. Similar levels of interferon were found to be produced at both these times.

RABBIT RETICULOCYTE SYSTEM: An mRNA dependent reticulocyte lysate protein synthesising system as devised by Pelham and Jackson (1976) was used by Dr. J Morser to assess the activity of all extracted RNA samples.

AGAROSE FORMAMIDE GEL ELECTROPHORESIS: The RNA samples which were injected into the oocytes were also examined in an agarose-formamide gel system using a 1.0% agarose gel and a tris/EDTA/ NaH_2PO_4 buffer (Dr. J Norton, this department, personal communication). Gels were stained in ethidium bromide (1.0 µg/ml) and examined under ultra violet illumination for the presence of ribosomal RNA bands.

SECTION ONE ---- THE CHARACTERISATION OF THE SINGLE CELL ASSAY

SECTION ONE ---- RESULTS

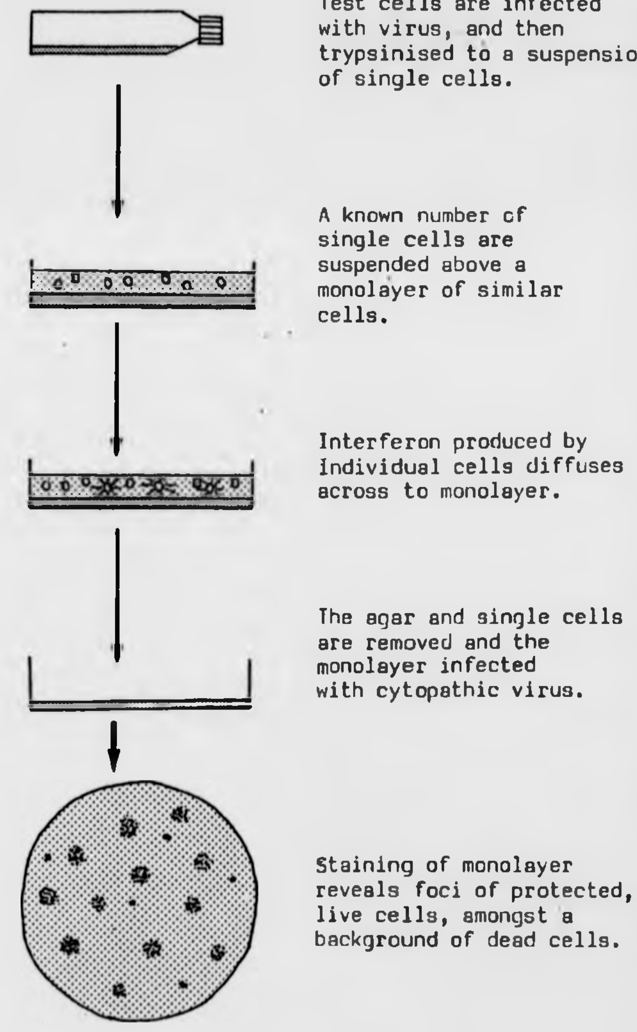
THE PUBLISHED SINGLE CELL ASSAY IS INACCURATE:

The single cell assay used throughout this study is fully described in the Methods; however, although it is similar, it does differ significantly from published descriptions of such assays (Osborn and Walker 1969, Kronenberg 1977). Because of this, section one will describe the reasons why changes to the published assays were thought necessary, provide a characterisation of the modified assay and also explore the use of this assay in the examination of interferon production from single cells.

A single cell assay which could qualitatively detect interferon production was first described by Osborn and Walker (1969), who used it to examine the behaviour of individual spleen cells taken from mice previously innoculated with virus. More recently, Kronenberg (1977) described a similar assay which was able to detect interferon production from individual culture cells previously infected with virus, or treated with dsRNA. The main features of these two assays are similar, and the assay described by Kronenberg is illustrated in figure 4.

This assay is analogous to the infectious centre assay for virus production, wherein virus yielding cells are detected by plaque formation in an indicator monolayer of cells. The difference between the single cell assay for interferon production and the infectious centre assay for virus production is that in the former, foci of live cells are seen amongst a background of dead cells in the indicator monolayer cells, whereas in the latter the reverse is true, plaques of

FIGURE 4. A SINGLE CELL ASSAY FOR INTERFERON (KRONENBERG 1977).



dead cells form amongst a background of live cells in the indicator monolayer cells.

Each of the foci produced in the single cell assay for interferon represents production from a single cell which has been suspended in agar above that area of the indicator monolayer. The number of foci counted, divided by the number of single test cells added to that assay dish, gives the percentage of cells in a culture able to produce interferon, under the inducing conditions used. Figure 5 shows photographs of the indicator cell monolayer after the single cell assay has been completed: foci of varying diameters (0.5 to 5.0mm) and also of varying intensity can be seen. Data obtained from four separate single cell assays are provided in figure 6 . This data shows that there is a linear relationship between the number of single test cells added to each assay plate, and the number of foci produced at the completion of the assay. Figure 6 shows two further features of the results obtained from early use of this assay; these were that consistently more foci were counted than the number of single cells added per assay dish, and also, that a wide variation was seen in the percentage of single cells found to be producing interferon (this varied from ninety-five to one hundred and fifty percent for a population of L929 cells infected with NDV-F virus).

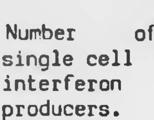
Such results may suggest errors in the addition of test cells to the assay plates; for in order to obtain appropriate cell numbers for use in the assay, a cell suspension of initially 500 000 cells per ml is diluted to approximately 500 cells per ml, and dilution errors are possible. However, such errors were not considered the most likely explanation, mainly because although the assay was repeated many times, the error was never negative. There were always the same, or more foci produced on completion of the assay, than the number of test cells

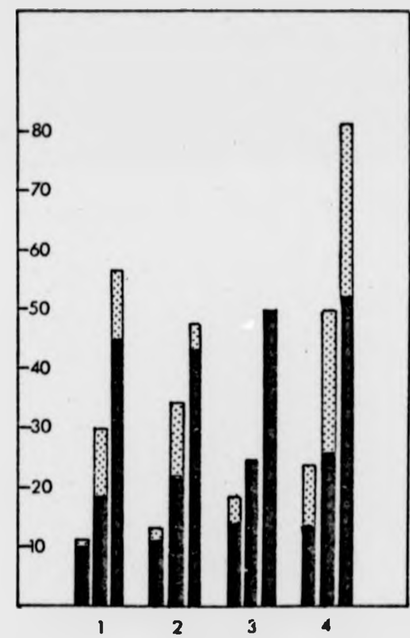
FIGURE 5. Appearance of assay plates at the completion of the single cell assay for interferon production. (Method as Kronnenberg 1977)

N° of cells added per assay dish.	N° of foci counted at end of assay.
74.0	106.0
37.0	53.0
19.0	30.0

The method for the single cell assay for interferon production is illustrated in figure **4**., the above cell monolayers were fixed in 100% methanol for one minute and stained with 0.1% crystal violet for ten minutes.

FIGURE 6. Single cell assay data (method as Kronenberg 1977)





Monolayers of L929 cells were induced to produce interferon by infection with NDV-F virus, washed three times with PBS and then trypsinised to single cells. Cell numbers from 20 - 100 were placed in the single cell assay as described in the text; the results from four separate experiments are illustrated. Each bar represents the mean of four dishes, (■) the number of cells added per assay dish, (▨) the number of foci counted per dish at completion of the assay.

originally added. This suggested that a second factor could be present in the assay system which was responsible for the production of foci similar to those caused by interferon production from single cells. It was possible that the virus used to induce the single test cells to produce interferon was being carried over, with the single cells, into the assay system and producing foci in the indicator cell monolayer (despite washing of the cells, trypsinisation and a one thousand fold dilution).

Thus the single cell assay for interferon production as described by Osbourn and Walker (1969) and Kronenberg (1977) appears to contain a systematic error, possibly due to virus eluting from the test cells.

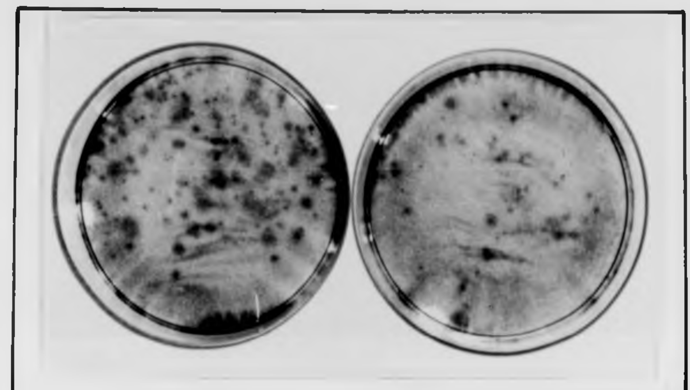
Can NDV-F directly cause the production of foci within the confines of the single cell assay system?

It has been shown by Brown *et al*. (1980) that a plaque forming strain of NDV, when added directly to a monolayer of L929 cells, can produce foci similar to that seen in the single cell assay. Figure 7 shows this to be true for NDV-F (a non-plaque forming strain of NDV); discrete foci could be seen when 10^{-3} to 10^{-4} HA units of virus were added directly to the indicator monolayer within the single cell assay system. The variation seen in diameter and intensity were similar to that obtained for the single cell assay as shown in figure 5. At amounts greater than 10^{-3} HA units discrete foci were not seen and the whole monolayer was protected from subsequent CPE by EMC virus; at amounts less than or equal to 10^{-6} HA units no foci were seen and complete cell death occurs. These data show that previous treatment of cells with NDV-F can protect cells from subsequent cell death caused by a lytic virus. Since NDV-F can induce interferon, and this NDV-F

FIGURE 7. Foci produced within the single cell assay system by various concentrations of NDV-F.

(a)

Amount of NDV-F.	10^{-3} HA	10^{-4} HA
Number of foci.	200.0	30.0



(b)

Foci produced by various concentrations of NDV-F.

Amount of NDV-F per assay plate.	Description of monolayer at completion of assay.
10.0 HA	Complete protection
1.0 HA	Complete protection
0.1 HA	Complete protection
0.01 HA	Complete protection
0.001 HA	149 - 200 foci
0.0001 HA	29 - 36 foci
0.00001 HA	2 - 3 foci
0.000001 HA	0
0.0000001 HA	0

100 ul of PBS containing various concentrations of NDV-F were added to 5 cm monolayers of L929 cells for one hour, the cells were then washed three times with PBS and then refed with 1.5 mls of agar-media. The proceedure then followed that of the single cell assay described in figure 4., (a), shows photographs of typical foci obtained with this method and (b), shows the data obtained with different dilutions of virus.

associated viral interference is probably due primarily to interferon production in the protected cells. These data also show that approximately 2.5×10^5 virus interfering (or possibly interferon-inducing) particles are present in one HA unit of NDV-F.

NDV-F is a large virus (approximately 500×10^6 MW), and it might be expected that the virus is relatively immobile within the assay. Therefore the ability of the virus to diffuse through the agar-media used in the single cell assay was examined. Figure 8 shows that NDV-F can diffuse through a one millimetre barrier of 0.3% agar during the period of time that the single test cells are suspended in the assay, and can induce protection in the underlying indicator monolayer cells. Since 1.25×10^5 viral interference particles were added to each dish (ie: 0.5 HA units) and complete protection was not seen (compare with figure 7), these data do show that 0.3% agar can inhibit the movement of NDV-F to a limited extent.

Does NDV-F replicate in, or desorb from L929 cells?

NDV-F is an avirulent strain of NDV (an avian paramyxovirus), it does not plaque, nor does it have a significant cytopathic effect in chick embryo fibroblasts or have a pathogenic action in young chickens. This is in contrast to the mesogenic and velogenic strains of NDV (Lomniczi et al. 1971). NDV-F does replicate and produce infectious virions in embryonated chick eggs (see methods) and the growth profile of NDV-F in such eggs is similar to that of the other strains which are grown in chick embryo fibroblasts (Lomniczi et al. 1971). None of the strains of NDV-F produce infectious virions when used to infect mouse cells, however, the velogenic strains can produce inhibitory effects on host cell synthesis and can also produce the major viral proteins

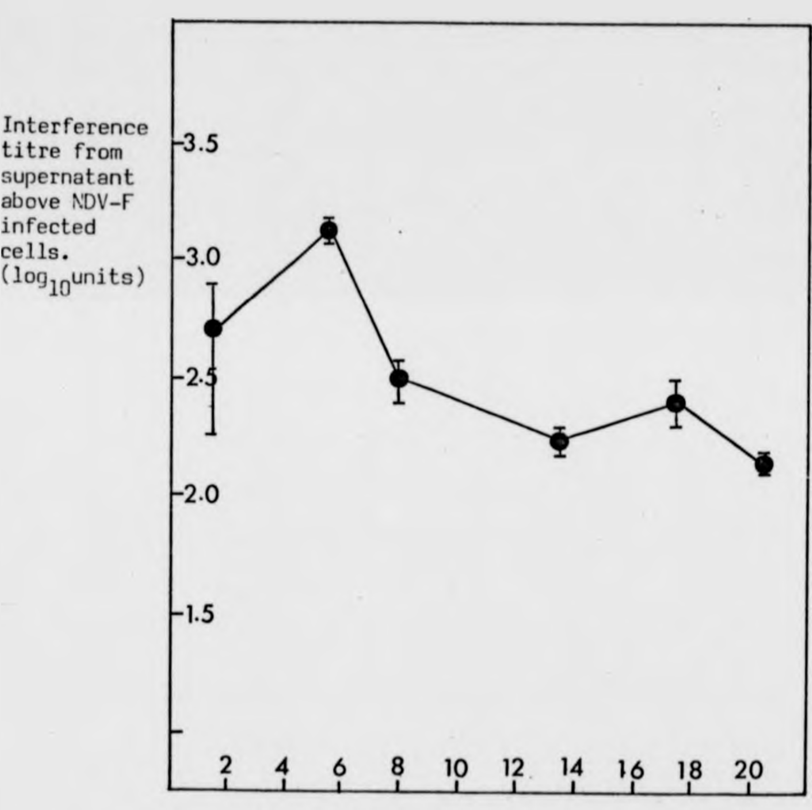
FIGURE 8. The effect of an agar barrier on the ability of NDV-F virus to infect an L_{929} cell monolayer.

Volume of agar barrier.	Description of L_{929} monolayer.
1.0ml	50% cell death no clear foci.
0.5ml	50% cell death no clear foci.
0.0ml	complete protection.

0.5ml - 1.0ml of agar-media (0.3%) was added to 5cm monolayers of confluent L929 cells, and allowed to gel. 0.5HA NDV-F in 50µl volume were added to the top of the gelled agar and the volume of agar-media made up to 1.5mls in total, the method of the single cell assay described in Figure 4 was then followed.

(ie: the haemaglutinin, neuraminidase) (Gandhi et al. 1971). In contrast NDV-F does not depress host cell RNA synthesis (as shown by incorporation of ^{3}H Uridine into infected cells) or host cell protein synthesis (as shown by polyacrylamide gel electrophoresis of infected cells), neither can viral proteins be detected (data not given). Because of the data presented in figures 7 and 8, it was of interest to examine the behaviour of NDV-F in L929 cells; this could not be done by conventional means, but since it was known that NDV-F could interfere with the growth of a second virus within the single cell assay, the behaviour of L929 cells infected with NDV-F was followed by measuring the levels of viral interference. Viral interference was assayed in the standard INAS assay (see methods) where the titre of viral interference of a solution is the reciprocal of the dilution which produced a fifty percent inhibition of SFV nucleic acid synthesis. This assay is also used to quantify interferon yields from samples in which virus has been inactivated (eg: by pH2 treatment). Since NDV-F will also induce interferon in the cells under study it is necessary when using this method to inactivate or remove any interferon present in the sample. Interferon was removed from the samples by spinning infected cell supernatants at 40 000 rpm for two hours, the molecular weights of mouse interferon range from 22 to 35 Kd and therefore remain in the supernatant. The resultant pellet was washed with PBS and resolubilised in media, and then assayed for viral interference. The percentage recovery of virus using this method is approximately 20% (data not given) and is sufficient to monitor the system. An analysis, using this method, of a culture of L929 cells infected with NDV-F is presented in figure 9. These data show the behaviour of virus infected L929 cells during the time course of the single cell assay; a base line reading (b) was taken immediately after the virus was washed off the cells, and can be

FIGURE 9 . The desorbtion of NDV-F virus from L929 cells.



Time (hours) post-infection

Duplicate monolayers of L929 cells were infected with 10 HAU NDV-F for one hour at 37^{0}C, then washed three time with PBS and refed with maintenance medium. The media was immediately resampled to obtain the first sample and the cells refed. Thereafter the supernatant was harvested at intervals, the cells being washed three times with PBS after each harvest. Samples were spun at 40 000 rpm for two hours and the supernatant discarded, the pellet was solubilised overnight in one ml of media. All samples were then assayed for interference using the INAS assay described in methods.

seen to contain a high titre of viral interference. This interference can be seen to increase up to 5 hrs post-infection, and then decrease to reach a plateau during the remainder of the experiment. Thus these data show that NDV-F infected L929 cells release particles capable of viral interference during the period of the assay.

Neutralisation of viral-interference produced by NDV-F infected cells by antiserum raised to partially purified NDV-F

It has been shown in figure 5, that NDV-F grown in embryonated chick eggs can directly induce foci within the single cell assay. Since it has been shown in figure 9, that NDV-F infected L929 cells can release viral-interference into the supernatant during the course of the assay, it is necessary to check firstly that this viral interference can produce foci within the single cell assay, and secondly that such foci are due to NDV-F.

Figure 10 shows the result of such an analysis, in which three parameters were measured from supernatant harvested from NDV-F infected L929 cells. These data show that the viral interference which is released into the cell supernatant could be 99.9% removed by treatment with antiserum raised to partially purified NDV-F. In addition, this viral interference, at a suitable dilution, was capable of producing foci of similar diameter and intensities to those described above; furthermore these foci could be completely removed by prior incubation with antiserum raised to NDV-F. The third parameter of interferon yield was measured to ensure that both the virus and the cells were behaving as expected.

FIGURE 10. Neutralisation of interference produced by NDV-F virus infected cells, by antiserum (AS) raised to partially purified NDV-F.

	TIME POST-INFECTION WITH NDV-F VIRUS.			
	1.5hrs		19hrs	
	nil AS	+AS	nil AS	+AS
Virus interference titre (log units) produced in INAS assay.	2.5	ND*	3.5	0.4 (99.9% reduction)
Virus interference the number of foci produced in single cell assay.				
---10^{-3} dilution	ND	ND	CP**	20-30
---10^{-4} dilution	ND	ND	160-175	0
Interferon yield titre (log units) produced in INAS assay	0.0	ND	3.9	ND

A confluent monolayer of L929 cells were infected with 500HA NDV-F for 1.5hrs at 37°C, washed three times with PBS, then refed with 5mls of maintenance media which was immediately sampled for the 1.5hr time point. The cells were then refed and the supernatant harvested at 19hrs post-infection.Each 5ml sample was divided into two; one half provided material for assay of virus interference and for use in the single cell assay, and was prepared as described in figure 9 . The remainder was used to provide material to assay the interferon yield; this material was dialysed at pH 2 for five days to inactivate virus and then at pH 7 for one day to readjust the pH, before being used in the assay. Neutralisations were performed by incubating samples with antisera for one hour at 37°C.

Both virus interference and interferon yield were assayed in the INAS assay (see methods), the single cell assay system used has been described in figure 6.

*ND = not done, **CP = complete protection, foci not discernable.

Production of foci within the single cell assay by NDV-F infected cells, which cannot produce interferon active upon L929 cells.

Interferon produced by virus infected cells can be species specific in action (see Introduction). Thus it is known that some species of interferon will not cross-react, ie: produce anti-viral or other effects, upon cells of another species. For example mouse L929 cell interferon induced by NDV-F will not induce anti-viral protection in chick embryo fibroblasts (CEF) or in human fibroblasts as measured by the INAS assay. It is also known that some cells are unable to produce interferon, for example embryonal carcinoma cells (see Introduction) do not produce interferon when infected by virus (Burke et al. 1978). Thus if either heterologous cells, or cells that do not produce interferon are infected with NDV-F and placed in the single cell assay performed as illustrated in figure 4, then no foci will be expected. Figure 11, shows the data obtained from such an experiment, these data show that NDV-F infected CEF, human fibroblasts and mouse ec cells did not produce interferon when assayed upon L929 cells in the INAS assay. But, all these cell types produced foci within the single cell assay when L929 cells formed the indicator monolayer; furthermore all these foci could be removed by prior treatment of the infected cells with antiserum raised to NDV-F.

Thus these data support the proposal that virus used to induce interferon in the test cells is carried over and probably desorbs from the single cells during the course of the assay, resulting in the production of foci which are similar to that caused by interferon production from single cells.

FIGURE 11. Production of foci within the single cell assay by virus infected cells; which either cannot produce interferon, or produce interferon which is inactive on the cells forming the indicator monolayer.

SINGLE TEST CELLS.

	Chick embryo fibroblasts (CEF)	Human fibroblasts (MG63)	Embryonal carcinoma (PC13 cl 5)
The number of foci produced in the SCA*			
---control sera:	32-54 (129)	65-80 (140)	39-43 (100)
---antisera:	0 "	0 "	0 "
Interferon yield titre (log units) produced in INAS assay.	0.0	0.0	0.0

The single test cells were grown as monolayers and infected with 500HA NDV-F for one hour at 37°C, washed three times with PBS and then either incubated with media containing antiserum raised to NDV-F, or with media containing control sera, for one hour at 37°C. The cells were then washed with PBS and trypsinised to a suspension of single cells, a small aliquot was diluted further to be used in the SCA; the remainder were returned to the culture dish for approximately 20hrs and the cell supernatant harvested for interferon yield.
The single cell assay data represents the range obtained from five plates, the number in brackets refers to the number of single cells added to each dish. The indicator monolayer of the single cell assay and the cells used in the INAS assay were mouse L_{929} cells.

* SCA = single cell assay.

A modified single cell assay for interferon

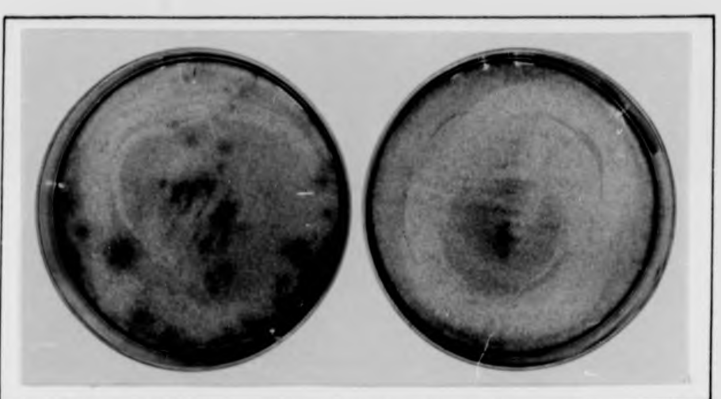
Using the information presented above a modified single cell assay was designed, which is fully illustrated in methods and differs from the assay illustrated in figure 4 in one feature. Test single cells are infected with virus for one hour at 37°C, then washed three times with PBS, as described previously. The modified assay differs in that the test cells are then incubated for a further hour with media containing antiserum raised to the inducing virus, before being trypsinised to single cells and placed in the assay. The data from figure 11 shows that this procedure is capable of preventing foci production from virus carried over with the single cells.

The foci produced at completion of the modified single cell assay were examined to ensure that they formed as a result only of interferon production by the single cells used in the assay. Figure 12 shows that single cells treated as for the modified assay, will not produce foci within the single cell assay if they are suspended in agar containing antiserum to mouse interferon. Thus all of the foci produced in the modified single cell assay are due to interferon production, and none are due to the direct actions of the inducing virus.

The percentage of an L929 cell population able to produce interferon upon infection by NDV-F, using the modified assay, was found to be approximately 30%, which is in contrast to the results obtained in figure 6 using the assay as described by Osborn and Walker (1969) and Kronenberg (1977). This figure of approximately 30% was however, found to be constant for relatively short periods of time only. Figure 13 shows that over the period of two years in which the assay was in routine use, the percentage of single cell interferon producers using this cell/inducer system, was found to vary between

FIGURE 12. Demonstration that all foci, produced in the modified single cell assay, are due to interferon production from the single cells used in the assay.

	A	B
N^{o} of cells/dish.	94	94
N^{o} of foci.	36	0



A monolayer of L929 cells were infected with 100 HA NDV-F for one hour, washed three times with PBS, and then incubated with media containing antiserum raised to NDV-F for a further hour. The cells werethen trypsinised to a suspension of single cells, a small aliquot of which was diluted furhter to provide cells for use in the single cell assay (performed as described in methods). Plate A shows the result of a single cell assay with single cells suspended in normal agar-media. Plate B shows the result of a single cell assay with cell suspended in agar-media containing antiserum raised to mouse interferon.

FIGURE 13. Variation in the percentage of single cell interferon-producers in a population of L929 cells infected with NDV-F virus.

Date	Percentage of single cell interferon producers.
March 1980	34
April "	49
May "	40
June "	24
July "	35
March 1981	36
April "	45
May "	43
June "	24
July "	33

The above data was obtained from examination of NDV-F infected single cells using the modified single cell assay as described in methods, and span a period of eighteen months. The L929 cells used were from the same batch of cells maintained in liquid nitrogen, and the virus (NDV-F) was from a batch grown in May 1979 and maintained at -70°C (the virus was not refrozen once thawed).
Each figure represents the average from at least four separate experiments occurring throughout the month, and each individual experimental result represents the mean from five dishes.

22% to 49%, the figures remaining constant only for a period of 1 - 2 months.

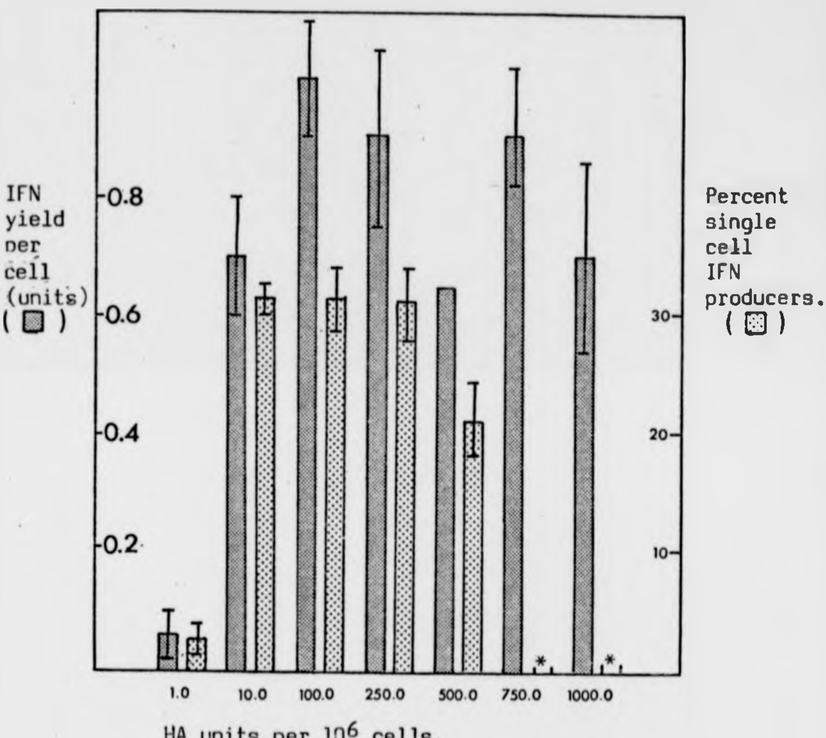
OPTIMISATION OF THE MODIFIED SINGLE CELL ASSAY.

