THE EFFECTS OF INTERFERON ON CULTURED CELLS PERSISTENTLY INFECTED WITH VIRUSES

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DECLARATION

I hereby declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Madeleine West

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ABSTRACT

An investigation was done to examine the role of IFN in viral persistence at the cellular level. For this purpose two types of persistent infections were chosen. The first type was cell lines which contained hepatitis B virus (HBV) DNA (PLC/PRF/5 and Hep 3B cells) uninfected control hepatoma cells, (Mahlavu, HA22T and Hep G2 cells) or simian virus 40 (SV40) DNA (C2, C6, C11 cells) and control uninfected (CV-1 cells). In the second type of infection Vero cells persistently infected with SSPE or Sendai virus were used.

The aim of this work was to determine what effect IFN had in these infections in terms of its antiviral and antiproliferative effects; which of the two major IFN-induced pathways, E enzyme or protein kinase were induced; whether there were any differences in sensitivity to IFN between the DNA and RNA virus persistent infections.

None of the cell lines produced endogenous IFN. The antiviral effect of IFN was examined by its ability to inhibit Sindbis virus replication using a radioimmunoassay system. The hepatoma and the CV cells were sensitive to the antiviral effect of IFN with low concentration of IFN ($\leq$80 IU/ml) inhibiting Sindbis virus replication. In Vero cells persistently infected with SSPE or Sendai virus a concentration of IFN equal to or greater than 1 600 IU/ml inhibited Sindbis virus replication, while in control Vero cells a concentration of 4 IU/ml of IFN was needed. This resistance appeared within a few passages

of the development of SSPE or Sendai persistent infection. Similarly the synthesis of measles virus polypeptides in lytically infected Vero cells was inhibited by an IFN concentration of less than 200 IU/ml, whereas the synthesis of SSPE and Sendai virus polypeptides were not affected by a concentration of 4 000 IU/ml of IFN.

The antiproliferative effect of IFN was determined by cell counting and ^{3}H-thymidine incorporation. All hepatoma and CV cells with the exception of Hep 3B cells were sensitive to the antiproliferative effect of IFN. The growth of control Vero cells were also inhibited by IFN whereas in SSPE-Vero and Sendai-Vero cells IFN had no inhibitory effect.

The induction of the two main IFN-induced enzyme pathways, the E enzyme and the protein kinase, was investigated.

The E enzyme was activated by IFN in all hepatoma and CV cells, from two-fold in Hep G2 cells to thirty-eight-fold in Cll cells. While increases in E enzyme levels were detected in SSPE and Sendai-Vero cells, these increases were regarded as being insignificant as the basal levels were very low. The activation of the ribonuclease F, determined by the inhibition of ^{3}H-leucine incorporation after introduction of 2-5 A into the cells, was variable, being activated in all cell lines with the exception of the PLC/PRF/5, Hep 3B and Hep G2 cells.

The activation of the protein kinase in extracts of cells, pretreated with IFN, was determined by the phosphorylation of α-eIF-2 subunit in the presence of dsRNA. Using a positive L-cells control extract it was shown that IFN did not activate the protein kinase in any of the cell lines studied.

Major differences between the two DNA persistent infections and the two RNA persistent infections were found. No correlation was found between the presence of HBV or SV40 persistent infections and the sensitivity of the cell lines to IFN. On the other hand both the SSPE and Sendai virus persistent infections were resistant to the antiviral and antiproliferative effect of IFN.

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CONTENTS

ABBREVIATIONS

A	actin
2-5 A	2'-5' oligoadenylic acid
BSA	bovine serum albumin
cpm	count per minute
DI	defective interfering
ds	double-stranded
DTT	diothiothreitol
DMEM	Dulbecco's minimum essential medium
DMSO	dimethylsulphoxide
EBV	Epstein Barr's virus
EIA	Equine infectious anaemia
eIF-2	eukaryotic initiation factor 2
EMCV	Encephalomyocarditis virus
EMEM	Eagle's minimum essential medium
E enzyme	2'-5' oligo A synthetase
FCS	foetal calf serum
Fig.	figure
FITC	fluorescein isothiocyanate
h	hour
H	haemagglutinin
HA	haemagglutination
HAU	haemagglutination unit
HBV	hepatitis B virus
HA22T	HA22T/VGH
HBsAg	Hepatitis B surface antigen
i.m.	intramuscularly
i.p.	intraperitoneally
IU	international unit
IFN	interferon
M	matrix
MW	molecular weight
MuLV	murine leukaemia virus
MuIFN	mouse IFN
N	nucleoprotein or nucleocapsid
NAD	nicotinamide adenine dinucleotide

NDV	Newcastle disease virus
NIV	National Institute for Virology
NP40	Nonidet-P 40
o.n.	overnight
P	phosphoprotein
P.	passage
P1	P1 protein phosphorylated by IFN-induced protein kinase
p.i.	post infection
pI	isoelectric point
PBS	phosphate buffered saline
pfu	plaque forming units
PPO	diphenyloxazole
PAGE	polyacrylamide gel
RT	room temperature
RIA	radioimmunoassay
rec	recombinant
RIPA	radioimmunoprecipitation assay
SDS	sodium dodecyl sulphate
SSPE	subacute sclerosing panencephalitis
SV40	simian virus 40
ts	temperature sensitive
TCA	trichloroacetic acid
$TCID_{50}$	Tissue culture infective dose that gives 50% cytopathic effect
VSV	Vesicular stomatitis virus

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CHAPTER 1

INTRODUCTION

Interferon (IFN) is by definition an inducible protein secreted by cells that induces an antiviral state in other cells (Stewart, 1979; Pestka and Baron, 1981). IFN was discovered by Isaacs and Lindenman (1957) who found that, upon exposure to heat-inactivated influenza virus, chicken chorioallantoic membranes released a substance into the surrounding fluid that, when added to the culture medium of other cells, inhibited the replication of active influenza virus. Since it interfered with virus replication, Isaacs and Lindenman called the substance IFN. Most of the cell types from a large variety of vertebrates can produce IFN (Lengyel, 1982; Sen, 1982). IFN is induced by a variety of agents which include all the major virus groups, protozoa, bacteria, fungal products and mitogens as well as natural and synthetic nucleic acids (Stewart, 1979; Pestka and Baron, 1981; Baron et al., 1985). Under normal growth conditions IFN concentrations are below detectable levels in most organs and cell cultures (Lengyel, 1982; Sen, 1982).

A concentration of 3×10^{-14} M of IFN may impair virus replication; therefore, IFN is one of the most potent biological agents known in cell culture (Sen, 1982). IFN is responsible for numerous other effects besides its antiviral activity. It may change the motility, proliferation or differentiation of cells (Stewart, 1979; Sen, 1982; Taylor et al., 1984); it may also affect various immunological processes such as antibody response, delayed hypersensitivity, graft rejection, enhancement of the

expression of certain surface antigens, recruitment of natural killer cells and activation of macrophages (Stewart, 1979; Hooks and Dietrick-Hooks, 1982). IFN also has an antitumour effect in experimental animal systems and against certain human tumours such as laryngeal papillomas and lymphomas (Stewart, 1979; Czarniecki and Friedman, 1982; Malpas, 1983). In the late 1970's human IFN became one of the "wonder" drugs due to its antitumour and antiviral effects.

It is the purpose of this chapter to review the characteristics of human IFN, the main metabolic pathways stimulated in in-vitro systems and IFN's importance in persistent viral infections.

1.1. BRIEF REVIEW OF HUMAN IFN

1.1.1. Natural IFNs

Three antigenically distinct types of human IFN have been described: leucocyte (α), fibroblast (β) and immune (γ). Their differences depend on the type of cell from which they originate and their inducing conditions (Pestka and Baron, 1981) (Table 1).

IFNs of the α-type are products of a multigene family sharing up to 80% homology (Collins, 1984; Gren et al., 1984). Whenever leucocytes are induced to produce α-IFN, a number of these genes are expressed resulting in a preparation which contains a mixture of different α-subtypes (Gren et al., 1984). In contrast to α-IFN, β-IFN seems to have only one or two genes (Weissenbach et al., 1980; Collins, 1984). The genes coding for the α-IFNs and for β-IFN show about 35% homology and are located on chromo-

some 9 (Collins, 1984). Chromosomes 2 and 5 have also been shown to contain β-IFN genes (Collins, 1984). The gene coding for γ-IFN, which is located on chromosome 12, shows little homology with the genes of β and α-IFNs. The γ-IFN gene contains two introns; introns are absent in the other IFN genes (Gray et al., 1982; Collins, 1984).

The main characteristics of α, β, and γ-IFN are shown in Table 1.

1.1.2. Lymphoblastoid IFN

One of the human IFNs used for experimental study in vivo and in vitro is lymphoblastoid IFN. The shortage of α-IFN from human leucocytes prompted research into producing IFN from transformed human cell lines (Finter and Fantes, 1980, 1984). Strander et al. (1975) and Finter and Fantes (1980) produced human IFN from the human lymphoblastoid cell line called "Namalwa". Namalwa cells are B lymphocytes containing the Epstein Barr virus (EBV) genome, and were isolated from a patient with Burkitt's lymphoma (Klein et al., 1972).

Namalwa cells produce large amounts of IFN after induction with Sendai or Newcastle disease virus (NDV) (Finter and Fantes, 1980, 1984). Lymphoblastoid IFN produced by Namalwa cells consists of 60 to 70% α-IFN and 10 to 20% β-IFN (Havell et al., 1977; Finter and Fantes, 1984). This IFN has been shown to induce antiviral activity and to inhibit cell growth in a number of cell types (Gresser et al., 1974; Gewert et al., 1981, 1983; Tovey et al., 1982).

TABLE 1. The properties of the three major groups of IFN produced in human cells

IFN TYPE	α	β	γ
INDUCING AGENT	dsRNA Viruses Mitogens B Lymphocytes Bacteria	dsRNA Viruses Inhibitors of RNA and protein synthesis	Foreign Antigens Mitogens T Lymphocytes
MAJOR PRODUCER CELL TYPE	Leucocytes B Lymphocytes Macrophages	Fibroblasts Epithelial cells Myeloblasts Lymphoblasts	T Lymphocytes
MOLECULAR WEIGHT (MW)	15-23 000	20-23 000	20-25 000
No. AMINO ACIDS IN PRIMARY PROTEIN	165-166	166	166
GENES	13	2	1
INTRONS	no	no	yes
CHROMOSOMAL LOCATION	9	2, 5, 9	12
ISOELECTRIC POINT (pI)	5.7-7.8	6.5	8.6
PROPERTIES	Stable at pH2 Stable with SDS Non glycosylated	Stable at pH2 Stable with SDS Glycosylated	Unstable at pH2 and to SDS Glycosylated
REFERENCES	Stewart et al., 1977 Stewart, 1979 Allen and Fantes, 1980 Hayes, 1981 Collins, 1984	Stewart, 1979 Tan et al.,1979 Hayes, 1981 Knight and Fahey, 1981 Weissenbach et al., 1980 Collins, 1984	Yip et al.,1981, 1982 Gray et al.,1982 Collins, 1984

1.1.3. Recombinant IFN

Using recombinant DNA techniques the genes of human α, β, and γ-IFN have been cloned into Escherichia coli, yeast and cultured cells (Nagata et al., 1980; Weissenbach et al., 1980; Hitzeman et al., 1981; Mory et al., 1981; Gray et al., 1982; Kingsman and Kingsman, 1983; Pestka, 1983; Devos et al., 1984; Arakawa et al., 1985). These recombinant (rec) IFNs have been shown to produce an antiviral and antiproliferative effect in vitro (Samuel and Knutson, 1981; Gray et al., 1982; Pestka, 1983; Czarniecki et al., 1984; Devos et al., 1984; Overall et al., 1984; Arakawa et al., 1985). Comparison of the physicochemical and biological properties of α-IFNs produced by human leucocytes and E. coli have shown that they are similar and have analogous properties; the main difference is that rec α-IFN preparations contain only one α-IFN subtype, while the naturally-derived α-IFN contains different subtypes (Stewart et al., 1980; Nagata et al., 1980; Kingsman and Kingsman, 1983).

1.2. OVERVIEW OF THE IFN-CELL SYSTEM

A common pathway of IFN production in different type of cells has been proposed. IFN genes that are normally switched off are activated by certain inducers and the genes transcribed (Burke, 1983). The mRNAs are transported to the cytoplasm where they are translated in the rough endoplasmic reticulum, and the resultant proteins are glycosylated (α-IFN excepted) and discharged into the extracellular milieu (Stewart, 1979; Burke and Shuttleworth,

1984). The amount of IFN produced has been shown to be directly proportional to the amount of IFN mRNA present (Morser et al., 1979) and can be enhanced by butyrate, 5-bromodeoxyuridine or by IFN pretreatment (priming phenomenon) (Adolf and Swetly, 1979; Stewart, 1979; Johnston, 1980). IFN production lasts for only a few hours following induction; the cause of the switch off of IFN production may be degradation or inactivation of the mRNA (Burke, 1983).

After liberation into the extracellular environment IFN recognizes and then binds to cells which possess specific IFN membrane receptors (Stewart, 1979). Studies of the binding of various IFNs to cells have led to the conclusion that there are separate receptors for α and γ-IFN (Branca and Baglioni, 1981) while β-IFN has been shown to share the same receptors as α-IFN (Branca and Baglioni, 1981). Some binding competition has been found between β and γ-IFN (Thompson et al., 1985).

The nature of IFN receptors is still obscure, a ganglioside and a protein structure having been postulated for the α and β-IFN receptors (Vengris et al., 1976; Besançon and Ankel, 1977; Gupta et al., 1984). Recently chromosome 21 has been shown to be responsible for the coding of α and β-IFN receptors (Raziuddin et al., 1984; Smith-Johanssen et al., 1984). The gene for the γ-IFN receptor has been located on chromosome 6 (Rashidbaigi et al., 1986).

Little is known of the events following receptor binding. Branca et al. (1982) have shown that after binding, human α-IFN is internalized and degraded. However, the need for internalization to produce a biological effect is still controversial

(Branca et al., 1982; Yonehara et al., 1983). It has been proposed that events at the cell membrane trigger signals to the nucleus which lead to the transcription of mRNA coding for the proteins responsible for mediating the effects of IFN (McMahon and Kerr, 1983; Smith-Johanssen et al., 1984). Cyclic AMP may also be involved in the transcription of certain proteins (Kerr et al., 1984). After IFN treatment, newly synthetized mRNA and proteins have been detected and these proteins are presumed to be responsible for the antiviral and antiproliferative effects (Lengyel, 1982; Smith-Johanssen et al., 1984; Kerr et al., 1984).

1.3. THE IN-VITRO EFFECTS OF IFN

Two effects produced by IFN in cells have been extensively studied. Firstly, the inhibition of cell proliferation (anti-proliferative effect) and secondly, the ability of IFN to protect cells against virus infection. These two effects have been shown to be separate and major differences exist in the mechanisms used (Taylor-Papadimitriou et al., 1985). The fact that long exposure to IFN and higher levels of IFN are required for effective growth inhibition in comparison to the lower levels necessary for the establishment of an antiviral state suggests that the receptor interactions leading to the antiviral and antigrowth effects may not be identical (Taylor-Papadimitriou, 1980; Taylor-Papadimitriou et al., 1985).

1.3.1. The antiproliferative effect

The cellular mechanism(s) induced by IFN which produce the

antiproliferative effect are still unknown (Sreevalsan, 1984). As the antiviral effect of IFN is mediated by more than one mechanism, the antiproliferative effect is probably also mediated by more than one mechanism (Sreevalsan, 1984).

IFN has been shown to produce an antiproliferative effect in a wide range of cell types (Taylor et al., 1984). The antiproliferative effect is mediated by receptors on the cell membrane (Kuwata et al., 1976; Taylor-Papadimitriou, 1980) and has been studied by either observing a reduction in cell number, or by a decrease in macromolecular synthesis where incorporation of radioactive precursors of DNA, RNA or proteins is used as a method of assay (Hilfenhaus et al., 1976; Fuse and Kuwata, 1977; Taylor et al., 1984).

IFN has been shown to act on the cell cycle by inhibiting the cell's entry into the S phase and by prolonging the G_1 and S + G_2 phases (Macieira-Coelho et al., 1971; Mataresi and Rossi, 1977; Sokawa et al., 1977).

The sensitivity of cells to the antiproliferative effect of IFN ranges from very sensitive to resistant depending upon the cell type studied (Taylor et al., 1984). Previously it was thought that transformed cells were more sensitive to the anticellular effect of IFN than non-tumour derived cells (Strander and Einhorn, 1977), but this is not the case. For example, X-ray transformed cells derived from C3H fibroblasts were less inhibited than the non-transformed cells (Brouty-Boyé et al., 1979). Various lymphoblastoid cell lines have been compared regarding their sensitivity to the antiproliferative effect of IFN and have been shown to be inhibited to differing degrees (Hilfenhaus et al., 1977).

The sensitivity of cells to the antiproliferative effect of IFN also depends on the type of IFN used (Taylor et al., 1984). α and β-IFN showed a similar antiproliferative effect when used in Daudi cells (Hilfenhaus et al., 1976). In contrast, Borden et al. (1982) have compared the antiproliferative actions of α and β-IFN in a number of cell lines and demonstrated that some cells were more sensitive to β-IFN than α-IFN. γ-IFN produced a significant cellular inhibition in HeLa and U-amnion cells while Daudi cells were insensitive (Tomita et al., 1982a; Rubin et al., 1983). Cloned α-IFNs also appeared to act differently on the same cell line, some producing a greater anticellular activity than others (Fish et al., 1983).

The timing and duration of IFN treatment have also been shown to produce variations in IFN's effects even when the same cell system was used (Taylor et al., 1984). Daudi cells reacted less if IFN was added to actively growing cells (Horoszewicz et al., 1976) and an inhibitory effect on DNA synthesis could be seen only when IFN was added in the late G_1 phase or early S phase in RSA cells (Fuse and Kuwata, 1977). The duration of treatment required for IFN to initiate an antiproliferative action also varied considerably in different cell systems. L-cells needed a minimum of 8 hours (h) of incubation with IFN (Gresser et al., 1970) while murine osteogenic sarcoma required more than 24 h before the antiproliferative effect was noted (Glasgow et al., 1978).

Gresser et al. (1970) found that in L 1210 and Ehrlich ascites cells IFN acted better when it was added to a lower concentration of cells and when a low serum concentration in the

growing medium was used.

1.3.2. The antiviral effect

As with the antiproliferative effect, the antiviral effect is mediated by receptors on the cell membrane (Smith-Johanssen et al., 1984). The antiviral action of IFN involves the induction and/or activation of certain cellular proteins which block virus replication, notably the synthesis of viral components and/or their assembly into new virus particles. The degree of inhibition varies from virus to virus and often is related to the dose of IFN and to the multiplicity of infection (Stewart, 1979).

Different assays have been established to measure the antiviral activity of IFN. The more common of these antiviral assays are the inhibition of viral cytopathic effect, plaque reduction, virus yield reduction, radioimmunoassays and immunofluorescence assays (Falcoff et al., 1973; Grossberg et al., 1984). Antiviral activity is also used to standardize different IFN preparations. The preparations are calibrated against reference standards and the concentration or titer of IFN is given as international units (IU) per milliliter of sample, one IU being the reciprocal of the dilution producing a 50% reduction in viral yield.

The mode of action of IFN varies with different virus. In fact, IFN may interfere with any stage of viral replication such as adsorption, penetration, uncoating, transcription, translation, assembly or release of the virus. Early experiments with infectious viruses suggested that IFN-treated cells were able to adsorb viruses normally, but later it was found that IFN induced a change

in the cell membrane suggesting that membrane changes may also play a role in inhibiting the adsorption and penetration of viruses (Stewart, 1979).

IFN has been shown to inhibit the penetration and uncoating of murine leukemia virus (MuLV) (Aboud et al., 1980; Salzberg et al., 1983a). Inhibition of early viral transcription has been observed with vesicular stomatitis virus (VSV) and simian virus 40 (SV40); IFN inhibited VSV RNA polymerase activity and the transcription of early mRNA of SV40 (Marcus et al., 1971; Oxman and Levin, 1971; Yamamoto et al., 1975; Revel, 1979).

The results of numerous studies concerning the effect of IFN on protein synthesis have shown that inhibition of translation is the major mechanism mediated by IFN (Marcus and Salb, 1966; Stewart, 1979). Depending on the conditions of cell growth, IFN dose and virus multiplicity, both host and viral or exclusively viral protein synthesis is inhibited (Kerr et al., 1984). This mechanism whereby viral protein synthesis can be inhibited may involve two minor pathways independent of double-stranded (ds) RNA or two major pathways dependent on dsRNA. In the minor pathways IFN acts directly on tRNA and mRNA. After IFN treatment it has been shown that cells lose their ability to translate mRNA and this may be overcome by adding tRNA (Gupta et al., 1974). This suggests that IFN may act by changing endogenous tRNA so it cannot accept newly made amino acids. Different species of tRNA were found necessary to restore translation in cell-free lysates (Falcoff et al., 1976; Mayr et al., 1977). The loss of aminoacylation activity of leucine, lysine and serine tRNAs was much faster in extracts of IFN-treated cells than in extracts of control cells (Sen et al., 1976). The inactivation of tRNA may be a consequence of an increase in the level of 2'-5' oligoadenylic acid (2-5 A) phospho-

diesterase upon IFN treatment; this enzyme, in fact, can remove the CCA terminus from tRNAs and inhibit tRNA aminoacylation (Lengyel, 1982; Sen, 1982).

IFN acts on mRNA by inhibiting cap methylation which is responsible for the binding of mRNA to ribosomes. Inhibition of cap methylation by IFN has been observed in reovirus and in VSV-infected cells (Sen et al., 1977; de Ferra and Baglioni, 1981). This impairment is due to the presence of an inhibitor of cap methylation in the extracts of IFN-treated cells. This inhibitor is very labile at 37°C and has been characterized only by its ability to impair cap methylation (Sen, 1982).

Inhibition of mRNA translation by IFN can also be mediated by the dsRNA-dependent pathways (Kerr et al., 1974, 1976). DsRNA is usually produced during replication of most viruses and has been shown to be an IFN inducer and a trigger for initiating the inhibition in IFN-treated cells (Stewart, 1979; Lengyel, 1982; Sen, 1982). The two IFN-activated pathways dependent on dsRNA are:

1) cAMP independent protein kinase

2) 2'-5' oligo A synthetase (E enzyme) (Kerr et al., 1984).

The two systems are independent of each other, although both need ATP and dsRNA (Farrel et al., 1978). Both enzymes are present constitutively in a wide variety of cells and tissues, but their levels increase in response to IFN (Kerr et al., 1984).

1.3.2.1. Protein kinase

Treatment of animal cells with IFN results in the induction of a dsRNA-dependent protein kinase which catalyzes the phosphorylation of the endogenous ribosome associated proteins, P1 and the α-subunit of eukaryotic initiation factor 2(α-eIF-2), and histones (Kimchi et al., 1979a).

P1 has a MW of 67-72 000 in human cells while the MW in murine cells is 65-68 000 (Lebleu et al., 1976; Kimchi et al., 1979a; Samuel, 1979a). There is a controversy regarding the function and identity of P1. P1 is thought to be the protein kinase itself or part of the enzyme as purification studies have failed to separate P1 from dsRNA-dependent protein kinase activity (Kimchi et al., 1979a; Berry et al., 1985). The MW of the protein kinase has been shown to be comparable with the MW observed for phosphorylated P1 (Berry et al., 1985) and P1 contains a dsRNA-dependent ATP binding site (Bischoff and Samuel, 1985). However, by using monoclonal antibodies specific for P1, it has been shown that a clean P1 preparation lacked the property of phosphorylation of itself or exogenous histones, suggesting that P1 and the protein kinase are two different entities (Laurent et al., 1985; Penn and Williams, 1985). Recently Galabru and Hovanessian (1985) have shown that the dsRNA-dependent protein kinase is a 100 000 MW complex of two IFN-induced subunits each having ATP binding sites; a 48 000 MW and a 68 000 MW subunit. The 48 000 protein is most probably involved with the phosphorylation of 68 000 protein which in turn becomes active and phosphorylates the exogenous substrate eIF-2 and histones (Galabru and Hovanessian, 1985).

The second endogenous protein phosphorylated by the protein kinase has been shown to be the α-subunit of eIF-2 which has a MW of 35-37 000 daltons (Lebleu et al., 1976; Zilberstein et al., 1976). The phosphorylation of α-eIF-2 inhibits the formation of the met-$tRNA_f$-40s ribosome complex, one of the first steps in mRNA translation (Jagus et al., 1981). This inhibition has

previously been proved *in vitro* by using ribosomal supernatant of rabbit reticulocyte lysate which contains an ATP-dependent protein kinase which phosphorylates α-eIF-2 (Jagus *et al.*, 1981). Exogenous added eIF-2 has also been shown to overcome the impairment of protein synthesis produced by an activated ribosomal preparation of ascites cells pretreated with IFN (Farrel *et al.*, 1978).

Evidence has been provided that the rate and extent of induction of the antiviral state in cells by IFN correlates with IFN's ability to induce the protein kinase system. Removal of IFN from the medium of L-cells infected with reovirus or VSV produced a corresponding increase in virus yield and a decrease in the level of the IFN-induced protein kinase (Samuel and Knutson, 1982a). Phosphorylation of P1 and α-eIF-2 was also observed in the HeLa cells after infection with reovirus and encephalomyocarditis virus (EMCV) and possibly the activation of the kinase was responsible for the inhibition of viral and cellular protein synthesis (Nilsen *et al.*, 1982; Rice *et al.*, 1985).

Only viral or synthetic dsRNA can activate the IFN-induced protein kinase (Lebleu *et al.*, 1976), but this activation is minimal if the kinase has not previously been stimulated by IFN (Zilberstein *et al.*, 1976; Samuel, 1979b). All types of IFN can induce the protein kinase and an increase of activity of five to ten-fold has been observed (Lebleu *et al.*, 1976; Samuel, 1979b; Hovanessian *et al.*, 1980; Sen, 1982). The ability of IFN to stimulate the protein kinase varies in cells. An increased level of protein kinase following IFN treatment can be detected after 3 h in L-cells (Kimchi *et al.*, 1979b) whereas in HeLa cells the

kinase level only increased after 10 h (Baglioni et al., 1979). In RD-114 cells, persistently infected with RD-114 retrovirus, and in human fibroblasts, IFN is able to activate the E enzyme but not the protein kinase (Meurs et al., 1981; Holmes and Gupta, 1982; Sen et al., 1983). In L-cells, HeLa, human amnion U cells and NIH-3T3 cells IFN activated the protein kinase (Baglioni et al., 1979; Kimchi et al., 1979a; Samuel, 1979b; Epstein et al., 1981).

Lack of protein kinase activity may be due to phosphoprotein phosphatases. A phosphoprotein phosphatase has been partially purified and it acts on the phosphorylated P1 and α-eIF-2; dsRNA was thought to stop the phosphatase degradation but it has been shown that the enzyme is independent of dsRNA (Kimchi et al., 1979a; Epstein et al., 1980; Samuel and Knutson, 1982b).

The stimulation of the protein kinase by IFN can be assayed in vitro by the phosphorylation of P1 and of α-eIF-2 in IFN-treated cell extracts in the presence of dsRNA. The phosphorylated P1 and α-eIF-2 are then detected by sodium dodecyl sulphate (SDS) polyacrylamyde gel electrophoresis (PAGE) followed by autoradiography (Lebleu et al., 1976; Zilberstein et al., 1976; Farrel et al., 1978).

1.3.2.2. 2'-5' oligo A system

The other major system stimulated by IFN is the 2'-5' oligo A system which involves three enzymes: the E enzyme which synthesizes 2-5 A, the 2-5 A dependent endonuclease (ribonuclease

F or L) and 2-5 A phosphodiesterase which degrades 2-5 A to ATP or AMP (Fig. 1).

Constitutive levels of all of these enzymes are present in a variety of cells and tissues (Kerr et al., 1984). The level of the first enzyme usually increases 10 to 10 000-fold in response to IFN while relatively minor increases of less than five-fold are normally observed in the other two enzymes (Kerr et al., 1984). Through the activation of the 2-5 A system IFN acts again on the protein synthesis mechanism by cleaving cellular and viral RNA (Nilsen et al., 1980).

E enzyme

The E enzymes from both mouse and human cells have been purified to homogeneity and these have been shown to have MWs of 105 000 and 100 000 respectively (Dougherty et al., 1980; Yang et al., 1981). Lately two groups have demonstrated the presence of at least two E enzymes (Revel et al., 1982; St. Laurent et al., 1983). St. Laurent et al., (1983) using IFN-treated Ehrlich ascites tumour cells, found one E enzyme fraction (85-100 000) predominantly in the cytoplasm and another fraction (20-30 000) mainly in the nucleus. Revel et al. (1982) reported two E enzymes in human cells, one of 60 000 MW and the other of 20-30 000 MW. In the intact cells the two E enzymes may regulate different events, depending on whether the activating dsRNA is nuclear or cytoplasmic (Silverman, 1984).