The results of the modified single cell assay using NDV-F infected L929 cells shows that less than half of the cell population can produce interferon; it is therefore necessary to assess whether or not this is a reflection of this particular inducer-cell system, or whether it reflects the limitations of the assay. By the latter is meant that the assay may for example, only be sufficiently sensitive to detect 30% of the interferon producers, or that the method of inducing and placing the cells in the assay does not allow the maximum number of cells to produce interferon. Following this, the various stages involved in the modified single cell assay were examined to assess their role in influencing the outcome of the assay.

a: Interaction between the test cells and the inducing virus.

The production of interferon at the population and the single cell level was measured in cultures of L929 cells infected with increasing amounts of NDV-F. These data are presented in figure 14, and show that both interferon yield per culture, and the percentage of single cell interferon producers, increased when more virus was applied to the culture. This increase reached a maximum for both parameters at approximately 10 HA units NDV-F per 10^6 cells. Therefore to ensure maximum interferon yields, interferon was induced in cell cultures using a ratio of 100 HA units per 10^6 cells throughout this study.

FIGURE 14. The production of interferon at the population and the single cell level in cultures of L929 cells infected with increasing amounts of NDV-F.



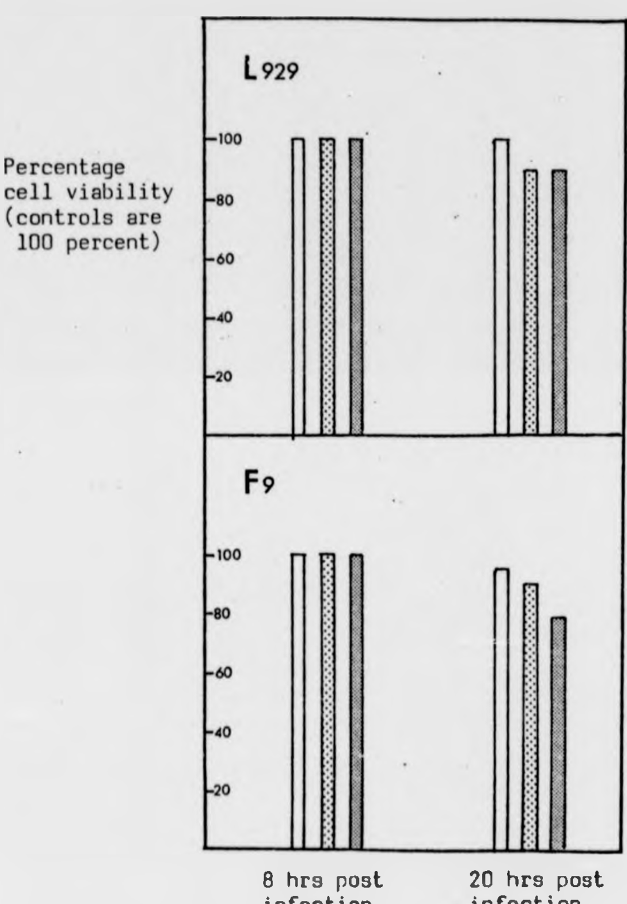
Duplicate monolayers of L929 cells were infected with different concentrations of NDV-F for one hour at 37 C, then washed three times with PBS. The cells were incubated for a further hour with media containing antiserum raised to NDV-F, then trypsinised to a suspension of single cells. A small aliquot of which was used to provide cells for the single cell assay (performed as descri--bed in methods), the remaining cells were returned to the culture dish. Media from the culture dish was harvested at 20 hrs post-infection, treated to inactivate virus and then assayed for interferon yield by INAS assay (as described in methods).

* = not done.

The percentage of single cell producers was reduced with a concentration of 500 HA NDV-F per 10^6 cells. This may have been due to cell death. As mentioned above, NDV-F is a lentogenic strain of an avian paramyxovirus which does not replicate significantly in mouse cells, however at high concentrations it can be toxic to some but not all mouse cell lines (my observation). If the virus is toxic at the concentration used to induce interferon in the single cell assay, then this could reduce the number of cells able to produce interferon. The viability of cells infected with different concentrations of NDV-F was examined (Figure 15). Two cell lines L929 and a teratocarcinoma cell line F9 ec were infected with increasing amounts of NDV-F. The viability of the two cell lines was not reduced by eight hours post-infection and was only slightly reduced by 20 hours post-infection. Interferon production is maximal in L929 cells at 10-12 hours post-infection and in ec cells at 5-6 hours post-infection (see later); thus it can be assumed that interferon production is not affected by the slight reduction in cell viability which occurs late in infection.

The influence of the density of the cells at the time of addition of virus, on subsequent interferon production, was also examined. Figure 16 shows that interferon production at the population and the single cell level is not influenced by the density of the cells at the time of infection. Cultures seeded at 0.9×10^6 per 3cm dish were approximately 75% confluent whilst those seeded at 3×10^6 per 3cm dish were overconfluent, rounded and poorly attached to the substrate. From this information test single cells were routinely seeded to a density of 1×10^6 per 3cm dish, before being used in the single cell assay.

FIGURE 15. The viability of cells infected with NDV-F virus.



Triplicate monolayers of L929 and F9 ec cells were infected with five (☐), fifty (☐) or one hundred (☐) HA NDV-F for one hour at 37°C. The cells were then washed three times with PBS then refed with maintenance media. At eight and twenty hours post-infection the cell number from each plate was counted, only trypan blue excluding cells were included, and the results are expressed as a percentage of control culture cell numbers.

FIGURE 16. The effect of cell density upon interferon production at the level of the population and the single cell.

	CELL NUMBER PER 3cm DISH.	
	0.9 10^6 cells	3.0 10^6 cells
IFN yield per culture cell (units/cell).	2.0 - 3.0	2.8 - 3.8
Percent single cell producers of IFN.	33 - 44	31 - 37

Triplicate 3cm dishes were seeded at two cell densities overnight, infected with 100 HA NDV-F for one hour at 37°C, washed three times with PBS and then incubated with media containing antiserum raised to NDV-F for a further hour. The cells were then washed and trypsinised to a suspension of single cells, a small aliquot of this was used to provide cells for the single cell assay (performed as described in methods), the remainder were returned to the culture dish. At 20 hours post-infection cell supernatants were harvested and the cell number counted; the supernatants were then treated to inactivate virus and then assayed for interferon in an INAS assay (this proceedure is described in methods).
The results show the range of results obtained from three dishes.

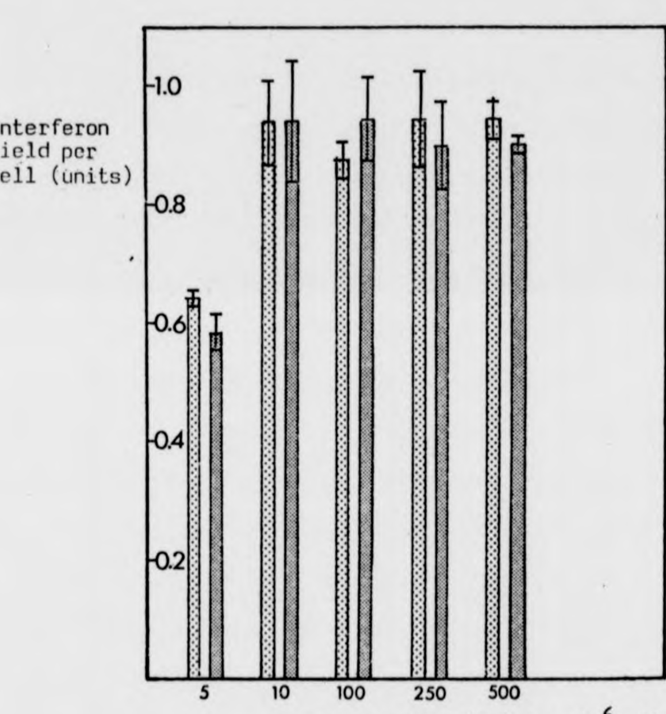
b: Interaction between virus infected test cells and antiserum raised to the inducing virus.

Test cells in the modified single cell assay are infected with virus for one hour, washed three times with PBS to remove surplus virus and then incubated with media containing antiserum raised to the inducing virus. This period of incubation is sufficient (see figure 10) to prevent virus desorbtion from the single cells during the period of the assay. As mentioned in the Introduction, it is not fully clear what comprises the actual interferon inducer in virus infected cells, although there is evidence that interferon production may be invoked by formation of dsRNA (Morser and Burke 1979). Therefore, it is necessary to check whether or not the period of incubation with antiserum raised to the inducing virus, can influence the production of interferon in virus infected cells. Figure 17 shows the interferon yield obtained from cultures of virus infected L929 cells treated either with control sera or with antiserum raised to the inducing virus. These data show that treatment with antiserum does not inhibit the production of interferon from virus infected cells.

c: The accuracy with which small cell numbers can be dispensed.

The result of a single cell assay is expressed as the percentage of cells able to produce interferon under the inducing conditions chosen. This figure is a ratio between the number of foci counted at completion of the assay and the number of cells originally added to each assay plate. The former can readily be counted macroscopically when the assay plates are stained, the latter are obtained from counting cells at a high concentration and then diluting a sample of

FIGURE 17. The yield of interferon in virus infected cells treated with antiserum raised to the inducing virus.



Duplicate monolayers of L929 cells were infected with various amounts of NDV-F for one hour at 37°C, and then washed three times with PBS. The cells were then incubated with media which contained either antiserum raised to NDV-F (▩), or control sera (▨). After a further hour the cells were washed and refed with maintenance media. Cell supernatants were harvested at 20 hrs post-infection , treated to inactivate virus (see methods) and assayed for interferon yield by INAS assay as described in methods. Each bar represents the mean of two determinations.

this suspension by approximately 1 000 fold. Thus the accuracy of the result of the assay depends primarily on the accuracy with which such dilutions can be made. Figure 18 shows data obtained from

dilutions of cell suspensions originally at different concentrations. These data show that dilutions of cell suspensions to small cell numbers can be made with a repeatable and high degree of accuracy. An additional check on the accuracy with which small cell numbers can be dispensed can be made by performing single cell assays on more than one culture of test cells simultaneously. Separate L929 cell cultures induced to produce interferon by the same means, should when trypsinised and diluted into the single cell assay, produce the same percentage of cells able to produce interferon. Figure 19 shows the result of single cell assays using three cultures of L929 cells, the single cells from each test plate being added to four assay plates. These data show that the mean percentage of cells able to produce interferon from all three cultures varied from 22% to 25%; thus confirming that small cell numbers can be dispensed with accuracy.

d: The timing of events within the single cell assay.

The test single cells, during the single cell assay, are suspended above the indicator monolayer for a period of approximately 18 hours. Because induction and other preparations of the test cells occupies two hours, the test cells are included in the assay only for 20 hours post-infection. This period in the assay was determined by following the kinetics of interferon production in virus infected cells. Figure 20 shows the rate of interferon production in virus infected L929 cell cultures which were either left *in situ* after addition of virus; or

FIGURE 18. Accuracy and repeatability of pipetting small cell numbers.

	CELL NUMBER PER MILLILITRE.		
	1 000	10 000	20 000
Aliquot dispensed.	1ul	5ul	5ul
Expected cell N^{O}.	1.0	50.0	100.0
N^{O} of drops counted.	96.0	16.0	11.0
Range of cell N^{O} counted in all drops.	0 - 5	40 - 79	85 - 112
Mean cell number per drop.	0.6	56.0	100.0

A monolayer of L929 cells was trypsinised to a suspension of single cells and cell numbers counted using a haemocytometer, the suspension was then diluted using a Gilson Pipetman to 1000, 10 000 and 20 000 cells per ml into separate containers. These containers were then agitated and dispensed as indicated above into one or five microlitre drops on to the base of plastic culture dishes. Each drop was then scanned, using a light micro--scope with a grid incorporated into an eyepiece, and the cell number per drop counted.

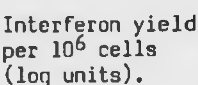
FIGURE 19. Repeatibility of results obtained from the single cell assay for interferon.

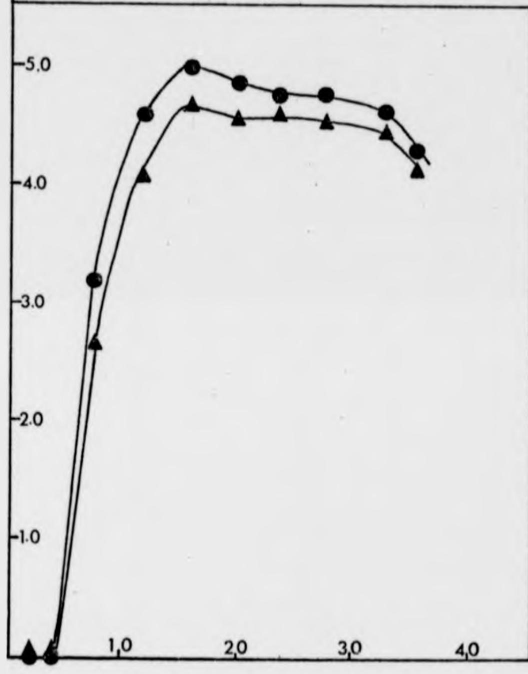
Culture dish	Number of cells added per SCA*	N^{o} of foci per SCA (4 dishes)	Mean foci N^{o}
A	95	25,23,20,31.	25
B	95	26,20,25,22.	23
C	95	22,23,30,14.	22

Three separate cultures (A,B and C.) of L929 were infected with NDV-F (100 HA) for one hour at 37°C, washed with PBS three times and incubated with media containing antiserum to NDV-F, for a further hour. Each culture dish was then separately assayed in the single cell assay (see methods for description).

* SCA = single cell assay

FIGURE 20. The rate of interferon production in a population of L_{929} cells infected with NDV-F virus.





Time (hours) post-infection.

Four monolayers of L929 cells were infected with 100 HA NDV-F for one hour at 37°C, then washed three times with PBS. Half the plates were refed with maintenance media, the remainder were trypsinised to a suspension of single cells and replaced in the culture dish with maintenance media. From both the trypsinised and reseeded cultures (▲), and the control infected cultures (●), supernatants were removed at various intervals. These samples were treated to inactivate virus and then assayed for interferon yield as described in methods. Each point represents the mean of two determinations.

trypsinised to a suspension of single cells and returned to the same culture dish. These data show that interferon production in both cultures began four hours post-infection and was maximal 10-12 hours post-infection. Trypsinisation of the infected cell monolayer did not effect the kinetics of production but did effect the yield. This reduction in yield was marked in the early hours post-infection (approximately 60%) but had recovered in the later hours following infection (approximately 20% reduction).

e: The role of the cells in the indicator monolayer and the cytolytic virus used to reveal the interferon-protected foci.

The role of the indicator monolayer cells is to detect interferon production from the single cells suspended above in agar. The presence of interferon-protected foci is indicated by a stain (0.1% crystal violet) which can clearly differentiate between foci of healthy cells amongst a background of dead cells.

It is thought that ageing (the length of time undisturbed in culture) and cell density can influence the sensitivity of some human cell lines to anti-viral action of interferon when used in the INAS assay (Carver and Marcus 1967). Ageing does not increase the sensitivity of L929 cells to the anti-viral action of interferon (data not given), possibly because the growth of L929 cells, unlike that of the human lines which do show the ageing phenomena, is not density dependent; L929 cells left for a period in culture will become over-confluent and then show reduced sensitivity to the action of interferon.

Figure 21 shows the anti-viral action of different concentrations of interferon, on cells seeded at different densities per surface area; all cell densities were infected with the same multiplicity of cytolytic

FIGURE 21. Effect of cell density on the sensitivity of cells to the antiviral effects of interferon.

	CELL DENSITY PER CM^2.			
	25 000	50 000	250 000	500 000
0.1u IFN/ml.*	-	-	++	++
1.0u "	-	++	++	++
5.0 "	-+	++	++	++
10.0 "	++**	++	++	++
50.0 "	++**	++	++	++

A monolayer of L929 cells was trypsinised to a suspension of cells to add the above cell densities into wells on a "micro-titer" plate. The cells were left overnight to attach, the growth media removed and maintenance media containing interferon at the concentrations indicated, was added per well for a further 24 hr period. The interferon containing media was then replaced with EMC virus at a multiplicity of 4 pfu per cell, for a final period of 12 hrs. All cells were then fixed using 100% methanol for one minute and then stained with 0.1% Crystal Violet. The cell in each well were then observed macroscopically and scored (-) no protection, (-+) partial protection, (++) complete protection.

* u IFN/ml = units interferon per ml.

** ++ = macroscopically complete protection, but microscopically some cell death seen.

virus (4pfu EMC per cell). The data shows that cells seeded at a density of 25 000 per cm^2 (no cell/cell contact) have reduced sensitivity to the action of interferon compared to cells seeded at higher densities. Although cell death could not be detected in cultures seeded at very high densities and treated with 0.1 units interferon, this was probably due not to interferon protection but to the inability of cell death to be detected in such cultures. The areas of cell death in the indicator monolayer at the completion of the assay, which contrasts with the foci of live cells, comprise 50-60% dead cells only, these cells round up leaving wide spaces between neighbouring cells, producing a pale background when the monolayer is stained, thus foci can be observed macroscopically. When cells are used at a high density, the live cells are rounded and with poor cell/cell contact and cell death cannot be clearly observed macroscopically even in virus infected cell cultures not previously treated with interferon. Foci produced in the single cell assay are most readily visualised by staining if cells in the indicator monolayer are just confluent; such cells are flattened, in close cell/cell contact and upon infection with a cytolytic virus such cultures round up leaving obvious areas of cell death. Therefore, because cell death cannot be accurately determined macroscopically in dense cell cultures, the indicator monolayer used throughout this study was seeded at a concentration of 50 000 cell/cm^2. This cell concentration (figure 21) can be protected from a multiplicity of 4 pfu EMC per cell by a concentration of 1.0 unit/ml interferon; these cells are not protected by 0.1 units/ml interferon.

The optimum multiplicity of EMC to reveal the interferon-protected foci in this assay was found to be 4 pfu per cell, the virus was left on the monolayer for a period of 12 hours.

THE OPTIMUM CONDITIONS FOR THE SINGLE CELL ASSAY

These conditions, based on information gathered from the above data, are fully described in methods and were used to obtain the single cell assay data presented in the remainder of this study. These data also show none of the manipulations involved in the assay inhibit the maximum possible production of interferon in NDV-F infected cells.

Sensitivity of the single cell assay to interferon.

Previous results (above) suggest that the procedures involved in the single cell assay do not inhibit interferon production from the single cells used in the assay. It is possible then that the reason why less than half the cell number in a virus infected culture produce interferon which is detectable in the single cell assay is that; the assay is only sensitive enough to detect cells which produce a sufficient quantity of interferon.

The amount of interferon produced by four cultures of L929 cells infected with NDV-F, is shown in figure 22. These data also relate the interferon yield per culture to the number of cells in the induced population. This assumes that all cells in the culture are producing equal amounts of interferon; if only a small percentage of the population were capable of producing interferon then the amount per producer cell would be larger. However the range of interferon production related to each culture cell can be seen to vary from 0.25 to 1.3 units per cultured cell.

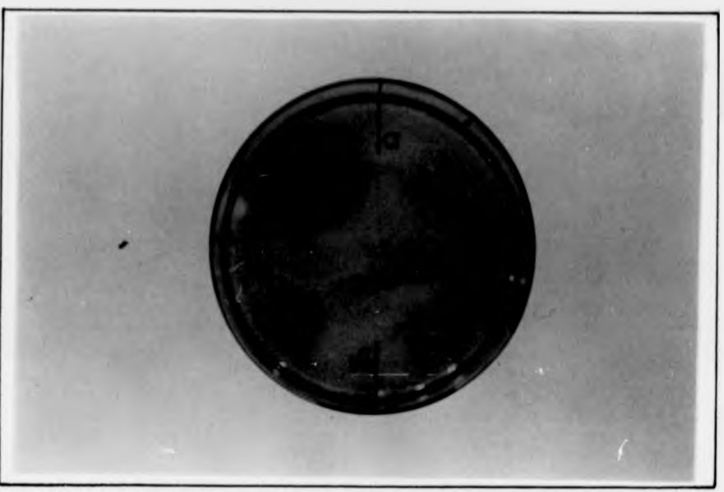
The sensitivity of the single cell assay is illustrated in figure 23. This shows photographs of a dish which had received interferon ranging from 0.5 to 50.0 units in one microlitre drops. The

FIGURE 22. The amount of interferon produced by cell populations infected with an interferon-inducing virus.

	EXPERIMENT NUMBER.			
	1	2	3	4
Total N^{o} of cells infected by NDV-F.	1 700 000	700 000	270 000	100 000
Total interferon yield (units)	2 000 000	630 000	63 000	130 000
Interferon yield per culture cell (units).	1.2	0.9	0.25	1.3

Various monolayers of L929 cells were infected with 100 HA NDV-F for one hour at 37°C, then washed three times with PBS and refed with maintenance media. Cell supernatants were harvested and cell numbers counted at 20 hrs post-infection; supernatants were treated to inactivate virus and assayed for interferon yield by INAS assay as described in methods.

FIGURE 23. The limits of sensitivity to interferon seen in the single cell assay.



1.5 mls of agar-media were added to a 5cm monolayer of L929 cells, the plates were transferred to 4°C for ten minutes to allow the agar to gel and harden. Using a ' Hamilton syringe ' (1 - 10 ul capacity) one microlitre aliquots of media, containing various amounts of mouse interferon, were injected into the agar into the four areas indicated in the above photograph. The plates were incubated for 20 hrs then the agar-media (plus interferon) was removed, the cell sheet washed three times with PBS, and infected with a cytopathic virus (EMC) for 12 hrs. Subsequent fixing and staining of the cell monolayer revealed areas of protected cells underneath the agar which contained the interferon sample.
a; 0.5 units IFN, b; 2.5 units IFN, c; 5.0 units IFN, d; 50.0 units IFN.

ability of these amounts of interferon to confer protection upon the indicator monolayer is shown by the areas of protected cells produced by each drop. The results show that amounts as low as 0.5 units can produce detectable foci within the assay, thus the sensitivity of the assay is within the range of yields as shown in figure 22. This data also shows that the size and the intensity of the foci are in proportion to the concentration of interferon applied. This latter point suggests that the foci produced in the single cell assay vary in area and intensity because the single cells used in the assay were each producing different amounts of interferon. If this suggestion is correct, ie: that interferon production is unequal amongst the cell population then it remains possible that some cells are producing amounts of interferon too low to be detected in the assay.

This point can be further clarified by examining the range of foci that are produced in an assay plate. Since it seems likely from the variation in area and intensity of the foci, that sub-populations of cells exist amongst a cell culture which each produce different amounts of interferon; the distribution of these sub-populations can be examined by measurement of the area of these foci. Figure 24 shows such an analysis. The variation in diameter of foci was examined in assay plates produced from four separate L929 cell assays, and the foci were grouped into one millimetre size groups. These data show that three out of four assay plates contain these size groups in an approximately normal distribution, while one assay plate shows a slightly skewed distribution. If the single cell assay was only sensitive enought to detect cells producing a minimum amount of interferon then the skewed distribution seen in one out of the four plates will have been expected in all assay dishes. However since three out of four plates show an approximately normal distribution then it seems more probable that

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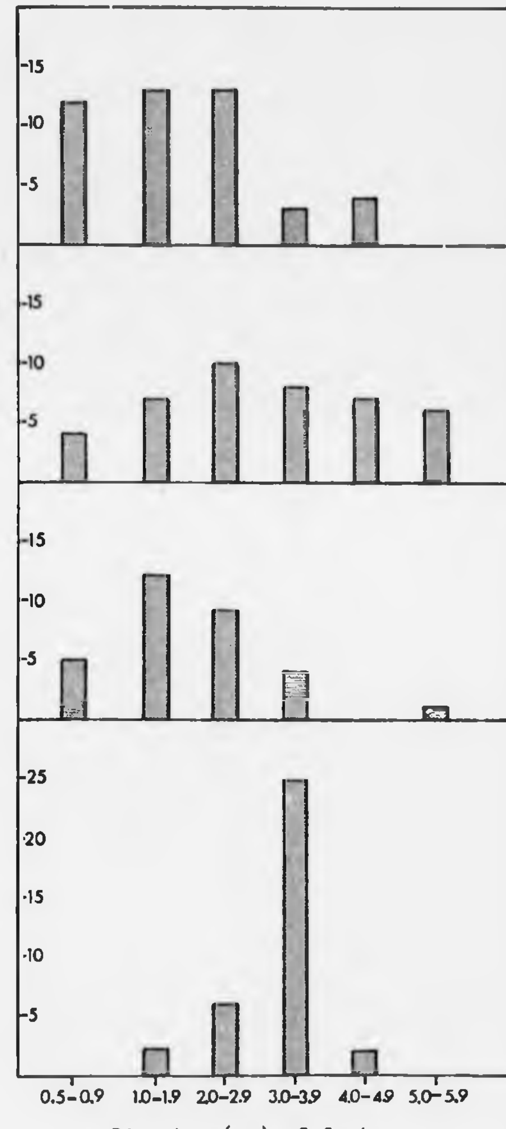
FIGURE 24. Variation of the size of foci seen in the single cell assay.



Single cell assay plates from four separate assays (using NDV-F infected L929 cells; see methods for details), were examined for the number and size of foci.

this assay is capable of detecting all of the cells which are producing interferon. This does not rule out the possible existence of a sub-population of cells which produce a range of very low amounts of interferon.

One further point which tends to support the argument that the assay can detect all the cells producing interferon is that if virus-infected L929 cells are placed in a single cell assay using indicator monolayer cells which are known to have greater sensitivity to interferon, (eg: C3H cells) then a larger number of foci are not seen (data not given). C3H cells frequently show an increase in sensitivity to the anti-viral action of interferon of approximately 0.5 log units (my observation); however these cells were not suitable for routine use as the indicator monolayer cells because their sensitivity to the cytolytic virus altered considerably with increasing passage.

Thus observations on the sensitivity of the single cell assay does suggest that the assay is capable of detecting all the cells capable of producing interferon in the NDV-F/L929 interferon-inducing system.

Can the percentage of virus infected cells able to produce interferon be increased ?

It has been shown that when L929 cells are infected with an optimum amount of NDV-F only a small percentage of cells produce interferon (figure 14), and furthermore, that this percentage of cells probably produces unequal amounts of interferon. It was therefore of interest to see if the interferon production at the population and the single cell level could in any way be increased. This was done for two reasons; firstly, the question of why all the cells in a population do not produce interferon is by itself interesting, and secondly, the

main aim of this study was to examine interferon production in differentiating cells which initially did not produce interferon. Therefore, to accurately observe the latter it is necessary to use interferon inducing conditions that optimised interferon production in 100% of the cells in the cell culture under study.