The E enzyme, in contrast to the ribonuclease F and the phosphodiesterase, is dsRNA-dependent (Sen, 1982) and in the presence of dsRNA can convert 97% of added ATP into $(2\text{-}5)(A)_n$ (where n ranges from 1 to 15) and pyrophosphates (Samanta et al.,

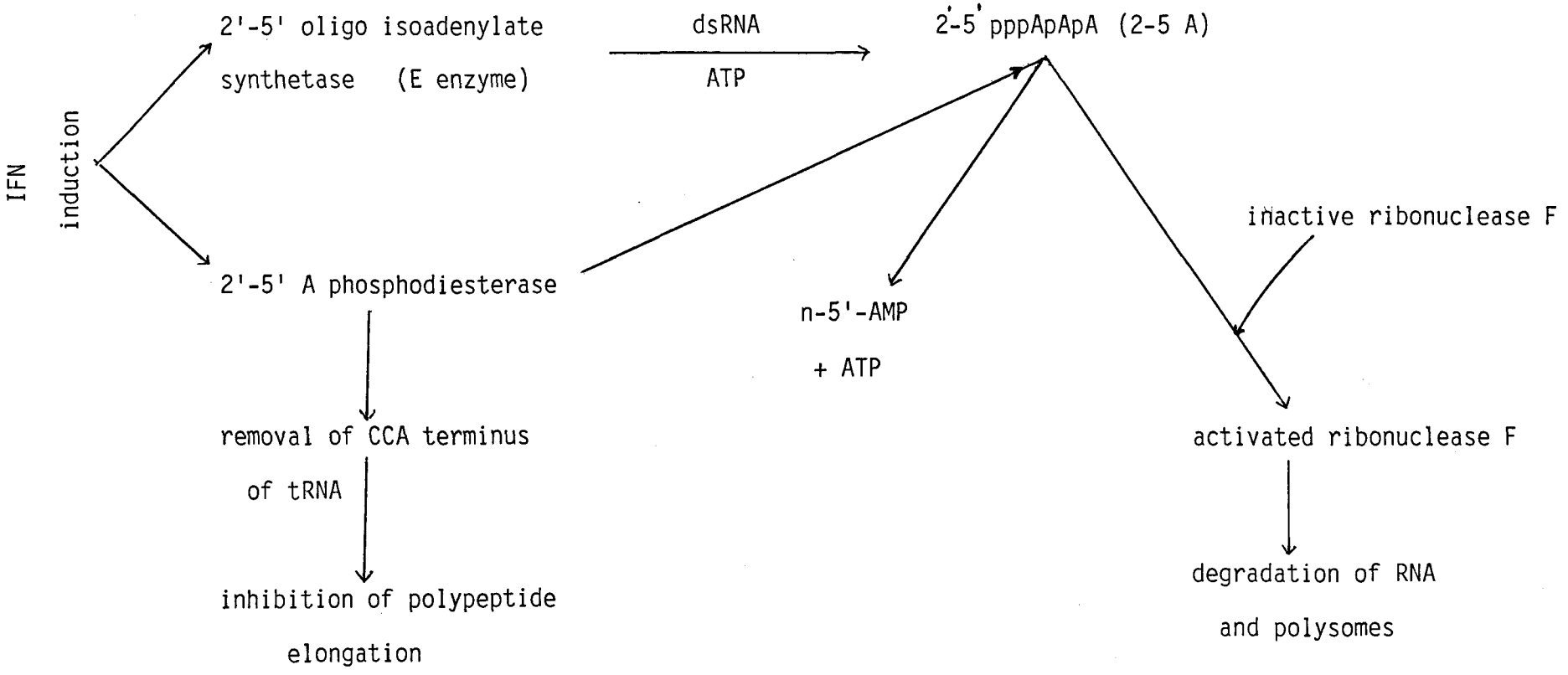

FIG. 1. Outline of the 2-5 A system induced by IFN.

1980). The stoichiometry of the reaction has been formulated as:

$$(n+1)ATP \rightarrow (2'\text{-}5')pppA(pA)_n + n \text{ pyrophosphate}$$

2-5 A trimers, tetramers and pentamers are usually the predominant products (Kerr and Brown, 1978). However, the E enzyme can also catalyse the synthesis of 2'-5' heteroligonucleotides with a structure of pppA2'(p5'A)$_n$2'p5'N where N stands for rC, rG, rU, rT, dC, dG, or dA (Ferbus et al., 1981). Other substrates such as NAD and tRNA can be used by the E enzyme (Ferbus et al., 1981). The ability of the E enzyme to adenylate tRNA has suggested the hypothesis that by inactivating the tRNA the E enzyme inhibits protein synthesis (Justesen et al., 1985).

The E enzyme has been shown to be maximally active when the concentration of dsRNA is about one half that of the enzyme (Samanta et al., 1980). Negligible synthesis of 2-5 A was observed with added single stranded RNA, dsDNA or DNA-RNA hybrids (Minks et al., 1979). 2-5 A has been shown to be sensitive to snake venom phosphodiesterase which cleaves 2'-5' bonds, and to bacterial alkaline phosphatase, which removes its terminal phosphates (Kerr and Brown, 1978). Micrococcal nuclease, nucleases P_1, T_2, U_2, pancreatic ribonuclease and spleen phosphodiesterase which cleave 3'-5' bonds are unable to degrade 2-5 A (Kerr and Brown, 1978). E enzyme activity has been found in a wide array of animals (Stark et al., 1979). The E enzyme has been found in serum, spleen, lung (Krishnan and Baglioni, 1980), in a variety of cultured cells (Torrence, 1982) and in virions of VSV and MuLV grown in IFN-treated cells (Wallach and Revel, 1980). The level of E enzyme can be increased by any of the three types of IFN (Baglioni and Maroney, 1980; Hovanessian et al., 1980) but the level of

the enzyme detected after IFN treatment varies among cell cultures. After IFN treatment the level of E enzyme could be increased in HeLa, L-cells, NIH-3T3 and lymphoblastoid cells (Minks et al., 1979; Hovanessian and Kerr, 1979; Johnston et al., 1980; Epstein et al., 1981), while MRC-5 cells were found to be resistant to any activation of the enzyme (Meurs et al., 1981).

Some cell cultures have high intrinsic levels of E enzyme (Verhaegen et al., 1980; Hovanessian et al., 1981) while others have very low levels (Meurs et al., 1981). Lymphoblastoid cells persistently infected with EBV, for example Daudi and Namalwa cells, have a high level of endogenous E enzyme (Johnston et al., 1980; Tovey et al., 1983).

The kinetics of the induction of the enzyme by IFN vary between cells. L-cells showed an increase in the enzyme level after a lag of 6 h while in BSC-1 cells the level increased after about 2 h, and a maximum peak was usually reached in both cell lines after 24 h of IFN treatment (Kimchi et al., 1979b).

The stimulation of the E enzyme was originally assayed in vitro by the ability of its product, 2-5 A, to inhibit protein synthesis in a cell-free system (Roberts et al., 1976; Hovanessian et al., 1977) but a more direct estimation of its activity was obtained by measuring the polymerization of ATP into 2-5 A oligomers. Labelled oligomers were then separated from ATP by DEAE-cellulose or after nuclease digestion by different methods such as thin-layer chromatography, high voltage paper electrophoresis or separation through alumina acid columns (Kerr et al., 1977; Minks et al., 1979; Merlin et al., 1981). 2-5 A concentra-

tions can also be determined by radiobinding and by radioimmunoassays (RIA)s (Knight et al., 1980).

2-5 A dependent ribonuclease

2-5 A is responsible for the activation of an endoribonuclease called ribonuclease F (Schmidt et al., 1978) or L (Floyd-Smith et al., 1981). In order to activate this enzyme the 2-5 A has to be a trimer or longer and must have two 5' terminal phosphates and two free 3' OH groups (Williams et al., 1978). The activation of the partially purified nuclease (MW 185 000) by 2-5 A is reversible; when 2-5 A is removed, the enzyme reverts to its latent form (Slattery et al., 1979). IFN can enhance the ribonuclease F activity but only very slightly (Silverman et al., 1982). However, Jacobsen et al. (1983) have described a cell line (mouse JLS-V9R) in which, after IFN treatment, the level of the ribonuclease F increased ten to twenty-fold.

The activated 2-5 A endonuclease cleaves cellular and viral single-stranded mRNA equally but does not cleave single or dsDNA, dsRNA or homopolyribonucleotides (Nilsen et al., 1980). Ribosomal RNA can be cleaved in the naked form or in situ in the ribosome (Baglioni et al., 1978). Experiments have shown that the enzyme cleaves predominantly at the 3' side of UA, UU and UG (Floyd-Smith et al., 1981; Wreschner et al., 1981).

The inhibition of protein synthesis and the degradation of polysomes by the ribonuclease F has been determined in cell-free systems and in cultured cells using exogenous 2-5 A (Clemens and Williams, 1978: Slattery et al., 1979). In cultured cells ribonuclease

F activity is determined by introducing 2-5 A by calcium coprecipitation, lysolecithin or hypertonic media followed by incorporation of labelled aminoacids (Williams and Kerr, 1978; Panet et al., 1981). The presence of ribonuclease F can also be detected by affinity labelling with radiolabelled 2-5 A (Wreschner et al., 1982).

In vitro, sensitivity to 2-5 A has been reported in L-cells, BHK-21, HeLa and RD-114 cells, while NIH-3T3 cells were resistant (Clemens and Williams, 1978; Williams and Kerr, 1978; Epstein et al., 1981; Panet et al., 1981; Sen et al., 1983; Silverman et al., 1983).

2-5 A can also inhibit RNA and DNA synthesis and inhibit cell growth but to a significantly lower level than protein synthesis inhibition (Hovanessian and Wood, 1980; Kimchi et al., 1981).

2-5 A phosphodiesterase

2-5 A is resistant to most cellular nucleases because of its unusual 2'-5' phosphodiester bonds (Kerr and Brown, 1978). Nevertheless an enzyme called 2-5 A phosphodiesterase, that degrades 2-5 A_n into AMP and ATP, has been partially purified from L-cells and has a MW of 40 000 (Schmidt et al., 1979). This enzyme is also able to cleave 3'-5' bonds, to degrade the CCA terminus of tRNA and to inhibit tRNA aminoacylation (Schmidt et al., 1979). By producing the latter actions the 2-5 A phosphodiesterase plays a role in the inhibition of protein synthesis in the IFN-dsRNA-independent pathway.

The ability of IFN to activate the enzyme in cells varies. A four to five-fold increase of 2-5 A phosphodiesterase activity after IFN treatment has been observed in L-cells while HeLa, RSA, HEC and Daudi cells did not show any increase (Schmidt et al., 1979; Silverman et al., 1982; Verhaegen-Lewalle and Content, 1982). The phosphodiesterase activity can be assayed by the degradation of 2-5 A and the degraded oligoadenylates separated by thin-layer chromatography or gel chromatography (Verhaegen-Lewalle and Content, 1982; Schmidt et al., 1979).

1.3.2.3. Other antiviral effects of IFN

Other ways in which IFN may reduce virus proliferation are by inhibiting virus budding from the cell membrane or by reducing virus particle infectivity. Inhibition of virus release by IFN has been demonstrated with MuLV, where IFN inhibited the formation of viral 4s RNA and caused a defect in viral assembly and release (Aboud and Hassan, 1983; Aboud et al., 1983). Biochemical changes such as reduction in glycoprotein and membrane protein in VSV particles grown in cells treated with IFN have been observed; these changes were responsible for the production of VSV with low infectivity (Maheswari et al., 1980).

1.4. PERSISTENT INFECTION AND IFN

In vitro a persistent, chronic infection is characterized by the continuous presence of viral genomes in the cells, which may or may not express antigens which are usually demonstrated

by immunofluorescence (Rustigian, 1966; Gluzman et al., 1977; Nishiyama, 1977; Sato et al., 1977; Weiss et al., 1980). In-vitro persistent infections are more readily established with RNA than DNA viruses and with negative-stranded RNA rather than positive-stranded RNA viruses, but examples of persistent infections can be found in all the major virus groups (Mahy, 1985).

Mechanisms by which persistence is induced and maintained can be studied more readily in vitro; such mechanisms usually involve the generation of virus mutants which interfere with wild-type replication and differ from parental virus in properties associated with structural proteins (Mahy, 1985). The virus mutant may be more or less resistant to inactivation by heat (ts mutants), pH, UV light, chemical agents or may have altered hemagglutinating activity (Rima and Martin, 1976; Friedman and Ramseur, 1979; Youngner and Preble, 1980). These changes reflect mutations in structural proteins with multiple functions in the virus replication cycle; the alteration may affect virus-specific RNA and protein synthesis as well as virion integrity and infectivity (Rima and Martin, 1976; Friedman and Ramseur, 1979; Youngner and Preble, 1980).

The presence of defective interfering (DI) particles, which are virus particles lacking part of the genome, has been shown to be important in many persistent infections (Huang and Baltimore, 1970; Holland et al., 1980). A persistent infection can also be established by integration of the virus genome into the host cell genome; this is the case with certain DNA viruses and retroviruses (Mahy, 1985). IFN has also been implicated in the production and maintenance of persistent infections by inhibiting

viral replication (Sekellick and Marcus 1978. 1979, 1980; Friedman and Ramseur, 1979).

1.4.1. DNA viruses

1.4.1.1. SV40

A number of cell lines have been transformed by SV40 virus where part of the SV40 genome has been found to be integrated into the cell genome (Sambrook, 1972; Suarez et al., 1972; Gluzman et al., 1977). The SV40-transformed cells are characterized by the production of T antigens and by the absence or very low levels of virus particles (Sambrook, 1972; Suarez et al., 1972; Gluzman et al., 1977).

The antiviral effect of IFN on SV40 replication has been studied. Pretreatment of the cells with IFN inhibited the cytopathic effect of SV40 (Brennan and Stark, 1983). IFN treatment prior to infection inhibited the onset of early SV40 proteins by acting on RNA transcription, while IFN given after infection acted by inhibiting mRNA translation (Oxman and Levin, 1971; Yamamoto et al., 1975; Metz et al., 1976; Yacobson et al., 1977; Revel, 1979; Brennan and Stark, 1983).

In transformed 3T3-SV40 cells, IFN was unable to inhibit the production of SV40 T antigen (Oxman and Black, 1966; Oxman et al., 1967) but could inhibit the transformation of 3T3 cells by exogenous SV40 (Todaro and Baron, 1965; Oxman and Black, 1966).

1.4.1.2. Hepatitis B virus (HBV)

Hepatoma cell cultures have been derived from primary liver

carcinomas and some of these cell lines produce hepatitis B surface antigen (HBsAg) (Prozesky et al., 1973; Macnab et al., 1976; Aden et al., 1979; Das et al., 1980; Oefinger et al., 1981; Chang et al., 1983). The presence of parts of HBV DNA integrated into the cell genome of HBsAg producer cell lines has been demonstrated by DNA hybridization in PLC/PRF/5 and Hep 3B cells (Marion et al., 1980; Twist et al., 1981).

IFN has been shown to have no effect on the production of HBsAg by PLC/PRF/5 cells and these cells did not produce endogenous IFN (Desmyter et al., 1981; Nakajima et al., 1982). However, IFN had an antiviral effect in these cells by inhibiting the replication of RNA viruses (Desmyter et al., 1981) and by increasing the level of the E enzyme (Nakajima et al., 1982).

1.4.2. RNA viruses

1.4.2.1. Retroviruses

A number of workers have studied the effect of IFN on cells persistently infected with retroviruses. Type C retroviruses such as MuLV and RD-114 were most commonly used for these studies. In cells chronically infected with MuLV, IFN has been shown to produce an accumulation of MuLV particles at the cell surface and to prevent the release of newly formed virus particles (Biliau et al., 1976; Pitha et al., 1976; Aboud et al., 1982; Sen, 1982). Inhibition of virus particle release by IFN was also found in cell lines persistently infected with type B or D retroviruses (Sen and Sarkar, 1980; Canivet et al., 1983).

In NIH-3T3 cells, persistently infected with MuLV, IFN was able to activate the protein kinase and E enzyme while the ribonuclease F was absent (Epstein et al., 1981). Extracts from IFN-treated RD-114 cells persistently infected with RD-114 virus showed no induction of the protein kinase (Tomita et al., 1982b; Sen et al., 1983).

IFN has been shown to be able to inhibit penetration and the uncoating process of MuLV in NIH-3T3 cells (Aboud et al., 1980; Salzberg et al., 1983a), and to prevent either the synthesis or integration of Kirsten murine sarcoma virus into the cell host genome and to stop cell transformation (Morris and Burke, 1979; Avery et al., 1980). This latter inhibition of proviral integration was not found with MuLV (Riggins and Pitha, 1982).

1.4.2.2. Togaviruses

Different factors have been implicated in the persistence of positive stranded RNA viruses such as Sindbis and rubella viruses (Maasab and Veronelli, 1966; Mifune et al., 1970; Inglot et al., 1973; Stanwick and Hallum, 1974; Norval, 1979; Weiss et al., 1980; Williams et al., 1981).

Infection with Sindbis virus is usually highly cytopathic but a persistent infection can be produced by DI particles, by other virus mutants or by IFN (Inglot et al., 1973; Weiss et al., 1980). Inglot et al. (1973) showed that a mouse cell line carrying Sindbis virus produced a small amount of IFN and that the presence

of IFN was necessary for the survival of the cells. When anti-IFN antibodies were added to the cell medium virus multiplication increased and the cell culture was destroyed.

Persistent rubella virus infections have been reported in different cell lines and may be caused by a ts mutant, by the presence of DI particles or by direct transmission of virus from cell to cell or through cell division (Maasab and Veronelli, 1966; Mifune et al., 1970; Stanwick and Hallum, 1974; Norval, 1979; Williams et al., 1981).

IFN was found unnecessary for the establishment or maintenance of chronic infections with rubella virus (Stanwick and Hallum, 1974; Sato et al., 1977; Williams et al., 1981), but was able to cure Vero cells of their persistent rubella infection (Stanwick and Hallum, 1974).

1.4.2.3. Rhabdoviruses

Persistent infections with VSV and rabies viruses have been described (Kawai et al., 1975; Holland et al., 1976; Nishiyama, 1977; Sekellick and Marcus, 1980; Andzhaparidze et al., 1981). DI particles, ts mutants and IFN have been shown to help the establishment and maintenance of persistent infection of VSV and rabies viruses (Kawai et al., 1975; Nishiyama, 1977; Sekellick and Marcus, 1980; Andzhaparidze et al., 1981).

Sekellick and Marcus (1980) proposed a model for the regulatory effect of IFN in persistent infections with VSV in which high concentrations of IFN could cure the cells while lower concentrations permitted the virus to replicate and to

kill the cell.

IFN has also been shown to regulate the establishment of persistent infections of rabies virus in human neuroblastoma cells where the addition of anti-IFN in the cell media favoured the replication of the endogenous rabies virus (Honda et al., 1984, 1985).

1.4.2.4. Paramyxoviridae

It has been possible to produce a persistent infection with a number of paramyxoviridae, e.g. NDV (Maeno et al., 1966), measles and subacute sclerosing panencephalitis (SSPE) viruses (Rustigian, 1966; Hallum et al., 1972; Stephenson et al., 1981; Carter and ter Meulen, 1983), Sendai virus (Ito et al., 1979, 1984) and mumps virus (Truant and Hallum, 1977; McCarthy et al., 1981; Ito et al., 1985).

Paramyxoviridae are negative stranded RNA viruses and persistence in cells is obtained using ts strains, by the presence of DI particles or by alteration of the viral-cellular system relationship (Rustigian, 1966; Truant and Hallum, 1977; Ito et al., 1979, 1984; Wechsler et al., 1979).

Production of IFN was noted in cells persistently infected with paramyxoviridae such as NDV, Sendai, measles and mumps viruses but the presence of IFN was not always considered critical for the establishment or the maintenance of the persistent infection (Hallum et al., 1972; Rima and Martin, 1976; Ito et al., 1976, 1985). Cell lines persistently infected with measles and Sendai viruses which do not produce IFN have been reported (Maeno

et al., 1966; Wild and Dugre, 1978); however, Ito et al. (1985) have shown the importance of IFN in the persistent infection of mumps virus in L-cells.

1.5. AIMS

After its discovery in 1957 by Isaacs and Lindenman, research on IFN as an antiviral drug multiplied, but later, as it was difficult to produce workable quantities, research was hampered. A few virologists continued, nevertheless, to work with IFN, and Strander and Cantell in 1966 brought IFN back to prominence by obtaining a large amount as a byproduct of blood banking. In 1979 Cantell predicted that by the year 2000 different kinds of IFNs would be used routinely for the treatment of a number of diseases and encouraged the production of lymphoblastoid IFN and α-IFN in every country where there was a blood banking system. In 1980, in South Africa, Schoub et al. announced the feasibility of mass production of IFN and the importance of further research with IFN if it was to be used as an antiviral drug.

With the advent of recombinant DNA technology and the production of recIFNs the drawback of the low amounts of IFN fell away and in-vitro and in-vivo studies on IFN and its effects multiplied (Dunnick et al., 1981).

One of the fields still to be investigated is the relationship between IFN and a persistent viral infection. Persistent infections with HBV lead to chronic liver disease or hepatoma while a persistent infection with measles virus may lead to SSPE. In South Africa chronic hepatitis and measles affect a large

percentage of the population and the knowledge of the role of IFN in chronic virus infection could bring about a better understanding of the mechanisms of persistence in viral diseases. The aim of this thesis was to examine the role of IFN in viral persistence at the cellular level. For this purpose two types of persistent infections were chosen. The first type was cell lines which contained integrated HBV or SV40 DNA. In the second type, persistent infection with two RNA viruses, SSPE and Sendai virus, were chosen. These virus systems were examined to determine:

1) what effect IFN has in these persistent viral infections in vitro in terms of its antiviral and antiproliferative effects and to compare the results obtained with control cells

2) which of the two major enzyme systems, E enzyme or protein kinase, was induced, and whether this correlated with the antiviral results

3) whether there were any differences, in terms of the sensitivity to IFN, between the RNA and DNA viruses in persistent infections.

CHAPTER 2

MATERIALS AND METHODS

2.1. REAGENTS AND MATERIALS

Analytical reagents were from Merck, Darmstadt, West Germany or from BDH Chemicals, England, and were of Analar or the highest available quality. Fine chemicals were from Sigma Chemical Co., MO, USA or from Boehringer, Mannheim, West Germany.

Radiochemicals were from Amersham International, Amersham, England, and fluorescein isothiocyanate (FITC) conjugated antibody products were from Cappel Laboratories, PA, USA.

RPMI 1640, Eagle's minimum essential medium (EMEM) plus non-essential amino acids, and Dulbecco minimum essential medium (DMEM) were from Gibco Lab., Grand Islands, NY, USA. Foetal calf serum (FCS), filtered and UV-irradiated, was from the State Vaccine Institute, Pinelands, South Africa.

The eIF-2 preparations were kindly provided by Prof. K.L. Manchester, University of the Witwatersrand, Johannesburg, South Africa, (Sample A), and by Dr. J. Siekerka, Roche Institute, NJ, USA, (Sample B).

Human IFN reference standard G023-901-527 and mouse IFN (MuIFN) reference standard G002-904-511 were from the National Institute for Allergy and Infectious Diseases, Bethesda, MD, USA. Recombinant leucocyte A IFN (R022-8181; IFL-rRA) was a gift from Roche, Basel, Switzerland.

Tissue culture flasks were from Costar, MA, USA, and Corning, NY, USA. Petri dishes (60 x 15 mm) and 6-multiwell plates were

from Costar, MA, USA, while 24 and 96-multiwell plates were from Linbro, Flow Lab., Scotland.

Centrifugation was performed in the following machines : Coolspin MSE, MSE Scientific Instr., Sussex, England, Beckman L88-55 Ultracentrifuge, Beckman Instr. Inc., CA, USA and Eppendorf Microfuge 5412, Eppendorf Geratebau, Hamburg, West Germany.

Photographs were taken with an Olympus OM-1 with a 50 mm lens; Ilford 125 ASA film was used and developed as directed by Ilford.

2.2. STOCK CULTURES

2.2.1. Cell lines

In Table 2 are shown the origin and source of cell lines used in this thesis.

2.2.1.1. Hepatoma cell lines

Hepatoma cell lines used in this study were derived from human hepatocellular carcinomas with the exception of Hep G2 which was derived from an hepatoblastoma. PLC/PRF/5 and Hep 3B cells are the only cell lines that produce HBsAg. Details of the hepatoma cells used in this study are shown in Table 3.

2.2.1.2. CV-1 cell lines

The effects of IFN on CV-1 cell lines with integrated SV40 DNA, (C2, C6, C11), were compared with a control, uninfected, CV-1

TABLE 2. The origin and source of cell lines used in this thesis

CELL LINE	ORIGIN	VIRUS PRESENT	SOURCE	REFERENCE
L-cells	Mouse	None	2	
NIH-3T3	Mouse	MuLV	2	Jainchill et al., 1969
PLC/PRF/5	Human	HBV*	3	Alexander et al., 1976
Hep 3B	Human	HBV*	4	Aden et al., 1979
Mahlavu	Human	None	5	Prozesky et al., 1973
Hep G2	Human	None	4	Aden et al., 1979
HA22T/VGH**	Human	None	6	Chang et al., 1983
CV-1	Primate	None	1	
C2	Primate	SV40*	7	Gluzman et al., 1977
C6	Primate	SV40*	7	Gluzman et al., 1977
C11	Primate	SV40*	7	Gluzman et al., 1977
Vero	Primate	None	1	
SSPE-Vero	Primate	SSPE	8	Stephenson et al., 1981
Sendai-Vero	Primate	Sendai	1	
Namalwa	Human	EBV	9	Klein et al., 1972

* Integrated viral DNA

** Referred to in the text as HA22T

1. National Institute for Virology (NIV), Johannesburg, South Africa.
2. Dr. A. Panet, Hebrew University, Jerusalem, Israel.
3. Dr. J.J. Alexander, Medical University of Southern Africa, South Africa.
4. Dr. D.P. Aden, Wistar Institute, Philadelphia, PA, USA.
5. Prof. O.W. Prozesky, University of Pretoria, Pretoria, South Africa.
6. Prof. C. Chang, National Yang Ming Medical College, Taipei, Taiwan.
7. Prof. E. Winocour, Weizman Institute, Rehovot, Israel.
8. Prof. V. ter Meulen, University of Wurzburg, Wurzburg, West Germany.
9. Prof. M. Revel, Weizman Institute, Rehovot, Israel.

TABLE 3. Hepatoma cell lines used in this thesis

CELL LINE	HBV PRESENT	HBsAg PRODUCTION
PLC/PRF/5	yes	yes
Hep 3B	yes	yes
Mahlavu	no	no
Hep G2	no	no
HA22T	no	no

cell line. Details of the CV-1 cells used in this thesis are shown in Table 4.

2.2.1.3. SSPE-Vero cell lines

A SSPE-Vero cell line derived from Vero cells co-cultivated with SSPE virus-infected brain cells was used in this thesis. Using the supernatant of this cell line, which contained a virus concentration of $6.2 \times 10^{1} TCID_{50}/ml$, secondary cell lines persistently infected with SSPE virus were established. Vero cells at 80% confluency were incubated with SSPE-Vero cell supernatant and when confluent were trypsinized and subcultured. After a few passages, usually at the second one, syncitia were seen and by the following passage the majority of the cells were detached. The few surviving cells were incubated with several changes of EMEM containing 5% FCS. Distinct colonies appeared after two to three weeks; the colonies were then trypsinized and subcultured. The monolayer, which was thus established, was trypsinized and subcultured when it reached confluency.

2.2.1.4. Sendai-Vero cell lines

A Sendai-Vero cell line was established by inoculating Vero cells with approximately 30 pfu/cell of Sendai virus and incubated for 1 h at 37°C. The viral fluid was then removed and replaced with EMEM containing 2% FCS. The cells were passaged when confluent and after few passages, the majority of the cells detached. The few surviving cells were incubated with several changes of EMEM

TABLE 4. CV-1 cells used in this thesis

CELL LINE	SV40 PRESENT	T Antigen PRODUCTION
CV-1	no	no
C2	yes	yes
C6	yes	yes
C11	yes	yes

containing 5% FCS. Distinct colonies were seen after fourteen days and these reached confluency fifteen days later. The cells were then trypsinized and subcultured.

2.2.2. Virus stocks and antisera

2.2.2.1. Viruses

The Edmonston strain of measles virus from the virus bank of NIV was passaged in Vero cells. When the Vero cells reached confluency of 80%, a 10^{-2} dilution of measles virus was added and the culture left for 1 h at 37°C on a rocker platform (Bellco Glass Inc., Vinelands, NJ., USA). The inoculum was then aspirated and replaced with EMEM + 2% FCS. After five days, when there was significant formation of syncitia, the virus was harvested by freeze-thawing. Measles virus was stored at -70°C and titrated by plaque assay and end-point dilution (Table 5).

SSPE virus produced by SSPE-Vero cells was titrated by the end point dilution test (Table 5). The presence of syncitia produced by the virus in SSPE-Vero cells could be seen by light microscopy (Figs. 2, 3).

Sindbis virus was a gift from Dr. J. Desmyter, Rega Institute, Leuven, Belgium. A stock preparation was produced using Vero cells. When Vero cells reached confluency of 70%, Sindbis virus at a dilution of 10^{-3} in EMEM was added and adsorbed for 1 h at 37°C. The inoculum was then aspirated and the monolayer incubated at 37°C; when the Vero cells showed a cytopathic effect, the virus was harvested by freeze-thawing. The virus was stored at -70°C and titrated by plaque assay and end point dilution (Table 5).

TABLE 5. Titres of viruses used

VIRUS	TYPE OF ASSAY	RANGE OF TITRES
Measles	Plaque assay	$3x10^5$ - $6.8x10^6$ pfu/ml
	End Point dilution	$5x10^6$ - $5x10^7$ $TCID_{50}$/ml
SSPE	End Point dilution	$6.2x10^1$ $TCID_{50}$/ml
Sindbis	Plaque assay	$1.8x10^6$ -$2.6x10^6$ pfu/ml
	End Point dilution	$6.9x10^5$ -$1.4x10^6$ $TCID_{50}$/ml
Sendai	Haemagglutination	960 - 1280 HAU
	Plaque assay	$4.8x10^7$ -$1.5x10^9$ pfu/ml

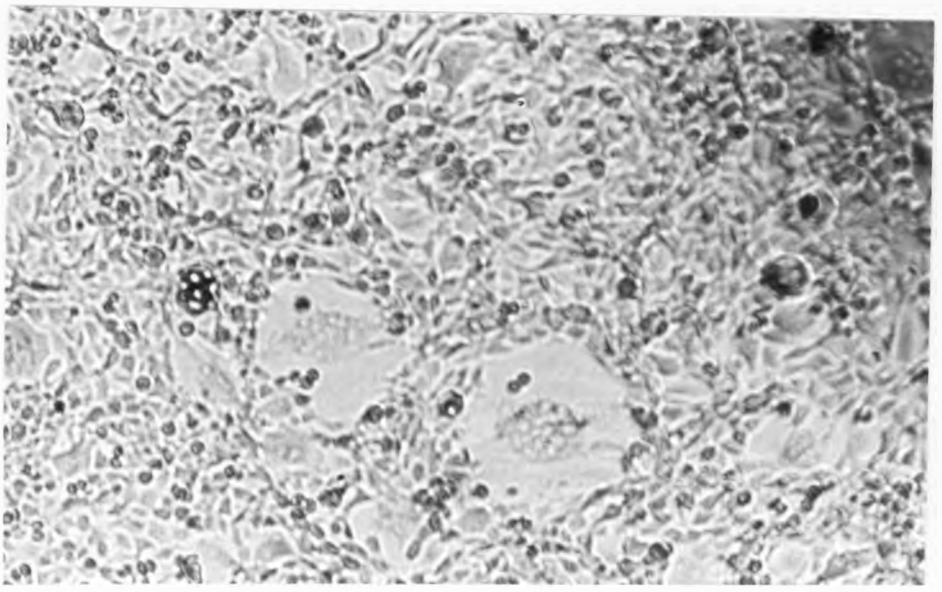

FIG. 2. Light microscopy of SSPE-Vero cells showing syncitia. Cells were examined in a Nikon microscope at 200x magnification.