Figures 25 and 26 illustrate two attempts to increase the interferon yield in L929 cells. Figure 25 shows the variation in interferon production per culture in cell cultures which had been pretreated with small amounts of interferon prior to being exposed to an interferon inducer (this is termed priming and has been described in the introduction). Cell cultures were primed with 3-12 units per ml of interferon and then induced with either virus or with dsRNA, the subsequent interferon yields showed that priming did not enhance production, therefore interferon production was not examined at the single cell level.

Figure 25, shows the effect of pretreatment of L929 cells with sodium butyrate. The interferon yield can be seen to increase by approximately six fold, but the single cell assay data show that this increase is not due to an increase in the number of cells able to produce interferon and therefore must be a result of an increased output per existing producer cell.

These results show that the L929 cell population can be induced to produce larger amounts of interferon by appropriate pretreatment, however, in the case of butyrate, this pretreatment does not alter the number of cells able to produce interferon.

Why cannot one hundred percent of culture cells produce interferon?

Two possibilities suggested themselves as to why all culture cells

FIGURE 25. The effect of pretreatment of cells with small amounts of interferon, on the yield of interferon obtained following subsequent induction.

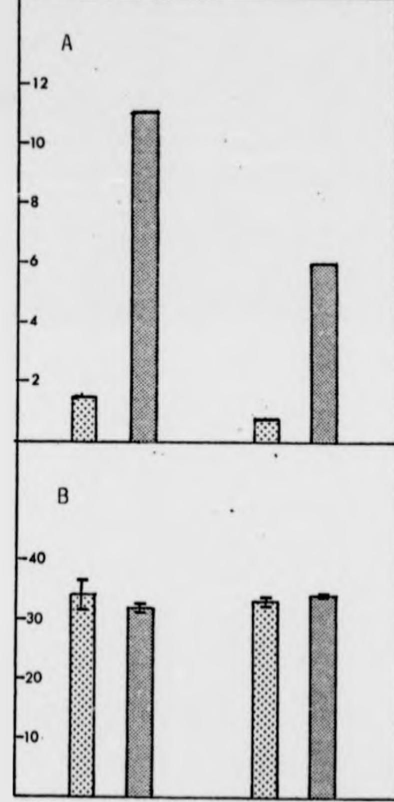
Interferon inducers	Amount of IFN used to pretreat cells (u/ml).			
	0.0	3.0	6.0	12.0
NDV-F virus (100 HA)	4.6*	4.7	4.6	4.6
Sendai virus (10 HA)	5.4	ND**	4.8	4.9
Synthetic ds-RNA, Poly rI:rC. (10µg + 100µg DEAE dextran)	5.4	ND	5.4	5.5

Confluent monolayers of L929 cells (containing approximately 10^6 cells) were incubated with maintenance media,containing interf--eron at various concentrations, for a period of six hours. The cells were then washed with PBS and virus or dsRNA added as ind--icated for a further hour. The cells were again washed three times with PBS and refed with maintenance media. At approximately 20 hours post-infection the cell supernatants were harvested and cell numbers counted; the supernatants were treated to inactivate virus (see methods) and to inactivate dsRNA (by addition of ribonucleases) and assayed for yield of interferon by INAS assay (as described in methods.). Triplicate monolayers yielded similar results.

* = Interferon yield expressed in log units.

** = Not done.

FIGURE 26. The effect of pretreatment of cells with Butyrate, on interferon yield per population and on the single cell production of interferon.



Four monolayers of L929 cells were seeded at a density of 5×10^5 per 3cm dish; two dishes were refed with growth media, the remainder with growth media containing one millimolar Sodium Butyrate. After 48 hours the cells were washed and infected with 100 HA NDV-F for one hour at 37°C, the cells were again washed three times with PBS and then trypsinised to a suspension of single cells. A small aliquot of this suspension was used to provide cells for the single cell assay*; the remainder were returned to the culture dish for approximately 20 hours. The supernatants were harvested and the cell numbers per culture dish counted; the supernatants were treated to inactivate virus (see methods) and then assayed for interferon yield by INAS assay as described in methods.

(▩), Control cultures. (■), Butyrate treated cultures.

* single cell assay is described in methods.

could not produce interferon upon virus infection.

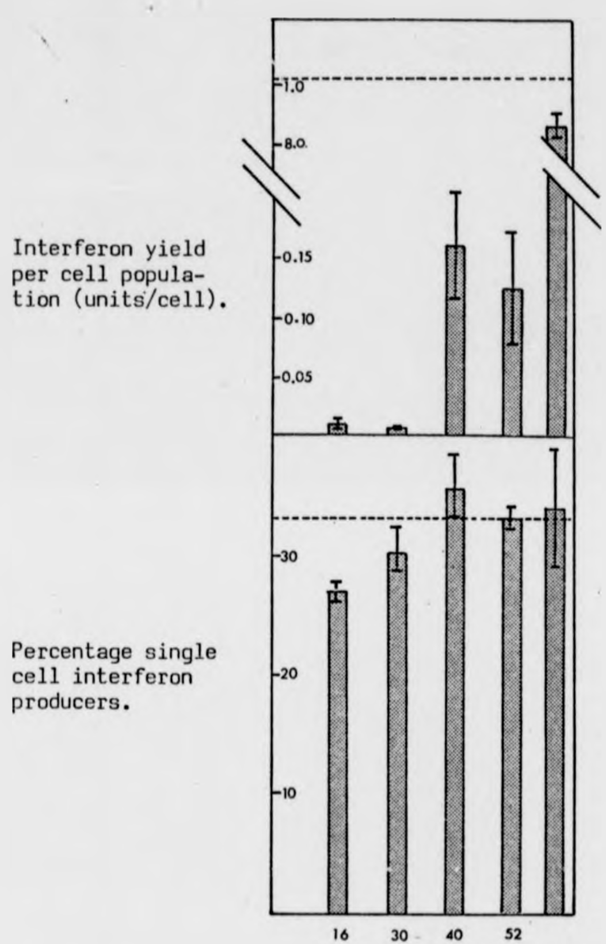
(a) The ability to produce interferon is dependent upon the stage of the cell cycle of the individual cell, this will vary between different cell lines.

(b) The cell population are heterogeneous in their ability to produce interferon, any cell line will contain multiple sub-populations of cells which differ slightly in their genetic constitution, which may influence interferon production.

Figure 27 shows the interferon production over a period of 52 hours, in cell cultures synchronised with respect to cell cycle. The interferon per culture can be seen to increase by approximately 15 fold at approximately 30 hours post-seeding, but no such increase was seen in interferon production at the single cell level. The interferon yields of the cell culture were reduced compared to control levels whereas the percentage of single cell producers did not differ from that seen in control cultures.

Figure 28 shows the results obtained from examination of interferon production at the single cell level in L929 cell populations which had been cloned from single cells. Fifty-four cloned colonies were obtained, the percentage of single cells able to produce interferon was examined in 24 of these (all contained approximately 50 000 cells). A variation was seen amongst these colonies in the percentage of single cells able to produce interferon, however, no colonies showed a greater percentage of single cell interferon producers than that seen in control cultures; but several of the colonies showed a reduction in the percentage of single cell interferon producers. These data suggest that the cell population is heterogeneous in its ability to produce interferon but, this heterogeneity does not explain the inability of some cells in the population to produce interferon.

FIGURE 27. Interferon production at the population and the single cell level in L929 cells synchronised with respect to cell cycle.



Interferon yield per cell population (units/cell).

Percentage single cell interferon producers.

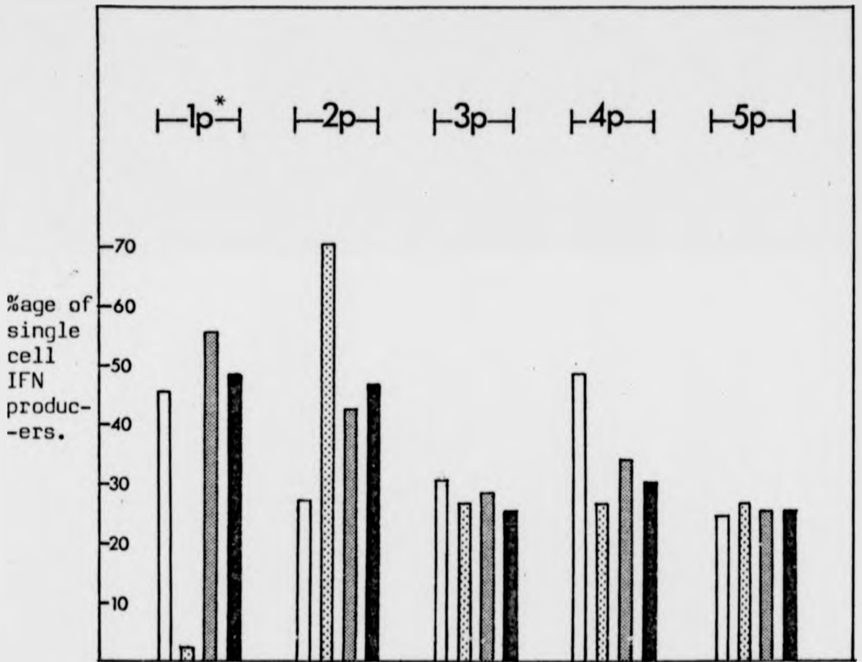
Time (hours) post-seeding.

L929 cells synchronised with respect to cell cycle, were obtained by gentle agitation of a recently confluent monolayer of cells. The suspended cells were sedimented by centrifugation at 2 000 rpm for five minutes and resuspended in fresh growth media at a density of 1×10^5 per ml, then seeded into 3cm dishes. Control cell cultures were obtained by trypsinisation of similar monolayers, these were seeded at the same density. At various intervals post-seeding duplicate cell cultures were induced to produce interferon by infection with 100 HA NDV-F for one hour. All cultures were then treated with antiserum raised to NDV-F for a further hour then trypsinised to a suspension of single cells; a small aliquot of which were used in the single cell assay (performed as described in methods), the remainder being returned to the culture dish to provide samples for assay of interferon yield (cell supernatants were treated to inactivate virus then assayed by INAS assay as described in methods).

(----) = mean values obtained from control cultures.

(▩) = cells synchronised with respect to cell cycle.

FIGURE 28. The percentage of single cell interferon producers in L929 cell populations cloned from single cells.



A monolayer of L929 cells were trypsinised to a suspension of single cells, this was diluted to a concentration of 1 000 cells per ml, and 1μl of this dilution added to separate wells in two "microtiter" plates. The wells were examined under light microscope and only those containing one cell were cultured in growth media. A random selection of cloned cell populations was examined in the single cell assay for interferon (as described in methods). The above histogram shows the variation in the percentage of single cells able to produce interferon; as the cloned cell populations are passaged. Three cloned populations are illustrated (□) C3, (▒) C5, (▓) C6; in addition results are shown from a population of non-cloned L929 cells (■), which were assayed simultaneously. Each bar represents the results from triplicate cultures which were tightly grouped around the mean.

* Passage number since cloning.

Interferon production at the single cell level was examined in three cloned populations during five subsequent passages and compared to control cultures (figure 28). The behaviour of the control cultures altered during the period of the experiment (this phenomenon has already been referred to in figure 13). However, these data do show that the cloned cell populations differ from the control cultures only for the first two passages, thereafter the cloned cell populations behave similarly to the control cultures.

SECTION ONE ---- DISCUSSION

The data presented above has shown that results from an assay capable of detecting interferon production from single cells as described by Osborn and Walker (1969) and Kronenberg (1977), was complicated by the presence of the interferon-inducing virus used initially to infect the test single cells (see figure 11). Cells infected with NDV-F produced material capable of producing foci within the single cell assay, and the production of these foci could be completely prevented by prior incubation with antiserum raised to NDV-F. during the time course of the assay, the virus infected cells released NDV-F particles capable of directly inducing interferon in the indicator monolayer cells, the diffusion of these particles was only slightly inhibited by the agar used to suspend the single test cells. Thus the assay as described by Osborn and Walker (1969) and Kronenberg (1977) was in reality capable of detecting two events, firstly, interferon production from the virus infected single cells suspended in agar and secondly, the ability of the cells in the indicator monolayer to respond to the virus which desorbed from these single cells.

A modified single cell assay was designed (and is fully described in Methods) which incorporates a stage wherein the interferon-inducing virus is neutralised by specific antiserum. This neutralisation stage involved treatment of the test cell monolayer immediately following the one hour infection period and prior to the trypsinisation of the test cells, and was sufficient to completely remove any foci caused by the inducing virus alone (see figures 10 and 11). The resultant foci seen in the modified single cell assay can be shown to be solely due

to interferon production from the single test cells. Incorporation of antiserum to mouse interferon completely removed all the foci produced in the modified assay by virus infected cells.

The modified single cell assay contains five main stages, and each stage was examined to assess its role in influencing the result obtained with the assay.

NDV is known to be a good inducer in mouse cells (Meager and Burke 1972, Wagner and Huang 1966, Fleischman and Simon 1974), and NDV-F is used routinely to induce interferon production in many different mouse cell types. In addition this strain has no pathenogenic effects and has less cytolytic effects on cells in culture than the other mesogenic and velogenic strains of NDV. The data presented above (figure 14) has shown that cultures of L929 cells infected with NDV-F produce a maximum interferon yield per culture at a particular virus/cell ratio, and that increasing the amount of virus does not increase the interferon yield per culture. Examination of the behaviour of single cells in this system, shows that only approximately one third of the cell population are producing interferon; this suggests that the majority of cells are not capable of producing interferon in response to infection by NDV-F. There are two possible reasons for this, either these non-producer cells are not infected by this virus (or the formation of the actual interferon inducer molecule does not occur) or, these cells do not produce interferon in response to infection by this virus (alternatively these cells could produce amounts of interferon too low to be detected by the assay). The first possibility (ie: that the virus does not infect the majority of cells) is unlikely, since Brown _et al_. (1980) have shown that one hundred percent of L929 cells can be infected by infectious NDV. In addition Fleischman and Simon (1973) have shown that one hundred percent of L929 cells can be

infected by Mengovirus. Thus it seems improbably that a large sub-population of culture cells cannot be infected by virus, which suggests that a large sub-population of cells are unable to produce interferon in response to virus infection. The finding of phenotypic variation amongst individual cells in a cell culture is not unexpected even in recently cloned cultures. The individual cell genotype can become abnormal in passage resulting in the existence of discrete sub-populations of cells which each respond differently to the same stimulus; the sum of the behaviour of these discrete sub-populations comprising the "normal" response shown by the cell population.

The modification made to the original single cell assay involves treatment of virus-infected cells with antiserum raised to the inducing virus. Treatment of virus infected cells prevented the desorbtion of virus from infected cells during the course of the assay (figure 11). It is not clear exactly how antisera is involved in the neutralisation of virus at the cellular level, nor is it known how or if neutralisation of virus at the cellular level affects subsequent interferon yield. Treatment of infected cells does act to prevent NDV-F from the single cells from reaching the indicator cells below; this might be because the antiserum acts to prevent the desorbtion of the virus or to neutralise the free virus. The results presented above do however show that treatment of virus infected cells with specific antiserum at one hour post-infection does not inhibit subsequent interferon yields. This latter point is relevant since it is proposed that the formation of dsRNA, an initial step in the replication of this virus, may constitute the actual interferon inducer (Morser and Burke 1979) and interference with this step by the neutralising antibody may have affected interferon yields.

It has also been shown above that it is possible to dispense cells into the assay with a reasonable degree of accuracy. The degree of inaccuracy increases with cell numbers of fifty, or less, but as shown in figure 18 cell numbers of one hundred can be dispensed with an error of ± 15%. The result of the assay relates the number of cells added to each assay plate, to the number of foci counted at the completion of each assay. The number of foci seen will depend on the sensitivity of the assay, and data presented above has shown that the assay can detect interferon amounts as low as 0.5 units per microlitre. The sensitivity of the conventional interferon assays (see Introduction and Methods) depends on the cells and the system in use and with a sufficiently sensitive system it is theoretically possible to detect one international reference research unit of interferon. Thus the two types of assay have a similar but not directly comparable sensitivity. Examination of the foci produced within the assay (figure 24) suggests that all of the interferon producer cells in a cell culture can be detected by the assay; although it still remains possible that cells producing vastly reduced amounts of interferon do exist but cannot be detected by the assay.

The timing of events within the assay are the same as those used by Kronenberg (1977); virus infected cells are left in the assay for approximately 18 hours. If this period of incubation is greatly extended then the foci become larger in area and less distinguishable from each other. However this period of 18 hours is not rigid; because similar results are obtained when the single cells are removed any time from 14-20 hours post-infection. The substitution of agarose for agar was not found to influence the result of the assay.

The monolayer used throughout the results presented in this study was composed of L929 cells and the condition of the monolayer was of

great importance in producing clear, easily discernable foci. The parameters which affected the behaviour of the monolayer were the cell density, the even distribution of cells across the dish and the attachment of cells to the substrate. The effect of cell density on the sensitivity of the cells to EMC has been described and this remained unchanged throughout use of the assay; the adhesive behaviour of the L929 cells was however, found to fluctuate. The assay involves a stage whereby the agar and single cells are removed, the monolayer washed and then infected with a cytolytic virus; this procedure periodically resulted in the partial or complete removal of the cell monolayer. To prevent this, the culture plates used for the single cell assay were coated with 0.1% gelatin, and the plates were air dried before use (see methods). The plates were further incubated with growth media for one hour at 37°C before the indicator monolayer cells were added. This ensured a more even distribution of these cells, and in particular prevented the central clumping of cells which always occurred without this pretreatment process. If the cells in the indicator monolayer were not evenly distributed, the cells tended to form clumps and swirls which obliterated much of the foci produced in the assay.

A variety of stains (Trypan Blue, Crystal Violet, Neutral Red, Giemsa) were used to identify the foci formed in the monolayer, and a 0.1% solution of Crystal Violet was found to give optimum results.

Thus the modified single cell assay (as described in Methods) can detect interferon production from single cells without detecting the virus used to induce interferon in the test cells. In addition the procedures involved in this assay do not hinder interferon production from the single test cells. The assay can be used for a variety of

cell types (human and mouse cell types have been assayed successfully) and for a variety of interferon inducers providing it can be ensured that the inducer does not directly affect the indicator cells. The procedures involved are straightforward, relatively simple and take an average total of approximately four hours to complete.

Interferon induction and production are usually examined in terms of the average response of a large population of cells. Data obtained from single cell studies can more suitably answer questions concerning these processes. For example, do procedures such as priming, superinduction and pretreatment with certain chemicals like butyrate, which increase the interferon yield; result from an increased output per cell or from an increase in the number of cells able to produce interferon? Such information can more accurately evaluate 'good' and 'poor' interferon inducers and may help to isolate cells which are high producers of interferon. In addition, the single cell assay can be used to identify interferon production in cell populations too small to produce amounts of interferon which could be assayed in the conventional assay systems, eg: cell populations which make up the early mouse embryo. Because of the lack of a suitable assay the previous work on interferon production in the mouse embryo has been performed using tissue explants taken from embryonic tissues, instead of performing direct analysis on the embryonic tissues themselves.

Using the modified single cell assay to examine interferon production in NDV-F infected L929 cells it has been shown (figure 13) that between 22% and 49% of cells can produce interferon. The percentage of cells able to produce interferon remained constant for periods of one to two months only and was found to oscillate between the two above extreme values during the course of this study. The source of this variation is not clear. The cells were obtained from stocks maintained

in liquid nitrogen, and further stocks were prepared from these cells and similarly maintained and used throughout this study. In addition a large quantity of NDV-F was also prepared at the begining of this study (see Methods), dispensed into 0.25 ml aliquots which were kept at $-70^{o}C$, the virus was not used once thawed and was sufficient for use throughout this study. The variations seen in interferon production at the single cell level also did not correlate with new batches of either media or of serum.

The fluctuations seen in the percentage of cells able to produce interferon using apparently constant inducing and assay conditions, may indicate that results obtained from different laboratories are difficult to compare directly; no clear pattern of the behavior of single cells in populations induced to produce interferon has yet emerged. The majority of the single cell assay data whether of the type described by Kronenberg (1977) or whether involving the examination of isolated single cells (Fleischman and Simon 1974) are all complicated by the fact that the virus used to induce interferon may also directly effect the indicatory cells. Brown et al. (1980) and Fleischman and Simon (1974) have shown that 100% of L929 cells infected by a plaque-forming unit of NDV will produce interferon, while Kronenberg (1977) using the assay illustrated in figure 4 has shown that only 33-41% of L929 cells will produce interferon when infected with excess amounts of NDV.

From the results presented above it can be seen that if the single cell assay method as published by Kronenberg (1977) is used with the L929:NDV cell/inducer system then the results show that between 100-150% of cells can produce interferon; such results are obviously wrong. Modification of the assay by introducing a neutralisation stage reduces these figures to 22-49%. It is not clear why the use of different methods (the original

and the modified assay) should produce such similar results with L929 cells. Kronenberg (1977) also examined the percentage of single cell producers in a population of NDV infected HFF (human foreskin fibroblast) cells and found that 50% of cells could produce interferon when infected with virus. When NDV-F infected HFF cells are placed in the modified single cell assay only 18% are found to produce interferon (data not given) using a variety of cells as the indicator monolayer cells including GM2504, a human cell line trisomic for chromosome 21 and therefore possessing greater sensitivity towards interferon. The discrepancy between the results obtained by Kronenberg (1977) and those obtained with the modified single cell assay may be due to the direct induction of foci in the indicator monolayer by NDV itself.

Interferon production in other mouse cell types infected with NDV-F is described in a later section; briefly three differentiated teratocarcinoma cell lines (F9Cl 9, PYS, PSA 5E) which have endoderm-like characteristics, were infected with NDV-F and placed in the modified single cell assay. The percentage of single cell interferon producers was found to be 18% (F9Cl 9), 3% (PYS), 0.4% (PSA 5E), the interferon yield per culture was found to correlate with the number of cells able to produce interferon (ie: PYS cell cultures produced approximately six times more interferon than PSA 5E cultures).

Thus it seems that various differentiated mouse cell types vary in their ability to produce interferon in response to NDV-F, and the source of this variation resides in the number of single cells which are able to produce interferon, and not in the variation in the output of interferon per cell.

An attempt was made to increase the interferon yield in L929 cells infected with NDV-F, by pretreatment of cells. Priming of cells with

small amounts of interferon did not increase yields. Although priming has been reported in L929 cells (Stewart 1979 p233-238), the lack of this effect with the cells under study may be because the cells were already producing maximum amounts of interferon. Pretreatment of the cells with sodium butyrate did however increase interferon yields by approximately six fold in the cell cultures (figure 26), but did not alter the number of producer cells. Butyrate has been shown to enhance interferon production in cultures of Namalwa cells, a human lymphoblastoid cell line (Baker *et al*. 1979); direct measurements of the amount of RNA produced in butyrate treated Namalwa cells has shown that the amount of interferon mRNA is increased in butyrate treated cells (Morser *et al*. 1980). Thus butyrate is thought to increase the yield of interferon per culture by increasing the rate of transcription of the mRNA. This observation is consistent with the finding in L929 cells that butyrate increases the output per cell without increasing the number of cells able to produce interferon.

Thus a situation exists in L929 and other mouse and human cell types wherein only a small proportion of cells will produce interferon upon virus infection of the culture. A similar situation can be seen with Sendai virus-infected cells; L929 cultures infected with Sendai contain approximately 10% single cell producers, while Namalwa cells similarly infected contain approximately 40% single cell producers (data not given). The production of interferon at the single cell level (and therefore the population level) in response to one virus does vary between cell types, and thus is influenced by the genotype, however the output per cell can be influenced in some cases by appropriate treatment of the cells.

Why cannot one hundred percent of the cell population produce interferon? Two possible reasons were suggested. The inability of the

major proportion of the cell population to produce interferon may be due to the stage of the cell cycle occurring at the time the individual cell was exposed to the virus. RNA and protein synthesis in eukaryotic cells are continuous except for the actual period of mitosis; nuclear RNA synthesis is inhibited completely during this stage and protein synthesis much reduced (reviewed in De Robertis et al. 1975). The production of interferon occurs by activation of a previously inactive gene, transcription and translation follow to produce the interferon protein (see Introduction), and this process could be envisaged as being delayed in cells undergoing mitosis. However the period of mitosis is relatively short compared to the remainder of the cell cycle, and the assay conditions are such that test cells in a monolayer are infected with virus but not trypsinised to a single cell suspension until two hours later; thus the viral interferon inducer will be present in the cell after mitosis has been completed and before the assay is performed. Cells which had been synchronised with respect to cell cycle (figure 27) do show an increase in interferon yield after approximately 30 hours seeding, but show no increase in the number of cells able to produce interferon. This suggests that the cell cycle does not influence the number of single cell producers. However, the method used to synchronise the cells was simplistic and the degree of synchrony within the culture was not checked by other means; this and also the reasons behind the reduced output seen in these cells need to be re-examined. It is interesting to note that Martin et al. (1969) have reported that the gene for tyrosine amino transferase cannot be induced in synchronised cell cultures during G2, M and early G1; although translation of the mRNA can occur during this period.

The inability of the major proportion of the cell population to produce interferon may reflect the phenotypic variation existing within

the cell population. No clear result was obtained from the examination of interferon production in colonies cloned from single L929 cells. A variation in the number of single cell interferon producers, different to that of control cultures, was seen in the cloned colonies (figure 28) but this variation was only negative, no cloned colonies were identified containing more than fifty percent single cell producers. In addition the difference between the cloned colonies and the control cultures decreased with increasing passage.

This experiment suggests that phenotypic variation amongst L929 cells does influence the number of single cells producing interferon upon virus infection, but only to a limited degree. The fact that even first passage cloned colonies did not contain some which were capable of one hundred percent single cell interferon production may suggest that production of interferon at the single cell level is controlled, not by one, but multiple factors; only some of which are controlled by the genome. De Maeyer and De Maeyer-Guignard (reviewed in De Maeyer and De Maeyer-Guignard 1979) have identified the presence of autosomal loci (which are not the structural genes for interferon) but which can influence the levels of circulating interferon in BalbC and C57B16 mice. Four loci have been identified so far each with two alleles regulating a high or low interferon response to a virus inducer. These four loci control the circulating interferon response to three viruses, NDV, Sendai and mouse mammary tumour virus (Sendai induced interferon production is controlled by two loci). In addition these two strains of mice, do not contain loci controlling interferon production in response to infection by Influenza or Sindbis virus. The alleles controlling circulating interferon levels have been shown to be expressed only in cell originating from the bone marrow stem cells, their effects

are not seen in mouse embryo fibroblasts established from these two strains are examined.

Thus interferon production is controlled not just by the presence of structural genes, but by genes which control the final yield of interferon in response to specific inducers, and it would be of interest to examine the number of single cell interferon producers present in macrophage cultures taken from high and low producer animals. However, similar genes controlling final interferon yields have not been identified in non-bone marrow cells although it is possible that they may exist.

The existence of such genes and also of the effects on interferon production by the cell cycle discussed above, might explain a feature seen in all the single cell assays performed. In all assays the foci produced varied in size and intensity, the diameter of the foci varied ten fold on average; this, is has been suggested, is due to differential production of interferon by the test single cells, and it is this feature rather than the number of cells able to produce interferon, which may be regulated by the cell cycle and any postulated genes which could influence interferon yields.

SUMMARY

A modified single cell assay has been designed which can detect only interferon production from single cells, this assay is probably sensitive enough to detect all cells capable of producing interferon within a given cell population. The question of why one hundred percent of cells do not produce interferon has been explored, but as yet no

clear answer is forthcoming. However the analysis of the NDV/L929 inducer/cell system has shown that alteration of the treatment of the cells in the assay does not alter the result of the assay; this therefore suggests that the assay can be used with confidence to analyse interferon production in the differentiated cell systems (described in the Introduction) which initially lack an interferon system.

SECTION TWO ---- THE INTERFERON SYSTEM IN DIFFERENTIATING TERATOCARCINOMA CELLS

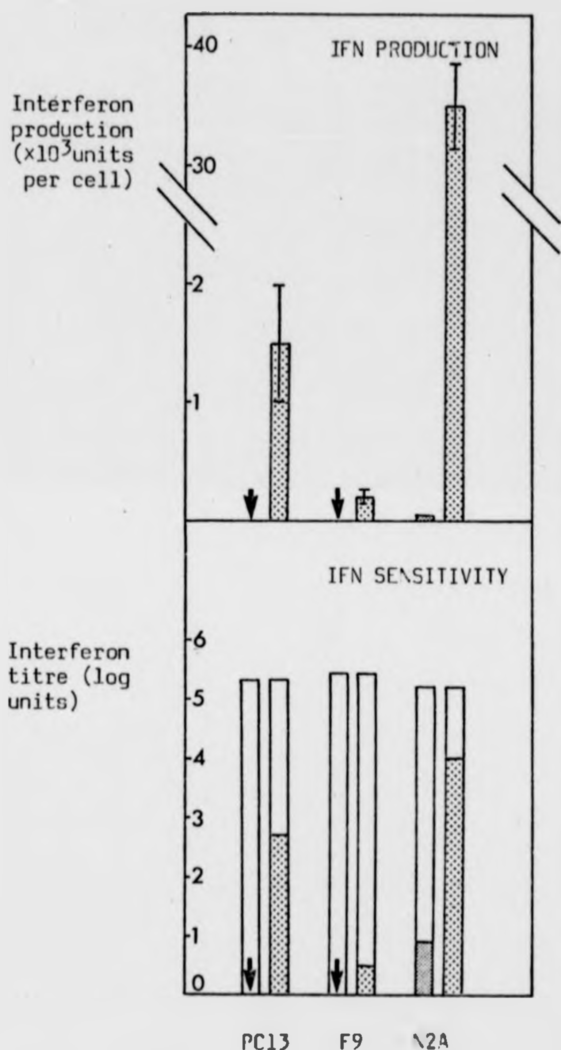
SECTION TWO ---- RESULTS

Appearance of interference inducibility and sensitivity in differentiating teratocarcinoma cells

The status of the interferon system in three 'nullipotent' teratocarcinoma cell lines was examined; 'nullipotent' cell lines are those which maintain an embryonal carcinoma cell morphology and phenotype when cultured in routine tissue culture media. Figure 29 shows the ability of teratocarcinoma cells, cultured with and without retinoic acid, to produce interferon in response to a virus inducer and also to develop an antiviral state in response to interferon treatment. These data show that the embryonal carcinoma (ec) cells of two of the three cell lines examined do not produce interferon and are not sensitive to its antiviral action. The ec cells of the third cell line (Nulli 2A) did show low levels of interferon inducibility and antiviral sensitivity which were found to increase considerably as the cells were caused to differentiate.

If the results from the three differentiated cell cultures are compared, it can be seen that the interferon system is most active in the Nulli 2A cells and least active in the F9 cells. All three cultures appear to contain 100% differentiated cells (see later), but are each capable of producing varied amounts of interferon. It has already been shown in section one that the interferon yield per cells from differentiated cell cultures varies considerably, this variation is also shown by the differentiating cultures in figure 29. The existence of this variation suggests that the absolute extent of the

FIGURE 29. The status of the interferon system in differentiated and undifferentiated teratocarcinoma cells.



Three teratocarcinoma cell lines (PC13 C15, F9 and Nulli 2A), were cultured for eight days either in growth media, or in growth media containing 3×10^{-6} molar retinoic acid.Cell cultures were then assayed for interferon production following virus induction (as described in methods); and also for sensitivity to the antiviral action of interferon. The latter was obtained by assaying a sample of IFN of known titre simultaneously upon the teratocarcinoma cells and upon control L929 cells, the difference in IFN titre between these cells indicates the sensitivity of the teratocarcinoma cells towards interferon.

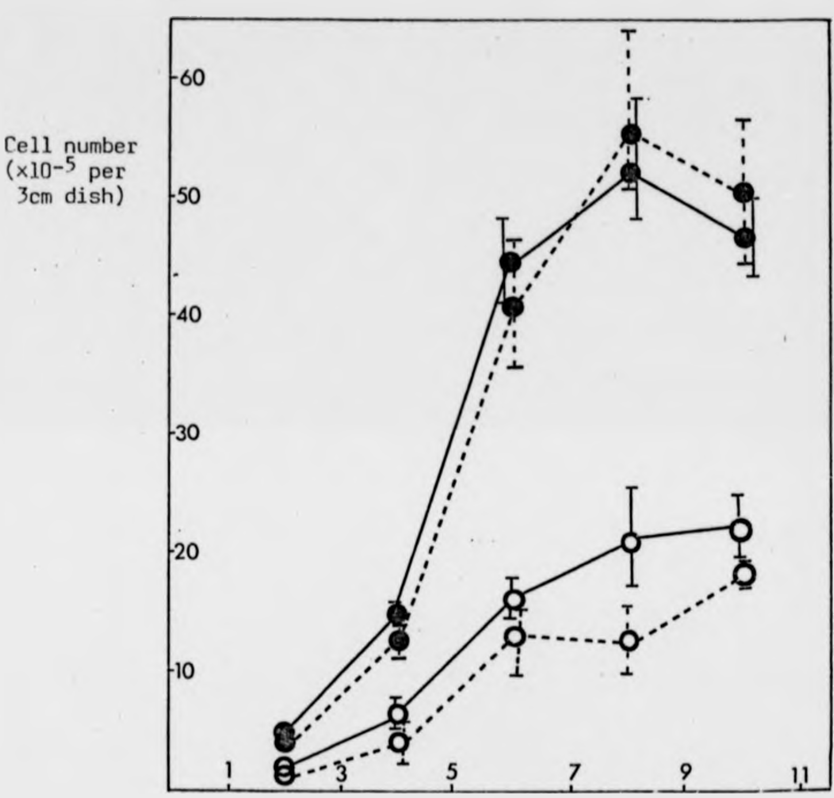
(▩) cells cultured in growth media
(⊡) cells cultured in media containing 3×10^{-6}molar retinoic acid.
(□) results obtained using control L929 cells.
↓ = zero result obtained.

production is not related to the extent of differentiation shown by the cells in the culture.

The sensitivity of teratocarcinoma cells to the anti-proliferative effects of interferon is illustrated in figure 30; PC13 Cl 5 embryonal carcinoma cells were cultured over a period of ten days in media supplemented with combinations of retinoic acid and interferon, or with neither. Ec cells cultured in normal growth media showed a sigmoid-shaped growth curve and a doubling time of approximately sixteen hours under the culture conditions used, and the growth curve of ec cells cultured in media containing interferon remained unchanged. Ec cells cultured in media containing retinoic acid showed both a reduced rate of growth and also a reduction in the density of the culture at completion of the experiment, when compared to control cultures. If ec cells cultured in media containing retinoic acid were treated with interferon, the initial rate of growth remained unchanged, but the final density of the culture can be seen to be slightly reduced, compared to cultures treated only with retinoic acid.

Thus the data from these two figures show that the ec cells of the 'nullipotent' cell lines PC13 cl 5 and F9 do not produce interferon and are not sensitive to its action. The differentiated cell type generated in these cultures by treatment with retinoic acid could produce interferon and were sensitive to the anti-viral action of interferon, and were probably marginally sensitive to the anti-proliferative action of interferon. The ec cells of the third cell line examined (Nulli 2A) produced small amounts of interferon in response to virus infection and showed slight sensitivity to the anti-viral action of interferon. Both these aspects of the interferon system increased greatly as the cells were caused to differentiate by treatment with retinoic acid.

FIGURE 30. The sensitivity of teratocarcinoma cells to the anti-proliferative action of interferon.



A teratocarcinoma cell line (PC13 C15) was seeded at a density of 2×10^4 per 3cm dish at day zero. Cultures were refed at day zero with growth media containing either 3×10^{-6} retinoic acid plus 10^4 units per ml interferon (--O--); or with 3×10^{-6} retinoic acid (—O—); or with 10^4 units per ml interferon (--●--); or with none of these (—●—). Cell numbers from triplicate dishes from each of the culture conditions were counted at the times indicated.(only trypan blue excluding cells were counted). All cultures were refed every two days.

Why is the interferon system inoperative in ec cells?

The interferon system has two components, these are production of the interferon molecule itself and the sensitivity of the cell to the pleiotropic actions of interferon. These two phenomena are distinct and separate cellular events, and, although they are usually present together in normal cells, they can be separately present or absent in some cells (see Introduction). The inhibition of the interferon system in ec cells may be due to one or more factors operating within the cell and an investigation of the nature of the block inhibiting one part of the interferon system may shed light on the degree of inhibition of the genome and its activities within the undifferentiated cell.

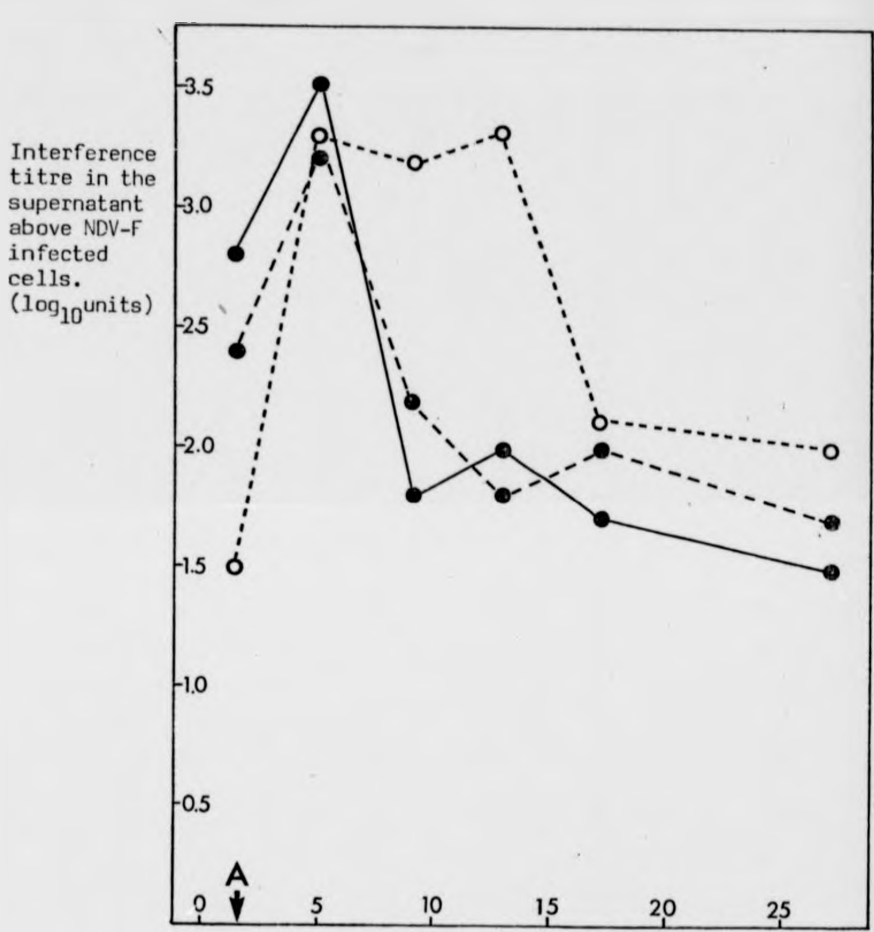
Interferon production is not seen in virus infected primed or unprimed ec cells, but it is seen in differentiated endoderm-like cells. Since the production of interferon is an inducible event there are two main processes which might be involved in the inhibition of interferon production in the ec cells. Firstly the molecule comprising the actual interferon inducer might be absent or reduced in the undifferentiated cell, and therefore the stimulus to activate the interferon gene might not be available, and secondly the ec cell may be unable to produce and secrete a functional interferon molecule in response to the production of the interferon gene inducer molecule.

It is not knownexactly how the transcription of the interferon gene is controlled. Because of the known efficiency to induce high yields of interferon, of both negative strand viruses and synthetic dsRNA molecules (eg: poly rI:rC); dsRNA itself has been proposed as the normal inducer in virus infected cells (Morser and Burke 1979). DsRNA

is formed in NDV-F infected cells via the action of an RNA polymerase and experiments using ultra violet irradiated NDV to induce interferon, have shown that loss of the ability to induce the production of interferon is associated with a loss in activity of the polymerase in chick embryo fibroblast cells (Meager and Burke 1972). However as mentioned above the growth of NDV-F in mouse cells cannot be easily measured, therefore it is not possible to ascertain whether or not the interferon inducer is present to the same degree in both the undifferentiated and the differentiated teratocarcinoma cell. However, if it could be shown that the virus behaved differently in the ec cell than in the differentiated endoderm-like cell then this might indicate that there could be differential production of the interferon gene inducer molecule between the two different cell types. NDV-F activity cannot be simply measured in infected mouse cells, since these cells do not show either an alteration in host cell synthesis or detectable viral synthesis after infections; however it is possible to measure indirectly the desorbtion and/or replication of NDV-F from such cells. Figure 31 shows the desorbtion and/or replication of NDV-F from virus-infected teratocarcinoma cells and from control L929 cells. The data presented in figure 31 shows that virus desorbtion and/or replication does occur for the whole of the 24 hour period following infection of the teratocarcinoma cells with NDV-F. The pattern of interference produced in the supernatant above the virus infected cells was similar for both the undifferentiated ec cell and for the differentiated endoderm-like cell. Thus these data show that both the undifferentiated and the differentiated teratocarcinoma cells behave similarly at the level of binding and release of the virus at the cell surface.

The alternative suggestion is that interferon production is inhibited in the ec cell because of a block in transcription or in

FIGURE 31. The desorbtion of NDV-F from teratocarcinoma cells.

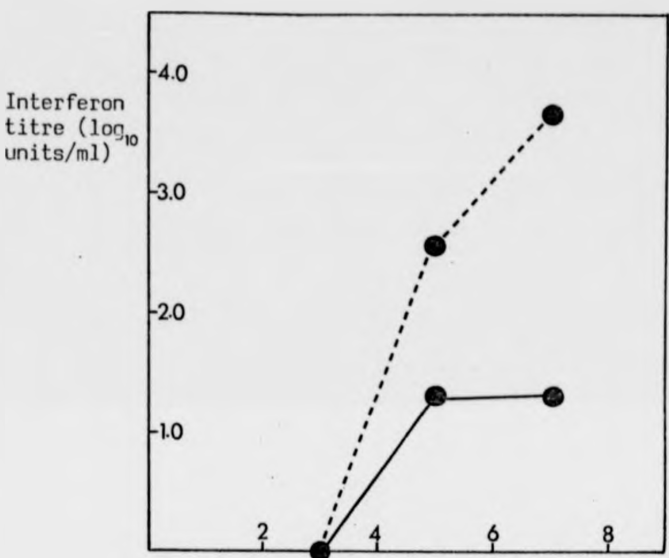


5 cm monolayers of F9 undifferentiated ec cells (--●--), F9 differentiated endoderm-like cells (—●—) and L929 cells (--O--) were infected with 100 HA NDV-F for one hour at 37°C. Cultures were then washed three times with PBS and refed with maintenance media, this was immediately harvested to provide the baseline reading (A). Cell supernatants from each culture were harvested at the times indicated, cell monolayers were washed after each harvest and refed with maintenance media. Samples were spun at 40 000 rpm for two hours, the supernatant discarded and the pellet solubilised overnight in four mls media; all samples were then assayed for interference using the INAS assay as described in methods.

in translation of interferon which is removed as the cells differentiate. This was investigated by a search for interferon mRNA in virus infected ec cells. The production of interferon mRNA can be assayed by translation of the message in Xenopus oocytes. RNA samples were injected into the nucleus and cytoplasm of the oocyte by Dr. A. Colman, and additional samples were injected into the oocyte cytoplasm by John Shuttleworth of this laboratory. The interferon protein secreted by the injected oocyte was assayed in the conventional INAS assay described in Methods.

The kinetics of interferon production in virus infected differentiated teratocarcinoma cells was examined to assess the optimum time post-infection to extract the induced interferon mRNA. Figure 32 shows that the synthesis of interferon mRNA or protein cannot be detected before 3 hours post-infection, and that the amount of mRNA synthesis does not increase beyond that seen at 5 hours post-infection although the synthesis of interferon protein from the infected cell continues to increase beyond 7 hours post-infection. Therefore it was decided to extract the cellular RNA at 6 hours post-infection and assay for the presence of interferon mRNA. The result from this experiment is shown in figure 33, RNA was extracted from virus infected teratocarcinoma cells in two ways: (A) extraction of the cytoplasmic RNA and (B) extraction of the total cell RNA. The data presented in figure 33 shows that virus infected ec cells do not produce interferon but the virus infected differentiated endoderm-like cell, can produce interferon. The RNA extracted from virus infected ec cells did not direct the synthesis of interferon when injected into Xenopus oocytes, in contrast to the RNA extracted from the virus infected differentiated cell.The RNA extracted from virus infected ec cells was injected into the nucleus of the oocyte therefore these results suggest that the ec cell is not able to produce interferon mRNA (or produces mRNA which cannot be 'matured' in the

FIGURE 32. The kinetics of interferon production in differentiated teratocarcinoma cells infected with NDV-F.



Time (hours) post-infection with NDV-F.

Three 125 cm^2 monolayers of differentiated PC13 cl 5 cells (ie: cultured for eight days in media containing retinoic acid) were infected with 2 000 HA NDV-F for one hour at 37°C. The cultures were then washed three times with PBS and refed with maintenance media containing 2µci/ml ^3H Uridine and incubated at 37°C. At the times indicated the cell supernatant was removed, treated to inactivate the virus and assayed for interferon using the INAS assay (as described in methods); the cells were washed three times with PBS at 4°C and removed from the culture flasks using the flat area of a rubber bung. The total cell RNA was then extracted from these cells (see methods for details), samples from this RNA were injected into the cytoplasm of Xenopus oocytes (by John Shuttleworth from this laboratory). The supernatants bathing the oocytes were removed at 24 hours post-injection and assayed for interferon yield using the INAS assay.

(- - ● - -) interferon yield from supernatant above infected cells.
(——●——) " " " " containing oocytes injected with RNA.

FIGURE 33. The production of interferon mRNA in virus infected undifferentiated (EC) and differentiated (END) teratocarcinoma cells.

	EXTRACTION A		EXTRACTION B	
	EC	END	EC	END
IFN titre (log_{10}units/ml) from infected cell supernatant.	0.0	3.6	0.0	3.0
IFN titre (log_{10}units/ml) from supernatant bathing oocytes injected with RNA extracted from infected cells	0.0	1.4	0.0	0.85
Activity of extracted RNA in rabbit reticulocyte system.(% stimulation over blank system)	170	220	140	140
Presence of bands on autoradiograph of poly-acrilamide gel of products from the RRL system.	++	+	++	+
Presence of ribosomal RNA bands in extracted cell RNA run on an agarose-formamide gel system.	++	++	++	++

Undifferentiated and differentiated teratocarcinoma cells were infected with NDV-F for one hour, washed with PBS and then refed with maintenance media and incubated at 37°C. At six hours post-infection cell supernatants were harvested, treated to inactivate virus and assayed for IFN yield using the INAS assay as described in methods. The infected cells were washed with PBS at 4°C and removed from culture dishes using the flat area of a rubber bung, Cellular RNA was then extracted using two methods A, phenol-chloroform extraction of cytosol RNA, and B, Guanidium Rhodamide extraction of total cell RNA (both methods are described in methods). The resultant RNA's were injected into the nucleus of Xenopus oocytes by Dr. A Colman , the supernatants bathing the oocytes were assayed for IFN yield at 24 hours post-injection using the INAS assay. The extracted RNA was translated in a rabbit reticulocyte system and the products run on a poly-acrylamide gel (see methods), and also examined using an agarose-formamide gel system for the presence of ribosomal RNA peaks.

oocyte nucleus). Figure 33 also includes data collected to ensure that the RNA extracted from the ec cell was as capable of directing translation as was the RNA extracted from the endoderm-like cell. RNA from both undifferentiated and differentiated teratocarcinoma cell stimulated protein synthesis in a rabbit reticulocyte system (RRL) to the same degree. When the products of the RRL system were run out on a poly-acrylamide reducing gel it showed that translation of the RNA had occurred, and that the RNA from the ec cell produced considerably more bands on the subsequent autoradiograph than the RNA from the endoderm-like cells (all RRL assays were kindly performed by Dr. J. Morser). In addition the extracted RNA was also run on an agarose-formamide gel system, and the extracted RNA from both cell types produced ribosomal RNA peaks of 18s and 28s, with no obvious signs of degradation. Although this observation does not prove that the mRNA is intact it does show that obvious degradation of the ribosomal RNA bands had not occurred.

Since interferon message cannot be detected in a situation when the oocyte would be expected to be able to splice and process immature message, it is possible that neither immature nor mature interferon message is produced in the embryonal carcinoma cells. Therefore there is no need to postulate either processing or translation mechanisms in the regulation of interferon production in teratocarcinoma cells.

In vitro differentiation of 'nullipotent' teratocarcinoma cells

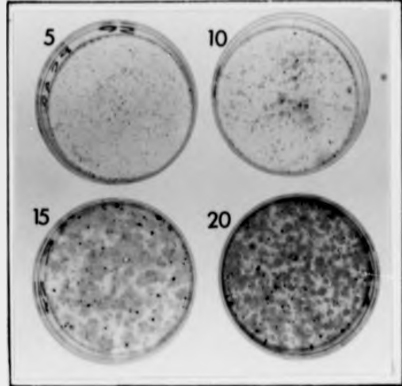
'Nullipotent' teratocarcinoma cells can be induced to differentiate to an endoderm-like cell by exposure to micromolar amounts of retinoic acid. The appearance of differentiation in these cultures can be

followed by observing changes in morphology and behaviour in the cells and also by quantitating changes in the synthesis of certain biochemical markers; of which the appearance of interferon inducibility and sensitivity is an example. The procedure used to obtain optimum differentiation is described in the Methods.

The morphological changes which occur as the undifferentiated teratocarcinoma cells are cultured in retinoic acid are illustrated in figures 34 a and b which show macroscopic and microscopic views of such cultures. The ec cell can be seen to be small in size, unevenly spaced and overgrown in some areas, and also poorly attached to the substrate (requiring only 30-60 seconds treatment with 8.0 µg/ml trypsin at 37°C to be removed). Because the ec cell is poorly attached to the substrate it cannot be passaged in glass culture dishes and instead is maintained *in vitro* using gelatinised culture plates (see Methods). The differentiating culture is seeded at a low cell density into retinoic acid containing media, and after two to three days treatment, colonies of morphologically differentiated cells are formed (see figure 34a). The differentiated endoderm-like cell is usually larger than the undifferentiated ec cell (the exact size depends upon the available space) and becomes more firmly attached to the substrate (requiring treatment for three to four minutes with 80.0 µg/ml trypsin at 37°C to be removed). The cells become extremely flattened and difficult to see by phase microscopy and form orderly arrays within three to four days exposure to retinoic acid. In contrast to the ec cell, the growth of the differentiated cell becomes contact inhibited, if the cuture remains in retinoic acid containing media, resulting in a much reduced cell density.

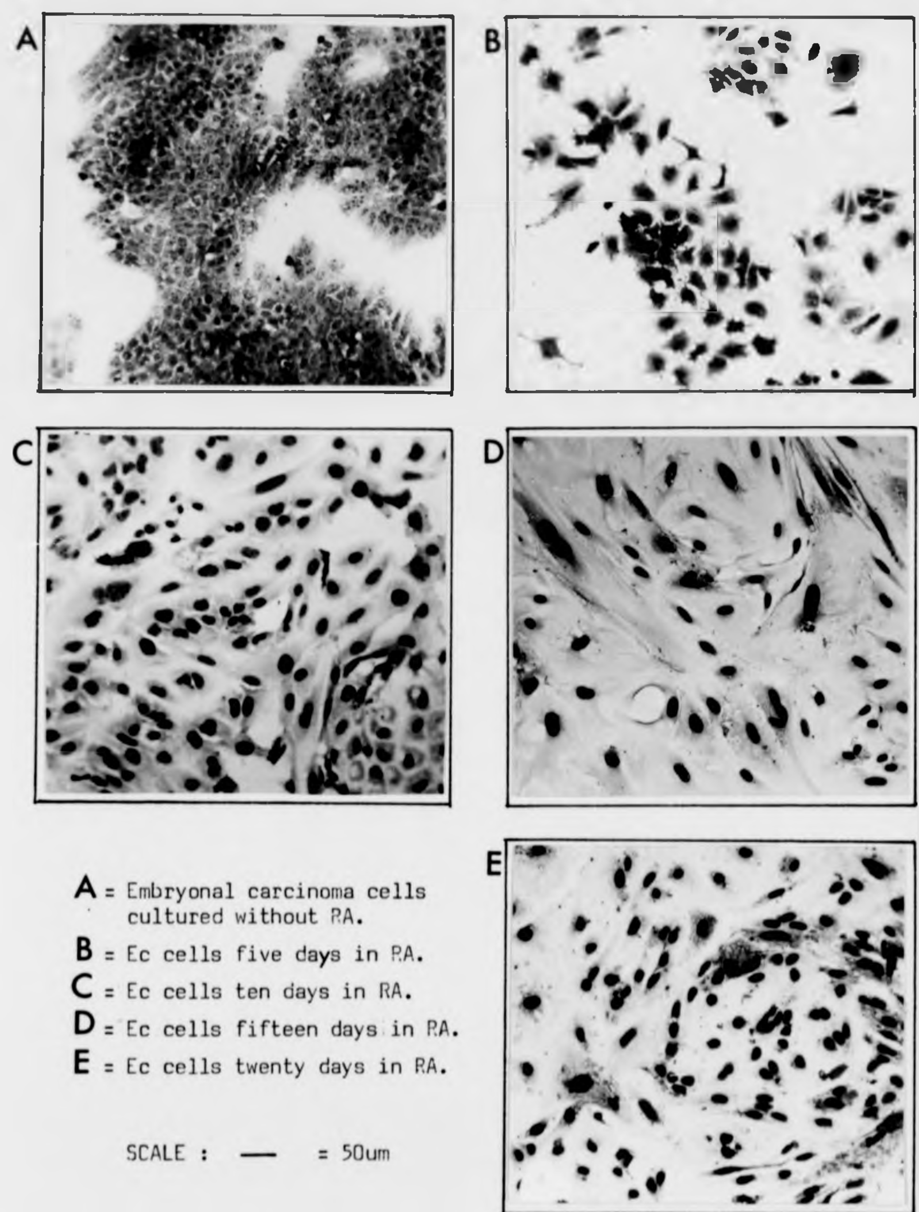
All three teratocarcinoma cell lines studied (PC13 Cl 5, F9 and Nulli 2A) showed most of the above changes upon treatment with retinoic

FIGURE 34a. Macroscopic view of embryonal carcinoma cells cultured for various periods of time in retinoic acid.