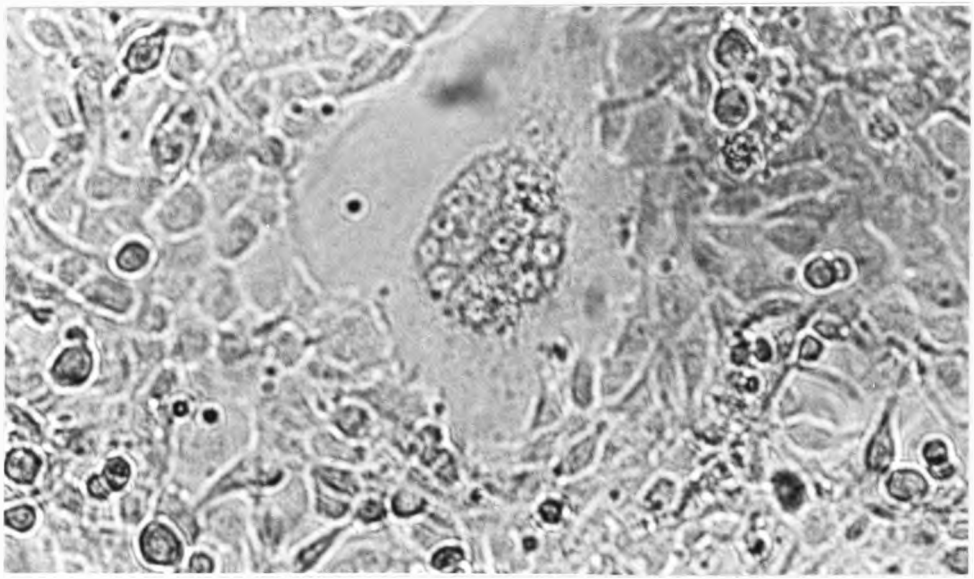

FIG. 3. Light microscopy of SSPE-Vero cells showing syncitia. Cells were examined in a Nikon microscope at 400x magnification.

Sendai virus E82 was a gift from Dr. K. Cantell, Central Public Health Lab., Helsinki, Finland, and was passaged in chicken eggs. Embryonated eggs were incubated for 11 days at 37°C, then inoculated with a 10^{-4} dilution of Sendai stock virus in phosphate buffered saline (PBS) pH7.4, into the allantoic cavity. The eggs were incubated for three days at 37°C and then chilled overnight (o.n.) at 4°C; the allantoic fluid was harvested and titrated using the plaque assay and the haemagglutination (HA) test (Table 4). The stock virus was stored at -70°C until used.

2.2.2.2. Antisera

Bovine anti-measles serum was obtained from Wellcome Diagnostics, Dartford, England; guinea pig anti-measles sera were obtained from the National Institute for Allergy and Infectious Diseases, Bethesda, MD, USA, and from the Behring Institute, Marburg, West Germany. Human anti-measles sera, with a titre $>$ 1:128 in the complement fixation test, were obtained from the NIV serum bank and from Prof. J. Moodie, University of Cape Town, South Africa.

Sindbis antiserum was prepared as immune ascitic fluid in mice (Lyons et al., 1982). A mouse brain suspension was prepared from suckling mice that had been injected intracerebrally with a 10^{-5} dilution of freeze-dried infected mouse brain. When the suckling mice became ill the brains were harvested (in lots of 5) and a 10% suspension of infected suckling mouse brain in saline was prepared. This suspension was mixed with an equal quantity of Freund's complete adjuvant (Gibco, NY, USA) and

inoculated intraperitonally (i.p.) into weanling mice (0.2 ml/mouse) at four weekly intervals. On the fifth week the mice were injected i.p. with 0.2 ml of sarcoma 180/TG ascitic fluid taken directly from a donor mouse with ascites and 10-12 days post-inoculation the ascitic fluid was collected by paracentesis. This was defibrinated with glass beads, centrifuged to remove the fibrin, inactivated at 56°C for 30 min and then recentrifuged. The antiserum was stored at -70°C until used.

Sendai antiserum was made by injecting adult mice intramuscularly (i.m.) with a 10^{-1} dilution of a stock preparation of Sendai virus (0.2 ml/mouse), four times over a two month period; the last injection was given i.p. A week later the mice were injected i.p. with 0.2 ml of sarcoma 180/TG ascitic fluid taken from a donor mouse with ascites. Thirteen to fourteen days later the ascitic fluid was collected and the fibrin removed by centrifugation. The antiserum was inactivated at 56°C and stored at -70°C.

2.3. CELL GROWTH AND SUBCULTURE

In this study all cells were grown as monolayer cultures except the Namalwa cells which were grown in suspension. Monolayer cells were grown in 75 cm^2 flasks in medium containing FCS (Table 6) and were subcultured when they reached confluency. The expended medium was decanted, the monolayers washed with serum-free medium and trypsin (0.25%) and EDTA (0.1%) (at a 1:1 ratio) were added and left until the cells started to detach from the flask. The cells were then resuspended in the growth medium indicated in Table 6. The frequency of subculturing is

TABLE 6. Culture media used for each cell line, and the number of subcultures per week for each cell line

CELL LINE	MEDIUM	NO. SUBCULTURES PER WEEK
L-cells	EMEM + 5% FCS	2
NIH-3T3	EMEM + 5% FCS	2
PLC/PRF/5	EMEM + 10% FCS	1
Mahlavu	EMEM + 10% FCS	1
Hep 3B	EMEM + 10% FCS	1
Hep G2	EMEM + 10% FCS	1
HA22T	EMEM + 10% FCS	1
CV-1	RPMI 1640 + 10% FCS	1
C2	DMEM + 10% FCS	1
C6	DMEM + 10% FCS	1
C11	DMEM + 10% FCS	1
Vero	EMEM + 5% FCS	2
SSPE-Vero	EMEM + 5% FCS	2
Sendai-Vero	EMEM + 5% FCS	1
Namalwa	RPMI 1640 + 20% FCS	1

also shown in Table 6. Viable cell counts were determined by adding 0.5% (w/v) trypan blue in PBS to the cell suspensions at a ratio of 3:1 and the cells were then counted using a haemocytometer.

2.3.1. Storage of cell lines

Confluent cells were trypsinized as above and resuspended in medium containing 20-40% FCS and 10% glycerol at a concentration of 2 x 10^6 viable cells/ml. Cells were dispensed in 1.5 ml aliquots in Nunc ampoules (Intermed, Denmark), left for 1 h at 4°C, followed by 1 h at -20°C in a polystyrene box and then stored at -70°C or in liquid nitrogen. Frozen cultures were thawed rapidly at 37°C and diluted slowly in 5 ml medium containing 10 or 20% FCS. The resulting cell suspensions were placed in 25 cm^2 flasks and subcultured after reaching confluency.

2.4. VIRUS TITRATION

2.4.1. End-point dilution

Cells, seeded in 96-multiwell plates using 0.1 ml of the appropriate cell concentration per well, were incubated with 0.1 ml of virus diluted serially two-fold or ten-fold; the plates were then incubated at 37°C in a 5% CO_2 incubator. The wells were observed daily for the presence of cytopathic effect, and after approximately 10 days the $TCID_{50}$/ml calculated according to the method of Reed and Muench (1938).

2.4.2. Plaque assay

Vero cells, seeded in Petri dishes, were inoculated with serial ten-fold dilutions of virus when they were 90% confluent. After 1 h adsorption at 37°C the virus was aspirated. The monolayers were covered with 8 ml of 1% Sea-agarose (FMC Corp. Rockland, ME, USA) in EBME buffer (0.8% Hepes, 0.11% $NaHCO_3$ containing 5 µg/ml of trypsin). The trypsin was omitted in the case of measles virus. The overlay was allowed to solidify and the plates incubated in an inverted position for 4 days. Neutral red (0.1% w/v in PBS) was added and left for 4 h at room temperature (RT) in the dark. The excess neutral red was poured off and the plates kept in the dark at RT. The next day the plaques were counted and the plaque forming units (pfu)/ml calculated.

2.4.3. Haemagglutination test

The HA test was done in 96-multiwell plates. To each well was added 100 µl of the appropriate dilution in PBS plus 100 µl of 0.5% fowl red blood cells in saline; the plate was left at RT for 45 min. The plate was then read and the highest dilution of the virus with complete haemagglutination was considered the end point of the titration (Salk, 1944) and this titre was given in HA units (HAU).

2.4.4. Immunofluorescence

Ten microliter of cells (1×10^6 cells/ml) suspended in

saline plus 5% FCS were fixed onto slides with cold acetone; the slides were then air-dried and stored at -70°C if not used at once. The fixed cells were incubated with the specified antibody for 15-30 min at 37°C in a humidified box. The slides were washed for 10 min in warm PBS and then incubated with FITC conjugated antibody in 0.05% Evans blue at 37°C for 15-30 min. The slides were then washed for 10 min in warm PBS, air-dried and covered with mounting fluid (10% glycerol in PBS) and a cover-slip. The immunofluorescence was read using the immunofluorescent microscope with a Fluoro 10x or 20x lens.

2.5. INTERFERON PREPARATION

2.5.1. Lymphoblastoid IFN

Lymphoblastoid IFN was prepared following the method of Lyons et al. (1982) using Namalwa cells which have been shown to produce high levels of IFN with Sendai virus and sodium butyrate (Strander et al., 1975; Baker et al., 1980; Johnston, 1980).

Namalwa cells were kept in suspension culture in RPMI 1640 plus 10% FCS and maintained at a concentration of 1 x 10^6 cells/ml by splitting two or three times per week. Duran Scott 1ℓ bottles, rolled on a cell production roller apparatus (Bellco Glass, Inc., Vinelands, NJ) at approximately 0.25 rev/min, were used to maintain the Namalwa cells in suspension. To produce IFN, the cells were adjusted to a concentration of 0.5 x 10^6 viable cells/ml in RPMI 1640 supplemented with 2% FCS and 100 ml aliquots were placed into tissue culture flasks (75 cm^2). Sodium butyrate was added to a final concentration of 1 mM. After incubation at 35°C for 48 h, the suspension was centrifuged and

each cell pellet resuspended in 30 ml of RPMI 1640 plus 2% FCS. Sendai virus was used for induction at a concentration of 100 HAU/10^6 cells. The cell suspension was incubated for 18-25 h at 37°C after which the supernatants were harvested and clarified by centrifugation. The pH of the supernatant fluid was adjusted to 2.0 with concentrated HCl, held at 4°C for 72 h to inactivate the virus, and then returned to pH 7 with 1 N NaOH. The IFN produced was assayed, using the RIA, for sensitivity to raised temperature (56°C) for 30 min, trypsin (100 µg/ml) for 30 min at 37°C, ultracentrifugation (100 000 g for 2 h) and to pH 2. The IFN preparation was found to be sensitive to 56°C and to trypsin, while IFN activity was found after a high speed centrifugation and after treatment at pH 2. The IFN was titrated against the human IFN reference standard and was found to have a titer of 16 000 IU/ml. The IFN preparation was kept at -70°C, and this preparation was used throughout this thesis if not otherwise indicated.

2.5.2. MuIFN

MuIFN was produced in L-cells induced with Sendai virus (Lyons et al., 1982). Confluent monolayer cells were washed twice with warm EMEM and then 200 HAU of Sendai virus per 10^6/cells were added. After incubation at 35°C for 1 h the inoculum was removed and replaced with fresh EMEM supplemented with 2% FCS. The cells were incubated for 18-24 h at 35°C after which the supernatant was harvested and clarified by centrifugation. The pH of the supernatant was adjusted to pH 2.0 with concentrated HCl and held at 4°C for 72 h to inactivate the virus, then returned to

pH 7 with 1 N NaOH. The IFN was titrated against the mouse reference standard and had an activity of 3×10^4 IU/ml. It was stored at -70°C in 100 µl aliquots.

2.5.3. Mock IFNs

Mock IFNs were prepared following the same procedure for lymphoblastoid or MuIFN but no virus inducer was added to the cells.

2.6. ANTIVIRAL ASSAYS

2.6.1. Radioimmunoassay

The indirect RIA following the method of Lyons et al. (1982 was used. Cells were seeded in 96-multiwell plates and incubate at 37°C in 5% CO_2 until the cells were confluent. The spent medium was removed by aspiration and replaced with 100 µl of diluted IFN. EMEM with 5% FCS was used as a diluent and a negative control. The plates were incubated at 37°C in 5% CO_2 for 18-24 h, the IFN removed and the cells washed twice with warm EMEM. To each well was added 100 µl of Sindbis virus at the appropriate dilution. The appropriate dilution of Sindbis virus was previously determined by titrating the virus in the same RIA system and by choosing the dilution which gave a value of approximately 1 000-2 000 counts/min (cpm). After adsorption of the virus for 1 h at 37°C the unattached virus was removed by aspiration, 100 µl of fresh EMEM containing 2% FCS added to each well and the plate incubated o.n. at 37°C in 5% CO_2. The medium

was then removed and the plates washed three times with warm wash-buffer [PBS, 0.1% bovine serum albumin (BSA), 0.02% sodium azide and 0.05% Tween 20]. After air-drying for 10 min the monolayer cells were fixed with chilled methanol and the plate placed at -20°C for 10 min. The methanol was then removed and the plate air-dried. After drying, the wells were washed three times with wash buffer and approximately 200 µl of the same buffer was added to each well. The plates were incubated at 37°C for 1 h, the wash buffer was then removed and replaced with 50 µl of anti-Sindbis antiserum which was used at antibody excess. After a further hour at 37°C the antiserum was removed and the plates washed three times with wash buffer. To each well was added 100 µl of ^{125}I-protein A previously adjusted to 30 000 cpm/100 µl, using wash buffer as diluent. The plates were again incubated for 1 h after which the wells were washed four times with wash buffer. The cells were solubilized with 0.1 N NaOH + 0.1% sodium dodecyl sulphate (SDS), 100 µl/well, for 1 h at 37°C. Fifty µl of solubilized material was then counted in a γ-counter. Each sample was tested in duplicate in each assay and the average count for two wells used in the calculations. The results are given in IFN IU/ml which is the equivalent of the concentration of IFN needed to produce a 50% Sindbis virus reduction in comparison to the virus control well, as measured by cpm/well.

2.6.2. Immunofluorescence

Cells were grown in multiwell Bellco chambers (Bellco Glass

Inc. Vinelands, NJ, USA) and when they were 80% confluent the EMEM was aspirated and replaced with dilutions of IFN. After 24 h at 37°C in 5% CO_2 the IFN was removed and replaced with the appropriate dilution of Sindbis virus and the chambers left for 1 h at 37°C in 5% CO_2. The appropriate dilution of Sindbis virus was previously chosen by titrating the virus in the same system, but without IFN, and taking the lowest dilution of the virus that produced immunofluorescence. The unattached virus was aspirated and EMEM + 2% FCS was added. After two to three days incubation at 37°C in 5% CO_2 the cells were examined for immunofluorescence, and the lowest dilution of IFN that inhibited virus replication determined.

2.6.3. Radioimmunoprecipitation (RIPA)

Cells were grown at 37°C and when they reached a confluency of 80%, IFN was added for 24 h. The IFN was then removed and virus added and left for 1 h at 37°C. The viral fluid was then discarded and replaced with EMEM + 2% FCS and the cells incubated at 37°C for 24 h or as indicated. The medium was replaced with methionine-free medium for 1 h and then for 2-24 h with methionine-free medium containing 20 µCi/ml of ^{35}S-methionine. The cells were washed once with chilled PBS, scraped off in 1 ml of PBS and pelleted by centrifugation. The cells were lysed in 0.5% w/v Nonidet-P 40 (NP40) in NETBSA-NP40 (0.5% w/v NP40, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, 1 mg/ml BSA, pH 7.4) and the nuclei removed by centrifugation at 3 000 rpm for 10 min at 4°C. The viral intracellular proteins in the supernatant were immuno-

precipitated with the specific antiserum using the method of Struthers and Swanepoel (1982). Cell extracts were incubated for 4-6 h at 4°C with the antiserum and then for 4 h or o.n. with 8 mg/ml of protein A-sepharose beads CL-4B (Pharmacia, Sweden) in NETBSA-NP40 at 4°C. The beads were then washed six times with NETBSA-NP40 buffer, resuspended in 100 µl dissociation buffer (10% SDS, 10% 2-mercaptoethanol, 15% glycerol, 0.01% bromophenol blue in 1 M Tris pH 6.8) (Laemmli, 1970) and boiled for 5-10 min. Virus specific polypeptides were then analysed by linear 12% SDS polyacrylamide slab gel electrophoresis (PAGE), run in parallel with MW markers and visualized by fluorography (see 2.11).

2.7. ANTIPROLIFERATIVE ASSAYS

2.7.1. ^{3}H-thymidine incorporation

Cells were plated in 24-multiwell tissue culture plates in EMEM supplemented with 10% FCS. Different dilutions of IFN or mock IFN were added at the time of plating and the cells incubated for 48 h at 37°C in a CO_2 incubator. Mitotic activity was then measured by pulsing with ^{3}H-thymidine at 2 µCi/ml in EMEM with 10% FCS for 1 h. The cells were then washed with cold PBS, 5% trichloroacetic acid (TCA) was added to each well and the plate held for 30 min at 4°C. After a further two washings with 5% TCA the cells were solubilized with 0.1 N NaOH containing 0.1% SDS and the plate incubated at 37°C for 1 h. The samples were neutralized with 1 M HCl and then counted in a scintillation counter. The percentage inhibition of TCA precipitable counts,

determined by comparing untreated cells and cells treated with various IFN dilutions, was recorded. Each cell line was tested three times and the mean of three counts was determined.

2.7.2. Cell counting

Cells were plated in Petri dishes (60 x 15 mm) in EMEM containing 5 or 10% FCS as indicated (Table 6). When the cells reached 80% confluency the medium was removed and 2.5 ml of IFN, at varying dilutions, was added. The Petri dishes were left at 37°C in 5% CO_2 for 48 h, then the cells were trypsinized and viable cells determined by trypan blue exclusion. Each IFN dilution was tested in duplicate and the assays repeated at least three times for each cell line.

2.8. E ENZYME ASSAY

The level of E enzyme in the cells was measured as described by Schattner et al. (1981). Cells were grown to confluency in 150 cm^2 flasks in EMEM containing 5 or 10% FCS with or without lymphoblastoid IFN (200 IU/ml) or mock IFN. After trypsinization the cells were washed with cold PBS and resuspensed in lysis buffer B [20 mM Hepes buffer pH 7.5, 5 mM $MgCl_2$, 120 mM KCl, 7mM dithiothreitol (DTT), 10% (v/v) glycerol, and containing 0.5% NP40]. Lysis was carried out at 4°C and the extract centrifuged for 6 min at 8 000 x g. The supernatant was stored at -70°C in aliquots if not used immediately. Cell extracts (10 µl) were mixed with agarose poly (I) (C) beads and incubated for 15 min

at 30°C. A reaction mixture (10 µl) of 10 mM Hepes pH 7.5, 5 mM $MgCl_2$, 7 mM DTT, 10% (v/v) glycerol, 2.5 mM α^{32}P-ATP (100-300 Ci/mmol), 3 mg/ml creatine kinase, 10 mM creatine phosphate and 40 µg/ml poly (I) poly (C) was added to the preparation. After 21 h incubation at 30°C, 20 µl of calf alkaline phosphatase (150 U/ml) in 1 M Tris base was added to the preparation and incubation was continued for 2 h at 37°C. Each extract mixture was then applied to an acid alumina column, which bound the free phosphate, and the cores of 2-5 A eluted with HCl-glycine, pH 2.3. The results were given in pmoles of 2-5 A/h/µg protein. The amount of protein in each extract was calculated using the BIO-RAD assay kit which is based on the shift in the maximum absorbance of Coomassie blue G250 from 465 to 595 when protein binding occurs (Bradford, 1976). Five ml of dye reagent was added to 0.1 ml of diluted sample and after 5-60 min the absorbance was read at 595 nm in a Pye Unicam spectrometer. A standard curve of absorbance versus concentration of protein was drawn using BSA (0-1 mg/l) and the unknown protein concentration read off the curve.

2.9. RIBONUCLEASE F ASSAY

Cells were plated in 24-multiwell plates in EMEM containing 5 or 10% FCS and incubated at 37°C in a humidified 5% CO_2 atmosphere. When the cell growth was approximately 75 to 80% confluency, the medium was aspirated, the cells washed with EMEM and then incubated for 1 h at 37°C with EMEM. Serial logarithmic dilutions of 2-5 A, from 10^{-6} to 10^{-9} M, were made in transfection buffer

(0.02 M Hepes, pH 7.08, 0.13 M NaCl, 5 mM KCl, 0.7 mM $Na_2HPO_4 2H_2O$, 6 mM glucose). The 2-5 A was introduced into the cells by calcium coprecipitation (Hovanessian and Wood, 1980); $CaCl_2$ (2.5 M) was added to the cells at a final concentration of 114 mM. After 10 min at RT, when a fine precipitate had formed, the 2-5 A dilutions were added to the cells. The plates were left at RT for 45 min and then transferred to 37°C for 90 min. The solution was aspirated, replaced with EMEM containing 10% FCS and the plate incubated for 105 min at 37°C. The medium was then changed to EMEM without leucine to which 1 µCi/ml of ^{3}H-leucine was added. After a further hour at 37°C the cells were washed with PBS, 1 ml of 5% TCA was then added to each well and the plate held for 30 min at 4°C. The cells were then washed twice with 5% TCA, digested with 1 ml of 0.1 M NaOH-0.1% SDS for 1 h at 37°C and then neutralized with 0.1 ml of 1 M HCl. The radioactivity of the samples was counted after dilution into liquid scintillant. The level of ribonuclease F activity was determined by the reduction in ^{3}H-leucine uptake.

2.9.1. 2-5 A uptake

The uptake of 2-5 A was tested by introducing into 80% confluent monolayers 0.1 mM of 2-5 A containing 2.5 µCi of 2-5 A tetramer 5'-triphosphate, 3'-(^{32}P)pCp (2-5 A-^{32}P) with transfection buffer containing 114 mM $CaCl_2$. After 45 min at RT the medium was removed and the cells washed twice with cold PBS. The cells were lysed with lysis buffer B containing 0.5% NP40, and the extracts counted in a scintillation counter. The ratio

of incorporation of labelled 2-5 A into the cells was calculated. A comparison of 2-5 A-^{32}P incorporation was made between cells treated with transfection buffer with and without $CaCl_2$. The permeability of the cells was also checked by trypan blue exclusion. Trypan blue was diluted in transfection buffer with or without $CaCl_2$ and incubated with the cells for 45 min at RT.

2.10. PROTEIN KINASE

The phosphorylation of P1 (MW 69 000) and α-eIF-2 were tested as described by Samuel (1981). A confluent monolayer was incubated at 37°C in the absence or presence of IFN (200 IU/ml) or mock IFN for 24 h before harvesting. The cells were then washed three times with chilled isotonic buffer (35 mM Tris-HCl pH 6.8, 146 mM NaCl, 1 mM glucose) and disrupted by the addition of lysis buffer B. After 10 min agitation, the cells were scraped off with a rubber policeman and centrifuged at 800 rpm for 10 min at 4°C. The cell extract was then centrifuged at 10 000 x g. The resulting supernatant was then stored at -70°C in aliquots. Cell extracts (10 µl) were incubated 30 min at 30°C with 20 mM Hepes (pH 7.5), 100 mM KCl, 4 mM $MgCl_2$, 1.5 mM DTT and 100 µM ATP containing 5-10 µCi of ^{32}P-ATP (30 Ci/mmol) and as indicated with 1 µg/ml of dsRNA and/or eIF-2 (2 µg). The reaction was stopped by the addition of 200 µl dissociation buffer (Laemmli, 1970) and the samples boiled for 5 min. The ^{32}P labelled products were analyzed by SDS-PAGE followed by autoradiography.

2.11. SDS-PAGE

Polypeptides were analyzed by PAGE using the discontinuous system of Laemmli (1970). The resolving gel (14 x 17 x 0.3 cm) contained 0.37 M Tris-HCl pH 8.8, 0.1% SDS and 12% bis-acrylamide (ratio of acrylamide : bisacrylamide 30 : 0.8). A 2 cm stacking gel containing 0.126 M Tris-HCl pH 6.8, 0.1% SDS and 4% bis-acrylamide was cast on top of the gel and 10 or 20 wells made in this gel using a perspex template. The tank buffer was 0.188 M glycine and 25 mM Tris containing 0.1% SDS. Electrophoresis was run at 40 mA. After electrophoresis, gels were fixed in 45% methanol and 10% acetic acid until they were processed for fluorography and autoradiography or they were stained in a solution of 0.2% (w/v) Coomassie brilliant blue in methanol : water : acetic acid (9 : 9 : 2) (v/v/v) and then destained in several changes of methanol : acetic acid : water (1 : 1 : 8) (v/v/v).

2.11.1. Estimation of polypeptides MW in SDS-PAGE

The MWs of electrophoresed viral polypeptides were calculated by the methods of Shapiro et al. (1967) and Weber and Osborn (1969). The labelled MW standards used were myosin (MW 200 000), phosphorylase B (MW 92 500), BSA (MW 69 000), ovalbumin (MW 45 000), carbonic anhydrase (MW 30 000) and lysozyme (MW 14 300). The unlabelled MW marker solution (Pharmacia Fine Chemicals, Uppsala, Sweden) contained the same MW standards as above except that myosin and lysozyme were omitted and contained trypsin inhibitor (MW 20 100) and α-lactalbumin (MW 14 400) were included. The

distance of migration of the standards and proteins were measured, and a plot of the distance migrated versus the logarithm of the MW of the standards yielded a line from which the unknown MWs were calculated.

2.11.2. Autoradiography and fluorography of SDS-PAGE

Gels which contained labelled proteins were prepared for fluorography by the method of Bonner and Laskey (1974). After electrophoresis, gels were fixed in the abovementioned fixer solution for 16 h or more. The gels were washed in two changes of distilled water (30 min each) and placed in two changes of dimethylsulphoxide (DMSO) (Riedel de Haen, Hannover, West Germany) for 30 min each and then soaked for 1 h 30 min in a solution of DMSO containing 22% diphenyloxazole (PPO). The gels were then washed for 1 h with two washes of distilled water and finally dried on filter paper using the BIO-RAD slab dryer (BIO-RAD, CA, USA). Dried gels were exposed to X-ray film (Cronex-4, Du Pont De Nemours & Co., Inc.) in an X-ray cassette and left at -70°C until developed. X-ray film was developed in Adefo X-ray developer (Adefo-Chemie GMbH, Nurnberg, West Germany) for 5 min at RT and fixed in Adefo X-ray fixer for 5 min at RT. The fixed film was then washed in water and air-dried.

CHAPTER 3

RESULTS

3.1. ESTABLISHMENT OF PERSISTENTLY INFECTED CELL LINES

3.1.1. SSPE-Vero cell line

After infecting Vero cells with the supernatant of SSPE-Vero cells, subcultures of the new SSPE-Vero cell lines were examined under light microscopy. From passage 5 (P.5) onward the cells formed a regular monolayer with syncitia accounting for 30 to 40% of the total monolayer. These cells were subcultured every four to five days, the rate of cell growth being faster than the parent Vero cell line (Fig. 4).

The new cell line was also examined by immunofluorescence using anti-measles antiserum. Figs. 5-8 show Vero cells before and after infection with SSPE. At P.1 only a few cells showed immunofluorescence, but by P.4 the giant cells which were present fluoresced strongly and the immunofluorescence persisted from this passage onwards.

3.1.2. Sendai-Vero cell line

After infecting Vero cells with Sendai virus and subculturing the monolayer formed, the passages were examined by immunofluorescence (Figs. 9-12). The percentage of cells fluorescing when anti-Sendai serum was used was approximately 40 to 70% from P.16 onwards. The growth rate of these cells was approximately half that of the parent cell line (Fig. 4).

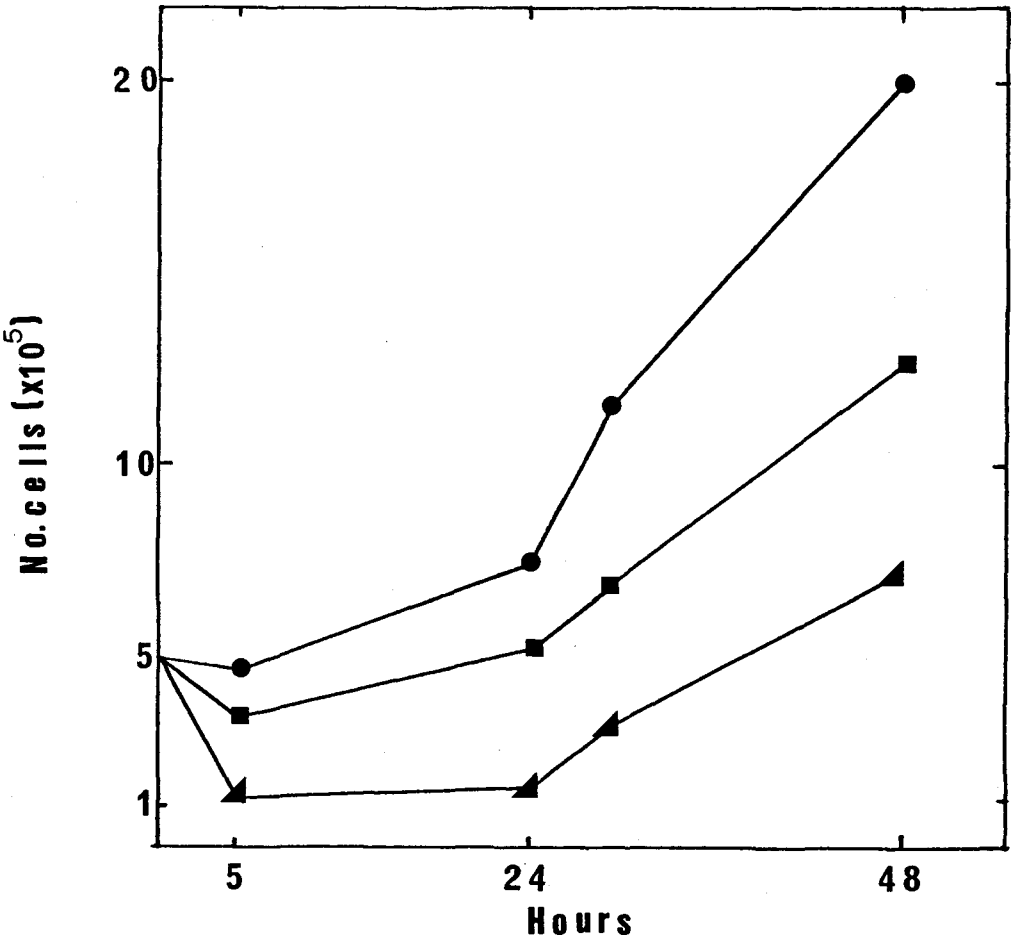

FIG. 4. Growth curve of Vero (■-■) SSPE-Vero (●-●) and Sendai-Vero cells (◢-◢).