2×10^4 PC13 cl 5 embryonal carcinoma cells were seeded into 3 cm gelatinised dishes containing growth media plus 3×10^{-6}M retinoic acid, and cultured over a period of twenty days. The media was changed every two days, at the intervals indicated on the above photograph (ie: 5, 10, 15 and 20 days) cultures were washed with PBS, fixed with 100% methanol and stained with Giemsa, before being photographed.

FIGURE 34b. Microscopic view of embryonal carcinoma cells cultured for various periods in media containing retinoic acid.



The cells were prepared as described in figure 34a; with the exception that the ec cells shown in A, were grown for eight days without retinoic acid (RA) before being fixed and stained. The cells were photograhed using a light microscope and brightfield illumination.

acid, the changes in morphology all occurring after two to three days exposure to retinoic acid.

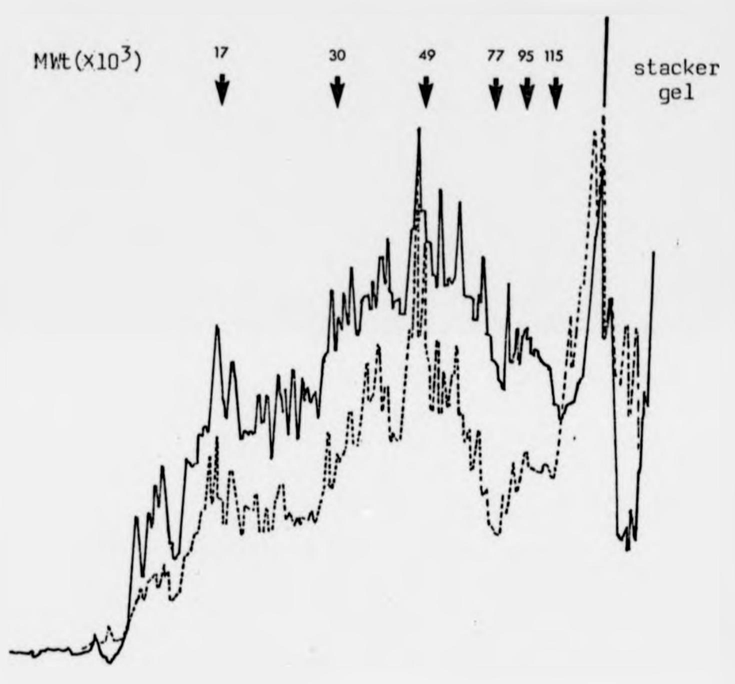
In figures 35 and 36 the changes in the pattern of protein synthesis which occur in a differentiating teratocarcinoma culture are examined. Both figures show microdensitometer scans of autoradiographs made from polyacrylamide gels in which cell lysates had been run; these lysates were of ^{35}S methionine-labelled teratocarcinoma cells treated for various periods with retinoic acid. Figure 35 shows two such scans which result from samples of ec cells grown for eight days in culture with and without retinoic acid. Considerable changes in protein synthesis profile can be seen. The relative amounts of many of the proteins synthesised is altered in the sample taken from differentiated teratocarcinoma cells; and in addition the area under this curve is 40% less than that obtained from samples of ec cells. It can be seen from figure 36 that these changes in protein synthesis profile are obvious after the ec cell has been treated with retinoic acid for two days, and become increasingly more pronounced the longer the time the cells are exposed to this vitamin.

Thus it can be seen that the changes in protein synthesis profile become apparent at approximately the same time as changes in the cells morphology and behaviour are observed. The onset of these structural changes will be discussed later in relation to the onset of production or changes in production of biochemical markers associated with differentiation.

The effect of retinoic acid on interferon yield from differentiated cells

Retinoic acid is used to induce differentiation in ec cells, and the ability of ec cells to respond to this vitamin has been correlated

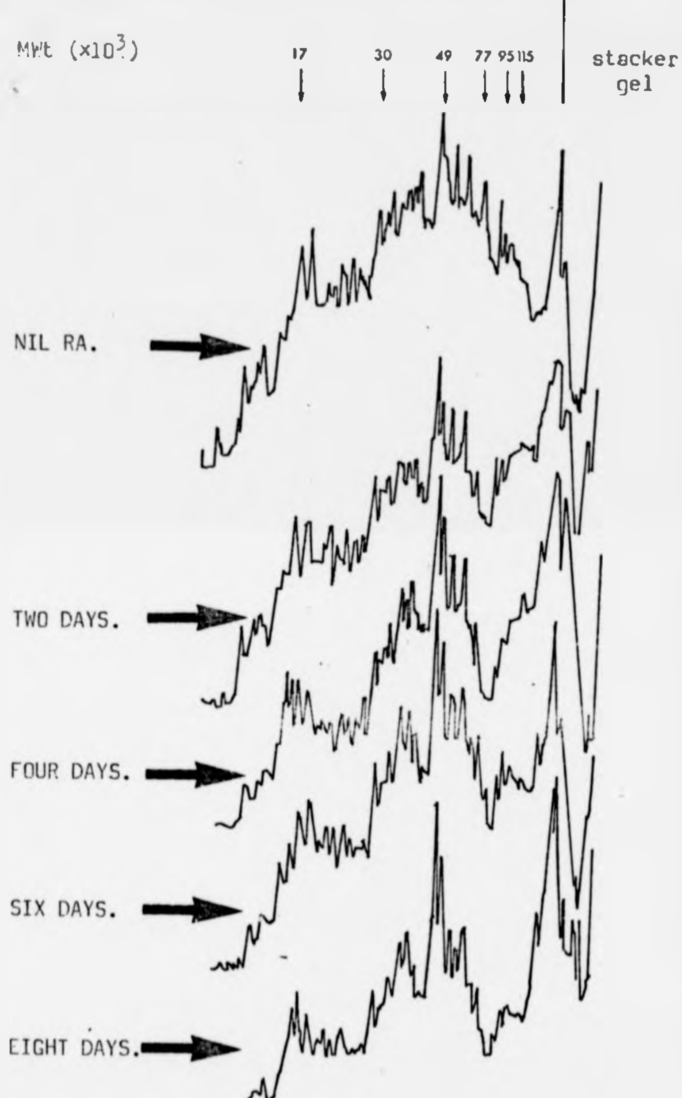
FIGURE 35. A microdensitometer recording from an autoradiograph of a poly-acrylamide gel containing samples of ^{35}S methionine labelled PC13 Cl 5 teratocarcinoma cells.



PC13 Cl 5 ec cells were seeded into dishes containing growth media or growth media plus retinoic acid, after eight days in culture at 37°C, the cultures were washed three times with PBS and then refed with serum and methionine-free media which contained 100μci ^{35}S methionine, for 30 minutes at 37°C. The cell monolayers were then washed several times with PBS kept at 4°C, scraped off the dish using the flat area of a rubber bung and solubilised in tris-SDS (as described in mehtods). Samples were then mixed with an equal amount 'blue buffer' (see methods) boiled for three minutes, spun for five minutes using an eppendorf microfuge. Volumes of the samples containing 100 000 cpm were loaded onto the poly-acrylamide gel (see methods for gel preparation). The gels were fixed and dried, autoradiographs were prepared and the relative densities of each track was scanned using a Joyce-Loebl microdensitometer.

(———) tracing of densitometer scan of samples from PC13 cl 5 cultured in growth media for eight days.
(-----) tracing of densitometer scan of samples from PC13 cl 5 cultured in growth media containing 3×10^{-6} molar retinoic acid, for eight days.

FIGURE 36. Microdensitometer scan of an autoradiograph of a polyacrylamide gel containing samples of teratocarcinoma cells cultured for various periods in retinoic acid.



PC13 cl 5 ec cells were cultured for 0, 2, 4, 6 and 8 days in retinoic acid, before being exposed for a brief period to ^{35}S methionine, cell samples were then prepared as described in figure 35 and the subsequent autoradiograph was scanned using the Joyce-Loebl microdensitometer. The recordings were photoreduced and then traced.

with the presence of a cytosol retinol-binding protein (Jetten and Jetten 1979, Schindler et al. 1981) and it is thought that retinoic acid may act in a manner analogous to that of the steroid hormones, by directly influencing transcription events in the genome. It was therefore of interest to see if differentiated cells treated with retinoic acid produced an altered yield of interferon. Three differentiated teratocarcinoma cell lines were examined. These are permanent cell lines in contrast to the differentiated cells established from embryonal carcinoma cells. Figure 37 shows the results obtained from three differentiated cell lines PSA 5E, PYS and F9 Cl 9. The cells of the former express markers characteristic of visceral endoderm. While the latter two lines express markers characteristic of parietal endoderm. The data from figure 37 shows three main points; firstly culture of these cells in 3×10^{-6} M retinoic acid has no significant effect on interferon production from these cells, secondly retinoic acid inhibited cell growth in the two parietal endoderm cell lines but showed no inhibition of the growth of the visceral endoderm cell line, thirdly each of the three cell lines showed a wide variation in interferon production at the population and the single cell level; in particular the number of cells able to produce interferon varied from 0.35% (PSA 5E) to 18% (F9 cl 9).

The kinetics of interferon production in differentiated teratocarcinoma cultures.

Cell differentiation in ec cell cultures treated with retinoic acid is marked by morphological and behavioural changes as described above, and also by the changes in production of certain biochemical markers. The kinetics of interferon production at both the population

FIGURE 37. The production of interferon in differentiated teratocarcinoma cell lines infected with NDV-F.

	PSA 5E (VE*)	PYS (PE)	F9 cl 9 (PE**)
IFN production after 8 days in culture.			
a: Population yield ($x10^3$ units/cell)	0.6	45.0	180.0
b: %age single cell IFN producers.	0.35%	3.1%	18.0%
IFN production after 8dys exposure to $3x10^{-6}$ molar retinoic acid.			
a: Population yield ($x10^3$ units/cell	0.4	60.0	169.0
b: %age single cell IFN producers.	ND	ND	ND***
% depression of cell numbers seen in cultures treated with retinoic acid.	0.0	78.0	88.0

Three differentiated teratocarcinoma cell lines PSA 5E, PYS and F9 cl 9 were seeded at an initial density of $5x10^4$ per 3cm dish into either growth media or growth media plus $3x10^{-6}$ molar retinoic acid. After eight days in culture the cultures were infected with 100 HA NDV-F for one hour at 37°C, washed three times with PBS and then cultured for a further hour with media containing antiserum raised to NDV-F. The cell monolayers were then trypsinised to a suspension of single cells, a small aliquot of which was used to provide cells for the single cell assay (performed as described in methods) the remainder were returned to the culture dish. After approximately 20 hours post-infection the cell supernatants were harvested and cell numbers were counted. The supernatants were treated to inactivate residual virus and assayed for interferon yield using the INAS assay as described in methods.

* (VE) cells produce biochemical markers characteristic of visceral endoderm.

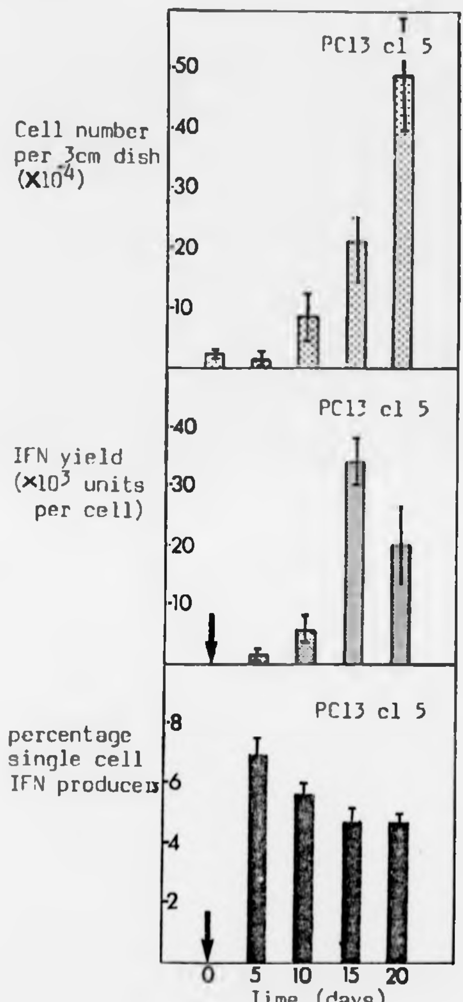
**(PE) cells produce biochemical markers characteristic of parietal endoderm.

*** ND not done.

and the single cell level was followed in the three teratocarcinoma cell lines described in figure 29. The results of this series of experiments are presented in figures 38 a, b and c. In this figure interferon production and cell growth were examined over a 20 day period after the ec cells were exposed to retinoic acid. These data show three general features; firstly, interferon production both at the population and the single cell level rises from an initial zero level (except for Nulli 2A), reaches a peak value and then declines rapidly. This decline in production is not due to obvious cell death only trypan blue excluding cells were counted (only rarely were non-trypan blue excluding cells seen), and in addition the cell number was still increasing in two out of the three cell lines after the decline in interferon production was seen. Secondly, the maximum percentage of single cells able to produce interferon varied between the three cell lines from 12% (Nulli 2A) to 7% (PC13 cl 5) and 4% (F9), and this variation was generally reflected in the population interferon yields. Thirdly, if the results from PC13 cl 5 and F9 are compared (which differentiate into endoderm-like cells with characteristics of visceral and parietal endoderm respectively), it can be seen that there are no differences in the kinetics of the development of interferon production. The differentiated PC13 cl 5 culture contained approximately twice as many single cell interferon producers when compared to the number seen in the differentiated F9 culture. In view of the variation seen in the differentiated cell lines (figure 37) this may represent an innate variation between cell lines rather than a difference between visceral and parietal endoderm cells.

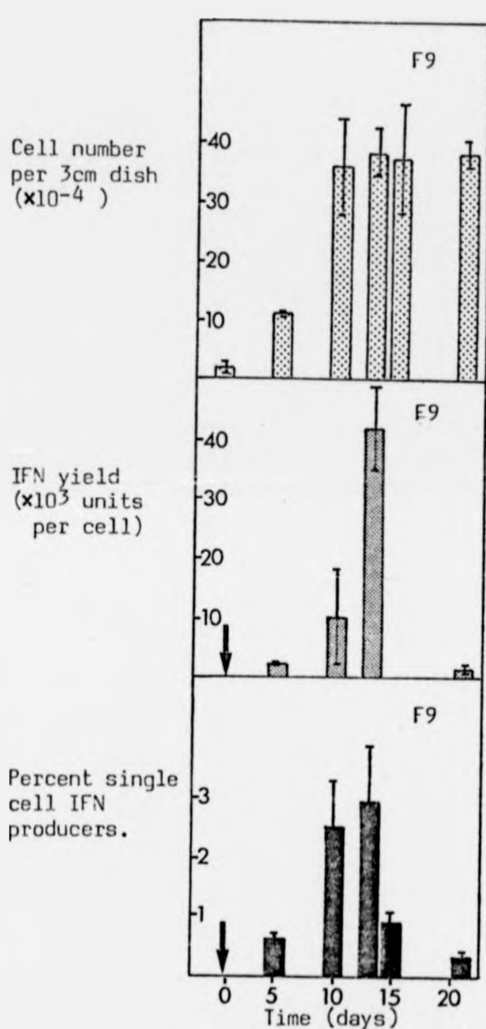
Of the three cell lines the growth of F9 was least affected by culture in retinoic acid, for these cells reached eight times the density of the PC13 cl 5 culture. However the rate of growth of cultures exposed to retinoic acid is not correlated with the number of single

FIGURE 3Pa The kinetics of interferon production in differentiating PC13 cl 5 teratocarcinoma cells.



2X10^4 PC13 cl 5 embryonal carcinoma cells were seeded in 3 cm dishes containing growth media plus 3X10^{-6} molar retinoic acid. At the times indicated duplicate cultures were infected with 100 HA NDV-F for one hour, the cells were then washed three times with PBS and then incubated for a further hour with media containing antiserum raised to NDV-F. The cells were then trypsinised to a suspension of single cells, a small aliquot of this suspension was used to provide cells for the single cell assay (performed as described in methods), the remainder were returned to the culture dish. At approximately 20 hours post-infection the cell supernatant was harvested, treated to inactivate virus and assayed for interferon yield using the INAS assay (as described in methods).
(↓) assay performed and zero result obtained.

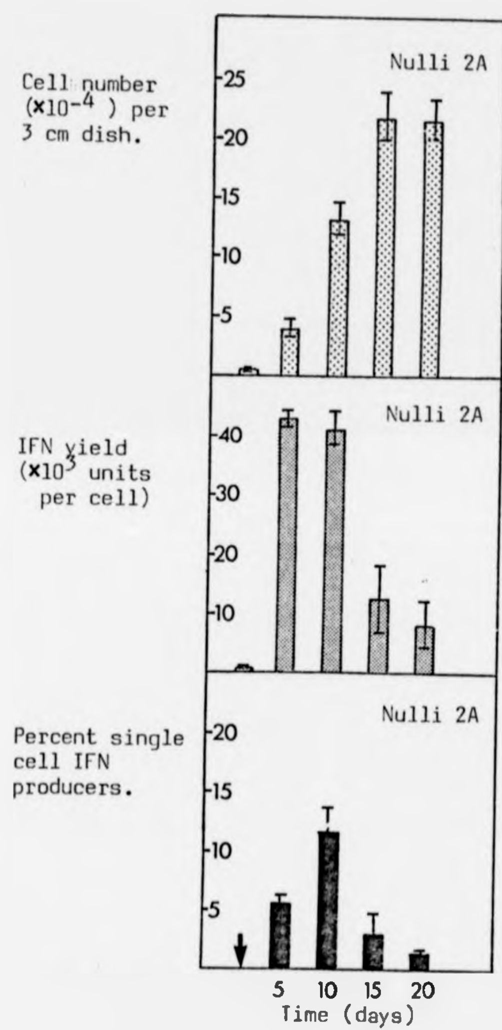
FIGURE 38b. The kinetics of interferon production in differentiating F9 teratocarcinoma cells.



1×10^{5} F9 embryonal carcinoma cells were seeded into 3cm dishes containing growth media plus 3×10^{-6} molar retinoic acid. Interferon production at the population and the single cell level was examined in the differentiating cell culture as described in figure 38a.

(↓) assay performed and zero result obtained.

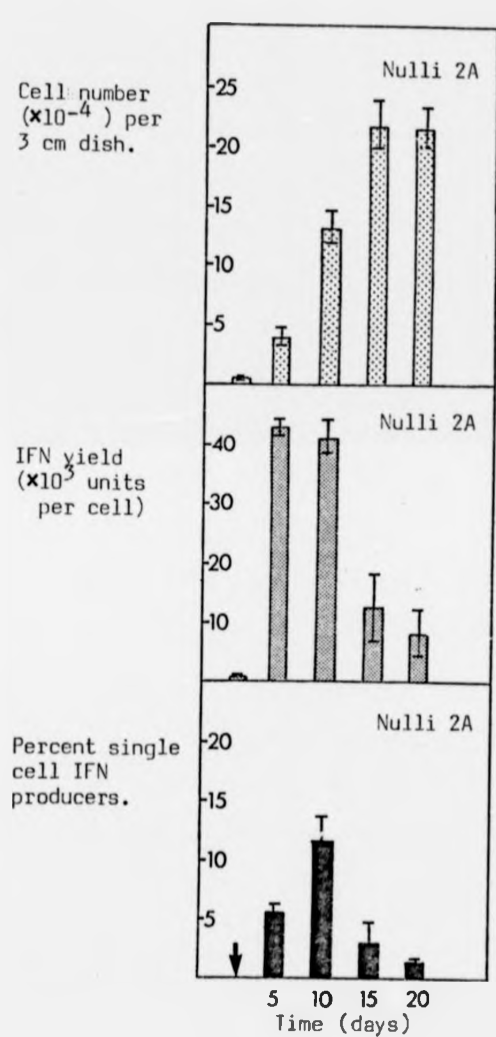
FIGURE 38c. The kinetics of interferon production in differentiating Nulli 2A teratocarcinoma cells.



5×10^{4} Nulli 2A embryonal carcinoma cells were seeded into 3 cm dishes containing growth media plus 3×10^{-6} molar retinoic acid. Interferon (IFN) production at the population and the single cell level was examined in the differentiating cell culture as described in figure 38a.

(↓) assay performed and zero result obtained.

FIGURE 38c. The kinetics of interferon production in differentiating Nulli 2A teratocarcinoma cells.



5×10^{4} Nulli 2A embryonal carcinoma cells were seeded into 3 cm dishes containing growth media plus 3×10^{-6} molar retinoic acid. Interferon (IFN) production at the population and the single cell level was examined in the differentiating cell culture as described in figure 38a.

(↓) assay performed and zero result obtained.

cell interferon producers seen in that culture; Nulli 2A differentiated cultures contained the highest percentage of single cell interferon producers but had a final cell density imtermediate between that of PC13 cl 5 and F9. If the plates resulting from the single cell assay are examined (see figure 39) it can be seen that the size of the foci produced as the cells differentiate is not constant. Foci produced from cells after five days treatment with retinoic acid are uniformly small (approximately 2-3 mm in diameter) whilst those from cells which have been in culture 10-15 days show a large variation in size (approximately 1-5 mm in diameter). These data suggests that the yield of interferon per cell is changing as the cells are treated for increasing lengths of time with retinoic acid.

It is possible to enrich the number of single cell interferon producers and therefore the population yield in differentiating cultures 24 hours prior to induction of interferon. The results of such an experiment are shown in figure 40. Differentiating teratocarcinoma cells were trypsinised (using trypsin of greater concentration than that used for ec cells) and reseeded onto non-gelatinised dishes, thus selecting for cells with increased adhesiveness. The effect of this procedure at all time intervals (except for ec cells not treated with retinoic acid) was to enhance the interferon yield at both the single cell and the population level. Thus these data suggest that the cell able to produce interferon is more able to withstand the trypsinisation procedure and to establish themselves on non-gelatinised dishes, than are the remaining cells in culture.

The differentiating teratocarcinoma cell lines can be seen to produce considerably less interferon (see figure 38) than that produced by L929 cells induced under similar conditions (see section one). Pretreatment of L929 cells with small amounts of interferon prior to

FIGURE 39. Embryonal carcinoma cells induced to differentiate by retinoic acid show an increase in yield of interferon per single cell.

Time (days) in retinoic acid.

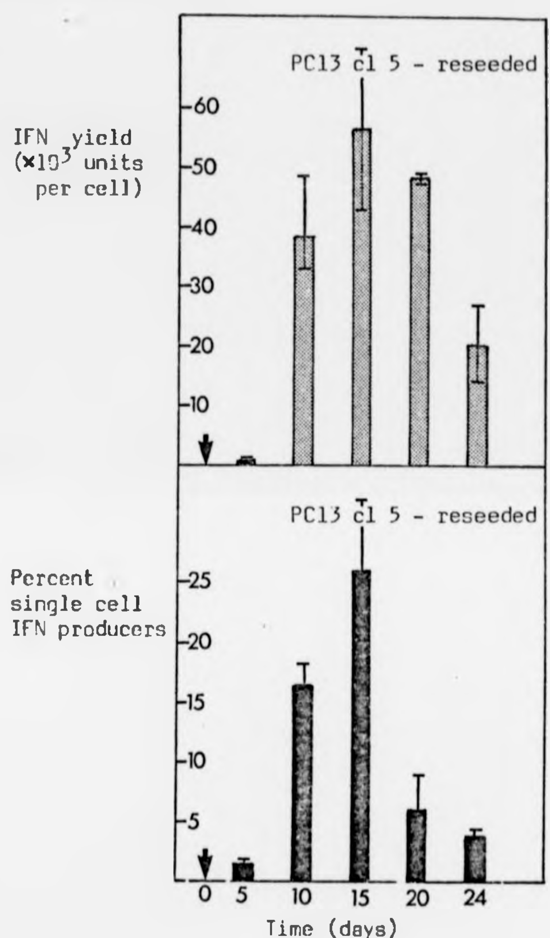
5

10

15

Interferon production from single cells was examined in a differentiating population of PC13 cl 5 teratocarcinoma cells as described in figure 38a. The above photograph shows typical single cell assay plates obtained from teratocarcinoma cells cultured for five, ten and fifteen days in retinoic acid and then induced to produce interferon.

FIGURE 40. Interferon production in populations of differentiating teratocarcinoma cells trypsinised and reseeded prior to interferon induction.



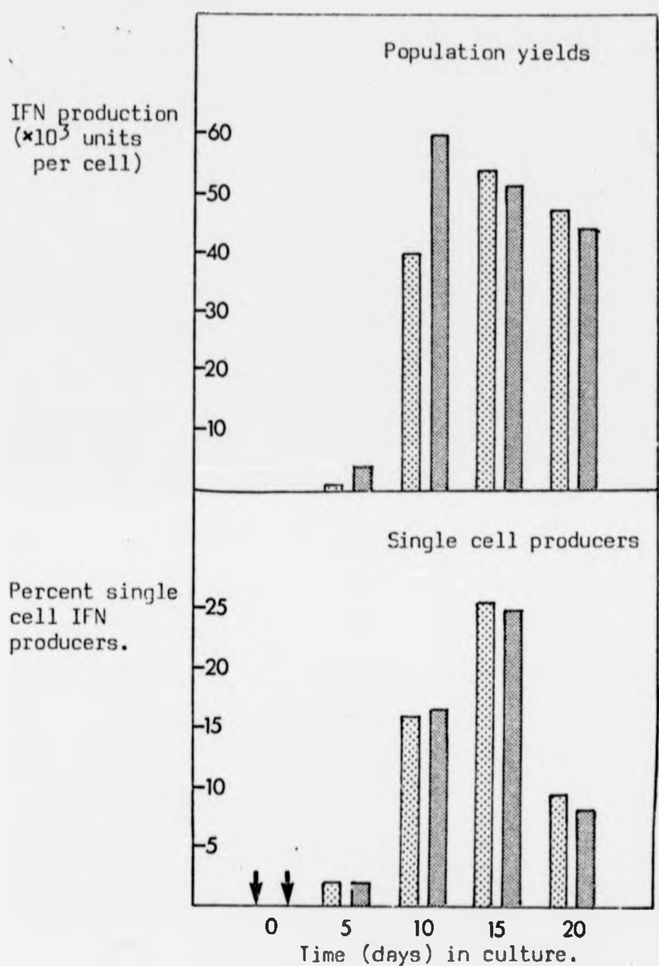
PC13 cl 5 embryonal carcinoma cells were seeded at a density of 3 000 per cm^2 into 9cm gelatinised plastic culture dishes into growth media containing 3×10^{-6} molar retinoic acid. 24 hours before the time intervals indicated the cultures were trypsinised (80µg/ml Trypsin) to a suspension of single cells and seeded into non-gelatinised 3cm plastic dishes at a density of 1×10^6 per 3 cm. At the times indicated the reseeded cultures were infected with 100 HA NDV-F for one hour at 37 C washed three times with PBS then cultured for a further hour with media containing antiserum raised to NDV-F. Interferon production was then examined at the population and the single cell level as described in figure .

(↓) assay performed and zero result obtained.

interferon induction (ie: priming) did not increase the subsequent yield; it was suggested that this was because the cells were already synthesising interferon at maximum levels . Differentiating teratocarcinoma cultures do not synthesise maximum levels of interferon until approximately 15-20 days after treatment with retinoic acid, and therfore it is possible that these cultures could be primed during their early exposure to this vitamin. Figure 41 shows the interferon yield at both the population and the single cell level in differentiating teratocarcinoma cultures primed prior to induction. Since it has been show that trypsinisation and reseeding the culture can enhance the number of single cell interferon producers, this procedure was used to obtain cells with the aim of increasing any result shown by priming. The data from figure 41 shows that differentiating teratocarcinoma can be primed to increase interferon yields but only for a limited period following exposure to retinoic acid. An enhancement of yields was seen in the period 5-10 days after treatment with retinoic acid in all the triplicate plates that were primed, and although some difference was seen between the primed and non-primed 15 and 20 day cultures, the range of results from these cultures did overlap. The single cell assay data shows that where an increase in yield was seen after priming, the number of single cells able to produce interferon did not increase; thus the action of priming is to increase the amount of interferon produced per cell and not the number of cells able to produce interferon. It is possible that cultures treated for 15 to 20 days with retinoic acid could not be primed because such cultures were already producing maximum amounts of interferon.

Ec cells cultured in retinoic acid differentiate, and since the culture then becomes able to produce interferon upon induction, it was of interest to discover the minimum period of exposure to retinoic

FIGURE 41. Priming of teratocarcinoma cells cultured in retinoic acid for various periods of time.



Cultures of differentiating PC13 cl 5 cells were prepared as described in figure 40., six hours prior to the times indicated cultures were either primed for six hours with maintenance media containing 10u/ml mouse interferon (▩), or refed with maintainance media only (▨). The cell monolayers were then washed with PBS and induced to produce interferon by virus infection. Interferon production at both the population and single cell level was examined as described in figure . Each bar represents the mean value from triplicate culture dishes, values were tightly grouped around the mean.

(↓) assay performed and zero result obtained.

acid needed to obtain the maximum production of interferon in such cultures. The results of this experiment are shown in figure 42. In this experiment two sets of cultures (A and B) were prepared, Culture A contained PC13 cl 5 embryonal carcinoma cells seeded into growth media containing retinoic acid; at daily intervals up to day 14, these cultures were induced to produce interferon by virus infection. Culture B, were similarly seeded but at daily intervals the retinoic acid containing media was removed and substituted by normal growth media. Interferon induction was performed on culture B cells only after a four day period in the absence of retinoic acid. Thus culture B represents results obtained from a series of dishes treated for periods of one to ten days with retinoic acid but then cultured for a period without this vitamin.

If the interferon production from cultures A and B are compared, for example at day 10, it can be seen that culture A cells (ie: cells in culture for ten days and exposed to retinoic acid for ten days) have a mean yield of 36×10^{-4}/cell, whilst culture B cells (ie: cells in culture for ten days but only exposed to retinoic for the first six days) have a mean yield of 4.0×10^{-4}/cell. The yield seen in culture B cells at day ten is similar to the range seen in culture A cells at day six, and this suggests that the development of maximum interferon yields requires continual exposure to retinoic acid for longer than six days. For the first 12 days in culture the results obtained from culture B cells are reduced compared to culture A. Thus a period of exposure to retinoic acid longer than eight days is required for maximum yields. After 12 days in culture the yields from both cultures reach a similar order of magnitude.

Thus the data from figure 42 shows three main points. Firstly exposure to retinoic acid for at least eight days is required for

FIGURE 42. The relationship between interferon yield in differentiating teratocarcinoma cells and the length of time the cells are exposed to retinoic acid.

Days in culture	←-----A------→		←-----B-----→	
	IFN yield*	N° of cells**	IFN yield	N° of cells
0	-	8	-	8
1	0	-	-	-
2	0	47	-	
3	0.5	-	-	-
4	0.7	129	-	-
5	1.2	-	0.03	-
6	1.2	170	0.6	335
7	11.0	-	3.5	-
8	12.8	160	5.0	275
9	30.0	-	7.1	-
10	36.0	142	4.1	250
11	37.0	-	12.0	-
12	200.0	178	20.0	250
13	281.0	-	225.0	-
14	300.0	186.	225.0	315

PC13 cl 5 embryonal carcinoma cells were seeded at a density of 8×10^{4} per 3 cm dish into growth media containing 3×10^{-6} molar retinoic acid. The cells in culture A were induced to produce interferon on the day in culture indicated , the cells in culture B were on the same day transferred to growth media lacking retinoic acid after copious washing. Culture B cells were maintained in media lacking retinoic acid for a period of four days, the media being changed daily, the cells then being induced to produce interferon in the same manner as used for culture A.
For interferon induction cell monolayers were infected with 100 HA NDV-F for one hour at 37 C then washed three times with PBS before being refed with maintenance media. At approximately 20 hours post-infection the cell supernatants were harvested and the cell numbers counted. The cell supernatants were treated to inactivate residual virus and then assayed for interferon yield using the INAS assay as described in methods. Each result represents the mean from triplicate dishes, values were tightly grouped around the mean.

* IFN yield 10^{4} units/cell

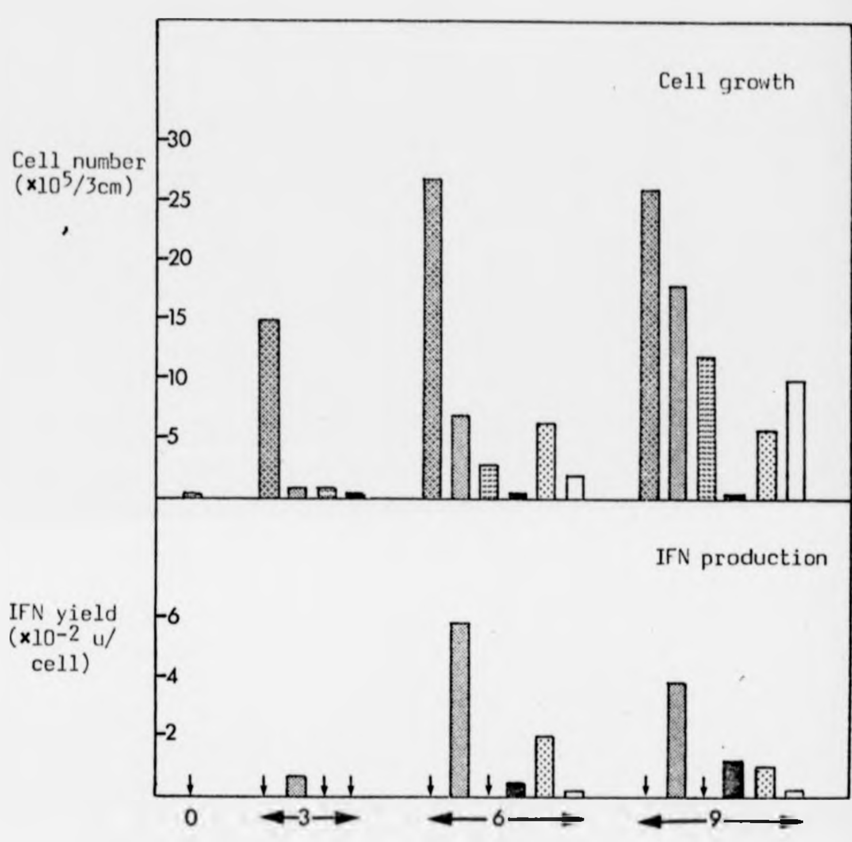
**Cell number 10^{-4} per 3cm dish.

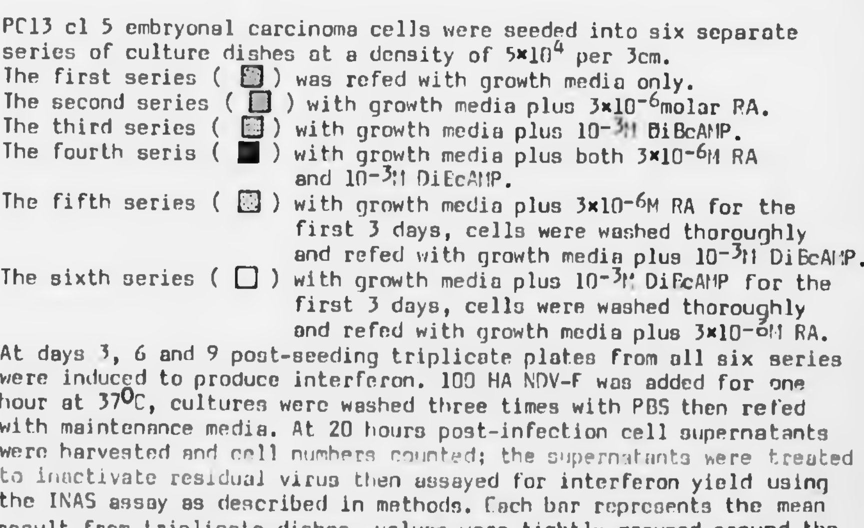
\- not done.

maximum interferon yields in teratocarcinoma cultures. secondly, ec cells develop a detectable interferon yield after only one days exposure to retinoic acid. Thirdly, there are cells which exist even after ten days treatment with retinoic acid which show an increased replication rate when retinoic acid was removed; the cell densities seen in culture B are all greater than that seen in culture A.

It has been shown by Strickland and Mahdavi (1978) that sequential treatment of F9 ec cells (with first retinoic acid then diButyrl-cAMP) increased the degree of differentiation seen in the resultant culture as measured by the expression of the biochemical marker plasminogen activator factor. The ec cells from both PC13 cl 5 (which differentiates into cells with biochemical characteristics of visceral endoderm) and from F9 (which differentiates into cells with biochemical characteristics of parietal endoderm) were treated in a manner which followed the Strickland and Mahdavi (1978) protocol, and examined for interferon production. The results from this experiment are shown in figures 43 a and b, which described results from six series of cultures. Only four sets of data are shown for day three but six sets of data are presented for days six and nine. This is because culture series 5 and six are treated for a total of six days before being induced to produce interferon. Comparison of the results obtained from both cell lines shows that while the growth of the two cell lines was differentially effected, the ability of the treated cultures to produce interferon was similar. The control ec cultures and those treated with retinoic acid behaved as expected (see figure 38); however diBcAMP produced different effects on the replication of the two cell lines. The replication of PC13 cl 5 was completely inhibited by this chemical whilst the replication of F9 was not affected. Activation of the interferon system in diBcAMP treated cells was seen in the F9 cultures but this was minimal (3 000

FIGURE 43a. Interferon production in PC13 cl 5 embryonal carcinoma treated with retinoic acid and dibutryl-cyclic-AMP.





PC13 cl 5 embryonal carcinoma cells were seeded into six separate series of culture dishes at a density of 5×10^4 per 3cm.
The first series (▨) was refed with growth media only.
The second series (▨) with growth media plus 3×10^{-6}molar RA.
The third series (▨) with growth media plus 10^{-3}M DiBcAMP.
The fourth seris (■) with growth media plus both 3×10^{-6}M RA and 10^{-3}M DiBcAMP.
The fifth series (▨) with growth media plus 3×10^{-6}M RA for the first 3 days, cells were washed thoroughly and refed with growth media plus 10^{-3}M DiBcAMP.
The sixth series (□) with growth media plus 10^{-3}M DiBcAMP for the first 3 days, cells were washed thoroughly and refed with growth media plus 3×10^{-6}M RA.
At days 3, 6 and 9 post-seeding triplicate plates from all six series were induced to produce interferon. 100 HA NDV-F was added for one hour at 37°C, cultures were washed three times with PBS then refed with maintenance media. At 20 hours post-infection cell supernatants were harvested and cell numbers counted; the supernatants were treated to inactivate residual virus then assayed for interferon yield using the INAS assay as described in methods. Each bar represents the mean result from triplicate dishes, values were tightly grouped around the mean.

FIGURE 43b. Interferon production in F9 embryonal carcinoma cells treated with retinoic acid and dibutryl-cyclic-AMP.

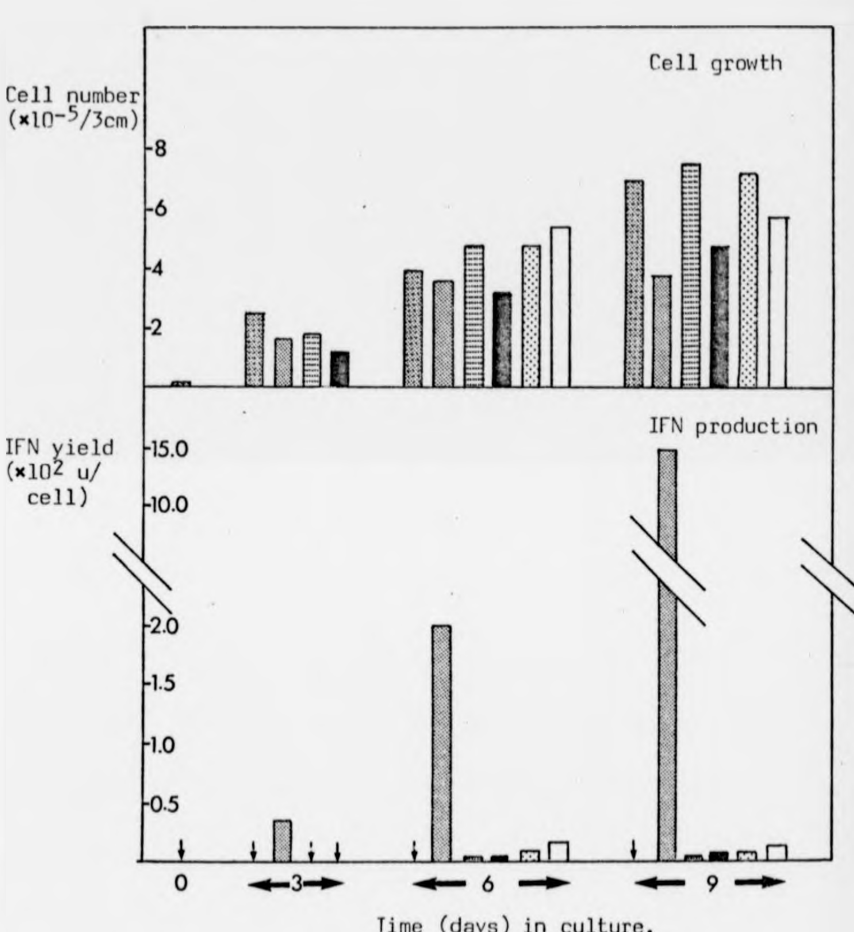


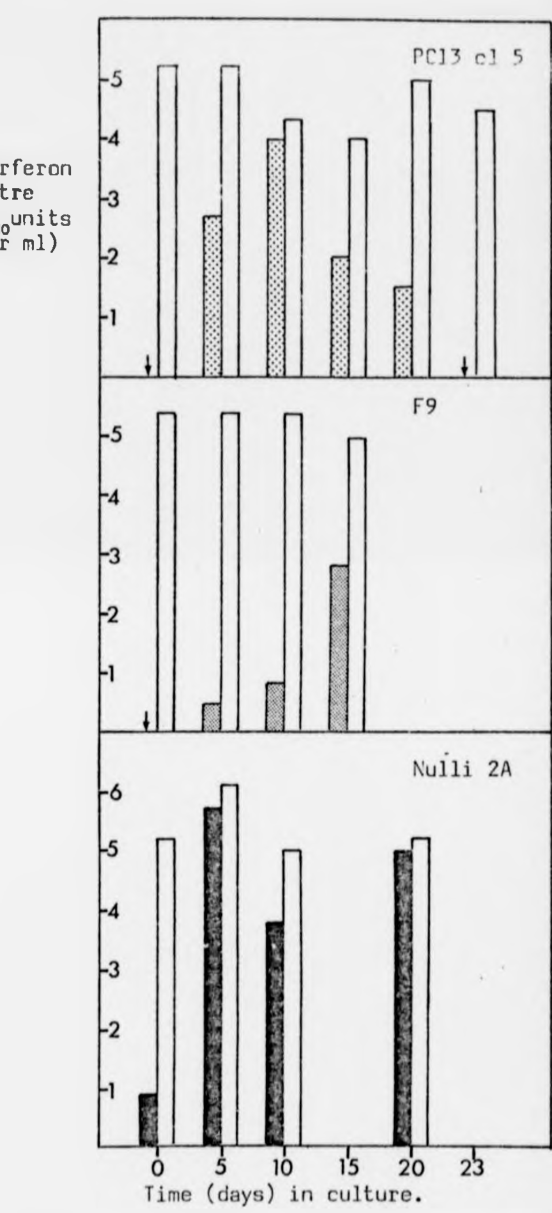
FIGURE 43b: legend as figure 43a.

fold less than seen in retinoic acid treated cultures) while no activation was seen in PC13 cl 5 cultures similarly treated. In neither cell type was an enhancement of interferon inducibility seen when retinoic acid and diBcAMP were applied to cells either simultaneously or sequentially, and the general effect of diBcAMP is to prevent further development of interferon inducibility in both cell types and to inhibit cell replication in PC13 cl 5 only.

Development of interferon anti-viral sensitivity in differentiating 'nullipotent' teratocarcinoma cells

It has been shown that interferon production develops over a period of time after the exposure of teratocarcinoma cells to retinoic acid. Figure 44 examines the development of anti-viral sensitivity to interferon in differentiating teratocarcinoma cultures. The sensitivity of these cultures to the anti-viral action of interferon was determined by assessing the extent of virus replication in the differentiating teratocarcinoma cells previously treated with a standard amount of interferon (performed as described for the INAS assay in Methods). The response of a control sample of L929 cells similarly treated was always included in the assay because of the known variation shown by this assay. Figure 44 shows that the development of interferon anti-viral sensitivity is similar to the development of interferon inducibility in the differentiating cultures; interferon anti-viral sensitivity increases during exposure to retinoic acid, and with PC13 cl 5 cells this can be seen to reach a maximum and then to decline. The decline in interferon anti-viral sensitivity was not seen in the F9 or the Nulli 2A cultures, although interferon

FIGURE 44. The development of interferon anti-viral sensitivity in differentiating teratocarcinoma cells.



Embryonal carcinoma cells from three teratocarcinoma cell lines were seeded into 9cm dishes at a density of 3 000 cells/cm^2 into growth media containing retinoic acid. 25 hours prior to the time intervals indicated the differentiating cultures were trypsinised (8ug/ml trypsin) and reseeded into 250µl 'microtitre' plates which had been treated with 0.1% gelatin (see methods); control L929 cells were also seeded at the same time into the microtitre dishes. For each time point a sample of interferon of known titre, was assayed simultaneously upon both the teratocarcinoma cells and the L929 cells using the INAS assay as described in methods.

(▒) IFN titre upon PC13 cl 5.
(▒) IFN titre upon F9 cells.
(■) IFN titre upon Nulli 2A cells.
(□) IFN titre upon control L929 cells.

inducibility (see figure 38) had declined in these cultures by day 13 and day 10 respectively. Thus although the general trend of the development of interferon anti-viral sensitivity is similar to the trend seen in the development of interferon inducibility, the actual kinetics do differ.

SECTION TWO ---- DISCUSSION

The data presented in figure 29 shows that the embryonal carcinoma cells (ec) of PC13 Cl 5 and F9 teratocarcinoma cell lines do not produce interferon in response to virus infection and are not sensitive to the anti-viral action of interferon. The differentiated progeny of these cell lines do show both interferon inducibility and sensitivity. The behaviour of the Nulli 2A teratocarcinoma cells differed slightly; the ec cells showed slight interferon inducibility and sensitivity which increased markedly upon differentiation. PC13 Cl 5 and F9 are recently recloned "nullipotent" cell lines that undergo little, if any spontaneous differentiation *in vitro* but can be induced to differentiate by treatment with retinoic acid (Rees *et al* 1979 and Strickland and Mahdavi 1978). Nulli 2A is similarly a "nullipotent" cell line but it has not been recloned since it was established (Martin and Evans 1975). The small amount of interferon inducibility and sensitivity seen in the ec cells of Nulli 2A suggest that this cell line undergoes some degree of spontaneous differentiation whilst in culture, or alternatively may suggest that this cell line has become heterogeneous since it was established. Despite this, data from the Nulli 2A cell line is included in this study because, firstly, the activity of the interferon system does increase greatly upon differentiation and secondly, the differentiated progeny show a greater level of interferon production and sensitivity than that shown by PC13 Cl 5 and F9 endoderm-like cells.

The activation of the interferon system in cultures of differentiated teratocarcinoma cells was first examined by Burke *et al* (1978). These workers were similarly unable to induce interferon production (using a wide range of inducers and conditions) in undifferentiated

cells. The cell lines examined were however, multipotent _in vitro_ and could be induced to differentiate by culture at low density. The data described above confirms that 'nullipotent' embryonal carcinoma cells also have an inactive interferon system which develops as these cells are caused to differentiate _in vitro_ to an endoderm-like cell. The use of such homogenous cultures capable of only limited differentiation _in vitro_ makes available a system wherein the development of the interferon system can be investigated as only one differentiated cell type is generated, and therefore any differences in the development of this system as different teratocarcinoma cell lines are caused to differentiate can also be compared.

The growth of ec cells in interferon-containing media was examined in figure 30 and it was found that cell replication was not affected by treatment with 10^4 units per ml of interferon. Taylor-Papadomitriou (1980) and Hicks _et al_. (1981) have both shown that transformed and normal cells will show an inhibition of growth if treated with interferon concentrations of between 10^3-10^4 units per ml. Ec cells cultured in retinoic acid and interferon simultaneously, did not show a reduction in the rate of replication but did show a reduced cell density at completion of this experiment (this effect was not noticed when the cells were treated with interferon at 10^3 units per ml). This minimal effect on the growth of the differentiated cell may be due to the fact that the growth of the culture is already markedly inhibited by retinoic acid, which would reduce any anti-proliferative action of interferon.

It has been reported that interferon production and sensitivity in a few cell lines, increases as these cells are 'aged' _in vitro_; ie: left undisturbed in culture for a period of eight to ten days (Carver and Marcus 1967, Morgan 1976). Since ec cells are cultured undisturbed for similar periods of time it is possible that the

observed increase in activity of the interferon system may be related to this ageing phenomenon. However, neither L929 cells nor differentiated ec cells show an increase in interferon production associated with ageing. Comparison of interferon yields from a culture of ec cells treated with retinoic acid for seven days in undisturbed conditions, with a similar culture trypsinised and reseeded onto gelatinised plates 24 hours prior to interferon induction, shows that ageing does not increase interferon yields (data not given).

The lack of interferon production and sensitivity can be attributable to many causes. Both these phenomena require the interaction of an inducer with the cell and the subsequent activation of previously inactive gene(s). Thus the cause of the inhibition of the interferon system in ec cells can lie at the level of cell/inducer interaction or at the level of transcription or translation of the induced gene product.

The behaviour of (lytic) virus interferon inducers in both ec and differentiated cells, is generally held to be similar. SFV (Burke et al. 1978) and EMC, Sindbis, Vacinnia, VSV (Teich et al. 1977) are lytic viruses which are all able to replicate equally well in undifferentiated and differentiated teratocarcinoma cells. Although the growth of the viral interferon inducer used in this study (ie: NDV-F) cannot be measured in mouse cells; it can nevertheless be shown (see figure 31) that the rate of desorbtion is similar in the ec and the differentiated cell during the time course of the assay for interferon production. Examination of the activation of the interferon gene in ec cells (by assay of the production of IFN mRNA, see figure 32) in response to virus infection suggests that transcription of the interferon gene does not occur in the ec cell. At the time the experiment was undertaken it was not known if the interferon gene contained introns, which would

require post-transcriptional maturation of the message. Because of this the RNA extracted from the infected cells was injected into the nucleus of the Xenopus oocyte, which is capable of maturation of such message. Recent examination of cloned human interferon genes has shown that neither the alpha nor the beta classes of interferon contain introns (see Introduction); although there are some workers (Sehgal et al. 1981) who consider that there exists an additional series of interferon genes, which contain intervening sequences, in addition to those already identified. The structure of the mouse interferon genes is not clear, but there are as yet, no reasons to consider that these sequences will differ from the form seen in human cells.

A system analogous to the lack of production of IFN-mRNA in induced ec cells, is that seen when these cells are infected with some integrating viruses. Wild-type SV40, Polyoma and ecotropic murine C-type (eg: Moloney leukaemia) viruses, which all require splicing of mRNA prior to translation, do not replicate in ec cells but will replicate in the differentiated cell type (Swartzendruber et al. 1977, Teich et al. 1977 and Segal et al. 1979). The lack of replication of these integrating viruses is thought to be due to the absence of a factor in the ec cell rather than a positive block on replication. Gautsch (1980) formed hybrids of ec cells with cells permissive for SV40 virus replication, and found that these hybrid cells were able to produce infective virus. SV40 virus has been shown to absorb, penetrate, uncoat and migrate to the nucleus in infected ec cells (Lehman et al. 1975, Swartzendruber et al. 1977 and Segal et al. 1979); thus the inhibition of replication of these types of virus in ec cells is considered to involve either integration or transcription of the viral genome. Swartzendruber et al. (1977) were unable to identify integrated

provirus in SV40 or Polyoma infected ec cells, but Segal *et al*. (1979) could identify the presence of non-spliced SV40 viral mRNA in infected cells, which was in contrast to the spliced SV40 viral mRNA seen in differentiated cells. In addition Knowles *et al*. (1980) have inserted a cloned single copy of the SV40 genome plus an adjacent thymidine kinase gene, into thymidine kinase negative F9 ec cells. These ec cells show transcription of the thymidine kinase gene in the absence of apparent transcription of the SV40 genome, and on differentiation of the ec cell, SV40 viral proteins can be detected. These experiments suggested that the ec cell was unable to mature the viral transcripts prior to translation. However the story is more complex for Kelly and Bocarra (1976) have shown that adenovirus 2 (a lytic virus requiring splicing of transcripts prior to translation) can replicate, albeit to a reduced degree, in ec cells. More recently, Katinka *et al*. (1980 a and b) have shown that mutants of SV40 and Polyoma (which have the same *in vitro* transformation ability and *in vivo* tumourigenesis as the wild type virus) are able to replicate in ec cells. The mutation in these variants are thought to affect the area of the viral genome associated with the initiation of transcription. Thus it seems more probable that an aberration in the initiation of transcription exists in ec cells preventing the replication of these integrating viruses. This explanation may also explain the lack of production of interferon and of the antiviral enzymes in the ec cells.