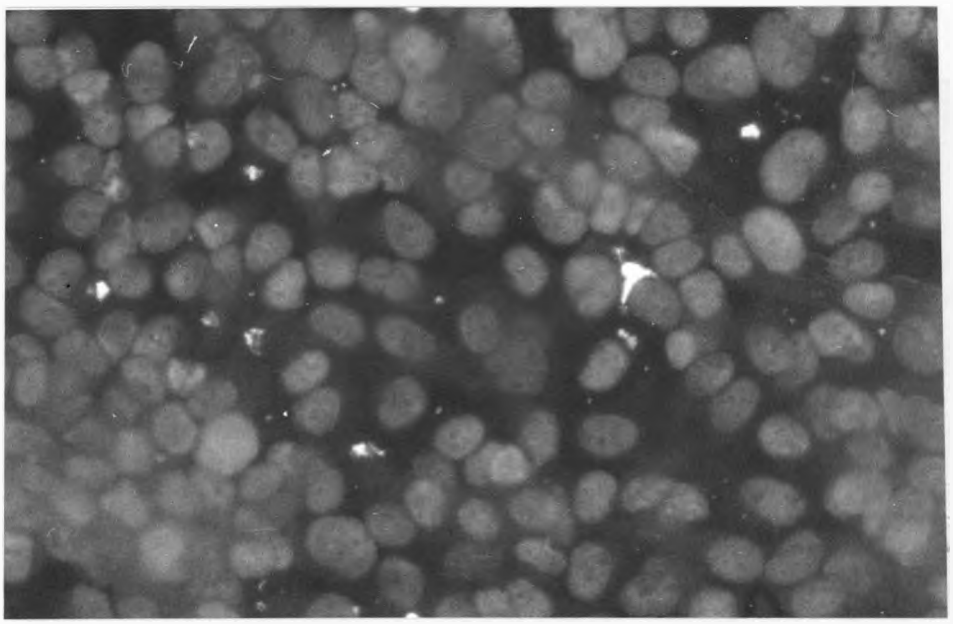

FIG. 5. Immunofluorescence of uninfected Vero cells. Cultures were washed in PBS, pH 7.4, fixed in cold acetone and stained by the indirect immunofluorescence technique using immune anti-measles human serum. Magnification 1 000x.

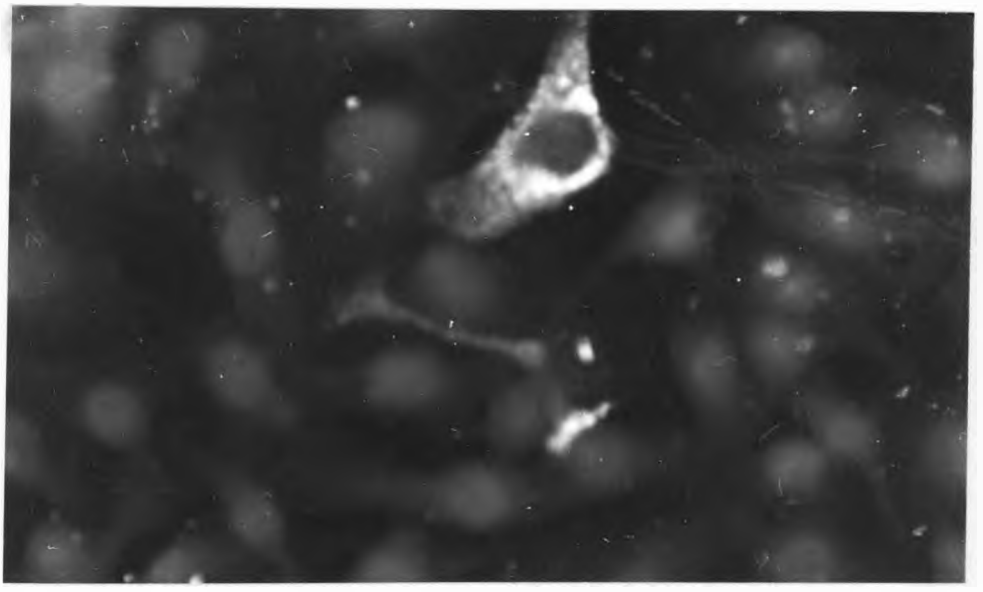

FIG. 6. Immunofluorescence of P.1 SSPE-Vero cells. Cultures were washed in PBS, pH 7.4, fixed in cold acetone and stained by the indirect immunofluorescence technique using immune anti-measles human serum. Magnification 1 000x.

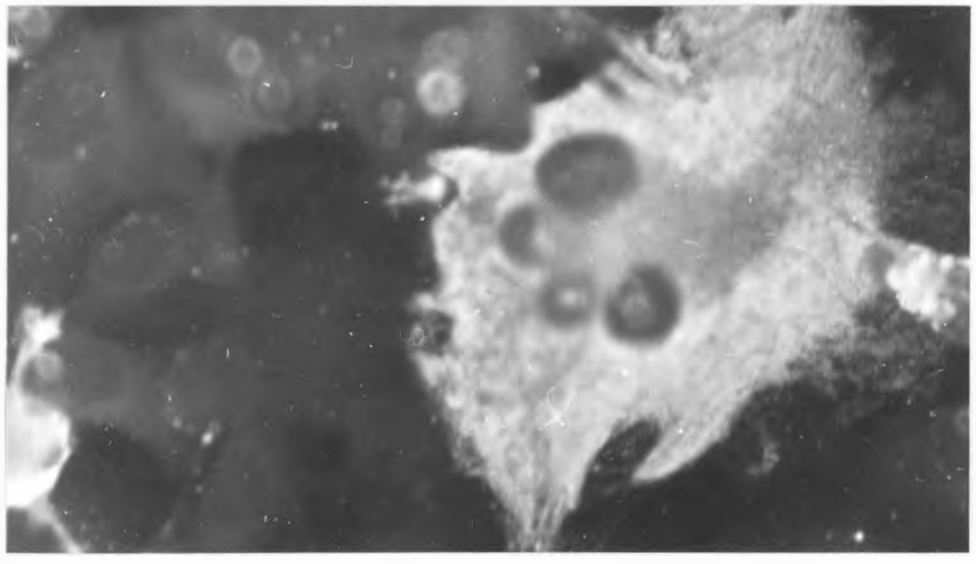

FIG. 7. Immunofluorescence of P.4 SSPE-Vero cells. Cultures *were washed in PBS, pH 7.4, fixed in cold acetone and stained by the indirect immunofluorescence technique using anti-measles human serum. Magnification 2 000x.*

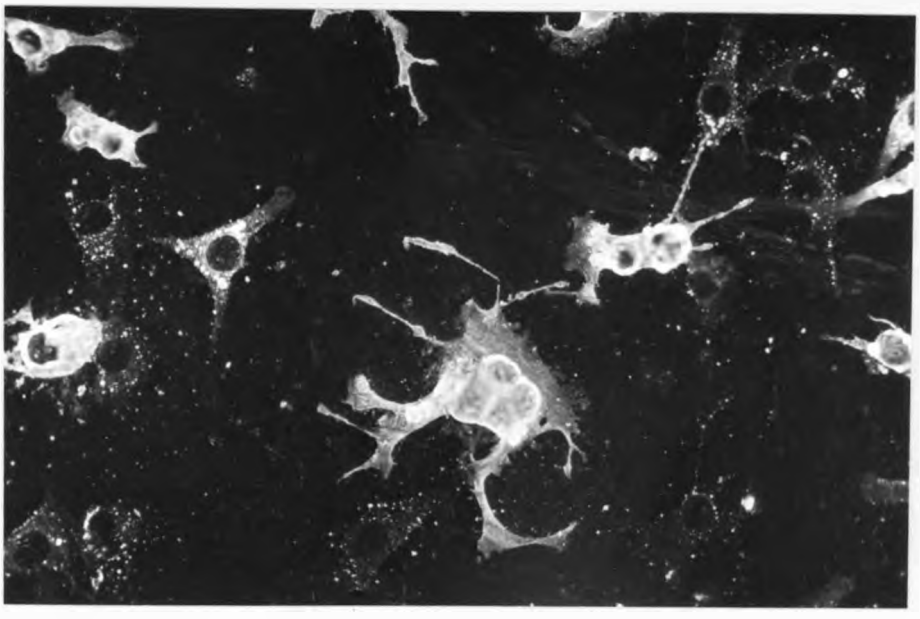

FIG. 8. Immunofluorescence of P.10 SSPE-Vero cells. Cultures were washed in PBS, pH 7.4, fixed in cold acetone and stained by the indirect immunofluorescence technique using immune anti-measles human serum. Magnification 630x.

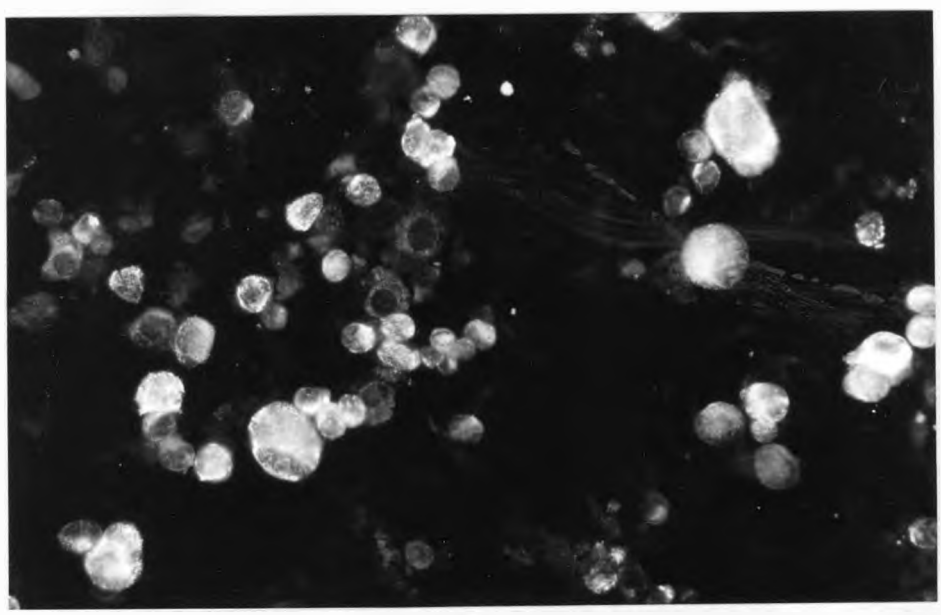

FIG. 9. Immunofluorescence of P.3 Sendai-Vero cells. Cultures were washed in PBS, pH 7.4, fixed in cold acetone and stained by the indirect immunofluorescence technique using immune anti-Sendai mouse serum. Magnification 630x.

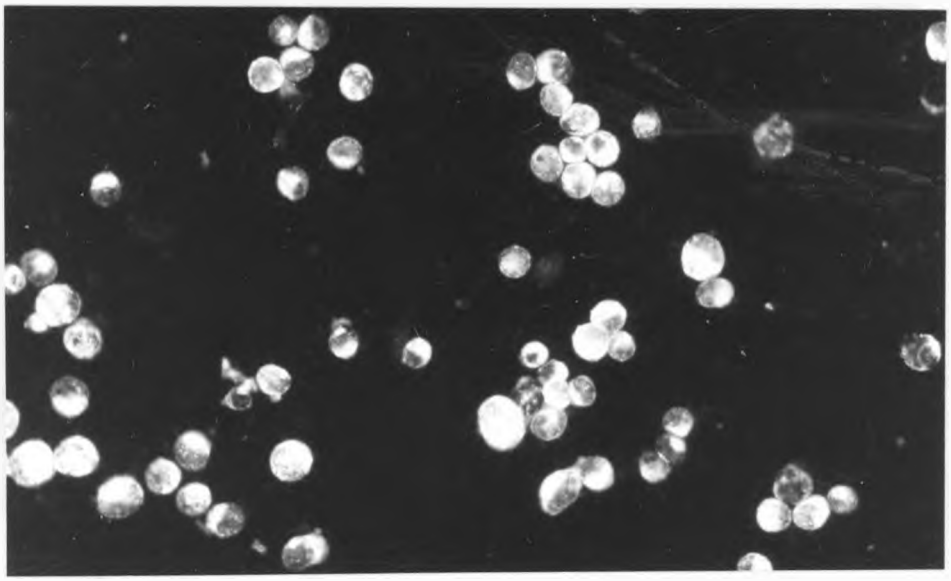

FIG. 10. Immunofluorescence of P.5 Sendai-Vero cells. Cultures were washed in PBS, pH 7.4, fixed in cold acetone and stained by the indirect immunofluorescence technique using immune anti-Sendai mouse serum. Magnification 630x.

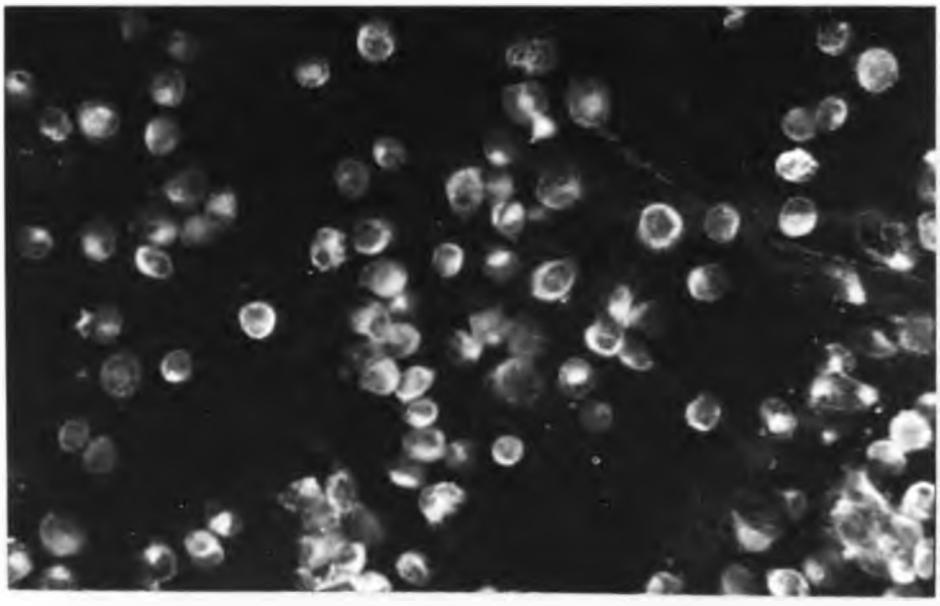

FIG. 11. Immunofluorescence of P.15 Sendai-Vero cells. Cultures were washed in PBS, pH 7.4, fixed in cold acetone and stained by the indirect immunofluorescence technique using immune anti-Sendai mouse serum. Magnification 630x.

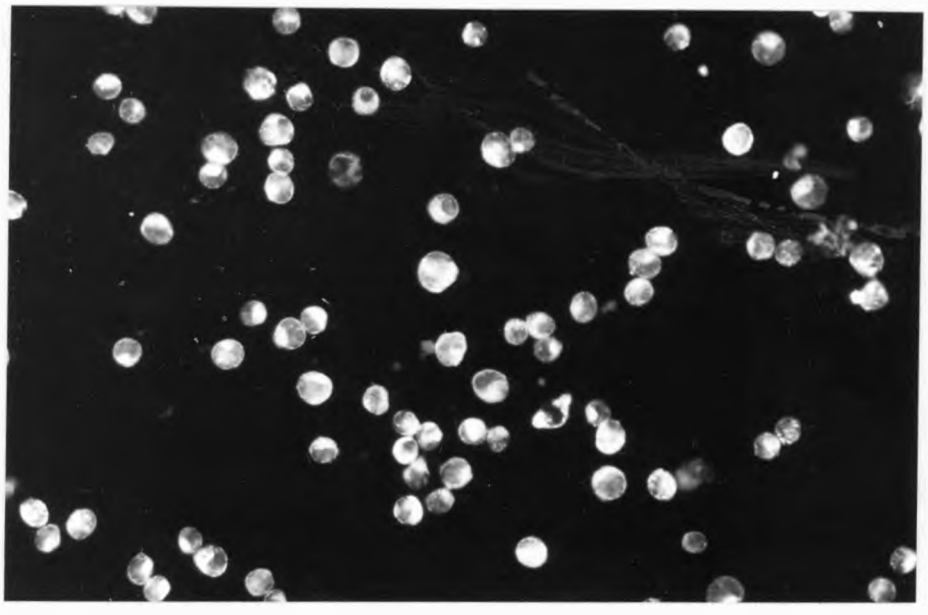

FIG. 12. Immunofluorescence of P.5l Sendai-Vero cells. Cultures were washed in PBS, pH 7.4, fixed in cold acetone and stained by the indirect immunofluorescence technique using immune anti-Sendai mouse serum. Magnification 630x.

At P.36 the Sendai-Vero cell line was examined for the presence of viral antigen in both the supernatant and cell homogenate using the HA test. While no haemagglutination could be found in the supernatant, the cell homogenate contained 15 HAU/10^6 cells. Even though the HA test did not detect any viral antigen in the supernatant, Sendai virus was present as a persistent infection could be established by infecting Vero cells with the supernatant from Sendai-Vero cells.

3.2. IFN PRODUCTION AND ANTIVIRAL ACTIVITY

3.2.1. Endogenous IFN production

The RIA system was used to monitor the presence of endogenous IFN in tissue culture supernatants of each cell line used in this study. Monolayers of Vero cells were incubated with 100 µl of each cell supernatant for 24 h and then challenged with Sindbis virus. No cell supernatant inhibited Sindbis virus replication and cell lines were therefore regarded as being negative for endogenous IFN production. The production of IFN by Vero cells infected with measles or Sendai virus was also determined; no IFN was produced by Vero cells infected with either type of virus. This confirms earlier results demonstrating that Vero cells are unable to produce IFN (Desmyter et al., 1968).

3.2.2. Antiviral activity by RIA test

The sensitivity of the different cells to IFN's antiviral activity was determined by the RIA test. Cells were incubated

with serial ten-fold or two-fold dilutions of IFN, or mock IFN for 24 h and then challenged with Sindbis virus. The sensitivity of the cells to IFN is given by the IFN IU/ml which inhibited Sindbis virus replication by 50%.

3.2.2.1. Hepatoma and CV-1 cells

Table 7 shows the sensitivity to IFN of cells infected with HBV or SV40 virus and the control, uninfected cell lines.

The hepatoma cells showed variable sensitivities to the antiviral effect of IFN as determined by the inhibition of Sindbis virus replication; PLC/PRF/5 and HA22T cells needed an IFN concentration of 2 IU/ml, Hep 3B a concentration of IFN of 4-16 IU/ml, while the Hep G2 were the least sensitive needing an IFN concentration of 80 IU/ml (Table 7). No significant difference in the antiviral effect of IFN against Sindbis virus was found between hepatoma cells which produce HBsAg, PLC/PRF/5 and Hep 3B, and the HBsAg non-producer HA22T cell line.

Difficulties were experienced in testing the antiviral effect of IFN in Mahlavu cells by the RIA test; during the processes numerous cells were lost. The antiviral effect of IFN in these cells was therefore tested using the immunofluorescence assay. Cells were pretreated with IFN before the addition of Sindbis virus and the presence of Sindbis antigens determined by fluorescence. The PLC/PRF/5 cells were also tested using the immunofluorescence assay so a comparison between the RIA and the immunofluorescence test could be made. Concentrations of 2-8 IU/ml and 8-16 IU/ml of IFN were needed to abolish Sindbis virus fluorescence in Mahlavu

TABLE 7. The antiviral effect of IFN in hepatoma and CV-1 cells

CELLS	IFN (IU/ml)
PLC/PRF/5	2
HEP 3B	4-16
HA22T	2
HEP G2	80
CV-1	4
C2	4
C6	2
C11	4

Cells were incubated with serial dilutions of IFN for 24 h and then challenged with Sindbis virus for 24 h. The IFN IU/ml value was then determined.

and PLC/PRF/5 cells respectively indicating that Mahlavu and PLC/PRF/5 cells have similar sensitivities to IFN.

All CV-1 control cells and cells infected with SV40, were equally sensitive to the antiviral effect of IFN with sensitivities of 2 to 4 IU/ml (Table 7).

Mock IFN did not inhibit Sindbis virus replication in any of the hepatoma and CV-1 lines used.

3.2.2.2. Cell lines persistently infected with SSPE or Sendai virus.

Control Vero cells and Vero cells persistently infected with SSPE or Sendai virus were also examined for their sensitivity to IFN or mock IFN. A value of 4 IU/ml of IFN was able to inhibit Sindbis virus in Vero cells, while a value of more than or equal to 1 600 IU/ml was required in SSPE-Vero and Sendai-Vero cell lines (Table 8). Mock IFN did not inhibit Sindbis virus replication in control Vero cells or in the two persistently infected Vero cell lines. These results show that there is a significant change regarding the sensitivity to IFN when Vero cells become persistently infected with either SSPE or Sendai virus.

The different passages (P.1 - P.11) obtained after infecting Vero cells with SSPE were examined for their sensitivity to IFN using the RIA test. The results are shown in Fig. 13. Control Vero cells were used as P.0. At P.2 a value of 4 IU/ml of IFN inhibited the growth of the challenge virus, while at P.5 1 600 IU/ml of IFN was required. This shows that the loss of sensitivity

TABLE 8. The antiviral effect of IFN in uninfected Vero cells or cells persistently infected with SSPE or Sendai virus

CELLS	IFN (IU/ml)
Vero	4
SSPE-Vero	1600
Sendai-Vero	>1600

Cells were incubated with serial dilutions of IFN for 24 h and then challenged with Sindbis virus for 24 h. The IFN IU/ml value was then determined.

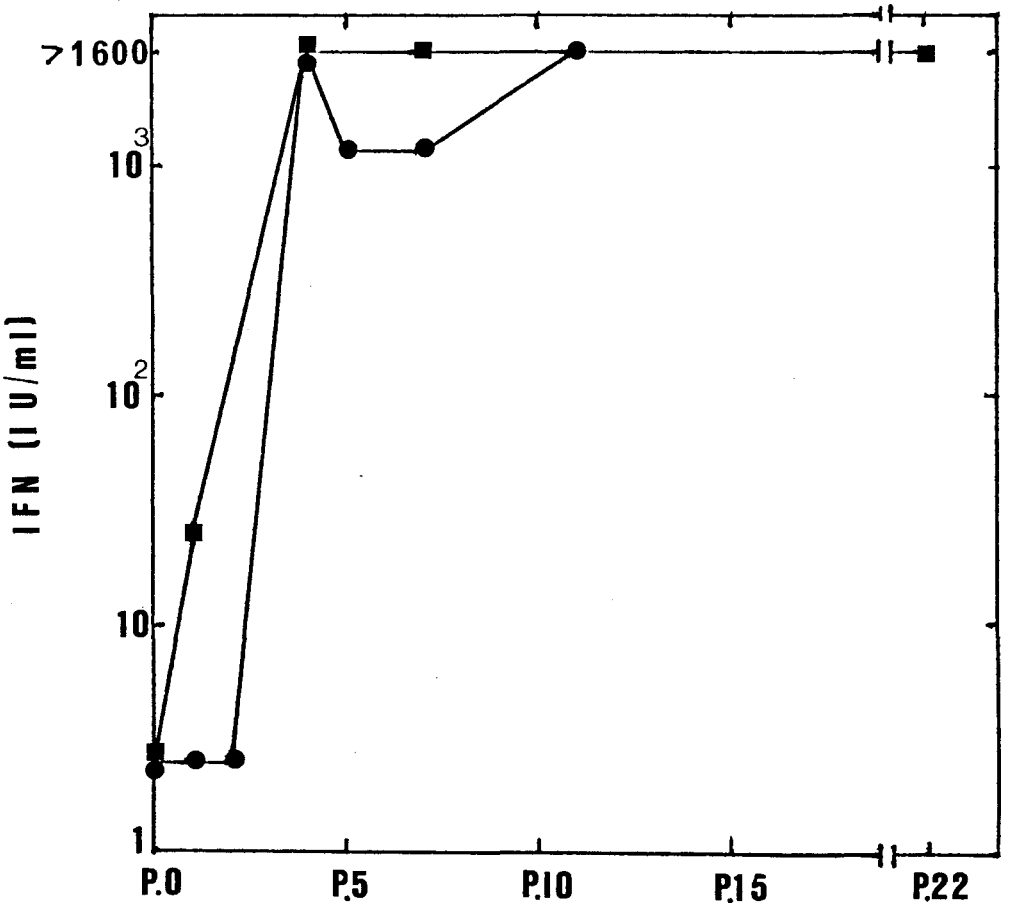

FIG. 13. Antiviral effect of IFN at different passages of SSPE-Vero (●-●) or Sendai-Vero cells (■-■). Cells were incubated with serial dilutions of IFN for 24 h then challenged with Sindbis virus. Results are expressed as IFN (IU/ml) which inhibited Sindbis virus by 50%.

to IFN's antiviral effect in these cells correlates with the percentage of fluorescence in the cells (see 3.1.1.).

Subcultures from P.1 to P.22 of Vero cells infected with Sendai virus were also examined (Fig. 13). At P.1 an IFN concentration of 40 IU/ml was needed to inhibit the growth of Sindbis virus and at P.4 an IFN concentration greater than 1600 IU/ml was required. The development of the Sendai-Vero persistent infection, like the SSPE-Vero persistent infection, is characterized by the resistance of the cells to IFN.

3.2.3. Examination of virus specific polypeptide synthesis in the measles, SSPE and Sendai systems

As SSPE-Vero and Sendai-Vero cells showed considerable resistance to the antiviral effect of IFN, as determined by the RIA assay, the effect of IFN on the synthesis of endogenous viral proteins was examined. The antiviral effect of IFN on measles, SSPE and Sendai viruses was examined by RIPA followed by SDS-PAGE.

3.2.3.1. Measles-SSPE system

By the RIPA method the major measles virus polypeptides were readily detected, these being haemagglutinin (H), nucleoprotein (N), matrix (M) and proteins with approximately 50 000 MW. The latter proteins have been previously detected by Wechsler and Fields (1978). On occasion the phosphoprotein (P) was also detected at low levels. Cellular actin (A) was detected in most gels.

Preliminary experiments were done to titrate measles virus by the RIPA system and the lowest virus concentration which showed the presence of the major viral proteins was used in the RIPA experiments (Fig. 14). A virus dilution of 1.7×10^5 pfu/ml was chosen for all experiments (Fig. 14, lane 5). Extracts of cells infected with measles at this concentration were also reacted with different measles antisera to determine which antiserum gave the best immunoprecipitation result based on the presence of measles viral polypeptides. Commercial antisera and antisera from patients with high antibody titers were used (Figs. 15a, 15b). The antiserum in lane 2 of Fig. 15b was chosen for all subsequent experiments.

Pretreatment of Vero cells with IFN had a significant effect on the synthesis of measles virus polypeptides (Fig. 16). The synthesis of H and N proteins was significantly reduced by 25 IU/ml of IFN, the M protein disappeared at 50 IU/ml, and at concentrations greater than 200 IU/ml negligible amounts of measles virus proteins were detected.

The synthesis of SSPE-viral proteins was also examined by RIPA, the H, and N, proteins being precipitated but not the M protein (Fig. 17). The lack of the M protein has been previously reported by Stephenson et al. (1981).

In contrast to the sensitivity of measles virus to IFN, SSPE virus was not affected by IFN as concentrations of 4 000 IU/ml did not inhibit the synthesis of SSPE viral proteins (Fig.17).

Mock IFN had no effect on the synthesis of either measles or SSPE polypeptides. The lack of an effect of mock IFN on measles or SSPE virus are shown in Figs. 18, 19.

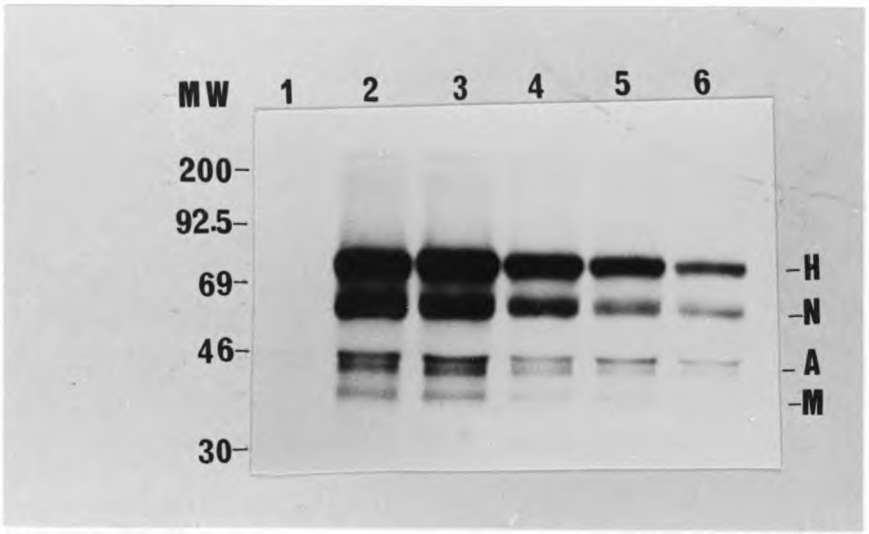

FIG. 14. Titration of measles virus polypeptides by radioimmunoprecipitation assay (RIPA). Vero cells were infected with dilutions of a stock culture of measles virus (6.8×10^6 pfu/ml). Following labelling with ^{35}S-methionine, cells were harvested at 24 h p.i. and polypeptides analysed by RIPA. Molecular weight markers (MW), uninfected Vero cells (lane 1), Vero cells infected with measles virus (lanes 2-6), undiluted measles virus (lane 2), dilution 1 : 10 (lane 3), 1 : 20 (lane 4), dilution 1 : 40 (lane 5), dilution 1 : 80 (lane 6). Haemagglutinin (H), nucleoprotein (N), actin (A), matrix (M).

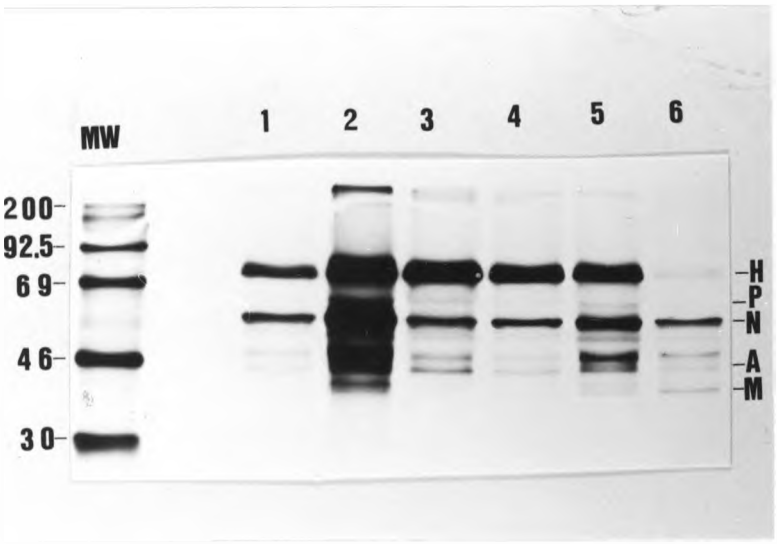

FIG. 15a. Immunoprecipitation of measles virus polypeptides using different antisera. Human antisera (lanes 1-3, 5-6), guinea pig antisera (lane 4).

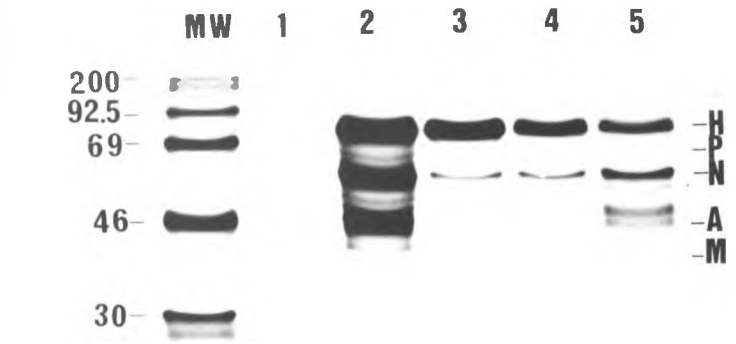

FIG. 15b. Immunoprecipitation of measles virus polypeptides using different antisera. Uninfected Vero cells (lane 1), human antiserum (lane 2), bovine antiserum (lane 3), guinea pig antiserum (lane 4), SSPE cerebral spinal fluid (lane 5).

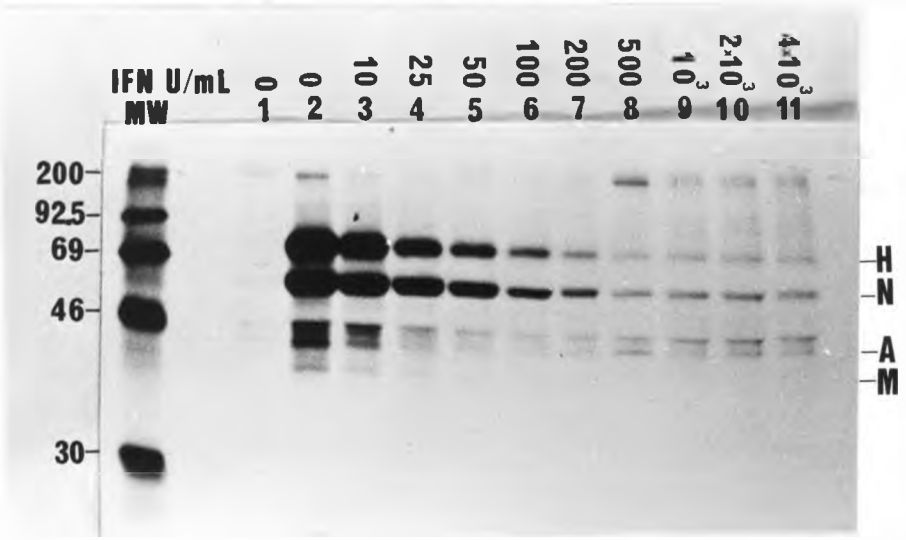

FIG. 16. Synthesis of measles virus polypeptides in the presence or absence of IFN. Vero cells were treated with serial dilutions of IFN for 24 h, infected with measles virus (1.7×10^5 pfu/ml) for 24 h then labelled with ^{35}S-methionine for 18 h and examined by RIPA. Uninfected (lane 1) or infected (lanes 2-11) cells were treated with EMEM alone (lanes 1, 2) or serial IFN dilutions (lanes 3-11).

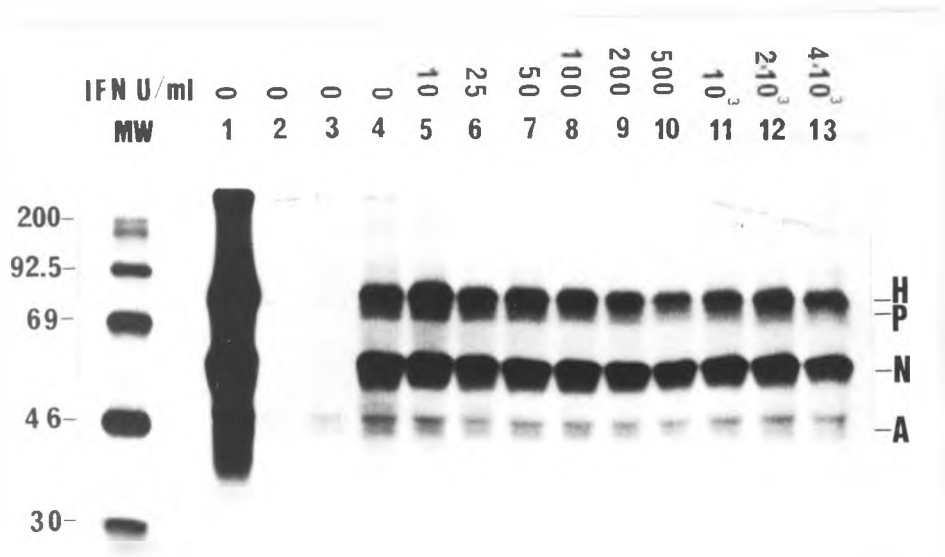

FIG. 17. Synthesis of SSPE virus polypeptides in the presence or absence of IFN. SSPE-Vero cells were treated with serial dilutions of IFN for 24 h, labelled with ^{35}S-methionine for 24 h then examined by RIPA. Immunoprecipitated measles virus proteins (lane 1), untreated Vero cells (lanes 2, 3), SSPE-Vero cells untreated and treated with serial IFN dilutions (lanes 4-13).

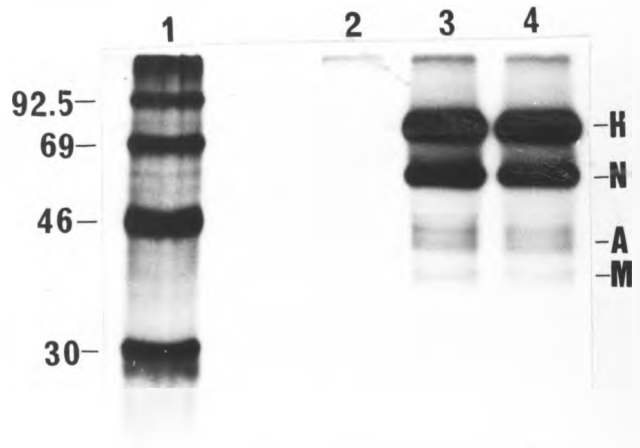

FIG. 18. Synthesis of measles virus polypeptides in the presence or absence of mock IFN. Cells were treated with mock IFN for 24 h, then infected with measles virus (1.7×10^5 pfu/ml) for 24 h then labelled with ^{35}S-methionine for 18 h and polypeptides examined by RIPA. MW markers (lane 1), uninfected (lane 2) or infected Vero cells (lanes 3, 4), Vero cells treated with mock IFN (lane 4).

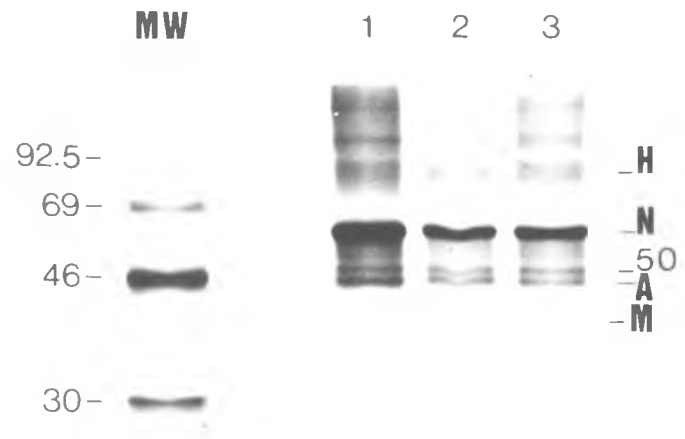

FIG. 19. Synthesis of SSPE virus polypeptides in the presence or absence of IFN or mock IFN. Cells were treated with IFN (200 IU/ml) for 24 h, then labelled with ^{35}S-methionine for 24 h and polypeptides examined by RIPA. Untreated SSPE-Vero cells (lane 1), SSPE-Vero cells treated with IFN (lane 2), SSPE-Vero cells treated with mock IFN (lane 3).

In sequential studies with measles virus, virus-induced polypeptides were detected by 12 h postinfection (p.i.) (Fig. 20), as previously reported by others (Wechsler and Fields, 1978). IFN at a concentration of 200 IU/ml inhibited H, N, and M protein synthesis at all time intervals studied (Fig. 20). These results show that IFN had an inhibitory effect on measles virus polypeptides synthesis at all stages of the virus replication cycle.

3.2.3.2. Sendai-Vero system

With Sendai virus the major polypeptides readily distinguished in gels were: phosphoprotein (P), nucleocapsid (N), matrix (M) and proteins with MWs between 48 000-53 000. Low levels of the haemagglutinin (H) were sometimes detected. These proteins were identified according to their MWs based on the paper of Lamb et al. (1976).

Sendai-Vero cells at P.30 and onwards were used to determine the effect of IFN on the synthesis of Sendai virus polypeptides. As shown in Fig. 21 IFN did not inhibit the synthesis of Sendai virus polypeptides even at a high concentration of IFN (4 000 IU/ml).

3.3. ANTIPROLIFERATIVE ASSAYS

3.3.1. Antiproliferative effect of IFN on hepatoma and SV40 infected cells

One of the standard methods used to assay the antiproliferative effect of IFN is by measuring the incorporation of radioactive labeled precursors into macromolecules, such as labeled thymidine

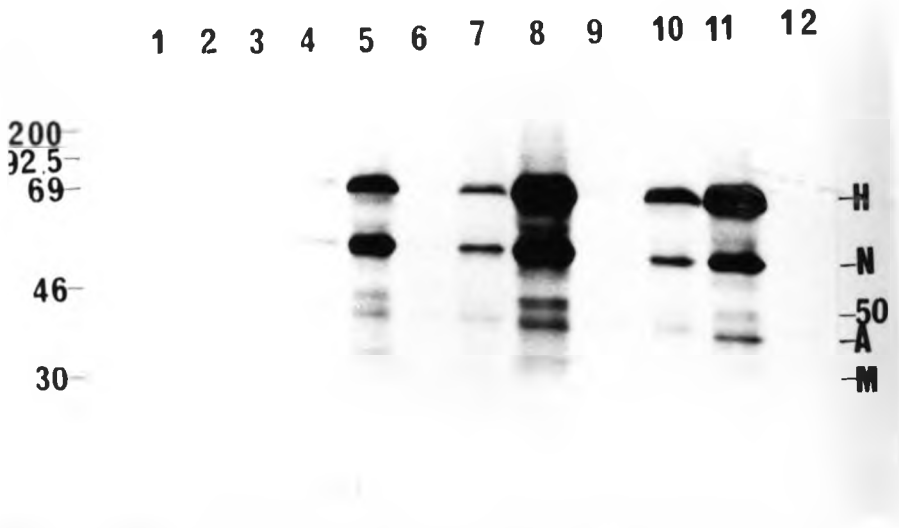

FIG. 20. Electrophoresis of ^{35}S-methionine-labelled proteins from Vero cells infected with measles virus after immunoprecipitation with measles antibody. Uninfected cells (lanes 3, 6, 9, 12), measles virus infected cells (lanes 1, 2, 4, 5, 7, 8, 10, 11), cells treated with IFN (200 IU/ml) (lanes 1, 3, 4, 6, 7, 9, 10, 12). Labelling was for 1 h from: 6 h p.i. (lanes 1-3), 12 h p.i. (lanes 4-6), 18 h p.i. (lanes 7-9), 24 h p.i. (lanes 10-12).

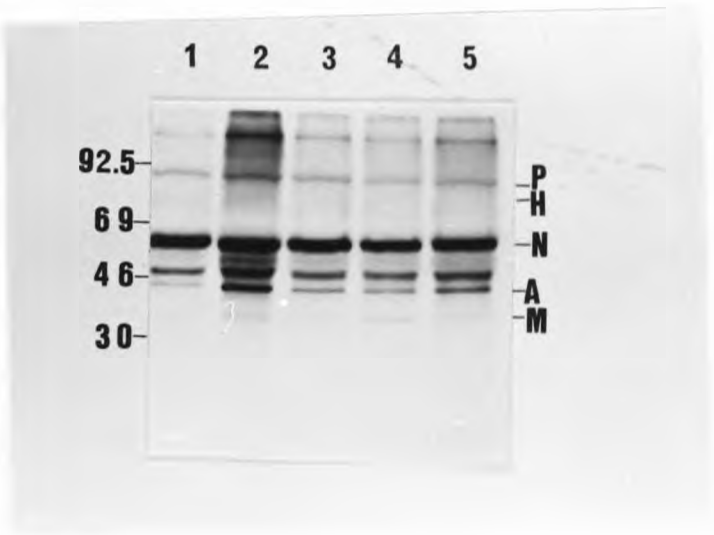

FIG. 21. Synthesis of Sendai virus polypeptides in the presence or absence of IFN. Sendai-Vero cells were treated with serial dilutions of IFN for 24 h, labelled with ^{35}S-methionine for 24 h then examined by RIPA. Sendai-Vero cells untreated (lane 1), treated with IFN (10 IU/ml) (lane 2), IFN (100 IU/ml) (lane 3), IFN (1 000 IU/ml) (lane 4), IFN (4 000 IU/ml) (lane 5).

into DNA (Hilfenhaus et al., 1976; Fuse and Kuwata, 1977; Evinger and Pestka, 1981).

PLC/PRF/5 and Mahlavu cells were treated with different concentrations of IFN and the incorporation of ^{3}H-thymidine was determined. IFN was able to inhibit ^{3}H-thymidine incorporation in both cell lines with the PLC/PRF/5 cell line being more sensitive than the Mahlavu cell line (Fig. 22). IFN at a concentration of 1 000 IU/ml inhibited ^{3}H-thymidine incorporation by 78% in PLC/PRF/5 cells and 45% in Mahlavu cells.

The meaning and the interpretation of ^{3}H-thymidine incorporation as a method of assay for the effect of IFN on cellular growth has lately been refuted by Gewert et al. (1981, 1983, 1984). These workers, in a detailed study on the incorporation of exogenous ^{3}H-thymidine in Daudi cells, showed that incorporation of exogenous tracer was not a true reflection of the effect of IFN on DNA synthesis and cell proliferation, and thus the method is not a reliable measure of the growth inhibitory activity. The antiproliferative effect of IFN was therefore determined by the more direct method of cell counting.

The IFN-induced inhibition of cell growth in hepatoma cells is shown in Figs. 23 and 24. The growth of the hepatoma cells, with the exception of the Hep 3B line, was inhibited by IFN. Using a concentration of 1 000 IU/ml of IFN for comparative purposes cell growth was inhibited by 36%, 47% and 52% in HA22T, PLC/PRF/5 and in Hep G2 cells respectively (Figs. 23, 24), whilst in Mahlavu cells the level of inhibition was 19%. Mock IFN did not have any inhibitory effect in Hep 3B and HA22T cells while in PLC/PRF/5, Mahlavu and Hep G2 cells produced an inhibition of 6%, 8% and 12% respectively (Figs. 23, 24).

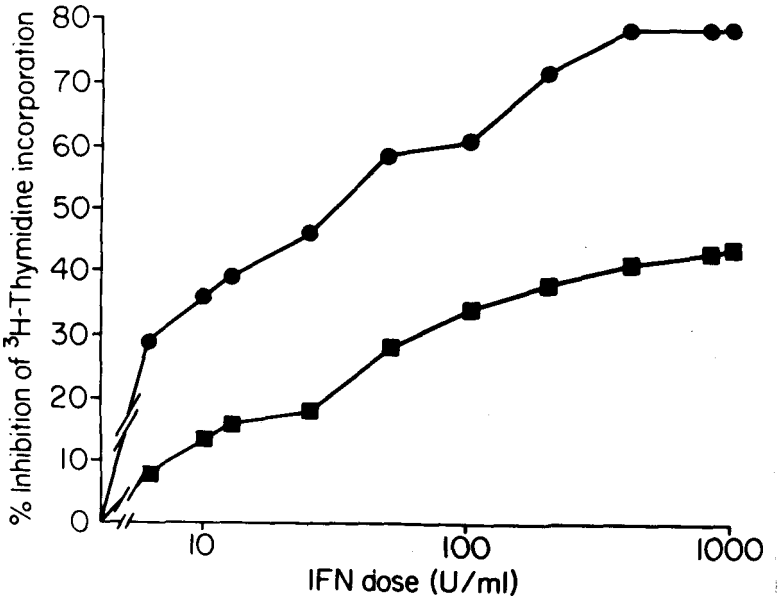

FIG. 22. Inhibition of ^{3}H-thymidine incorporation by IFN in PLC/PRF/5 and Mahlavu cells. Monolayers were incubated with different dilutions of IFN for 48 h and then ^{3}H-thymidine was added for 1 h. Incorporation was determined by TCA precipitation. PLC/PRF/5 (●-●), Mahlavu (■-■).

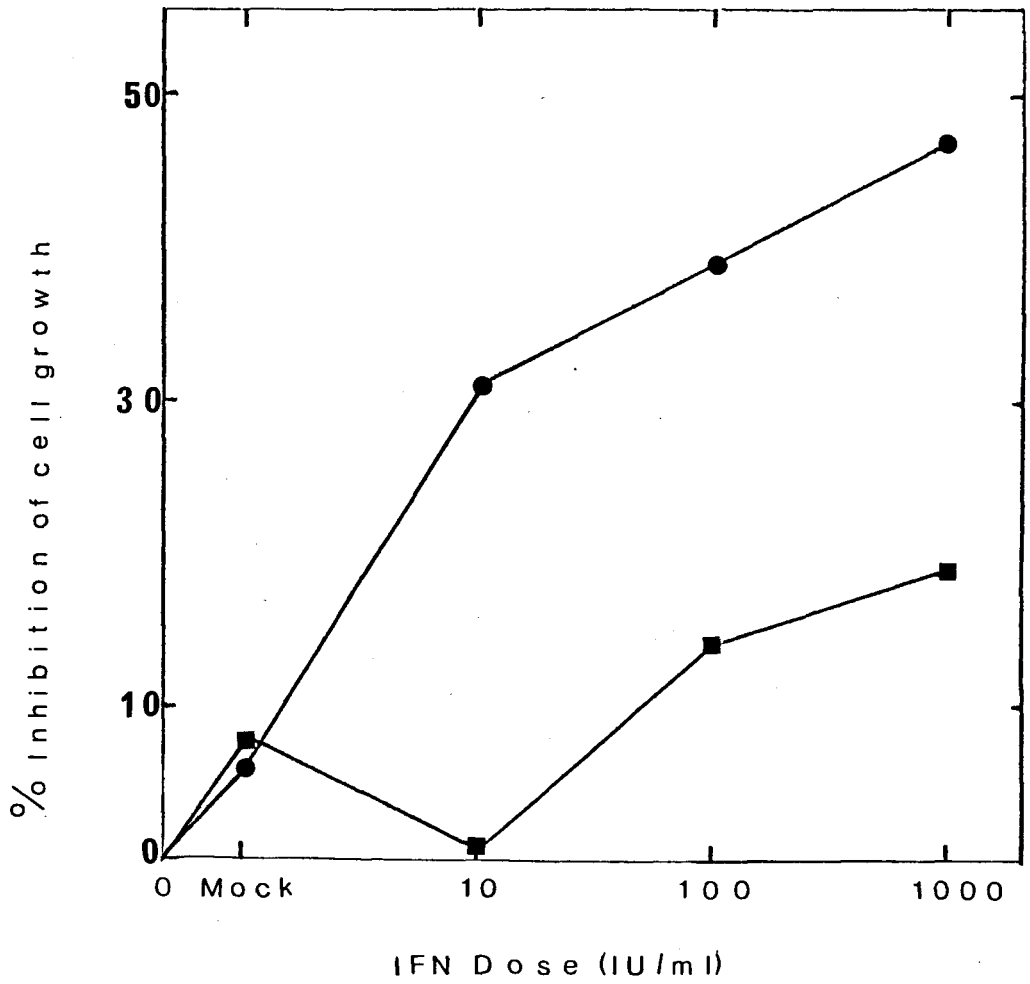

FIG. 23. Antiproliferative effect of IFN on PLC/PRF/5 and Mahlavu cells. Monolayers which were 80% confluent were treated with serial dilutions of IFN for 48 h, harvested and counted in trypan blue. Inhibition of cell growth of IFN and mock IFN treated cells was compared with untreated controls. PLC/PRF/5 (●-●), Mahlavu (■-■).

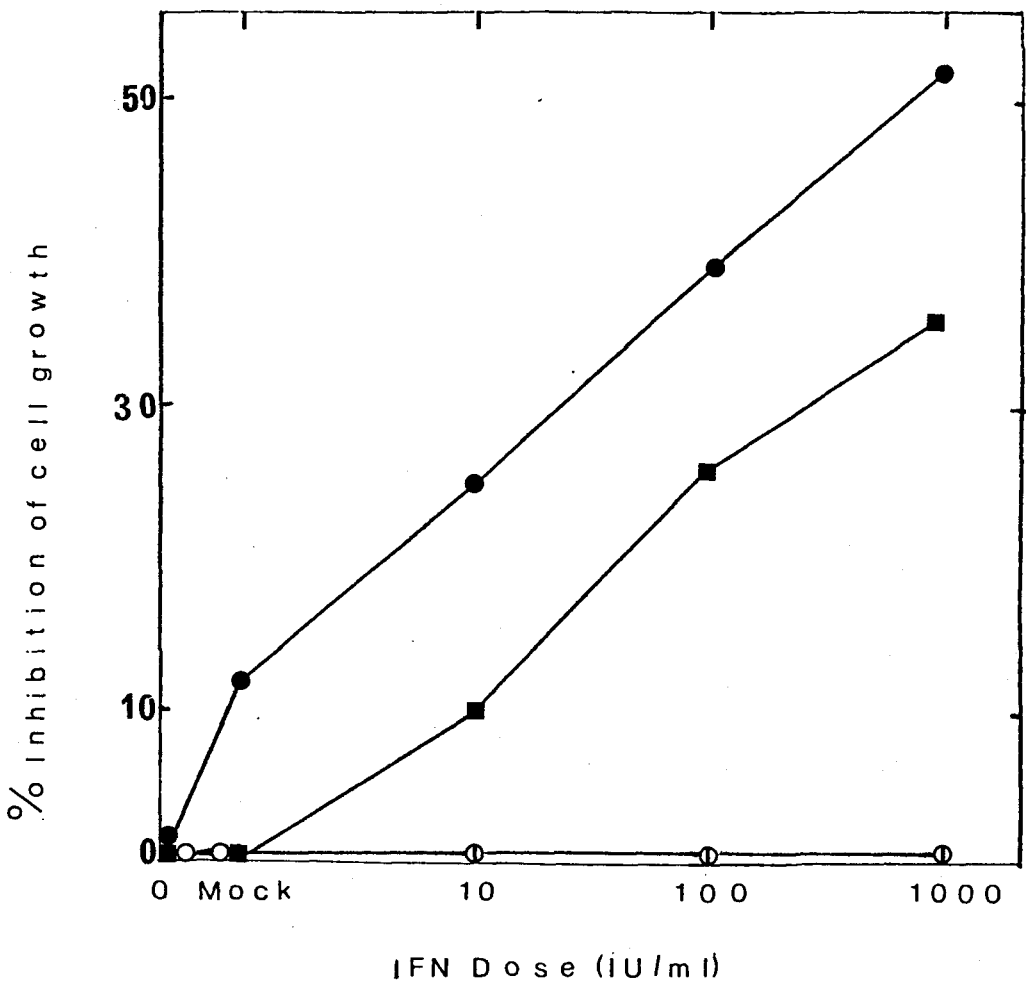

FIG. 24. Antiproliferative effect of IFN on Hep G2, HA22T and Hep 3B cells. Monolayers were incubated with different dilutions of IFN for 48 h, harvested and counted in trypan blue. Inhibition of cell growth of IFN and mock IFN treated cells was compared with untreated controls. Hep G2 (●-●), HA22T (■-■) Hep 3B (o-o).

By comparing the above two methods in determining the antiproliferative effect of IFN, the inhibition of ^{3}H-thymidine uptake would appear to be more sensitive than cell counting. This has been previously reported by Gewert et al. (1981, 1983, 1984), despite the fact that these authors queried the reliability of this method.

The antiproliferative effect of IFN on control CV-1 cells and CV-1 cells infected with SV40 ranged from 18 to 30% at an IFN concentration of 1 000 IU/ml (Fig. 25). Mock IFN did not have any antiproliferative effect in control CV-1 cells while in C2, C6 and C11 it produced an inhibition of approximately 10%.

3.3.2. Antiproliferative effect in Vero cells persistently infected with SSPE or Sendai virus

The results of the antiproliferative effect of IFN are shown in Fig. 26. IFN, at a concentration of 1 000 IU/ml inhibited the proliferation of Vero cells by 29%, while there was no inhibition of cell proliferation when SSPE or Sendai virus was present. Mock IFN did not produce any inhibition in the uninfected Vero cells or those persistently infected with SSPE or Sendai virus. Vero cells thus become significantly less sensitive to the antiproliferative effect of IFN when they are persistently infected with SSPE or Sendai virus.

3.4. E ENZYME ASSAY

All cells were assayed for E enzyme before and after treatment

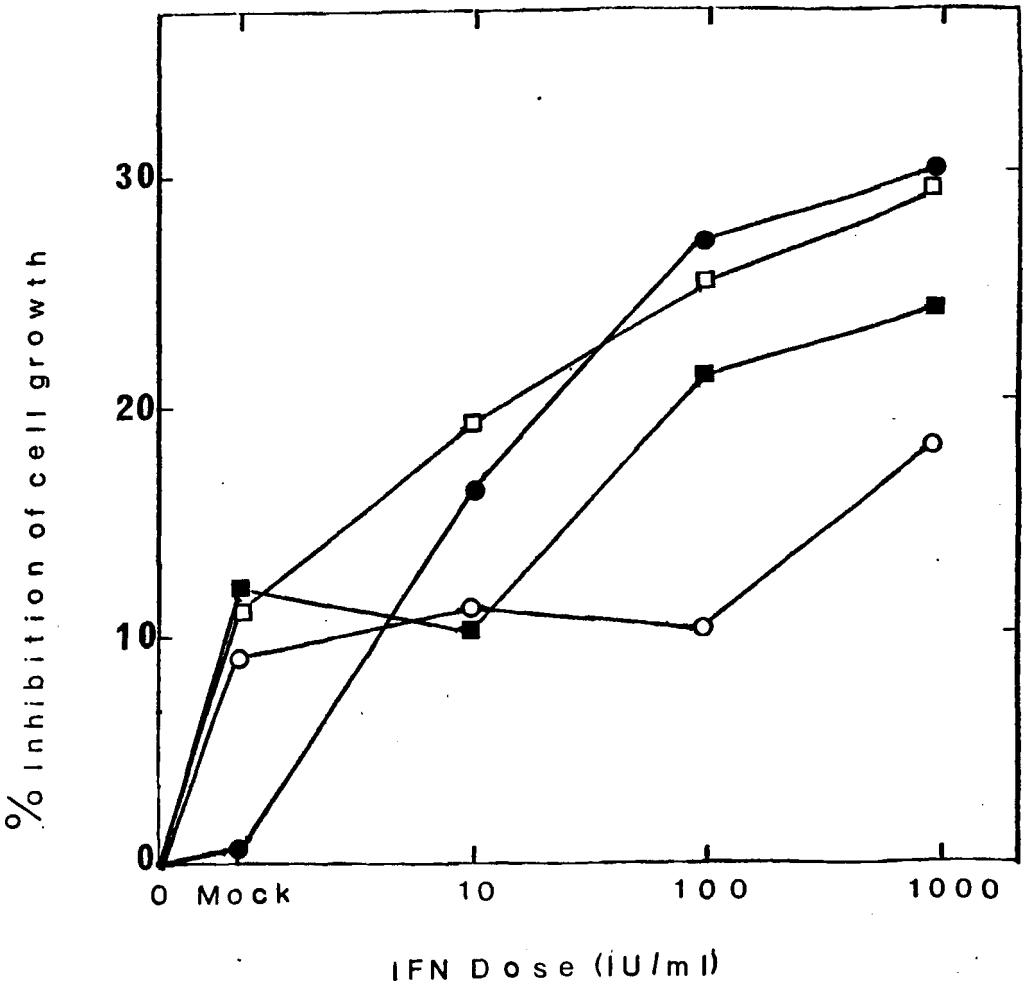

FIG. 25. Antiproliferative effect of IFN on CV-1, C2, C6 and Cll cells. Monolayers were incubated with different dilutions of IFN for 48 h, harvested and counted in trypan blue. Inhibition of cell growth of IFN and mock IFN treated cells was compared with untreated controls. CV-1 (●-●), C2 (o-o), C6 (■-■), Cll (□-□).