Work by Wood and Hovanessian (1979) and Hovanessian *et al*. (1981) has shown that in interferon-treated cells only one of the antiviral enzymes normally associated with interferon sensitivity is produced. PCC4 ec cells treated with interferon show an elevation of 2'5' oligo A synthetase butnot an elevation of the protein kinase. Wood and Hovanessian (1979) proposed that the lack of interferon sensitivity seen

in ec cells was due to lack of production of one of the anti-viral enzymes and not due to a failure of transduction of the inducer signal to the appropriate genes. However, as has been described in the Introduction, although the presence of these anti-viral enzymes is associated with the anti-viral state in interferon treated cells, direct measurement of these enzymes can be misleading. Meurs et al. (1980) have examined a human cell line which develops an anti-viral state in response to interferon treatment, but does not show elevation of the two anti-viral enzymes. In addition Epstein et al. (1981) have reported an NIH3T3 cell line which fails to develop an antiviral state in response to interferon treatment but shows elevated levels of these enzymes. This cell line has been shown to lack production of the endonuclease which is activated by 2'5' A oligonucleotide synthetase. Recently Nilsen et al. (1980) have shown that although interferon treated PCC4 ec cells do not exhibit an anti-viral state when infected with VSV virus, they do exhibit this state when infected with picornovirus (EMC or Mengo) or with temperature sensitive mutants of VSV. These results support the view that interferon inhibits viral replication in a variety of ways, and that interferon sensitivity needs to be examined using a range of viruses. Ec cells from PC13 cl 5 and from F9 cell lines were examined to see if these cells could exhibit an antiviral state when infected with EMC. Such a state was not found in either of these cell lines, in addition, the minimal sensitivity shown by Nulli 2A was not increased when the ec cells were infected with EMC (data not given). These results suggest that the PCC4 line, on which many interesting features have been demonstrated, may differ considerably from the 'nullipotent' ec cell lines used in this study.

Differentiation of 'nullipotent' ec cells treated with retinoic acid

Examination of the morphology of retinoic acid treated ec cells (see figure 34 b) shows that although changes in phenotype are apparent by day five, the fully differentiated phenotype does not appear until between days five to ten. The differentiating cells show an increase in cell size until a confluent cell culture is obtained. A decrease in cell area is then seen as the cell density increases. The photographs presented in figure 34 b were taken from a culture of PC13 cl 5 cells treated with retinoic acid (shown in figure 34 a) which showed that although 100% of cells were differentiated by morphological characteristics, a nevertheless small percentage of cells did not assume the elongated, flattened shapes seen in the latter photographs of gigure 34 b, but instead continued to resemble the cells which formed after five days treatment with retinoic acid. The behaviour of F9 and Nulli 2A differed slightly from that of the PC13 cl 5 cells; Nulli 2A showed a more rapid transition to the flattened, elongated phenotype and no morphologically undifferentiated cells could be detected. F9 cultures, in contrast, contained many scattered foci of apparently undifferentiated cells which persisted despite repeated trypsinisation and reseeding of the differentiating cultures onto non-gelatinised plates; the undifferentiated cells in the reseeded cultures were found growing in clumps on top of the differentiated cell, which were able to attach to the substrate. Strickland and Mahdavi (1978) report that morphological changes can be seen in the cells after 24 hours treatment with retinoic acid, when cells were observed to move apart and become better attached; such changes were not observed in the cells used in this study until after two to three days treatment with retinoic acid. As described above, the attachment of the ec cells to the substrate is

poor, and these cells when confluent can be removed from the culture dish by simply pipetting in a versene solution. The attachment of the cells to the substrate increases during differentiation, for ec cells cultured for a few days in retinoic acid require incubation with trypsin (80.0 μg/ml for 3-4 minutes at 37°C) to remove the cells.

The microdensitometer scans presented in figures 35 and 36 show that considerable changes in protein synthesis occur as ec cells differentiate. These changes are already noticable by day two and are in advance of observable changes in morphology. The autoradiograph scans presented in these figures were from gel tracks which received an equal loading of acid-precipitable counts. Despite this procedure the weight of the area under the scans from the tracks which contained ec cells treated with retinoic acid, are considerably reduced compared to that from ec cells not treated with retinoic acid. There is no simple explanation for this. It is possible that more labelled protein was loaded onto the tracks containing the ec cells and the cells treated for short periods in retinoic acid than was indicated from the counts per minute obtained after acid precipitation. This might occur if the ec cells contained a large amount of unlabelled protein which quenched the emissions from the labelled protein. However this is just speculation and it is not clear why this phenomenon should be observed. This behaviour does not seem to be an aberration of the gel system since all samples were run on the same gel. The effect was greater the longer the cells were exposed to retinoic acid and also the effect was obtained repeatedly with different samples and different gel systems. It has been reported by Paulin *et al*. (1978) that undifferentiated and differentiated teratocarcinoma cells contain similar amounts of actin and tubulin which undergoes reorganisation to form microfilaments and microtubules during differentiation. The only polypeptide seen not to

alter in relative amount in figure 35, was one slightly smaller than 49Kd MWt, which is probably actin. Lovell-Badge and Evans (1980) have examined the change in protein synthesis in differentiating teratocarcinoma cells using a two-dimensional gel system. Changes in protein synthesis were seen to occur in advance of morphological change in the cells, but the alterations in protein synthesis were few in number compared to the total number of polypeptides involved.

The process of differentiation in teratocarcinoma cells can be followed by measuring the levels of expression of some biochemical products. There have been some reports however that retinoic acid can influence the levels of some of these biochemical markers in 'normal' differentiated cells not undergoing differentiation. Wilson and Reich (1979) report that retinoic acid increases the level of plasminogen activator factor (PAF) ten fold in chick embryo fibroblasts. Jetten et al. (1979) have shown that retinoic acid increases the cell surface incorporation of fibronectin and glycosaminoglycans, and Jetten (1980) has shown that retinoic acid increases the number of epidermal growth factor (EGF) receptors on the surface of mouse fibroblasts. All of these products have been shown to increase as teratocarcinoma cells are caused to differentiate (Linney and Levinson 1977, Wartiovaara et al. 1978 a, b, Rees et al. 1979). The action of retinoic acid in influencing the yield of these products from 'normal' differentiated cells differs from its action on differentiating cells in that the action is reversible; Jetten (1980) reports that the numbers of EGF receptors returns to normal three days after the retinoic acid is removed.

Pretreatment of 'normal' differentiated cells with retinoic acid does not alter subsequent interferon yields, but addition of this vitamin after the cells have been induced to produce interferon has been shown to influence yields. Blalock and Gifford (1976, 1977)

report that retinoic acid inhibits interferon synthesis if applied soon after the interferon inducer, and this inhibition is thought to occur via an effect on transcription. Abb and Dienhardt (1980) also report that retinoic acid inhibits the production of gamma interferon from mitogen stimulated lymphocytes whilst enhancing DNA synthesis in the same cells. Thus the reports that exist show that interferon synthesis is supressed only if retinoic acid is applied after the inducer; therefore all cells treated with retinoic acid in this study were induced to produce interferon in the absence of retinoic acid.

The use of interferon production and sensitivity as a marker of cell differentiation in teratocarcinoma cell cultures

Treatment of ec cell cultures with retinoic acid allows the development of interferon inducibility and sensitivity over a period of approximately 15 days (see figure 38). The pattern of interferon production in these cultures follows the pattern observed in the change in cell size (see figure 34). Both interferon production and sensitivity and cell size are found to be maximal at approximately day 15, when the culture has just reached confluence, and then decrease thereafter. The increase in cell size is difficult to quantify exactly as the cell borders are indistinct in older cultures and the cells become extremely flattened, but approximate measurements can be made. During the period five to ten days after the addition of retinoic acid, cell size increases two fold whilst interferon production increases five fold. For the period five to fifteen days after the addition of retinoic acid cell size increases three fold while interferon production increases thirty-four fold. And finally, during the period five to twenty days after the addition of retinoic acid cell size increases

two fold while interferon production increases twenty-fold. Thus although the pattern of change in interferon yield does follow that of the change in cell size, the degree of change does not correlate. This comparison suggests that a proportion of the observed changes in interferon production are secondary to the retinoic acid induced change in cell size (the larger the cell the greater the synthetic ability). However because the changes in interferon production do not correlate exactly with the changes in cell size other factors must be involved in regulating the increased yield.

The maximum level of interferon production at both the single cell and the population level in a differentiating teratocarcinoma culture, can be seen to vary greatly (see figure 38); this variation was also seen in results obtained from differentiated teratocarcinoma cell lines (see figure 37). Thus it would seem that this variation in interferon production does not reflect the degree of differentiation obtained in the culture but can be attributed to the biological variation normally seen between different cell lines.

The majority of the reports concerning the measurement of bio-chemical products, during teratocarcinoma differentiation, measure levels at any time between three to twelve days after the addition of retinoic acid, and therefore an understanding of the kinetics of the development of the differentiated state is not available. There are however, some exceptions. Rees _et al_. (1979) and Adamson _et al_. (1979) report that maximum activity of EGF receptors and of collagen synthesis in differentiating cultures of PC13 cl 5, occurred at approximately six days after the addition of retinoic acid to these cultures.

The numbers of single cell interferon producers is seen to increase during differentiation to reach maximum levels which varied from 3-26%

of the cells in culture (see figure 38). With any assay it is not possible to say that no product is present, merely that the assay system in use can detect no product. With this in mind, the results from the single cell assay suggest that as the ec cells are caused to differentiate the number of single cells able to produce interferon increases to reach a maximum level. Since interferon production is not expressed in all single cells in a differentiated culture (see section one) this assay system can only provide limited information on the differentiation of individual teratocarcinoma cells.

There are many parameters used to follow the differentiation of ec cells _in vitro_. These have been described in the Introduction in general terms. As described above, the ec cell lines used in this study differentiate into endoderm-like cells which have characteristics resembling either visceral or parietal endoderm. These two types of endoderm are generated in the six day old mouse embryo from the primitive endoderm. It has been shown (Dziadek and Adamson 1978) that visceral endoderm cells secrete alphafetoprotein (AFP), whilst cells from the parietal endoderm do not secrete AFP but instead secret plasminogen activator factor (PAF) (Strickland and Sawey 1980). It has however been demonstrated by Bode and Dziadek (1979) that PAF can be detected in the visceral endoderm of the mouse embryo and it is thus not a unique marker for parietal endoderm; but this secretion is apparently not detected in cultures of differentiating ec cells which produce visceral endoderm (Strickland _et al_. 1980). Appropriate use of AFP (and possibly PAF) can be used to examine the generation of these two types of endoderm _in vitro_; Hogan _et al_. (1981) have demonstrated that F9 ec cells (known to produce parietal on exposure to retinoic acid) will generate cells with a visceral endoderm morphology which secrete AFP if allowed to differentiate in retinoic acid media in the

form of cell aggregates. The same cells treated as a monolayer with retinoic acid plus Dibutryl-cAMP will differentiate into parietal endoderm-like cells which do not secrete AFP.

The information obtained from examination of the interferon system in differentiating teratocarcinoma cell cultures shows that there is no difference in the activation of the interferon system between cultures that produce differentiated cells resembling either visceral or parietal endoderm. The activation of the interferon system can therefore be used as a marker of differentiation in all cultures of teratocarcinoma cells.

The experiment presented in figure 41, which shows that trypsinisation and passage of the differentiating cultures onto non-gelatinised dishes can increase the percentage of single cell interferon producers; indicates that the differentiated population is heterogeneous. At all times during the differentiation period (ie: 5 to 20 days) passage of the cells increased the number of single cell producers in the culture. The procedure involved in passage would be expected to select against the undifferentiated cell, because the ec cell is less tolerant of the concentration of trypsin used (80.0 µg/ml) and cannot readily attach to a non-gelatinised dish. However because this enrichment was seen in cultures already producing maximum levels of interferon (ie: day 15) this experiment suggests that at this period there exist a population of cells which differ in their reaction to passage when compared to the cells producing interferon. By morphological and other biochemical criteria two cell populations do not exist, but as judged by the behaviour of the cells on passage, two cell populations do exist.

Examination of the reversibility of the effect of retinoic acid on the interferon system in differentiating teratocarcinoma cells (see figure 43), has shown that the increase in interferon yield in these

cultures is not reversible. Removal of retinoic acid does not cause a decrease in interferon yield but, it does prevent the full development of the interferon system; the ec cells were shown to require treatment for up to eight days with retinoic acid before maximum interferon yields were obtained. In contrast to the effect of retinoic acid upon interferon production, the effect of this vitamin upon growth of these cells was seen to be reversible. At all times up to ten days treatment with retinoic acid, removal of the vitamin allowed a burst of cell replication. This increase in cell number may be due to the presence in the culture of a sub-population of cells susceptible to the growth inhibitory effects of retinoic acid, but unable to differentiate. The effect of retinoic acid upon interferon production in these cultures is not reversible and is therefore a consequence of differentiation itself.

Treatment of differentiating teratocarcinoma cells with combinations of retinoic acid and of DiButrylcAMP was not found to enhance interferon production in either F9 or PC13 cl 5 cells. However this experiment did highlight a difference in behaviour between these two cell lines. The replication of undifferentiated and differentiated PC13 cl 5 cells was completely inhibited by addition of DiButrylcAMP, whereas the growth of F9 was unaffected by treatment with this chemical. This suggests that both the ec and differentiated cells of these two cell lines are widely different. Strickland et al. (1980) has reported that F9 ec cells treated sequentially with first retinoic acid and then DiButrylcAMP show an enhanced differentiation; laminin synthesis, in addition to increases in PAF and collagen synthesis can be detected. Kuff and Fewell (1980) also report a similar increased differentiation as measured by acetyl cholinesterase activity, in F9 cells similarly treated.

Role of interferon in the control of growth and differentiation

Reports were described in the Introduction concerning the possible biological role for interferon in the regulation of normal growth and differentiation *in vivo*. Some evidence has been presented to support this role for interferon in regulating the behaviour of cells derived from the bone marrow stem cells (Kimchi *et al*. 1981 a and b, Dolei *et al* 1980). The production of spontaneous interferon in cultures of differentiating teratocarcinoma cells was examined during a period of ten days in culture and found to be negative (data not given). Since it has been shown earlier that the ec cells are insensitive to the anti-proliferative and anti-viral actions of interferon, this result although not surprising is worth obtaining, for interferon has frequently been shown to produce paradoxical effects when applied at high or low concentrations (Rossi *et al* 1979b and 1980, and Dolei *et al* 1980). Thus these results show that interferon is probably not involved in regulating growth and differentiation in teratocarcinoma cells. It is possible that the explanation for the lack of involvement of interferon in these processess in ec cells, lies in the fact that interferons can be induced under various pathological conditions. Therefore the production of interferon is not under adequate control, and it is possible that embryonic cells (as typified by the ec cells) have evolved to lack an interferon system to avoid any possible external and uncontrolled influence upon crucial differentiation stages.

SUMMARY OF SECTION TWO

It has been shown that cloned 'nullipotent' embryonal carcinoma cells do not possess an active interferon system and that the lack of this system may be related to a block at the level of transcription in these cells. When these ec cells are treated with retinoic acid there is a development of interferon inducibility and sensitivity which extends over a period of fifteen to twenty days, and is coincident with changes in cell morphology. The kinetics of the development of the interferon system coincide, but are not absolutely correlated, with changes in the cell size within the differentiating culture. It has been shown from the use of the single cell assay for interferon production that as the culture differentiates, there is an increase in both the percentage of cells able to produce interferon and in the yield of interferon per cell. Thus the process of differentiation is marked in these cultures by the recruitment of differentiated (interferon producing) cells and also by processes which modulate interferon yields from those cells, this latter involving both changes in the cell size and in the activity of the genes regulating the interferon system within the cell. The structure of the differentiating population is seen to be more complex than previously thought, for not only can the percentage of 'single cell interferon producers' be increased by means which select for a more differentiated phenotype, but a proportion of cells exist in these cultures whose growth is only temporarily affected by retinoic acid. Thus it would seem that the response of the cell population to retinoic acid is not uniform, despite the many accepted published reports that 100% of teratocarcinoma cells are

changed by treatment with this vitamin.

The activation of the interferon system can thus be used as a marker of cell differentiation in a culture of, but not in individual, teratocarcinoma cells. The regulation of the developing interferon system in differentiating teratocarcinoma cells, may differ to that regulating production of other biochemical markers of differentiation in this system since the activity of the interferon system is not increased by a chemical which has been shown to increase production of some other biochemical markers; these differences could be related to the fact that interferon production and sensitivity is an inducible system. In addition the development of the interferon system does not distinguish between embryonic carcinoma cells caused to differentiate into either visceral or parietal endoderm-like cells, this may be related to the use of interferon *in vivo* which is not restricted to any one tissue type but is active throughout the body.

SECTION THREE --- THE INTERFERON SYSTEM IN THE DEVELOPING MOUSE EMBRYO

SECTION THREE ---- RESULTS

The work described in this section was performed in collaboration with B. R. Randle, of the Zoology department, Oxford University.

Interferon production was examined in embryonic mice at various stages of development. These mice were the progeny of matings between 129J females and F_1 C57BL/CBA males. Pregnant mice of four to fifteen days of gestation (day one of embryonic development is the day that the fertilisation plug is observed), were killed by cervical fracture and the conceptus removed into alpha media containing 5% (v/v) fetal calf serum. (All dissections were performed by B. R. Randle.) Small pieces of embryo tissue and transferred to 10 μl wells containing fresh media (and 5% fetal calf serum) in a 'Terasaki' microtitre plate. The plate was flooded with sterile liquid paraffin and placed in a gassed (with 5% CO_2 in air (v/v)) and humidified lunch box; the samples were then transferred to Warwick University contained inside a large polystyrene box to prevent dramatic temperature changes. Dissections were undertaken in the morning and the samples were transferred to Warwick by mid-afternoon for assay in the early evening. By this time, the majority of samples had rounded up and attached loosely to the base of the plates but had not obviously increased in size. It was found that samples needed to be approximately 0.5 mm in diameter to survive and remain intact during the procedures involved in the assay, and to give repeatable results within the assay.

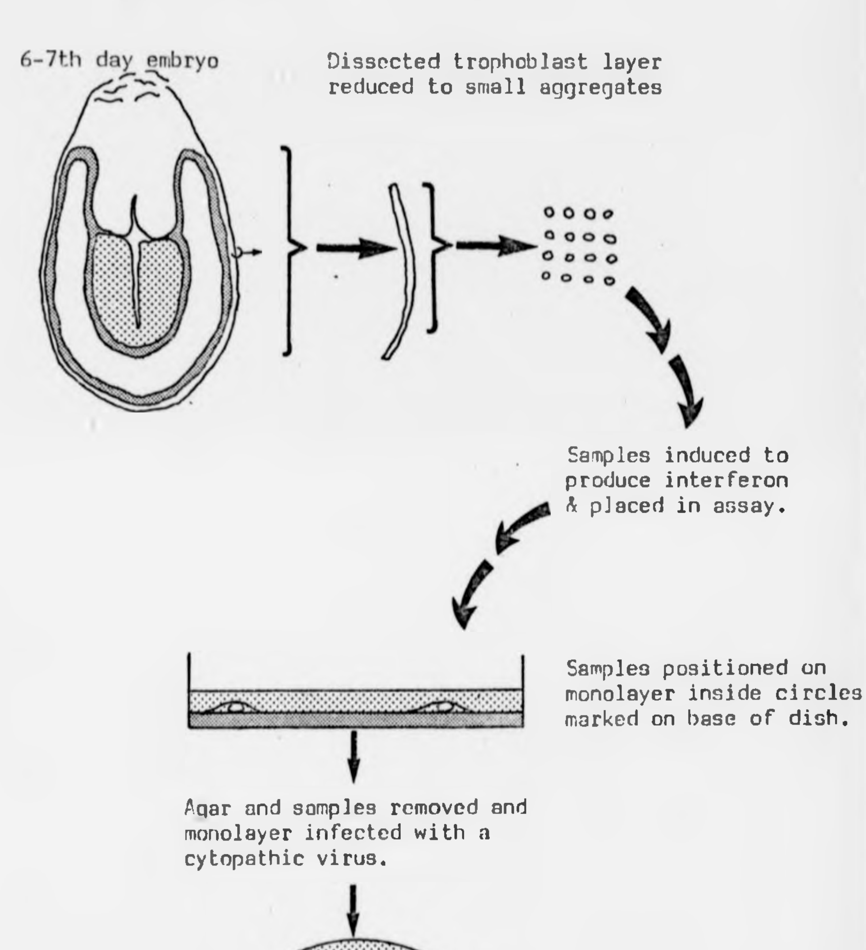
The presence of interferon inducibility in the various embryonic tissues was examined using the modified single cell assay described in

section one. The samples were manipulated during the assay procedure using finely drawn capillary tubes and a mouth pipette, with the aid of a dissecting microscope. On arrival at Warwick, all samples were induced to produce interferon and incorporated into the single cell assay system; the samples were placed in the centre of a circle drawn on the reverse of the assay dish and held in place with a drop of 0.3% (v/v) agar/media. When the agar holding the samples in position had set the whole plate was flooded with 0.3% (v/v) agar/media and the assay continued with as described in Methods. As previously, the presence of interferon production from the samples was marked by the production of a foci of protected cells within the area of the circle containing the sample. The procedures involved in the assay of embryo samples for interferon are illustrated in figure 45.

Since the use of the modified single cell assay to detect interferon production from embryo samples necessitated the use of large aggregates of cells and frequently involved obtaining negative results, it was necessary to run several control experiments concurrently with the embryo samples. It was thought necessary to demonstrate three things with each assay; firstly, that the release of inducing virus from the embryo cell aggregate was inactivated by the antiserum stage; secondly, that the negative results obtained with the assay were not due to a lack of viability of the sample or to a failure of the assay, and thirdly, that the principle under assay was the result of virus induction and not due to spontaneous production of non-interferon interfering factors.

The three control experiments which were incorporated into the assay are illustrated in figure 46. Plate A, in this figure shows the result obtained from an assay performed as illustrated in figure 45 but using aggregates of embryonal carcinoma cells instead of the embryo

FIGURE 45. The proceedures involved in the assay for interferon production from embryonic tissue.



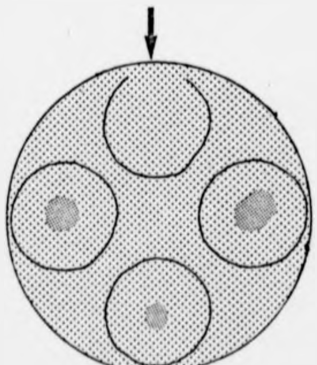


FIGURE 46. Control experiments run concurrently with the single cell assay used to examine interferon inducibility in mouse embryo tissue.

Plate A.

Plate B.

Plate C.

Plate A: virus induced embryonal carcinoma cell aggregates incorporated into the assay as described in figure 45, the incomplete circle contained a non-virus induced sample-

Plate B: virus induced uterus samples taken from a 13day mouse and incorporated into the assay as described in figure 45, the incomplete circle contained a non-virus induced sample.

Plate C: virus induced parietal endoderm taken from a 13 day embryo and incorporated into the assay as described in figure 45, the incomplete circle contained a non-virus induced sample.

samples. As demonstrated in section two, embryonal carcinoma cells do not produce interferon, and therefore virus infection of aggregates of these cells and their subsequent incorporation into the single cell assay system should not produce foci. As can be seen from plate A, no foci are produced when embryonal carcinoma cell aggregates are placed in the assay, the small dark spot present in the centre of each circle are the remains of the ec cells themselves. Thus the results from this assay demonstrated that the cell aggregates do not release additional amounts of inducing virus, and that the foci obtained within the assay are the result of interferon and not the result of a direct interaction between the inducing virus and the indicator monolayer. Plate B, from figure 46 shows the result of an assay in which the samples were taken from the pregnant mouse uterus and incorporated into the assay as described in figure 45. All adult tissues are thought capable of producing interferon (see Introduction) and positive results from these samples would indicate that the samples had survived until placed in the assay, and it would also indicate that the assay was functioning correctly. This plate also illustrates a commonly found feature. Frequently some virus infected samples which were expected to produce interferon did not produce foci in the assay. The proportion of positive control samples which failed to produce interferon was approximately 20% for the control samples used (ie: uterus and decidua), and for this reason ten or more samples from each tissue were included in each assay, and all the results are expressed as the percentage of foci obtained. The last plate C from figure 46, shows typical results obtained from an embryo sample. Parietal endoderm from a 13 day embryo was induced to porduce interferon and placed in the assay. The incomplete circle contained a non-virus induced sample, the remainder contained virus induced samples. Only

virus induced samples of embryonic or maternal origin produced foci throughout the time the assay was in use.

Data from an examination of interferon production in the 7th to 8th day embryo are presented in figure 47; this data is transposed onto diagrams of the embryo in figures 48 a and b. Figure 47 shows the data obtained from embryos aged from seven to eight days. In this figure interferon inducibility is indicated by a percentage obtained from the number of foci produced in the assay divided by the number of samples added to the assay. Values of between 70% to 100% indicate maximum interferon inducibility and those of between 10% to 20% indicate minimum inducibility. It was found that when samples of smaller size were unavoidably used in the assay, the percentage figure obtained was variable and frequently reduced. Consistent results could only be obtained with samples of approximately 0.5 mm diameter. The data in figure 47 shows that the extent of interferon inducibility changes considerably in the embryo during the seventh to eighth day of development. The early seventh day embryo shows only minimal interferon inducibility which is limited to the trophoblast cells. These cells are the first differentiated cells to appear during embryogenesis and constitute the fetal maternal barrier during the first half of gestation. The remainder of the embryo is seen to be unable to produce interferon in response to a viral infection. In addition it can be seen that the inducibility of the control uterus and decidua samples is depressed. The mid-late seventh day embryo shows a spectrum of interferon inducibility. The periphery of the embryo consisting of extra-embryonic tissue (ie: the ectoplacental cone, trophoblast and parietal endoderm) show maximum interferon inducibility. The interior extra-embryonic tissues, the extra-embryonic ectoderm and the visceral embryonic endoderm show minimal interferon inducibility, whilst the

Figure 47. Interferon production in the early post-implantation mouse embryo.

<table>
<tr><th></th><th>Early 7th dy embryo</th><th>Mid-late 7th day embryo</th><th>Late 7th dy embryo</th><th>early 8th dy embryo</th></tr>
<tr><td>Ectoplacental cone:</td><td>0%,0%</td><td>75%</td><td>71%</td><td>ND</td></tr>
<tr><td>Trophoblast ------:</td><td>25%,40%</td><td rowspan="2">75%</td><td>33%**</td><td rowspan="2">80%</td></tr>
<tr><td>Parietal endoderm :</td><td>ND,0%</td><td>ND</td></tr>
<tr><td>Visceral extra-embryonic endoderm:</td><td>0%,0%</td><td rowspan="2">11%</td><td>0%</td><td rowspan="2">91%</td></tr>
<tr><td>Extra-embryonic ectoderm ---------:</td><td>0%,0%</td><td>ND</td></tr>
<tr><td>Visceral embryonic endoderm ---------:</td><td>0%,0%</td><td>7%*</td><td>73%*</td><td rowspan="2">82%</td></tr>
<tr><td>Embryonic ectoderm:</td><td>0%,0%</td><td>0%</td><td>0%</td></tr>
</table>

Control experiments

	Early 7th dy embryo	Mid-late 7th day embryo	Late 7th dy embryo	early 8th dy embryo
Uterus (virus induced):	33%,40%	92%	73%	90%
Uterus (not induced) -:	0%,0%	0%	0%	0%
Decidua(virus induced):	0%,40%	100%	70%	90%
Decidua (not induced)-:	0%,0%	0%	0%	0%
Embryo tissue (not induced) -------------:	0%,0%	0%	0%	0%
embryonal carcinoma cell aggregates ------:	0%,0%	0%	0%	0%

Samples of embryonic tissue, and the control samples, were induced to produce interferon and placed in the single cell assay, as described in figure 45. Results are expressed as the percentage of interferon protected foci produced in the assay per number of samples added to the assay. Results bracketed together were obtained from two adjacent tissues which could not be cleanly separated during the dissection.