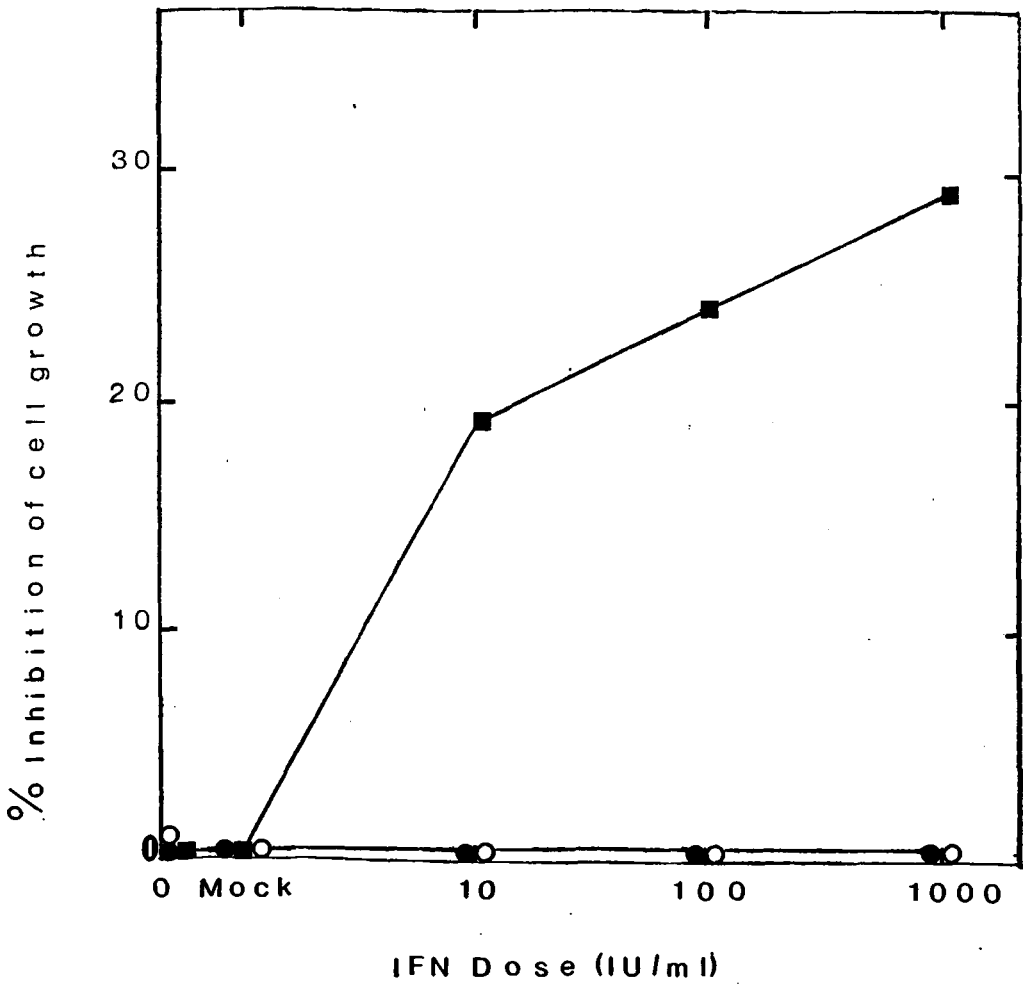

FIG. 26. Antiproliferative effect of IFN on Vero, SSPE-Vero, Sendai-Vero cells. Monolayers were incubated with different dilutions of IFN for 48 h, harvested and counted in trypan blue. Inhibition of cell growth of IFN and mock IFN treated cells was compared with untreated controls. Vero (■-■), SSPE-Vero (●-●), Sendai-Vero (o-o).

with IFN or mock IFN. The results are given by the amount of 2-5 A produced during 21 h incubation with ATP adjusted to the protein concentration in each extract. The amount of protein contained in each cell extract was determined using the Bio-Rad protein assay kit. The amount of protein in the cell extracts ranged between 2 700-9 900 µg protein/ml.

3.4.1. E enzyme in hepatoma and in SV40 infected cells

Fig. 27 shows the amount of 2-5 A produced by the various hepatoma cells after IFN treatment. No significant difference in the activation of the E enzyme was seen between the two hepatoma cells that contain integrated HBV DNA and produce HBsAg, the PLC/PRF/5 and the Hep 3B cell lines, and the Mahlavu, HA22T and Hep G2 which do not produce HBsAg. After IFN treatment the level of E enzyme in the PLC/PRF/5 and Hep 3B cells increased from five to seven-fold compared to untreated cells while in the Mahlavu, HA22T, and Hep G2 cells the increase was from two to five-fold.

The CV-1 cell lines infected with SV40 showed a significant increase in the E enzyme level after IFN treatment (Fig. 28). Increases of twenty-seven-fold, eighteen-fold and thirty-four-fold were seen in C2, C6 and C11 cells respectively in comparison to their homologous untreated cells. An increase of six-fold was observed in IFN-treated control CV-1 cells (Fig. 28). The presence of SV40 integrated into the cell genome might be responsible for making the CV-1 cells very sensitive to the IFN-mediated induction of E enzyme.

The effect of mock IFN in stimulating the E enzyme varied

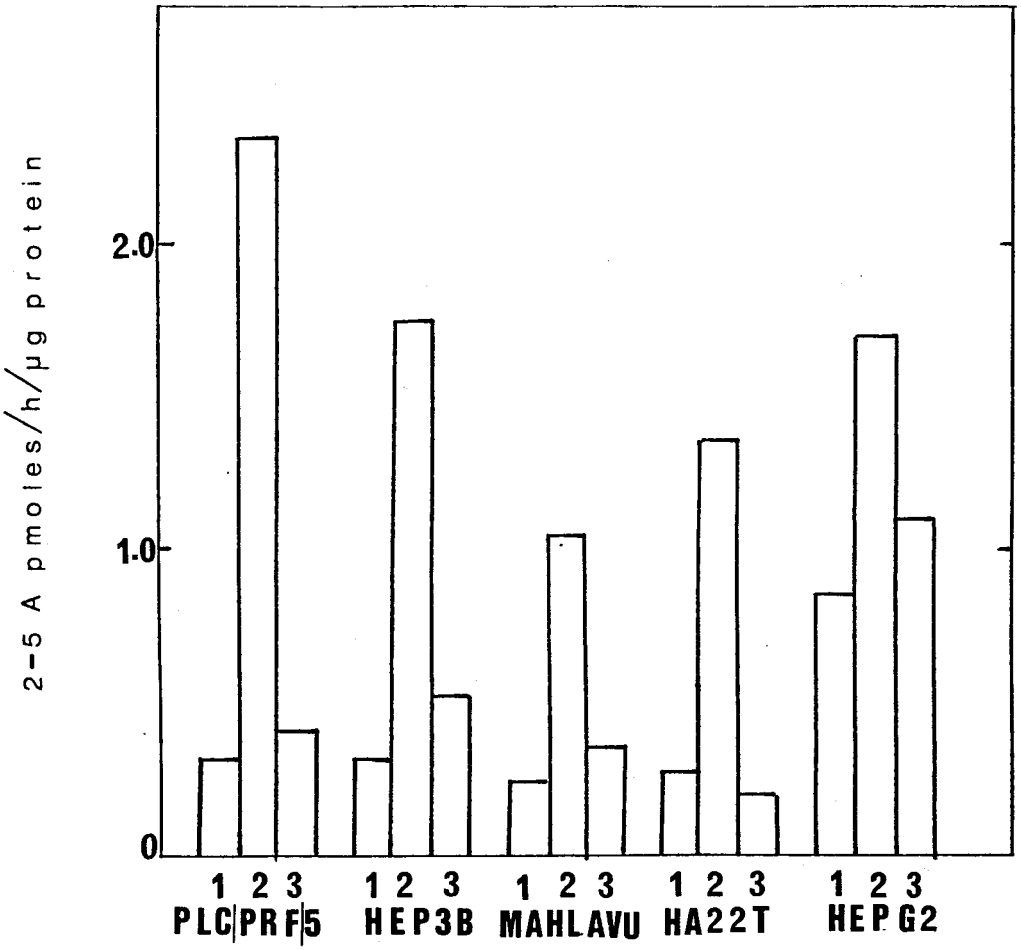

FIG. 27. Histograms of E enzyme levels (2-5 A pmoles/h/µg protein) in extracts of hepatoma cells untreated (1), treated with IFN (200 IU/ml) (2), treated with mock IFN (3).

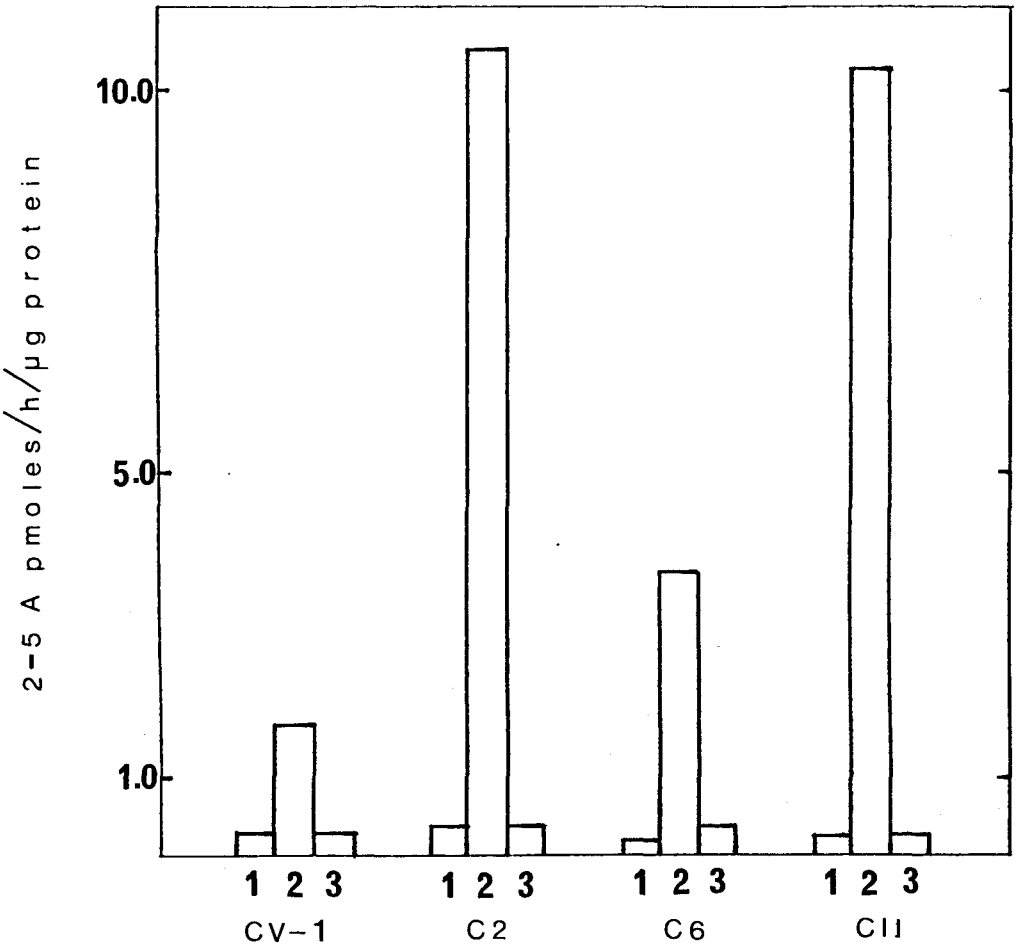

FIG. 28. Histograms of E enzyme levels (2-5 A pmoles/h/µg protein) in extracts of CV-1, C2, C6 and C11 cells untreated (1), treated with IFN (200 IU/ml) (2), treated with mock IFN (3).

between hepatoma and CV-1 cell lines but the level of E enzyme induced was always considerably lower than that induced by IFN (Figs. 27, 28).

3.4.2. E enzyme in Vero cells infected with SSPE or Sendai virus

Uninfected Vero cells showed an eighteen-fold increase in the E enzyme level after IFN treatment, while Vero cells persistently infected with Sendai virus had a very low basal level of the E enzyme and the enzyme increased by only 1.8-fold after IFN treatment (Fig. 29). An increase of nine-fold of the E enzyme level after IFN treatment was seen in SSPE-Vero cells, but this increase is regarded as being insignificant considering the very low basal level of the enzyme in this cell line (Fig. 29). In the Vero and SSPE-Vero cell lines mock IFN increased the level of the E enzyme only very slightly.

The development of persistent infections in the Vero cells was followed using the E enzyme assay. As is shown in Fig. 30, IFN's ability to induce the E enzyme was significantly reduced when the Vero cells became persistently infected with SSPE or Sendai virus.

3.5. RIBONUCLEASE F ASSAY

Hovanessian and Wood (1980) have previously shown that activation of the ribonuclease F could be measured by the addition of exogenous 2-5 A to cells. 2-5 A is introduced into the cells by $CaCl_2$ coprecipitation and is responsible for activating the

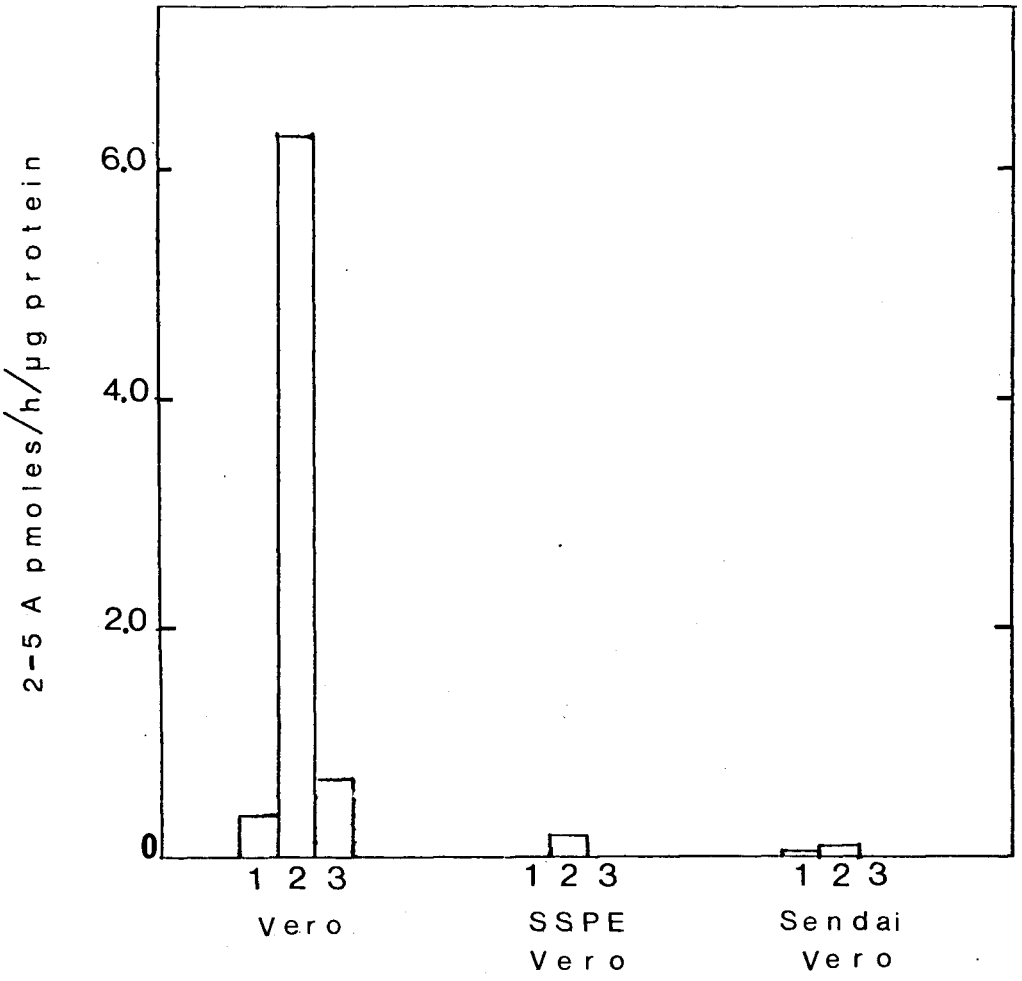

FIG. 29. Histograms of E enzyme levels (2-5 A pmoles/h/µg protein) in extracts of Vero, SSPE-Vero, Sendai-Vero cells. Untreated cells (1), treated with IFN (200 IU/ml) (2), mock IFN (3).

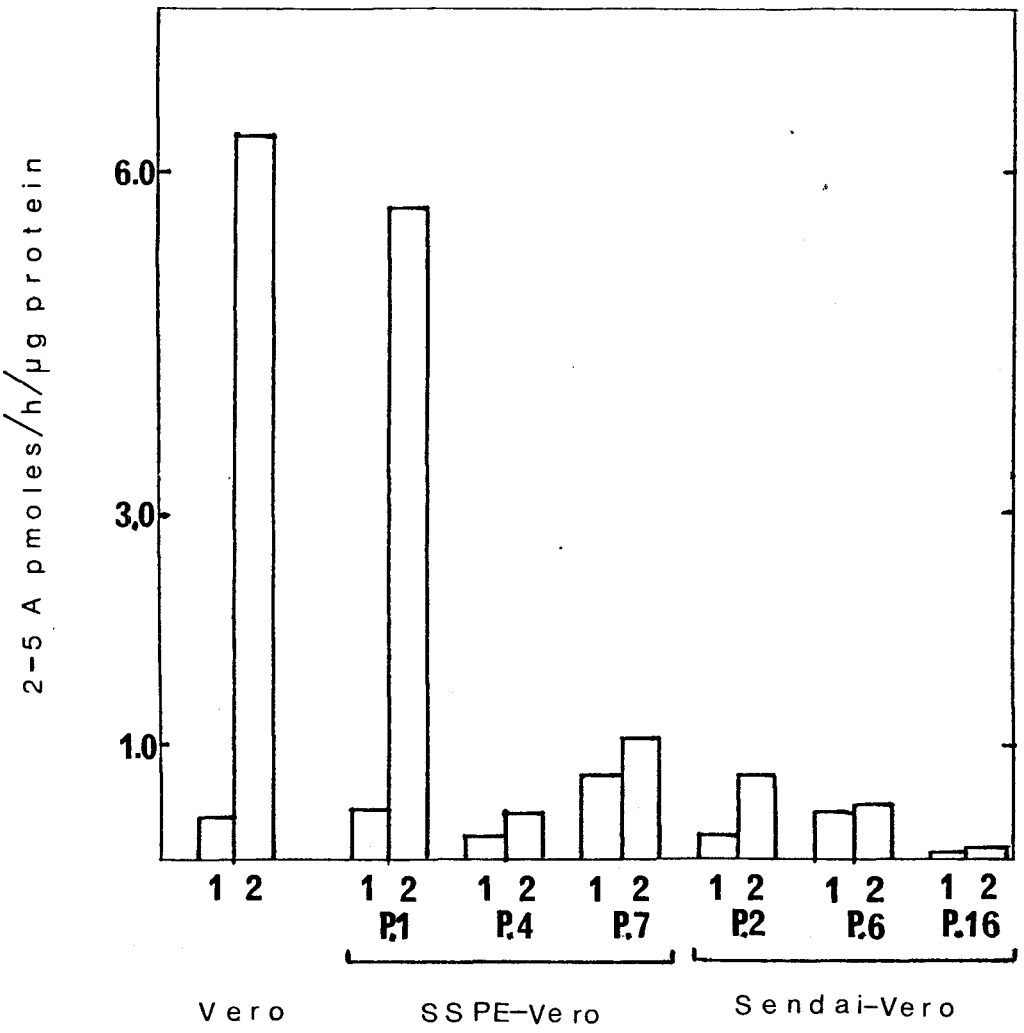

FIG. 30. Histograms of E enzyme levels (2-5 A pmoles/h/µg protein) in extracts of different passages of SSPE-Vero and Sendai-Vero cells. Untreated cells (1), treated with IFN (200 IU/ml) (2).

ribonuclease F, which in turn degrades mRNA and rRNA (Baglioni et al., 1978; Nilsen et al., 1980). By stimulating the degradation of RNA, 2-5 A is indirectly responsible for the inhibition of protein synthesis and this inhibition can be measured by the incorporation of ^{3}H-leucine.

Each cell line was assayed for ribonuclease F activity at least three times and the percentage of ^{3}H-leucine incorporation was determined. A ribonuclease F positive cell line, L-cells (Panet et al., 1981), and a negative ribonuclease F cell line, NIH-3T3 (Panet et al., 1981) were also tested and used as controls (Fig. 31). Inhibition of 61% and 14% can be seen in L and NIH-3T3 cells respectively at 10^{-6}M of 2-5 A.

3.5.1. Ribonuclease F in hepatoma and SV40 infected cells

The three hepatoma cell lines, PLC/PRF/5, Hep 3B and Hep G2, were largely insensitive to the addition of 2-5 A with a maximum 10% inhibition of ^{3}H-leucine incorporation in the PLC/PRF/5 cells with a concentration of 10^{-6}M of 2-5 A (Fig. 32). To check if an increase in the activation of the ribonuclease F could be seen, these three cell lines were pretreated with rec α-IFN (200 IU/ml) for 24 h before the 2-5 A was added. The ribonuclease F activity after IFN treatment did not increase in PLC/PRF/5 or Hep G2 cells, while in Hep 3B cells pretreated with IFN a concentration of 10^{-6}M of 2-5 A produced an inhibition of ^{3}H-leucine incorporation of 19%, equal to an increase of 2.7-fold in comparison to Hep 3B cells non-treated with IFN. HA22T and Mahlavu cells were sensitive to 2-5 A with a maximum of 56% and

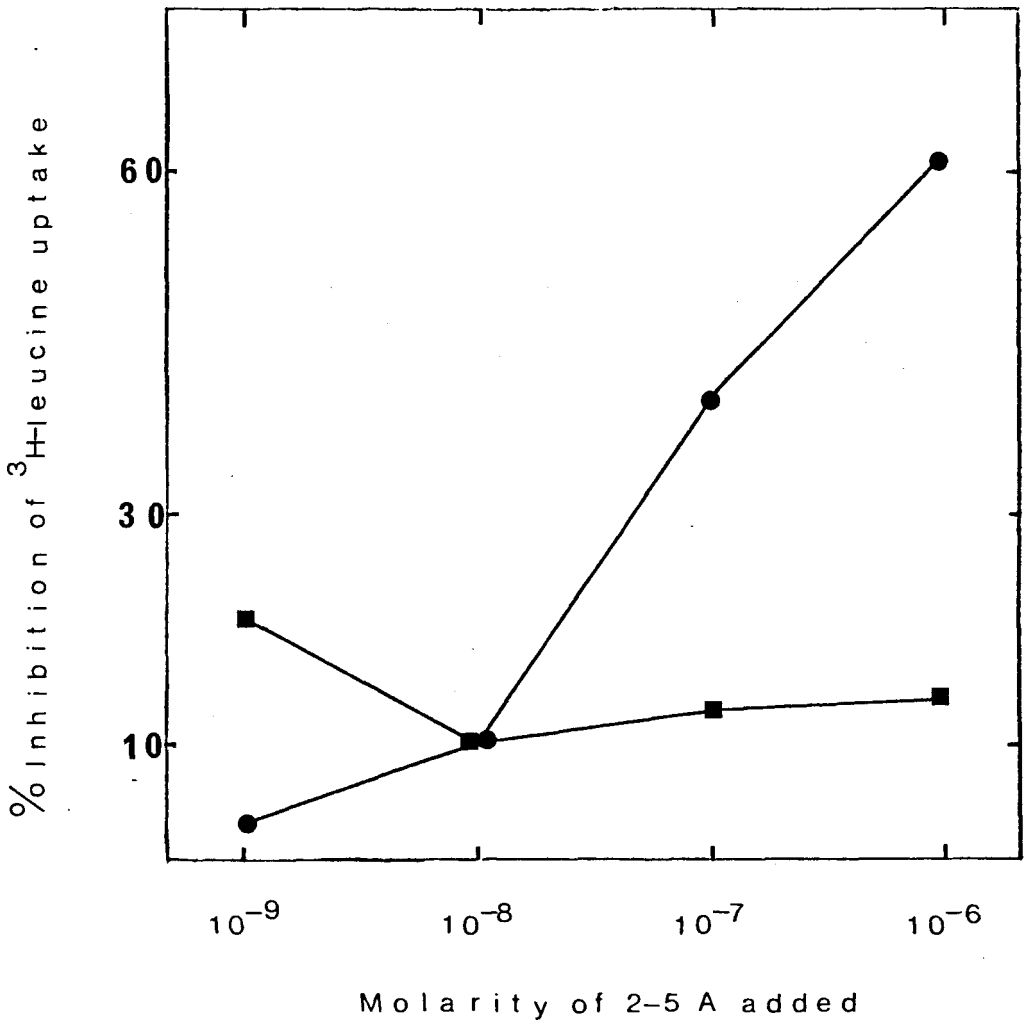

FIG. 31. Effect of 2-5 A on ^{3}H-leucine incorporation in L and NIH-3T3 cells. Monolayer cells were treated with serial dilutions of 2-5 A in transfection buffer containing $CaCl_2$ for 135 min. Medium was changed and after 105 min ^{3}H-leucine was added for 1 h. Incorporation was determined by TCA precipitation. L-cells (●-●), NIH-3T3 cells (■-■).

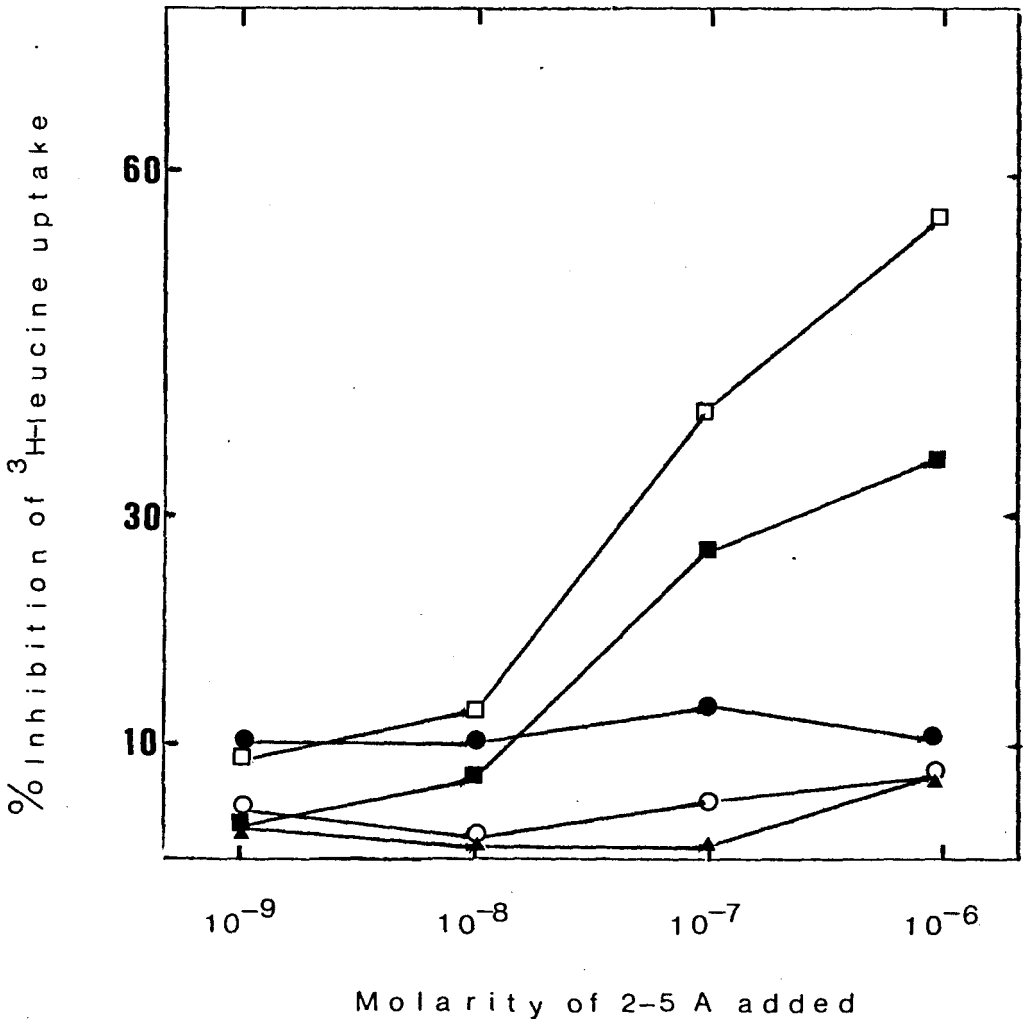

FIG. 32. Effect of 2-5 A on ^{3}H-leucine incorporation in hepatoma cell lines. Monolayers were treated with serial dilutions of 2-5 A in transfection buffer containing $CaCl_2$ for 135 min. Medium was changed and after 105 min ^{3}H-leucine was added for 1 h. Incorporation was determined by TCA precipitation. PLC/PRF/5 (●-●), Hep 3B (○-○), Mahlavu (■-■), HA22T (□-□), Hep G2 (▲-▲) cells.

35% inhibition respectively when a similar concentration of 10^{-6}M of 2-5 A was used (Fig. 32).

An investigation was done to find out if the reason for the lack of sensitivity to 2-5 A in PLC/PRF/5 hepatoma cell line was caused by a reduced uptake of 2-5 A. The ribonuclease positive, Mahlavu, and the ribonuclease negative, PLC/PRF/5, cell lines were compared in their ability in incorporating 2-5 A.

The permeability of the hepatoma cells to the transfection buffer was first studied using trypan blue uptake and then by incorporation of labelled 2-5 A. No differences in permeability were found between PLC/PRF/5 and Mahlavu cell lines using trypan blue; in fact 90% of both these hepatoma cell lines were permeable when transfection buffer contained $CaCl_2$, whereas only 2% were permeable when $CaCl_2$ was omitted. The percentage of labelled 2-5 A incorporated by the cells was then calculated. In the presence of $CaCl_2$ the PLC/PRF/5 cells incorporated 34% of the labelled 2-5 A and the Mahlavu cells 30% whereas in the absence of $CaCl_2$ an incorporation of approximately 4% was observed in both cell lines (Table 9).

From these results it can be concluded that the lack of ribonuclease F activity in PLC/PRF/5 cells was not due to a defect of 2-5 A uptake but might be due to some intracellular defect.

2-5 A significantly inhibited the incorporation of ^{3}H-leucine in CV-1 control cells and CV-1 infected with SV40 with a maximum of inhibition between 67 to 83% at 10^{-6}M of 2-5 A (Fig. 33). These results show that both the CV-1 control and C2, C6, and C11

TABLE 9. Uptake of labelled 2-5 A by the PLC/PRF/5 and Mahlavu cells

CELLS	TREATMENT	% LABELLED 2-5 A
PLC/PRF/5	$-CaCl_2$	4
	$+CaCl_2$	34
MAHLAVU	$-CaCl_2$	4
	$+CaCl_2$	30

Monolayers were treated with labelled 2-5 A in transfection buffer in the presence or absence of $CaCl_2$ for 45 min and the amount of labelled 2-5 A present at 45 min compared to 0 min is given as a percentage. The mean of three experiments is given.

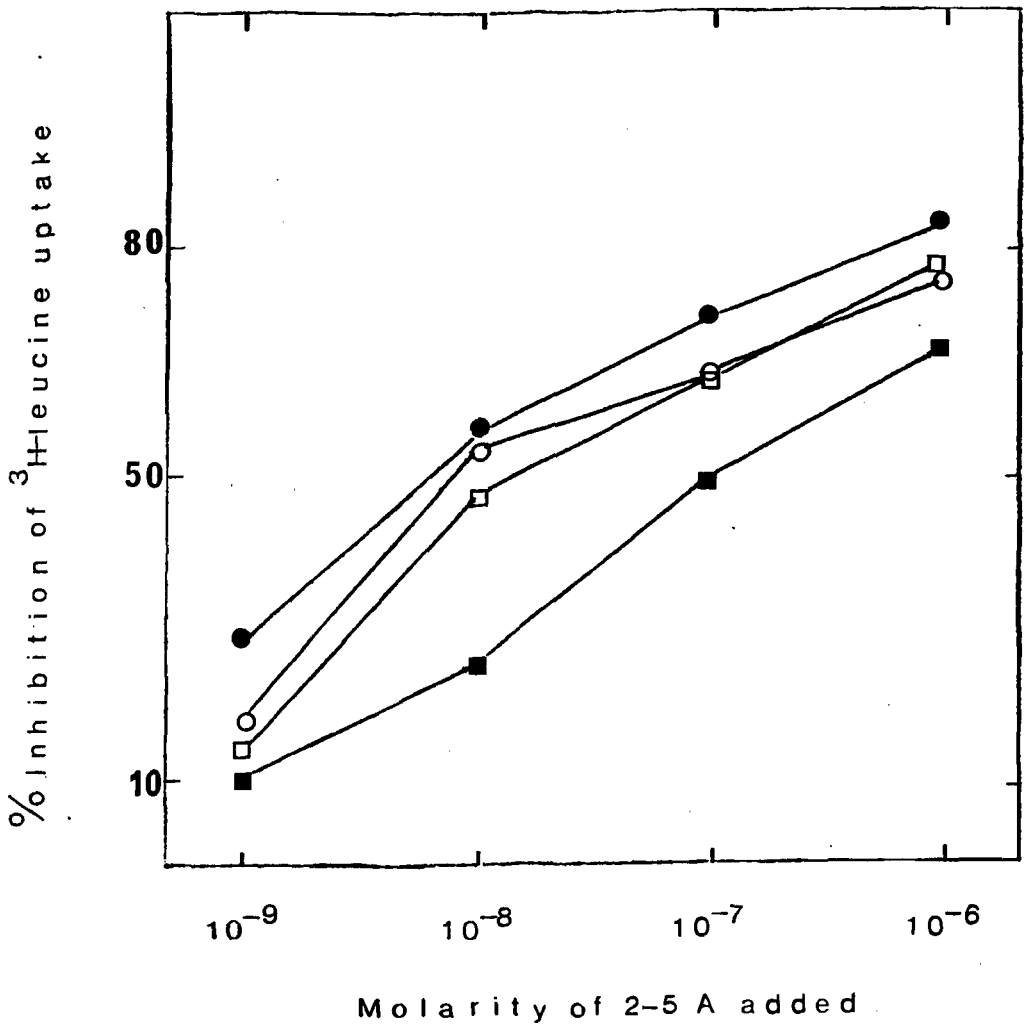

FIG. 33. Effect of 2-5 A on ^{3}H-leucine incorporation in CV-1, C2, C6 and Cl1 cells. Monolayers were treated with serial dilutions of 2-5 A in transfection buffer containing $CaCl_2$ for 135 min. Medium was changed and after 105 min ^{3}H-leucine was added for 1 h. Incorporation was determined by TCA precipitation. CV-1 (●-●), C2 (o-o), C6 (■-■), Cl1 (□-□) cells.

cell lines are sensitive to 2-5 A and that they have an endogenous ribonuclease F enzyme.

3.5.2. Ribonuclease F in Vero persistently infected with SSPE or Sendai virus

Vero, SSPE-Vero and Sendai-Vero cell lines responded positively to 2-5 A as is shown by a similar decrease in the rate of ^{3}H-leucine incorporation (Fig. 34). Incorporation of ^{3}H-leucine was inhibited by 79% on Vero cells, 84% in SSPE-Vero cells, and 65% in Sendai-Vero cells.

3.6. PROTEIN KINASE

Stimulation of the protein kinase by IFN was assayed using the enzyme's ability to phosphorylate the α-subunit of eIF-2 as the phosphorylation of the endogenous P1 (69 000 MW) could not readily be detected due to strong background in this region of the gels. Murine L-cells were used as a positive control as these cells have been shown to activate the protein kinase (Zilberstein et al., 1976). These cells were treated with muIFN or mouse mock IFN, while all the other cells were treated with lymphoblastoid IFN.

Extract of cells before or after IFN treatment were reacted with exogenous eIF-2. Two samples of eIF-2 were used (see Materials and Methods) and their purity compared by SDS-PAGE (Fig. 35). This figure shows that both samples of eIF-2 contain the α-subunit and are contaminated with the β-subunit (50 000 MW);

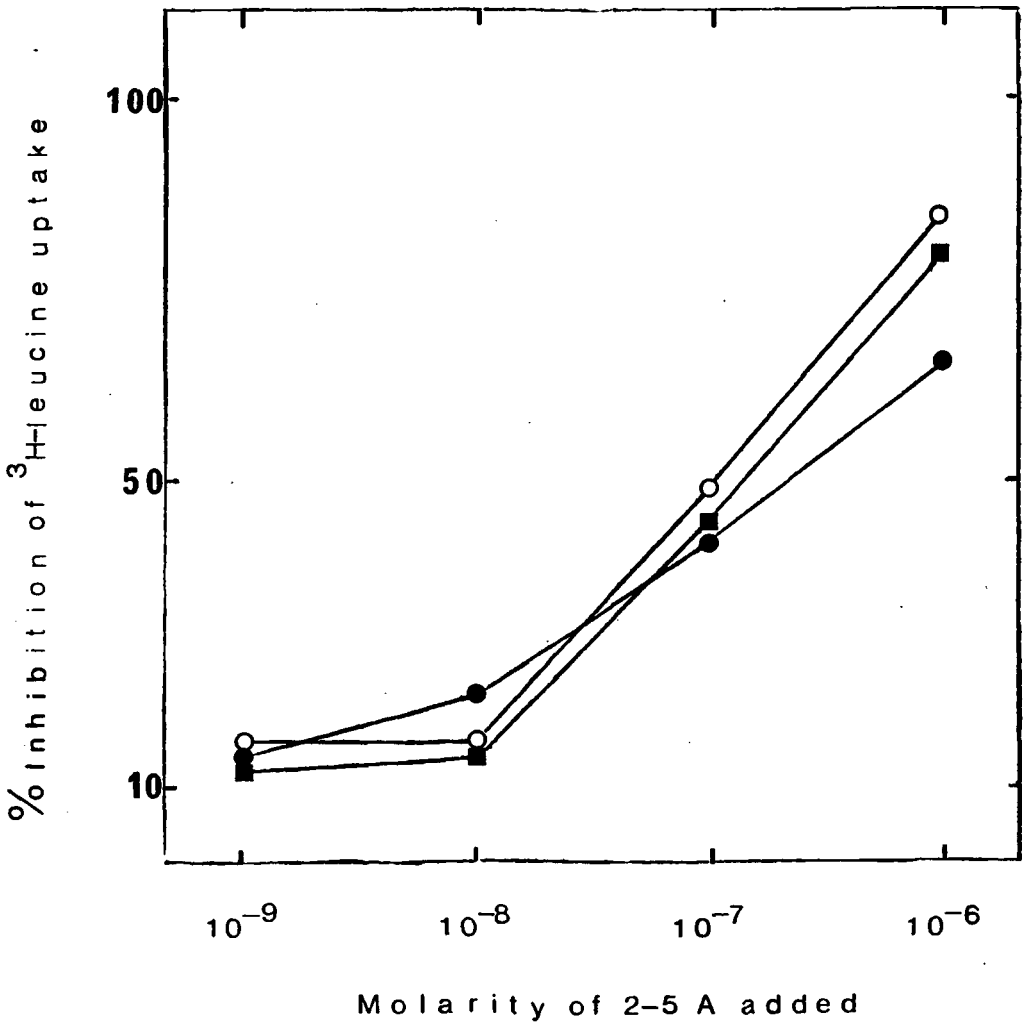

FIG. 34. Effect of 2-5 A on ^{3}H-leucine incorporation in Vero, SSPE-Vero and Sendai-Vero cells. Monolayers were treated with serial dilutions of 2-5 A in transfection buffer containing $CaCl_2$ for 135 min. Medium was changed and after 105 min ^{3}H-leucine was added for 1 h. Incorporation was determined by TCA precipitation. Vero (■-■), SSPE-Vero (o-o), Sendai-Vero (●-●) cells.

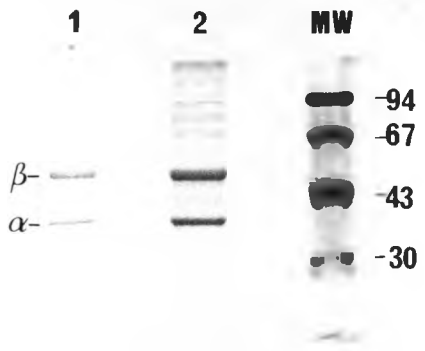

FIG. 35. Electrophoresis of eIF-2 preparations used. Gels were stained with Coomassie blue. Batch B (lane 1), Batch A (lane 2).

the phosphorylation of the β-subunit appeared in most gels when eIF-2 was added and this has been previously reported by Samuel (1979b).

The L-cells were reacted with the two different samples of eIF-2 (Figs. 36, 37). The α-subunit in each sample of eIF-2 was phosphorylated to the same extent by the L-cell extracts. In sample B a contaminant with a MW of 33 000 was present (Fig. 37). This contaminant which was previously seen (Palomo et al., 1985), runs very close to the α-eIF-2 protein which, in some instances, made the results difficult to interpret. The two eIF-2 samples were titrated in the protein kinase system; the titration of sample A is shown in Fig. 38.

As seen in Figs. 36 and 37 the activation of the protein kinase by IFN could be confirmed by the phosphorylation of the endogenous P1 (67 000 MW). The latter protein appeared phosphorylated every time an extract of L-cells treated with muIFN was assayed in the presence of dsRNA.

3.6.1. Protein kinase in hepatoma cell lines and SV40 infected cells

Extracts of PLC/PRF/5 and Mahlavu cells were tested using sample A of eIF-2 and no increase in phosphorylation of α-eIF-2 was observed after IFN treatment (Figs. 39, 40). Extracts of Hep 3B, Hep G2 and HA22T cells were tested with sample B of eIF-2. In the Hep 3B cell extract no increase in phosphorylation of α-eIF-2 could be observed after IFN treatment as the same level of phosphorylation is seen before treatment (Fig. 41 lanes 7, 12) and for this reason IFN was considered unable to activate the protein kinase in Hep 3B cells. Extracts of Hep G2 and HA22T cells,

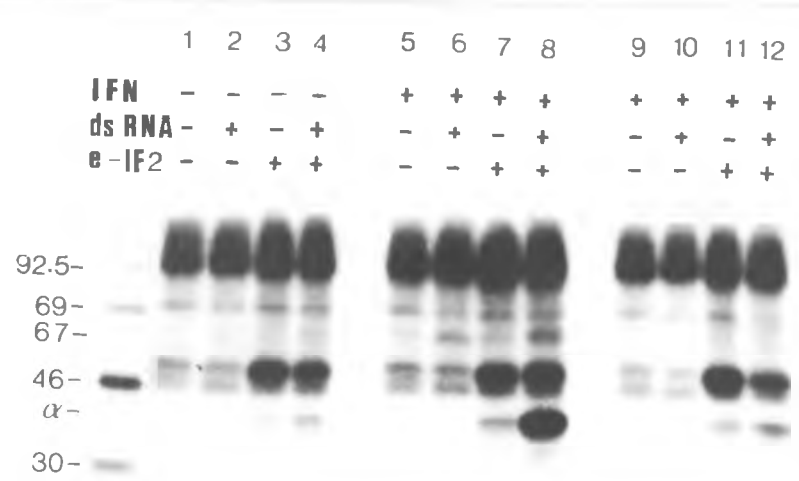

FIG. 36. Autoradiogram showing the effect of IFN on the protein phosphorylation of L-cells. L-cell extracts from cells treated in the presence or absence of IFN were incubated for 30 min in the presence or absence of dsRNA (1 μg/ml), eIF-2 (2 μg) (Batch A) in buffer containing ATP-^{32}P. The extracts were then resuspended in dissociation buffer, run on SDS-PAGE and the gel fluorographed. Untreated cells (lanes 1-4), treated with muIFN (200 IU/ml) (lanes 5-8), treated with mock IFN (lanes 9-12).

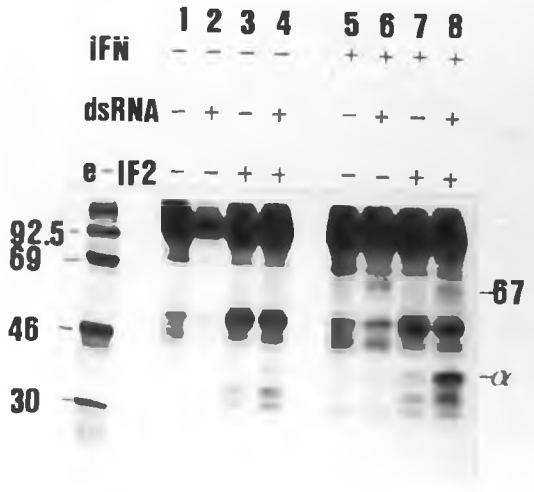

FIG. 37. Autoradiogram showing the effect of IFN on the protein phosphorylation of L-cells. L-cell extracts from cells treated in the presence or absence of muIFN were incubated for 30 min in the presence or absence of dsRNA (1 μg/ml), eIF-2 (2 μg) (Batch B) in buffer containing ATP-^{32}P. The extracts were then resuspended in dissociation buffer, run on SDS-PAGE and the gel fluorographed. Untreated cells (lanes 1-4), treated with muIFN (lanes 5-8).

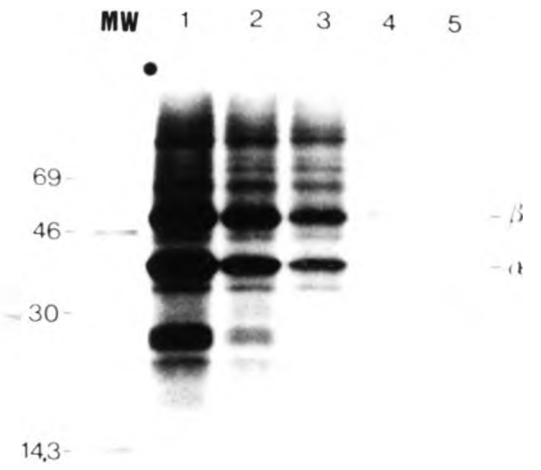

FIG. 38. Autoradiogram of extracts of L-cells pretreated with muIFN (200 IU/ml) and then incubated in the presence of different concentrations of eIF-2. Extracts were incubated for 30 min in the presence of dsRNA (1 µg/ml), of different concentrations of eIF-2 (Batch A) in buffer containing ATP-^{32}P. The extracts were then resuspended in dissociation buffer, run on SDS-PAGE and the gel fluorographed. Treated with eIF-2 (5 µg) (lane 1), eIF-2 (2.5 µg) (lane 2), eIF-2 (2 µg) (lane 3) without eIF-2 (lanes 4, 5). The presence of α and β -eIF-2 are indicated.

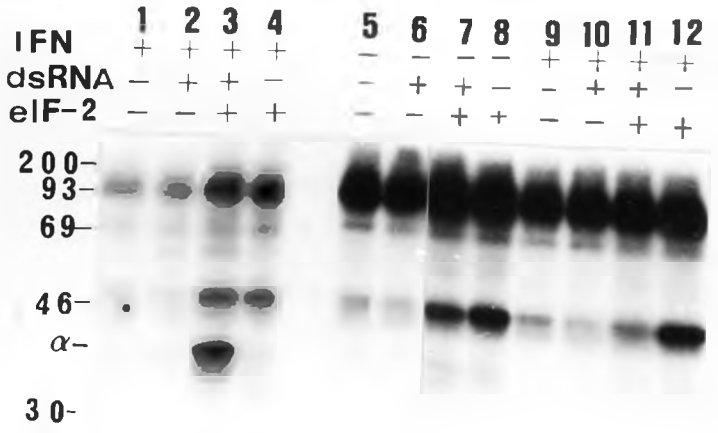

FIG. 39. Autoradiogram showing the effect of IFN on the protein phosphorylation of extracts of PLC/PRF/5 cells. Extracts from cells treated in the presence or absence of IFN (200 IU/ml) were incubated for 30 min in the presence or absence of dsRNA (1 μg/ml), of eIF-2 (2 μg) (Batch A) in buffer containing ATP-^{32}P. The extracts were then resuspended in dissociation buffer, run on SDS-PAGE and the gel fluorographed. L-cell extracts (lanes 1-4), PLC/PRF/5 cell extracts (lanes 5-12).

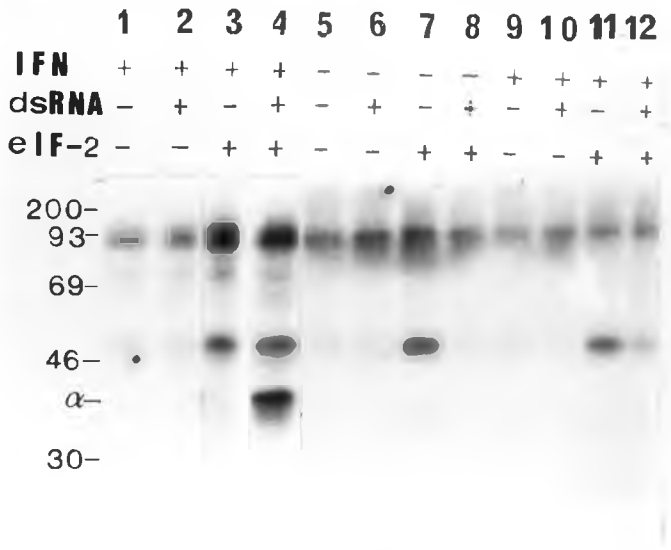

FIG. 40. Autoradiogram showing the effect of IFN on the protein phosphorylation of extracts of Mahlavu cells. Extracts from cells treated in the presence or absence of IFN (200 IU/ml) were incubated for 30 min in the presence or absence of dsRNA (1 μg/ml), of eIF-2 (2 μg) (Batch A) in buffer containing ATP-^{32}P. The extracts were then resuspended in dissociation buffer, run on SDS-PAGE and the gel fluorographed. L-cell extracts (lanes 1-4), Mahlavu cell extracts (lanes 5-12).

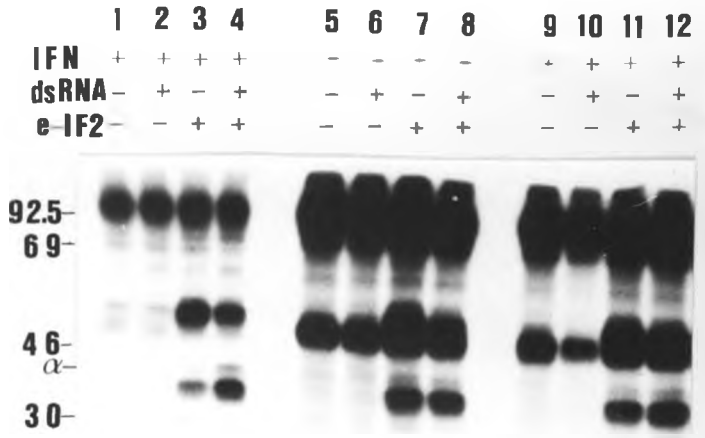

FIG. 41. Autoradiogram showing the effect of IFN on the protein phosphorylation of extracts of Hep 3B cells. Extracts from cells treated in the presence or absence of IFN (200 IU/ml) were incubated for 30 min in the presence or absence of dsRNA (1 µg/ml), of eIF-2 (2 µg) (Batch B) in buffer containing ATP-^{32}P. The extracts were then resuspended in dissociation buffer, run on SDS-PAGE and the gel fluorographed. L-cell extracts (lanes 1-4), Hep 3B cell extracts (lanes 5-12).

as well, did not increase the phosphorylation of the α-eIF-2 after IFN treatment (Fig. 42).

All CV-1 cell extracts, uninfected and infected with SV40, were tested with sample B of eIF-2 and did not show any increase in phosphorylation of α-eIF-2 after IFN treatment (Figs. 43-45). It appears that the protein kinase system is not used by the hepatoma and CV-1 cells.

3.6.2. Protein kinase in Vero cells infected with SSPE or Sendai virus

Vero and SSPE-Vero cell extracts were tested with sample A of eIF-2. In Vero cells α-eIF-2 was phosphorylated, but no increase in phosphorylation was seen after IFN treatment, while in SSPE-Vero cells no phosphorylation of α-eIF-2 was found (Figs. 46, 47).

Vero and Sendai-Vero cell extracts were subsequently tested using sample B of eIF-2 and in both cell extracts α-eIF-2 was phosphorylated before and after IFN treatment. No increase in the phosphorylation could be seen after IFN treatment or in the presence of dsRNA (Fig. 48). Sendai-Vero extracts were also run in parallel with L-cells (Fig. 49). These results show that as in the Vero cells, in Sendai-Vero the protein kinase stimulated by IFN is not activated.

3.7. SUMMARY

The summary of the results of the experiments done in this thesis are shown in Table 10.

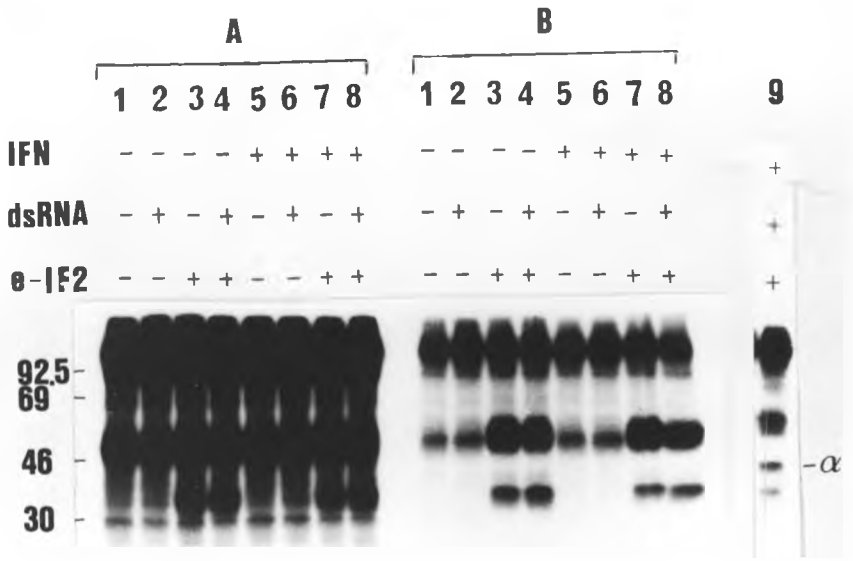

FIG. 42. Autoradiogram showing the effect of IFN on the protein phosphorylation of extracts of Hep G2 and HA22T cells. Extracts from cells treated in the presence or absence of IFN (200 IU/ml) were incubated for 30 min in the presence or absence of dsRNA (1 μg/ml), of eIF-2 (2 μg) (Batch B) in buffer containing ATP-^{32}P. The extracts were then resuspended in dissociation buffer, run on SDS-PAGE and the gel fluorographed. Hep G2 cell extracts (A), HA22T cell extracts (B). L-cell extract (lane 9).

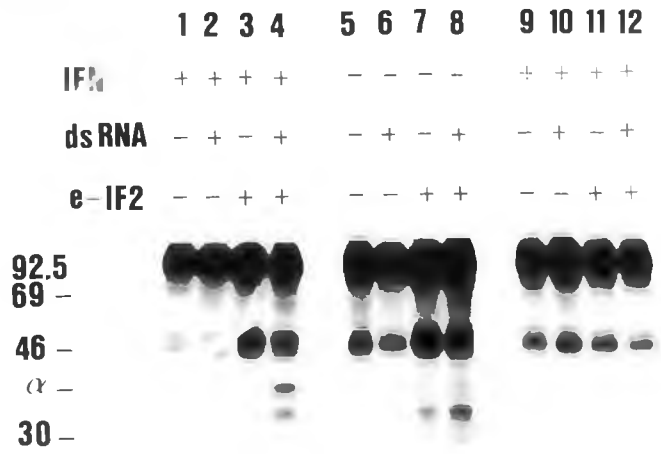

FIG. 43. Autoradiogram showing the effect of IFN on the protein phosphorylation of extracts of CV-1 cells. Extracts from cells treated in the presence or absence of IFN (200 IU/ml) were incubated for 30 min in the presence or absence of dsRNA (1 μg/ml), of eIF-2 (2 μg) (Batch B) in buffer containing ATP-^{32}P. The extracts were then resuspended in dissociation buffer, run on SDS-PAGE and the gel fluorographed. L-cell extracts (lanes 1-4), CV-1 cell extracts (lanes 5-12).

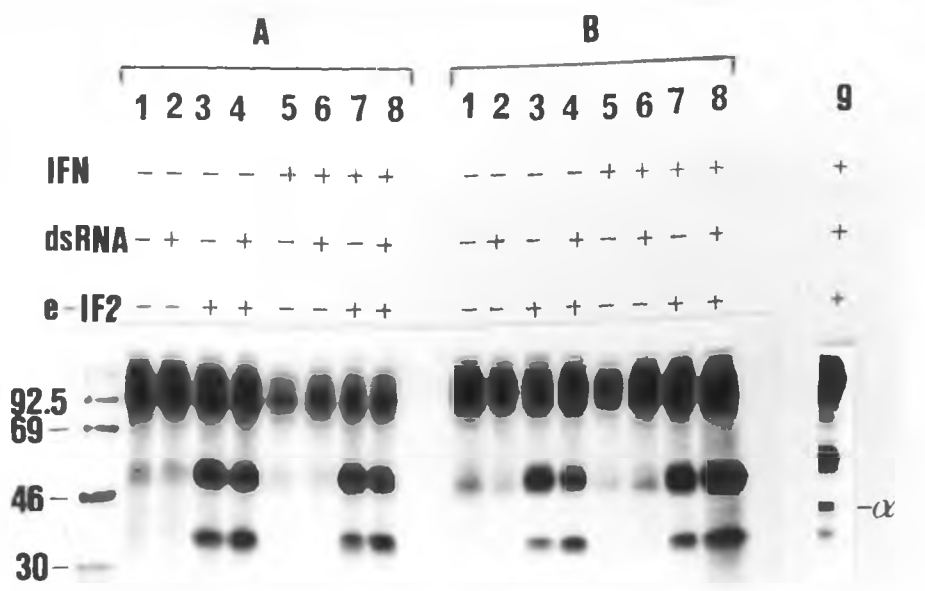

FIG. 44. Autoradiogram showing the effect of IFN on the protein phosphorylation of extracts of C2, C6 cells. Extracts from cells treated in the presence or absence of IFN (200 IU/ml) were incubated for 30 min in the presence or absence of dsRNA (1 μg/ml), of eIF-2 (2 μg) (Batch B) in buffer containing ATP-^{32}P. The extracts were then resuspended in dissociation buffer, run on SDS-PAGE and the gel fluorographed. C2 cell extracts (A), C6 cell extracts (B), L-cell extracts (lane 9).

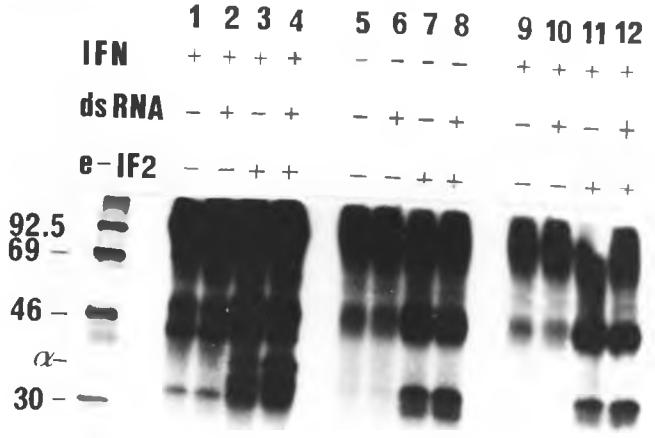

FIG. 45. Autoradiogram showing the effect of IFN on the protein phosphorylation of extracts of Cll cells. Extracts from cells treated in the presence or absence of IFN (200 IU/ml) were incubated for 30 min in the presence or absence of dsRNA (1 μg/ml), of eIF-2 (2 μg) (Batch B) in buffer containing ATP-^{32}P. The extracts were then resuspended in dissociation buffer, run on SDS-PAGE and the gel fluorographed. L-cell extracts (lanes 1-4), Cll cell extracts (lanes 5-12).

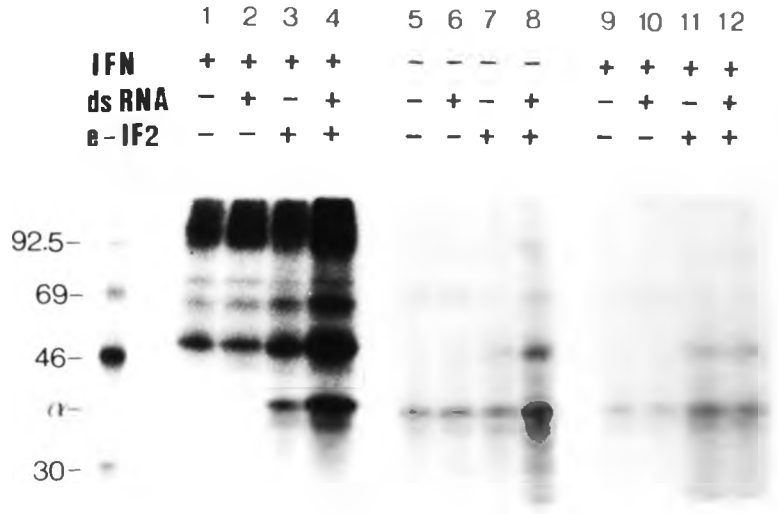

FIG. 46. Autoradiogram showing the effect of IFN on the protein phosphorylation of extracts of Vero cells. Extracts from cells treated in the presence or absence of IFN (200 IU/ml) were incubated for 30 min in the presence or absence of dsRNA (1 μg/ml), of eIF-2 (2 μg) (Batch A) in buffer containing ATP-^{32}P. The extracts were then resuspended in dissociation buffer, run on SDS-PAGE and the gel fluorographed. L-cell extracts (lanes 1-4), Vero cell extracts (lanes 5-12).

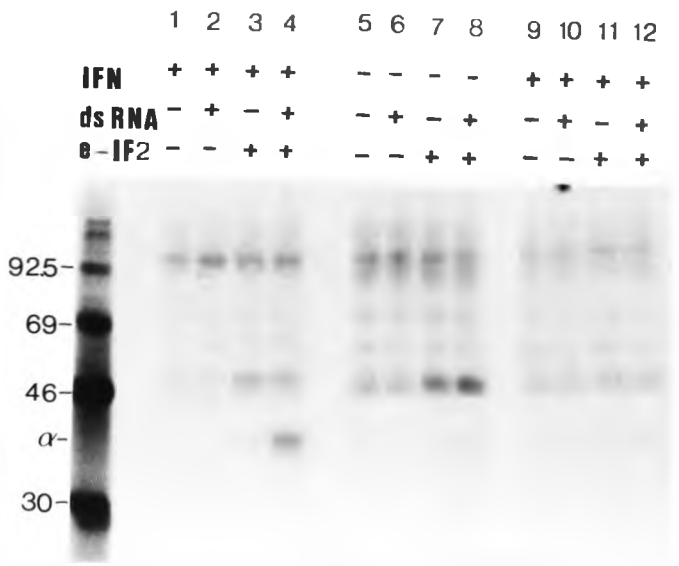

FIG. 47. Autoradiogram showing the effect of IFN on the protein phosphorylation of extracts of SSPE-Vero cells. Extracts from cells treated in the presence or absence of IFN (200 IU/ml) were incubated for 30 min in the presence or absence of dsRNA (1 μg/ml), eIF-2 (2 μg) (Batch A) in buffer containing ATP-^{32}P. The extracts were then resuspended in dissociation buffer, run on SDS-PAGE and the gel fluorographed. L-cell extracts (lanes 1-4), SSPE-Vero cell extracts (lanes 5-12).