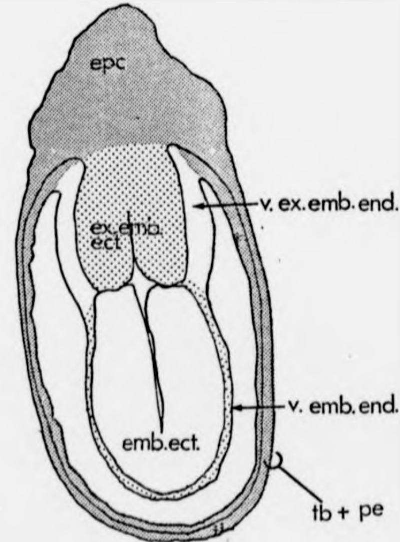
ND : not done. * : visceral embryonic endoderm plus mesoderm.
** : samples smaller in size than those previously assayed.

Figure 48a. Interferon production in the early post-implantation mouse embryo.

Early 7th day embryo.

epc
ex.emb. ect.
v. ex. emb. end.
pe
v. emb. end.
emb. ect.
tb

Mid-late 7th day embryo.

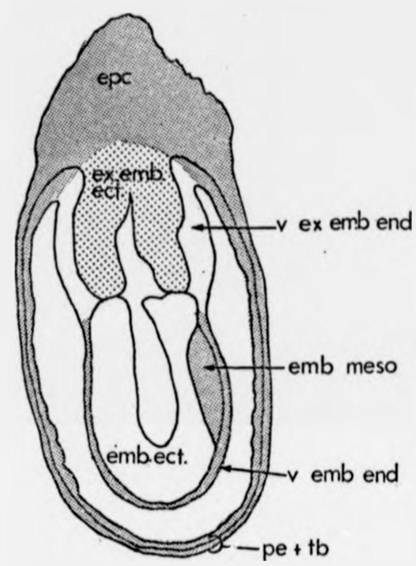


(■) Maximal interferon inducibility.
(▒) Minimal interferon inducibility.
(□) Nil interferon inducibility.

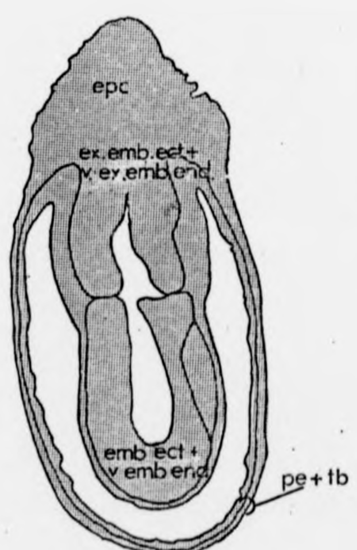
epc: ectoplacental cone, ex.emb.ect: extra embryonic ectoderm, emb.ect: embryonic ectoderm, pe: parietal endoderm, tb: trophoblast, v.ex.emb.end: visceral extra embryonic endoderm, v.emb.end: visceral embryonic endoderm.

Figure 48b. Interferon production in the early post-implantation mouse embryo.

Late 7th day embryo.



Early 8th day embryo.



(▩) Maximal interferon inducibility.
(⬚) Minimal interferon inducibility.
(☐) Nil interferon inducibility.

KEY: as figure 48a.

embryonic ectoderm (the source of the embryo proper) and the visceral extra-embryonic endoderm (which form part of the second extra-embryonic membrane) show nil interferon inducibility. The control samples of uterus and decidua for this and later stages of development show the expected maximum interferon inducibility.

The late seventh day embryo is unchanged except for the increased interferon inducibility shown by the visceral embryonic endoderm and mesoderm. It was not possible to separately obtain results for the extra-embryonic ectoderm from this stage and it is possible that an increase in inducibility in this tissue went unnoticed. The data obtained from the early eighth day embryo was obtained from combinations which contained two neighbouring tissues. All tissue combinations showed maximum interferon inducibility, thus indicating that the degree of inducibility has increased at least in the extra-embryonic ectoderm. The results from this stage cannot however confirm that interferon inducibility can be seen in the embryonic ectoderm and in the visceral extra-embryonic endoderm, but they suggest that this is so.

The diagrams presented in figures 48a and 48b illustrate the onset of interferon inducibility during these stages of embryogenesis and demonstrate clearly that this phenomenon originates in the outer extra-embryonic tissue and develops progressively inwards. The situation presented for the early eighth day embryo was not explicitly identified and remains an interpretation from the data obtained; this data confirmed that functionally the whole embryo is capable of producing interferon in response to a virus infection at this stage of development.

Interferon inducibility in response to virus infection was also examined in the 13th day mouse embryo. The embryo by this stage of gestation has undergone inversion of the germ layers (see Introduction)

and it is surrounded by three embryonic derived membranes. The data presented in figures 49 and 50 show the extent of interferon inducibility in the embryonic membranes and from various parts of the embryo. These data show that tissue representative of the three embryonic germ layers (ie: ectoderm, mesoderm and endoderm) are capable of maximal interferon inducibility, and suggest that the whole of the embryo can similarly produce interferon in response to virus infection. The three extra-embryonic membranes can also be seen to show maximum interferon inducibility. The trophoblast which forms the outer layer of the third membrane is however seen to be unable to produce interferon. The trophoblast was the first embryo derived tissue shown to be capable of producing interferon (see figure 47) and the ability to produce interferon extended over a period of six days in this tissue.

The results presented in figure 47 suggested that the uterus and decidua of the early 7th day gestation mouse is acting either to inhibit the production or action of interferon. Since there has been an isolated report that the mouse embryo may contain an inhibitor to the action of interferon (Cembrzymska-Nowak 1977), it was decided to examine the ability of various extracts of reproductive tissue to inhibit the anti-viral action of interferon. The results of this experiment are presented in figure 51, and it can be seen that all samples whether from pregnant or non-pregnant animals were able to depress the anti-viral action of interferon. The extent of this depression was approximately 40% for the majority of the samples (including the decidua from 7th day pregnant animals) but increased to approximately 80% for the sample obtained from 7th day pregnant uterus.

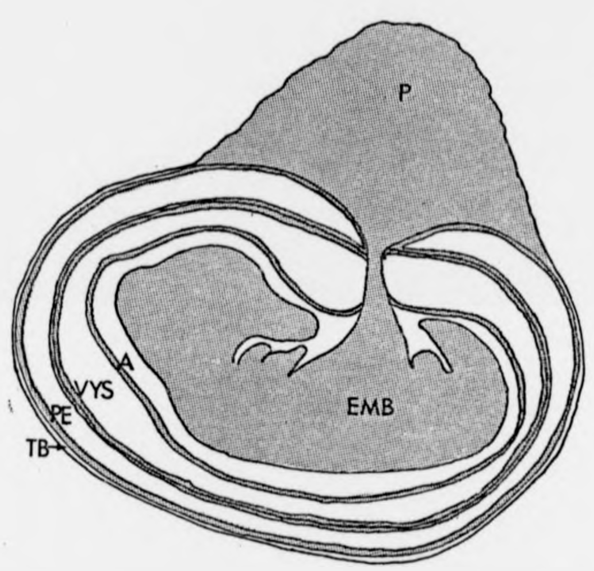
Figure 49. Interferon production in the mid-gestational mouse embryo.

	13th day mouse embryo.		
Trophoblast------------:	0.0%	11.0%	0.0%
Parietal endoderm------:	90.0%	40.0%*	100.0%
Visceral yolk sac------:	90.0%	20.0%*	100.0%
Amnion ----------------:	100.0%	0.0%**	100.0%
Brain------------------:	80.0%	70.0%	50.0%
Heart------------------:	100.0%	0.0%**	ND
Gut region-------------:	89.0%	75.0%	ND
Placenta --------------:	ND	ND	100.0%
Control experiments			
Uterus (virus induced)-:	78.0%	71.0%	75.0%
Uterus (not induced)---:	0.0%	0.0%	0.0%
Embryo tissue (not induced).--------------:	0.0%	0.0%	0.0%
Embryonal carcinoma (virus induced)--------:	0.0%	0.0%	0.0%

Samples of embryonic and control tissues were induced to produce interferon and incorporated into the single cell assay, as described in figure 45. Results are expressed as the percentage, of interferon protected foci compared to the number of samples added per assay.

ND: not done, *: reduced result due to use of very small samples.
**: large and apparently healthy samples used, but negative result obtained.

FIGURE 50. Interferon production in the mid-gestational mouse embryo (13days)

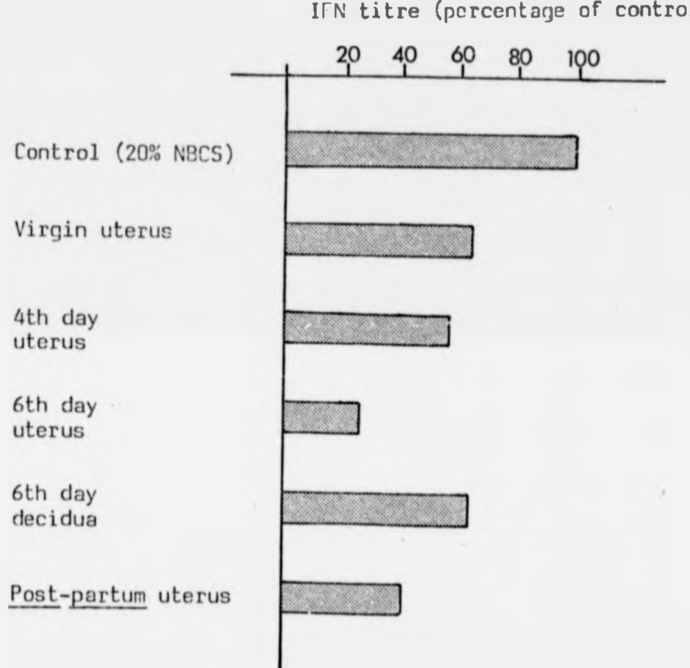


(■) Maximal interferon inducibility.

(□) Nil interferon inducibility.

P: Placenta, EMB: Embryo, A: Amnion, VYS: Visceral yolk sac (ie: visceral extra embryonic endoderm plus extra embryonic mesoderm), PE: Parietal endoderm, TB: Trophoblast.

FIGURE 51. Depression of the anti-viral action of interferon by the reproductive tissues of the mouse.



Female pregnant and non-pregnant mice were killed by cervical fracture and the uteri removed. Samples were homogenised to 20% (w/v) with alpha media. The homogenates were spun at 4 000 rpm for 20 minutes and the supernatants harvested and stored at $-20^{o}C$ until assay. The control sample consisted of alpha media plus 20% new born calf serum (NBCS), each bar represents the mean from two samples.

To assess the inhibitory effects of the homogenates an equal volume of each homogenate were added to replicate dilution series made from a sample of interferon of known titre. A reduction of interferon titre below that of the control indicates that the experimental sample depressed the anti-viral activity of interferon.

SECTION THREE ---- DISCUSSION

The data presented in figures 48 and 50 which illustrate the degree of interferon inducibility of various embryonic tissue, shows that samples which produced less than 20% foci are considered to have minimum interferon inducibility whilst those producing more than 70% foci are considered to have maximum inducibility. It has been demonstrated in sections one and two that the level of interferon production from various tissue culture cell lines was correlated with the number of single cell interferon producers in that population. Thus if interferon production is examined in an aggregate of cells taken from a cell population capable only of low interferon production, it might be expected that such aggregates will produce a smaller number of foci than that seen from aggregates taken from a cell population capable of high interferon production. This is because, with the former population, the probability of the cell aggregates containing sufficient single cell interferon producers to cause production of a foci will be reduced. Thus the designation of the embryonic tissues in figures 48 and 50 as those capable of either maximum or minimum interferon inducibility is based upon the known correlation between the level of interferon production and the number of single cell interferon producers in that population.

The data presented in figure 47 shows that the early post-implantation embryo is capable only of minimum interferon production and this is limited to the trophoblast layer. This layer consists mostly of primary giant trophoblast cells which originated from the trophectoderm cells of the pre-implantation blastocyst. At the early blastocyst developmental stage the embryo consists of two cell

populations, these are the undifferentiated inner cell mass and the surrounding differentiated trophectoderm cells. The former give rise to the extra-embryonic endoderm and mesoderm plus the three germ layers of the embryo, and the latter give rise to the primary giant trophoblast cells, the ectoplacental cone and the extra-embryonic ectoderm (see Introduction).

Examination of interferon production from intact blastocysts (data not given) has shown that no interferon can be detected from this developmental stage. Since only minimal production is seen in the post implantation trophoblast cells, it does however, remain a possibility that the trophectoderm cells are producing interferon in amounts too low to be detected in the single cell assay. Despite this the results do suggest that interferon production in response to viral infection, develops after implantation of the mouse embryo, and that this phenomenon initially is seen in a derivative of the first differentiated embryonic tissue to form during development.

It can be seen from the results of the control experiments described in figure 47 that the interferon inducibility of the control maternal tissue was transiently depressed in the earliest stage examined. These results may suggest that the maternal uterus and decidua was capable of producing a substance which could inhibit either the production or the action of interferon. Mendelson _et al_. (1970) have reported that interferon production from tissues of a 12 day rat embryo was completely inhibited _in utero_, in contrast to interferon production from the tissues of a 19 day rat embryo. In addition, Cembrzynska-Nowak _et al_. (1976) and Cembrzynska-Nowak (1977) claim to have identified an interferon inhibitor in the tissue of a 15 day Porton strain mouse embryo. Examination of reproductive tissues from non-pregnant and pre and early post-

implantation development stages (see figure 51) revealed that homogenates from all these tissues could substantially inhibit the action of interferon. The uterus from the 7th day pregnant animal was seen to exert a greater inhibition than that seen with the other tissue samples. Two features of these results suggest that this depression of the anti-viral action of interferon may not be due to a specific inhibition of interferon. Firstly all samples showed a substantial depression of the activity of interferon. Secondly, although interferon production in the decidua was seen to be transiently decreased in figure 47, the depression of the anti-viral action of interferon was similar to that shown by the remaining tissues. It is possible that the inhibitory activity from these tissue homogenates is due to the presence of substances, which could be carrier proteins for reproduction associated hormones, which bind interferon non-specifically and remove it from solution.

The results obtained from the pre-implantation and the early post-implantation embryo suggest that the interferon system is inactive during the first third of pregnancy in the mouse. Since the embryonic cell and humoral mediated immune response is not seen until the latter part of the second third of pregnancy (Ritter, 1975), the early embryo is itself not able to respond to the presence of a virus pathogen by either interferon production or antibody production. Thus any viral pathogen will potentially multiply unchecked within early embryonic tissues, causing damage to tissue organisation and organogenesis. It is known that virus infections of the embryo which occur during the first trimester of pregnancy in humans and in other animals will result in teratological damage and even death of the fetus (Mims 1968, 1977). It has been reported that the maternal immune response is slightly depressed during pregnancy (Billington and Wild

1979, Mims 1977). However production of maternal antibody will occur during intra-uterine infections and these can reach the embryo via the extra-embryonic membranes. However it has been shown, through a study of viral infections of adult mice, that the early production of interferon, which occurs in these infections, is an important and necessary component of host anti-viral resistance. Without this early production of interferon many, normally harmless, viral infections become fulminating causing rapid death. Gresser et al. (1976 a, b) have shown that innoculation of adult mice with EMC, VSV, NDV or Herpes simplex type II virus whilst the animals are under treatment with anti-serum to mouse interferon, results in enhanced replication of the virus and accelerated death of the experimental animal. It is not clear whether or not maternal interferon can be absorbed via the extra-embryonic membranes in a similar fashion to maternal antibody absorbtion. The results from figure 51 indicate that the maternal reproductive tissue may remove a substantial amount of interferon from uterine fluids, and thus prevent it from reaching the embryo. However, whether or not the embryo has access to maternal interferon is most likely irrelevant. The data from section two showed that interferon production and sensitivity arose together in differentiating embryonal carcinoma cells, this would suggest that these two aspects of the interferon system are similarly linked in embryonic cells. Thus, since interferon is absent from embryonic cells during the first third of pregnancy, it would be expected that these cells also lack sensitivity to the anti-viral effects of interferon.

Interferon inducibility can be seen to develop rapidly during the 7th and 8th day of development, maximum induction is initially shown by the trophoblast and parietal endoderm cells and also by the ecto-placental cone. By late 7th day, the visceral extra-embryonic endoderm

(v.ex.emb.end.) and the embryonic ectoderm (emb.ect.) are the only tissues in the embryo not capable of producing interferon in response to virus infection. Thus at this stage a significant difference is seen in the behaviour of the visceral endoderm, for the visceral embryonic endoderm (v.emb.end.) shows maximum interferon inducibility whereas the v.ex.emb.end. shows nil inducibility.

There have been reports in the recent literature describing similar differences between these two visceral endoderms during the 7 to 8 day period of development Dziadek and Adamson (1978) report that Alphafetoprotein synthesis can be detected in the v.emb.end. but not in the v.ex.emb.end., and Bode and Dziadek (1979) describe a similar difference between these two forms of endoderm in the secretion of plasminogen activator. The production of certain biochemical products from the visceral endoderm is now thought to be modulated by the cell contacts experienced by the v.emb.end. and the v.ex.emb.end., for the former is exposed to the embryonic ectoderm and the latter to the extra-embryonic ectoderm. Dziadek (1978) has reported that although the v.ex.emb.end. cannot produce alphafetoprotein at the 8th day gestation stage, but this tissue becomes able to synthesise this protein after a period in culture in isolation from the extra-embryonic ectoderm. Furthermore, if the v.emb.end. is mixed with the extra-embryonic ectoderm in culture, then alphafetoprotein synthesis is inhibited in this tissue; this inhibition has been shown not to be due to the release of a soluble factor by the extra embryonic ectoderm but is thought to be mediated via cell contacts with this tissue. Hogan and Tilly (1981) report that the phenotype of the v.ex.emb.end is labile whilst in contact with the extra-embryonic ectoderm; for when cultured in proximity to primary giant trophoblast cells this tissue differentiates into cells which are characteristic of parietal endoderm.

Thus the above reports all suggest that the expression of certain biochemical products (previously shown to mark cell differentiation in teratocarcinoma cells), by the visceral endoderm, is regulated by cell interactions with neighbouring tissues. These reports also suggest that contact with the extra-embryonic ectoderm inhibits the expression of these markers of cell differentiation and therefore could be described as inhibiting the actual process of differentiation to endoderm in the v.ex.emb.end cells. The data presented in figure 47 also indicates that the expression of interferon inducibility is, like the expression of alphafetoprotein and plasminogen activator, modulated by cell contact. Visceral endoderm in contact with extra-embryonic ectoderm cannot be induced to produce interferon whilst visceral endoderm in contact with embryonic ectoderm can be so induced. Since the data from section two shows that the activation of the interferon system coincides with cell differentiation, this data can be interpreted as suggesting that the extra-embryonic ectoderm acts to prevent differentiation in the v.ex.emb.end. cells.

The data from figure 47 shows that interferon production is first seen in the trophoblast cells and arises next in two areas; in tissues thought to be derivatives of trophectoderm (ie: the ectoplacental cone and extra-embryonic ectoderm) and also in tissues known not to be derivatives of trophectoderm (ie: v.emb.end.). Thus the activation of the interferon system, like the production of alphafetoprotein and plasminogen activator, is not associated with a particular cell lineage but is associated both with specific cell interactions and with cell differentiation. The activation of the interferon system in teratocarcinoma cells coincides with the differentiation of the embryonal carcinoma cells to an endoderm-like cell, this stage is considered to represent the transition of embryonic ectoderm to endoderm in embryo-

genesis. The results from figure 47 partially support this in that the v.emb.end is shown to be capable of interferon production whilst the embryonic ectoderm lacks this function. However, the v.ex.emb.end. is unable to produce interferon at the same stage of development. This anomaly may suggest that the cells designated as v.ex.emb.end. have not yet differentiated into endoderm proper (which may only occur as these cells are removed from the influence of the extra-embryonic ectoderm during gastrulation), or alternatively this may suggest that some forms of endoderm do not produce interferon.

The results from the early 8th day embryo extends the previous results slightly and suggests that functionally the whole embryo is capable of producing interferon in response to a virus infection. In addition the data confirm that tissues shown to be unable to produce interferon do not exert an inhibitory effect on interferon production from neighbouring tissues.

Thus at the begining of the second third of pregnancy it is probable that the whole embryo can produce interferon in response to a virus infection. It was assumed above, from the information obtained from section two, that sensitivity to the pleiotropic effects of interferon arose coincidentally with interferon production in differentiating cells. Drasner et al. (1979) have shown that sensitivity to the anti-proliferative effects of interferon is not present in in vitro cultures established from pre and early post-implantation mouse embryos, and is first detected in cultures established from 8 day gestation embryos. It is therefore reasonable to assume that the development of sensitivity to the anti-viral effects of interferon follows a similar pattern as that established for interferon production,and sensitivity to the anti-proliferative effects of interferon. Thus if the mid-gestational embryo is exposed to a virus the interferon system will be

activated, resulting in the inhibition of virus replication and also of cell replication. The embryo, at the beginning of the mid-gestational period undergoes gastrulation and organogenesis, and inhibition of cell growth by interferon, induced through virus infection of the embryo, may result in teratological damage similar to that caused by an uncontrolled virus infection of earlier embryo stages. The effect of interferon upon this development stage is unknown. However, Gresser et al. (1978 a, b) has shown that neonatal mice, in contrast to adult mice, can suffer irreversible damage to the liver and kidney tissue resulting in death, if these animals are treated with high levels of interferon (approximately 20 000 units/gm body weight) from birth. This result implies that embryogenic production of interferon during the second and third periods of pregnancy, when the embryo is known to be sensitive to this agent, could produce similar damage to the developing fetus.

It has been shown by Fowler et al. (1980) that there exists a pregnancy-associated elevation of interferon in the reproductive tissues only, of NIH mice. This spontaneously produced interferon was detected from ten days gestation and was present until term, and could also be detected in pathogen free animals. Extremely high levels of interferon were detected in homogenates of placental tissue (up to 3 000 units per ml), and the levels found in the homogenates of the uterus and decidua were reduced compared to the placenta. Thus this report suggests that in normal uterine life the embryo is exposed to interferon during the second and third period of gestation when the embryo is known to be sensitive to the effects of interferon. Thus these results conflict with the known effects of interferon on the later embryos. Furthermore the data presented in figures 47 and 49 demonstrates that none of the embryonic or maternal samples used in the assay

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spontaneously produced interferon. In addition samples were obtained from homogenates of reproductive tissue from an NIH mouse; serum and uterus of pregnant and non pregnant animals, and samples of embryo, placenta and amniotic fluid from 15 day pregnant animals, were all found not to contain spontaneously produced interferon (data not shown). It remains possible that production of interferon during pregnancy indicates an abnormality in that pregnancy, which may or may not represent a pathogenic infection of embryo or maternal host.

It is not yet clear whether or not maternal interferon is able to pass via the placenta into the embryonic circulation. Overall and Glasgow (1970) have found that the placenta was an effective barrier to exogenous interferon whether administered to the foetus or the maternal host. However, Williams and Reed (1981) have reported the presence of the interferon-induced anti-viral enzyme 2'5' oligo A synthetase in mononuclear cells from paired maternal-cord blood samples. This was interpreted to suggest that either interferon or the anti-viral induced enzymes were able to cross the placenta. However the work of Shimizu and Sokawa (1979) and of Kimchi et al. (1979, 1981) has shown that cells of bone marrow origin contain elevated levels of this enzyme when in a quiescient state, and thus the presence of this enzyme in embryonic and maternal mononuclear cells may indicate that the growth of these cells is inhibited.

The data presented in figure 49 shows that all three extra-embryonic membranes, and probably all embryonic tissue from the mid-term embryo is capable of producing interferon in response to virus infection. The parietal endoderm can be seen to produce interferon at this stage, the visceral endoderm together with the extra-embryonic endoderm is also positive. The trophoblast layer of the third embryonic

membrane was however found to show nil interferon inducibility at this stage; as described in the introduction, both layers of this membrane atrophy and disappear at approximately days 14 - 15 gestation but despite this, the trophoblast cells appeared microscopically healthy when used in the assay. Thus interferon inducibility appears only transiently in the trophoblast cells, which are positive by early day seven but negative on or before day 13 of development.

The presence of maximum interferon inducibility in the three embryonic membranes, implies that these membranes would function as a multiple barrier to potential viral pathogens during the latter half of pregnancy. Virus replication could be envisaged as being successively inhibited by the induced interferon production from each layer, thus reducing the probability of infective virus reaching the embryo from a uterine source.

SECTION THREE --- SUMMARY

The data presented in this section demonstrates that maximum interferon inducibility, in response to virus infection, can be seen in the mouse embryo during the second and third period of pregnancy. The interferon system is thus active before the development of the foetal cell and humoral mediated immune response, and probably plays a vital role in the protection of the foetus from viral pathogens during this period of development. The early onset of the interferon system, and the known inhibitory effect of interferon on cell replication suggests that induction of this molecule during morphogenetic events could result in teratological damage to the embryo.

Interferon inducibility is not present in the tissues of the pre-implantation mouse embryo, and is first detected in the trophoblast cells of the early 7th day embryo,becoming established in all extra-embryonic tissues by the end of the 7th day. The pattern of development of interferon inducibility showed that this was not limited to cells derived from any one cell lineage, but instead, arose simultaneously in the tissues derived from the two cell populations present at the blastocyst stage. Production of interferon was found to be absent in the trophoblast cells of the 13th day embryo, but could be demonstrated in the remaining extra-embryonic and embryonic tissues from this embryo. The production of interferon from the maternal 'control' tissues was found to be transiently depressed during the early part of the 7th day of development, and it was suggested that this was due to the non-specific binding of interferon. In contrast to a published report (Fowler et al 1980) spontaneous production of interferon was not detected in any of the maternal or embryonic samples used in this assay.

During the 7th to 8th day of development the visceral extra-embryonic endoderm and the visceral embryonic endoderm were shown to behave differently with respect to interferon production. This is in accord with the published reports on the behaviour of these tissues during this period (Dziadek and Adamson 1978, Bode and Dziadek 1979), which have demonstrated that contact between the visceral endoderm and the extra-embryonic ectoderm inhibited the expression of various markers of differentiation from the former tissue.

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