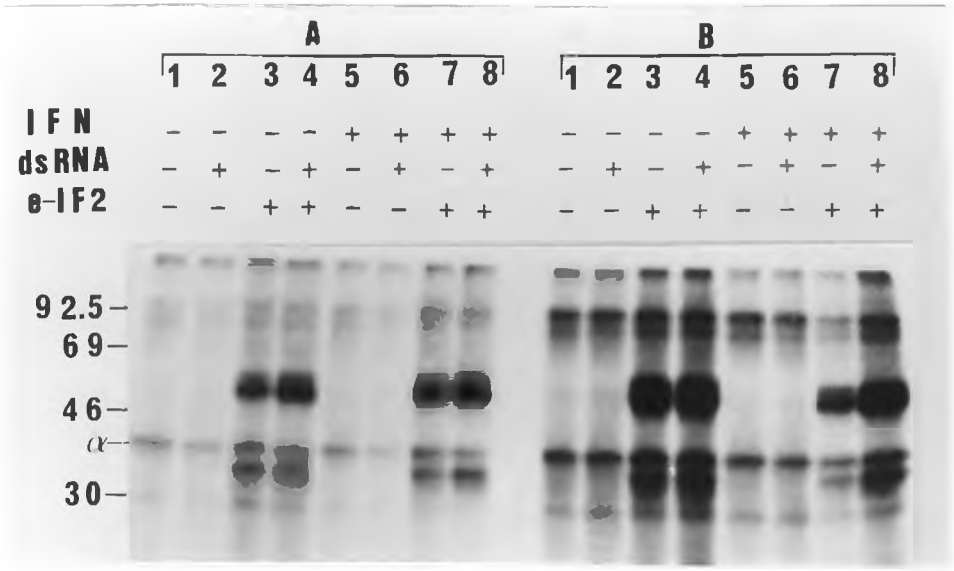

FIG. 48. Autoradiogram showing the effect of IFN on the protein phosphorylation of extracts of Vero and Sendai-Vero cells. Extracts from cells treated in the presence or absence of IFN (200 IU/ml) were incubated for 30 min in the presence or absence of dsRNA (1 μg/ml), eIF-2 (2 μg) (Batch B) in buffer containing ATP-^{32}P. The extracts were then resuspended in dissociation buffer, run on SDS-PAGE and the gel fluorographed. Vero cell extracts (A), Sendai-Vero cell extracts (B).

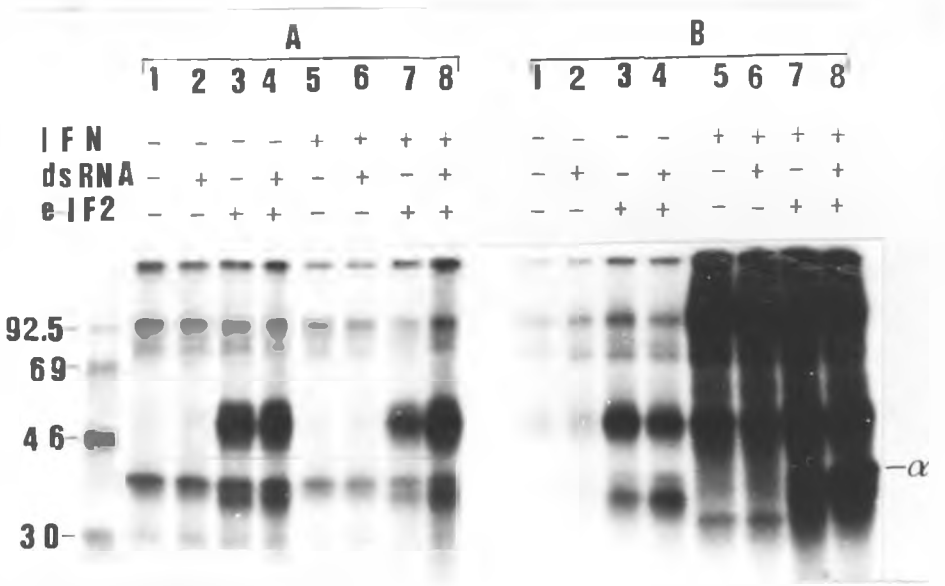

FIG. 49. Autoradiogram showing the effect of IFN on the protein phosphorylation of extracts of Sendai-Vero cells. Extracts from cells treated in the presence or absence of IFN (200 IU/ml) were incubated for 30 min in the presence or absence of dsRNA (1 μg/ml), eIF-2 (2 μg) (Batch B) in buffer containing ATP-^{32}P. The extracts were then resuspended in dissociation buffer, run on SDS-PAGE and the gel fluorographed. Sendai-Vero cell extracts (A), L-cell extracts (B).

TABLE 10. Summary of results of IFN's assays in the cell lines used in this thesis

	ANTIVIRAL (a) (RIA) IFN IU/ml	ANTI- (b) PROLIFERATIVE	E ENZYME ACTIVATION (c) -IFN	+IFN	RIBONUCLEASE F (d) ACTIVATION	PROTEIN KINASE (e) ACTIVATION
PLC/PRF/5	2	47	0.31	2.35	10	-
HEP 3B	4-16	0	0.32	1.76	7	-
MAHLAVU	2-8 *	19	0.24	1.06	35	-
HA22T	2	36	0.28	1.36	56	-
HEP G2	80	52	0.86	1.7	7	-
CV-1	4	30	0.28	1.72	83	-
C2	4	18	0.39	10.6	76	-
C6	2	24	0.20	3.74	67	-
C11	4	29	0.27	10.35	78	-
VERO	4	29	0.35	6.3	79	-
SSPE-VERO	1600	0	0.02	0.19	84	-
SENDAI-VERO	1600	0	0.05	0.09	65	-

(a) concentration of IFN which inhibits Sindbis virus replication by 50%
(b) % inhibition of cell growth after treatment with 1 000 IU/ml of IFN
(c) pmoles of 2-5 A/h/µg protein produced before and after IFN (200 IU/ml)
(d) % inhibition of ^{3}H-leucine incorporation after addition of 2-5 A $10^{-6}M$
(e) phosphorylation of added α-eIF-2

* determined by immunofluorescence

CHAPTER 4

DISCUSSION

Many viruses from a wide variety of families have evolved strategies whereby they can persist in a host without destroying it. In virology the term "persistence" is used with remarkable looseness to cover a wide variety of situations which could not conceivably be grouped together under any single, properly defined heading (Epstein, 1982).

A recent appraisal of virus persistence is the paper by Mahy (1985) in which he divided persistence into that occurring in the individual and that occurring in the cell.

Regarding persistence in the individual, he defined three mechanisms which are listed below; however, it is important to point out that these are not distinct groupings, as for example, mechanisms 1 and 3 are both exhibited by a number of virus groups. The mechanisms are:

1) integration into cellular DNA
2) latent infection of neuronal cells
3) persistent infection with virus shedding

A virus family for which genome integration into cellular DNA is well documented as a means of persistent infection is the Retroviridae (Topp et al., 1981). One retrovirus subfamily, the lentiviruses, is of particular interest. Examples in this subfamily are Visna and Equine Infectious Anaemia (EIA) viruses (Youngner and Preble, 1980; Stroop and Baringer, 1982). Visna virus produces a slow progressive disease in sheep affecting the central

nervous system (Gudnadottir, 1974) while EIA infects horses and produces immunological-mediated lesions (Henson and McGuire, 1974). In both viruses infection can persist for many years despite the host immune response. It is clear that the mechanism of persistence in these two viruses involves integration of viral DNA into the host genome and antigenic variation (Mahy, 1985). As these lentiviruses also produce periodic viraemia they could be classified in the third group of persistent infection.

Haase et al. (1977) have found high levels of Visna DNA, low levels of viral RNA and no virus in sheep choroid plexus during persistent infection. This suggests that viral DNA is maintained in cells in vivo but that the expression of viral genetic information is blocked at the transcriptional level. Therefore the infected cells do not express viral antigens and are not recognized as foreign by the host immune system (Stroop and Baringer, 1982).

Another way in which Visna and EIA viruses can persist is by mutations of the major envelope glycoprotein. These mutations are responsible for the production of antigenic variants which enable the virus to circumvent the host immune response (Narayan et al., 1977; Stroop and Baringer, 1982). The latter phenomenon has been termed antigenic drift (Youngner and Preble, 1980).

The Herpesviruses are the best example of latent infection of neuronal cells. These viruses, after producing a primary infection, can persist in a latent state for the host's lifetime (Mahy, 1985).

Herpesviruses most commonly establish a persistent infection in the central nervous system by infecting the spinal ganglia (Stevens and Cook, 1971). Virus reactivation may occur when the

virus migrates along the peripheral nerve fibre of a tissue (ter Meulen et al., 1984). No expression of viral antigens in latently infected ganglion cells has been found, suggesting that in this way the virus avoids the host immune defence mechanism (Stroop and Baringer, 1982).

Whether the Herpesvirus DNA is in the integrated or episomal form is still uncertain (Rock and Fraser, 1983; Mahy, 1985). Herpes virus DNA can be detected in latently infected tissue by hybridization, but little or no virus specific RNA can be found (Galloway et al., 1982; Rock and Fraser, 1983).

The third type of persistent infection in humans and animals is characterized by the recovery of the virus or parts of the virus in the serum and tissue throughout the life of the host (Mahy, 1985). Two examples of this type of persistent infection are HBV and measles virus. After a primary infection with HBV, 4-5% of the cases become disease carriers and a proportion of these carriers continue to shed infectious virus and maintain significant levels of HBsAg in their sera (Szmuness, 1975; Zuckerman, 1975).

HBV virus DNA may become integrated into the liver cell genome (Shafritz et al., 1981) and these integrated HBV sequences often remain after all forms of episomal DNA have disappeared (Mason et al., 1985). The integration of HBV DNA into the cell genome preferentially uses a single stranded region of the viral genome and is probably random (Koshy et al., 1983). Integrated sequences often contain multiple deletions, inversions and/or duplications and rearrangements have been detected in flanking cellular sequences (Koch et al., 1984). Based on these facts it

is clear that HBV also falls into the first group defined by Mahy (1985), that of integration of virus into the cellular genome.

Measles virus infection, after causing a primary disease may lead to a complication of the central nervous system, termed subacute sclerosis panencephalitis (SSPE), which only appears a few years later (Wechsler and Meissner, 1982). High levels of measles antibody occur in the serum and the cerebrospinal fluid of patients with SSPE (Connolly et al., 1967). No detectable extracellular viral particles are found in the brain (Dubois-Dalcq, 1979); however the virus can be recovered from the brain cells by cocultivation with cells permissive for measles virus (Wechsler and Meissner, 1982). The mechanism of measles virus persistence in SSPE is not completely understood, but it seems that a defect in expression of the virus matrix protein in infected brain cells may prevent the formation of mature viral particles (Hall and Choppin, 1979). Other factors may be related to the persistent measles infection such as the accumulation of large numbers of nucleocapsids in the nuclei of SSPE infected brain cells rather than in the cytoplasm (Dubois-Dalcq, 1979). In latent infections the nucleocapsid has also been shown, in electron micrographs, to have a smooth appearance as opposed to the fuzzy appearance observed in acute measles infection; also, they do not align with the cell membrane and no budding virus has been observed in brain cells (Dubois-Dalcq, 1979). The theory of antigenic modulation has also been put forward as a cause of persistent measles infection (Joseph and Oldstone, 1975). In the latter case measles virus antibody may

strip or modulate viral antigens from the surface of infected cells, thus protecting the cells from immunological injury (Joseph and Oldstone, 1975).

Mahy (1985) has defined four mechanisms whereby viruses persist at the cellular level. These mechanisms, which have been studied *in vitro*, are:

1) carrier culture
2) steady-state infection
3) intracytoplasmic persistence
4) latent infection

In carrier cultures only a small proportion of the cell population is infected. The infected cells release virus and die and the virus then infects only a small proportion of the remaining cells (Mahy, 1985). An example of a carrier culture infection is lymphocytic choriomeningitis virus which in L-cells shows regular fluctuations in the amount of virus released into the medium (Weber *et al.*, 1983; Lehman-Grube *et al.*, 1983).

The second type of in-vitro persistent infection is the steady-state infection where both virus and cell multiplication are in equilibrium (Mahy, 1985). This equilibrium probably involves both viral and cellular genetic factors. Many RNA viruses produce a persistent steady-state infection by the selection of mutants which are less cytopathic (Youngner and Preble, 1980).

Examples of virus mutants such as DI and ts mutants are numerous (Rima and Martin, 1976; Holland *et al.*, 1980; Youngner and Preble, 1980). The evolution of measles and Sendai virus mutants which replicate in cells without a significant cytopathic

effect has been studied in several cell lines (Youngner and Preble, 1980). A decrease in the level of expression of the haemagglutinin and matrix proteins has been observed in many cellular systems (Roux and Waldvogel, 1982, 1983; Giraudon et al., 1984; Roux et al., 1984; Sheshberadaran et al., 1985).

The third type of in-vitro persistent infection termed intracytoplasmic persistence has only been observed with lymphocytic choriomeningitis virus in C13/SV28 and in BHK-21F cells (Mahy, 1985). In this case the medium of the infected cells did not contain any virus or DI particles (van der Zeijst et al., 1983). In-vitro infection can be established using virus-infected cell extracts or by cell-cell contact; the infectious material is probably contained in large vesicles (van der Zeijst et al., 1983).

The fourth type of in-vitro persistent infection defined by Mahy (1985) is a true latent infection where the viral genome or part of the genome is integrated into the host cell genome. This type of persistent infection is usually established by retroviruses and DNA viruses (Mahy, 1985). Examples of retroviruses that establish such latent infections are EIA and Visna viruses (Cheevers et al., 1982), while examples of DNA viruses are HBV and SV40 (Marion et al., 1980; Topp et al., 1981; Twist et al., 1981).

Integrated DNA viruses have been shown to be responsible for the transformation of cells and for the induction of cancer (Grodzicker and Hopkins, 1981; Hadziyannis, 1980). In most cell lines transformed with SV40 or infected with HBV the presence of integrated viral DNA is evident by the continuous production of viral proteins such as T antigen and HBsAg (Grodzicker and Hopkins, 1981; Koshy et al., 1981).

SV40 can become integrated into the cellular genome at several sites and this integration is stable for many generations (Topp et al., 1981). Virus specific RNA has been found in both SV40 transformed cell lines as well as in the hepatoma PLC/PRF/5 cells (Chakraborty et al., 1980; Topp et al., 1981). No episomal HBV DNA has been found in PLC/PRF/5 or Hep 3B cells and while transformed SV40 cells may or may not contain free copies of viral DNA they can contain single or multiple copies of integrated DNA and may also contain either less than one whole genome or tandem duplicated copies (Marion et al., 1980; Twist et al., 1981; Griffin, 1982).

In this study five cell lines derived from primary liver carcinoma were examined. Two cell lines containing HBV DNA were compared with three control cell lines which did not contain HBV DNA. The ability of IFN to inhibit the replication of an exogenous virus, to stimulate the antiproliferative effect and to activate the two IFN-induced enzymes was examined.

The characteristics of the hepatoma cell lines used have been previously studied. All these hepatoma cell lines produced the characteristic liver enzymes and hepatocyte plasma proteins (Hammond et al., 1975; Alexander et al., 1976; Aden et al., 1979; Knowles et al., 1980; Chang et al., 1983). α-fetoprotein was produced by the Hep G2, Hep 3B and by the PLC/PRF/5 cells while all the hepatoma cell lines, except Hep G2, produced tumours in mice (Bassedine et al., 1980; Daemer et al., 1980; Sattler et al., 1982; Shouval et al., 1981, 1985; Chang et al., 1983). Hep G2 was the only cell line that was derived from an hepatoblastoma (Knowles et al., 1980). The cell structure of most of

the hepatoma cells is polygonal and only the Mahlavu cells have a fibroblast-like morphology. HBsAg is produced by the Hep 3B and PLC/PRF/5 cells and both these cell lines had HBV DNA integrated into the cellular genome (Macnab et al., 1976; Oefinger et al., 1981; Aden et al., 1979; Koshy et al., 1981; Twist et al., 1981; Ziemer et al., 1985).

The sensitivity to IFN has only been studied previously in the PLC/PRF/5 cells (Desmyter et al., 1981; Nakajima et al., 1982) and their results correlated with those obtained in this study:

1) PLC/PRF/5 cells did not produce IFN constitutively.
2) After IFN treatment PLC/PRF/5 cells could inhibit exogenous RNA virus replication at low levels of IFN such as 2 IU/ml (Table 7).
3) After IFN treatment the level of endogenous E enzyme increased (Fig. 27).
4) IFN produced an antiproliferative effect in PLC/PRF/5 cells (Figs. 22, 23).

Desmyter et al. (1981) showed as well that PLC/PRF/5 cells were unable to induce IFN when they were challenged with Newcastle disease virus or with poly(I)poly(C), a synthetic IFN inducer. IFN could not inhibit the formation of tumours in mice injected with PLC/PRF/5 cells, and could not stop the production of HBsAg in PLC/PRF/5 cells (Desmyter et al., 1981; Nakajima et al., 1982). In conclusion, IFN could not inhibit the synthesis of HBV products such as HBsAg or the development of hepatocellular carcinoma, but could prevent the replication of exogenous virus

and stimulate an antiproliferative effect.

In this thesis it was also shown that IFN did not induce either the ribonuclease F or the protein kinase in the PLC/PRF/5 cells. The sensitivities of the other hepatoma cell lines to IFN did not always follow the same pattern as the PLC/PRF/5 cell line although similarities were found. In all the hepatoma cells studied no endogenous IFN could be detected in cell supernatants. Variable sensitivity to the antiviral effect of IFN against Sindbis virus was found (Table 7), the least sensitive being Hep G2 cells which needed an IFN concentration of 80 IU/ml to inhibit Sindbis virus replication. In general, low amounts of IFN could inhibit Sindbis virus replication in hepatoma cells and there was no difference with the control HBsAg non-producer cell lines. IFN did not induce the protein kinase in any hepatoma cell line studied as shown in Figs. 39-42. However the E enzyme was activated in all the hepatoma cell lines after IFN treatment (Fig. 27); in PLC/PRF/5 and Hep 3B cells the level of E enzyme increased approximately six-fold while an increase of approximately four-fold was seen in the control Mahlavu and HA22T cells. The level of E enzyme in the Hep G2 cells reached a level equal to that of the Hep 3B cells, but their basal level, i.e. before stimulation, was much higher than the basal level of E enzyme in the Hep 3B cells. In fact, Hep G2 cells had the highest basal level of all the hepatoma cell lines and an increase of only two-fold in the E enzyme level was seen after IFN treatment (Fig. 27).

The ability of the hepatoma cell lines to respond to the activation of the ribonuclease F, the second enzyme activated in the E enzyme pathway, was studied by measuring the inhibition

of ^{3}H leucine incorporation after exogenous 2-5 A had been introduced into the cells. In Hep 3B, PLC/PRF/5 cells and in Hep G2 cells, 2-5 A was unable to activate the ribonuclease F, while in Mahlavu and HA22T cells the ribonuclease F was activated (Fig. 32). Pretreatment of PLC/PRF/5 and Hep G2 with rec α-IFN (200 IU/ml) did not increase the ribonuclease F level while with Hep 3B cells there was a partial activation of the enzyme as shown by a 2.7-fold decrease in ^{3}H-leucine incorporation at $10^{-6}M$ of 2-5 A. The lack of activation of the ribonuclease F in PLC/PRF/5 cells was not caused by an inability to introduce 2-5 A into the cells as the same amount of 2-5 A was introduced into these cells as in the Mahlavu cells (Table 9).

The antiproliferative effect of IFN on hepatoma cells showed that the growth of all the hepatoma cells studied with the exception of the Hep 3B cells could be inhibited (Figs. 22-24). The sensitivity of the different hepatoma cell lines to the antiproliferative effect varied as Mahlavu cells reacted to a lesser extent than the PLC/PRF/5, HA22T or the Hep G2 cells.

It therefore appears that the PLC/PRF/5 and the Hep G2 cell lines use neither the protein kinase nor the second part of the E enzyme pathway to inhibit virus replication, whereas the Mahlavu, the HA22T and possibly Hep 3B cells could use the E enzyme pathway as both the E enzyme and the ribonuclease F are activated. However, as Sindbis virus replication is inhibited in all hepatoma cell lines following IFN treatment (Table 7) and as IFN induces an antiproliferative effect in all hepatoma cell lines, with the exception of Hep 3B cells (Figs. 22-24), indicates that there might be other pathway(s) available whereby

IFN acts. These results also show that there is no distinct correlation between the induction of the two main IFN-enzyme systems and the presence of integrated HBV DNA.

The results with Hep 3B cells where there is activation of the antiviral effect but no activation of the antiproliferative effect show that there must be two distinct and dissociable intracellular pathways whereby IFN affects its antiviral and antiproliferative actions as previously proposed by Taylor-Papadimitriou et al. (1985).

The other DNA virus system that was studied was the persistent infection of CV-1 cells with SV40 virus. SV40 virus is a papovavirus which has been shown to produce tumours when it is injected into newborn hamsters (Eddy et al., 1962; Girardi et al., 1962) and to transform cells (Todaro and Green, 1964; Black, 1966). In SV40-transformed cells IFN has been shown to be unable to inhibit the synthesis of the early T antigen, but inhibits the replication of exogenous VSV and the production of T antigen in a superinfection with SV40 (Oxman et al., 1967; Revel, 1979; Garcia-Blanco et al., 1985). The inability of IFN to inhibit the production of SV40 T antigen in SV40-transformed cells is similar to the inability of IFN to prevent the production of HBsAg in hepatoma derived cell lines containing integrated HBV DNA. This lack of IFN's effect may be due to the fact that IFN does not recognise viral mRNA as the viral DNA in both cases is integrated into the cell genome (Revel, 1979; Nakajima et al., 1982).

Three SV40-transformed cell lines, C2, C6 and C11, were used in this study. All three cell lines produce SV40 T antigen

and have the growth characteristics of a non-permissive transformed cell line such as reduced cell density inhibition, reduced serum dependence, ability to overgrow normal cells and colony formation in soft agar (Gluzman et al., 1977; Prives et al., 1978). No spontaneous release of infectious virus occurs however, and SV40 DNA is integrated in these cell lines (Gluzman et al., 1977; Prives et al., 1978). Each cell line has a single incomplete insert of viral DNA sequence and the bulk of the SV40 sequences are associated with the high molecular weight chromosomal DNA (Gluzman et al., 1977; Krieg et al., 1981). Restriction mapping of the inserted SV40 DNA indicated that the early viral DNA region is retained in all cells and alterations are found in the late regions (Krieg et al., 1981).

CV-1 cells were used as controls and the effects of IFN on CV-1, C2, C6 and C11 cells were compared. Neither CV-1 control nor infected cells spontaneously secreted IFN, but all were sensitive to the antiviral effect of IFN (Table 7) with a low concentration of IFN (2-4 IU/ml) inhibiting replication. All control and infected cells were sensitive to the antiproliferative effect of IFN with 30% inhibition of cell growth at an IFN concentration of 1 000 IU/ml (Fig. 25). As with most of the hepatoma cells, IFN was able to activate the antiviral and antiproliferative pathways.

The stimulation of the E enzyme after IFN treatment (200 IU/ml) was six-fold in the control CV-1 cells (Fig. 28) while a significantly higher increase in the level of E enzyme after IFN treatment was seen in C2, C6 and C11 cells. The presence of ribonuclease F in CV-1, C2, C6 and C11 cells was shown by a significant decrease in the

incorporation of ^{3}H-leucine after introduction of 2-5 A (Fig. 33). These results indicate that both the E enzyme and ribonuclease F are active in the SV40-infected cells, and therefore this pathway is functioning in these cells. A large increase in the synthesis of 2-5 A in cells pretreated with IFN and then infected with SV40 has previously been reported (Revel et al., 1980; Hersh et al., 1984). However, Hersh et al. (1984) have analysed by high performance liquid chromatography the 2-5 A products and have found that most of these products were largely non-functional 2-5 A cores or non-phosphorylated 2-5 A and that these products were unable to activate the ribonuclease F or to degrade rRNA and virus RNA. It is possible that the 2-5 A produced in the C2, C6 and C11 cells examined in this study also contained a large percentage of non-functional 2-5 A. The protein kinase was not stimulated in any of the CV-1 cells uninfected or infected with SV40 virus (Figs. 43-45).

The presence of SV40 appears to significantly increase the level of E enzyme following IFN treatment and presuming that the 2-5 A produced is functional and the fact that the ribonuclease F is induced, would indicate that this pathway is active.

The effect of IFN on persistent RNA virus infections was studied in SSPE and Sendai viruses.

In this study the effect of IFN on a Vero cell line persistently infected with SSPE was compared with the uninfected Vero cell line. Vero cells have previously been shown to be unable to produce IFN (Desmyter et al., 1968) and the presence of measles or SSPE virus in the cells did not change this characteristic.

Very low levels of IFN (4 IU/ml) inhibited Sindbis virus replication in Vero cells while a concentration of 1 600 IU/ml of IFN was needed to inhibit Sindbis virus replication in SSPE-Vero cells; this represents a 400-fold decrease in the sensitivity of SSPE-Vero to IFN. A possible reason for this decreased sensitivity was investigated by examining the ability of IFN to activate the E enzyme and protein kinase pathways. Protein kinase was not induced in either the Vero or SSPE-Vero cells (Figs. 46-47), while there was decreased activation of E enzyme in SSPE-Vero cells as compared to the control Vero cells (Fig. 29). SSPE-Vero cells also had a very low basal level of E enzyme, which was approximately ten times lower than the uninfected Vero cells (Table 10). Thus a major difference between the two cell lines would appear to be the level to which the E enzyme was activated. The second part of the E enzyme pathway in both Vero and SSPE-Vero cells could be activated as shown by inhibition of the incorporation of ^{3}H-leucine by approximately 80% after 2-5 A was introduced into the cells.

SSPE-Vero cells were not only less sensitive to the antiviral effect of IFN but were also less sensitive to the antiproliferative effect of IFN when compared to the control Vero cells. In fact, no inhibition on cell growth in SSPE-Vero cells was found while Vero cells were inhibited by 30% at an IFN concentration of 1 000 IU/ml (Fig. 26).

As SSPE-Vero cells produce SSPE virus, a new Vero cell line persistently infected with SSPE was established. Passages from P.1 onwards were examined for their sensitivity to IFN. While early passages were sensitive to the antiviral effects of IFN and the levels of E enzyme induced were similar to control Vero

cells (Fig. 30) by P.4 the cells needed a 400-fold greater concentration of IFN to inhibit Sindbis virus replication. At P.4 large syncitia were present and these showed strong viral immunofluorescence in the cytoplasm. The E enzyme level determined at P.7 was very low following IFN treatment (Fig. 30).

The effect of IFN on the synthesis of measles and SSPE viral proteins was also examined in this study. While an IFN concentration of 25 IU/ml inhibited the synthesis of measles H and N proteins and by 50 IU/ml the M protein had disappeared (Fig. 16), a concentration of 4 000 IU/ml of IFN did not inhibit the synthesis of any SSPE viral proteins (Fig. 17). The effect of IFN in inhibiting measles polypeptide synthesis was present from the beginning of the replication cycle (Fig. 20).

From these results it can be concluded that the actions of IFN are considerably reduced in SSPE-Vero cells, with IFN having negligible antiviral and antiproliferative effects. It is most likely that this decreased sensitivity to IFN is due to the presence of SSPE virus. The fact that the E enzyme is induced in these cells, albeit at a very low level, would indicate that IFN does have a slight activity in stimulating at least one of its antiviral pathways.

The other persistent RNA virus infection examined was Sendai virus. As with measles virus, Sendai virus is a good inducer of IFN in lymphoblastoid cells and IFN reduces cytolysis in HeLa cells infected with Sendai virus (Finter and Fantes, 1980; Sato et al., 1980).

A monolayer of Vero cells was infected with Sendai virus and a persistent infection was obtained by subculturing the

colonies that survived the lytic effect of Sendai virus. The new colonies established a monolayer which had the morphology of Vero cells and more than 50 sequential subcultures have been done with continuous presence of viral antigens. Immunofluorescence showed that 50% of these cells were infected with Sendai virus. No IFN could be detected in the culture media, but low levels of virus were produced as the culture media could induce a persistent infection in Vero cells. Absence of IFN in the medium of HeLa cells persistently infected with Sendai virus (HVJ-strain) has been reported (Maeno et al., 1966).

An IFN concentration of more than 1 600 IU/ml was needed to inhibit the replication of Sindbis virus in this newly established Sendai-Vero cell line; this is at least a 400-fold higher concentration than the one needed in Vero cells. Ito et al. (1979) have also found a decreased sensitivity to the anti-viral effect of IFN in Vero cells persistently infected with HVJ Sendai.

The activation of the main enzymes induced by IFN was examined. The level of E enzyme was only increased 1.8 fold and the protein kinase was not activated (Figs. 29, 48, 49). As with SSPE-Vero cells the basal level of endogenous E enzyme was lower than in the control Vero cells (Table 10). The Sendai-Vero cell line was sensitive to the action of 2-5 A as there was activation of the ribonuclease F enzyme. Similar activation of the ribonuclease F was seen in control Vero cells (Fig. 34).

No antiproliferative effect of IFN in the Sendai-Vero cells was seen at an IFN concentration of 1 000 IU/ml (Fig. 26).

The effect of IFN on endogenous Sendai virus protein synthesis in these persistently infected Vero cells was also studied. No

inhibition of the synthesis of the Sendai virus protein P, H, or N, was detected even at 4 000 IU/ml of IFN (Fig. 21). It can thus be concluded that the antiviral and antiproliferative actions of IFN in Sendai-Vero cells are considerably reduced in comparison to the control Vero cells. Therefore, as with SSPE-Vero cells, a persistent infection with Sendai virus changes the sensitivity of Vero cells to IFN.

CONCLUSIONS

The results of this thesis show that there are major differences between the two persistent DNA virus infections and the RNA virus infections studied as regards to their sensitivity to IFN. The presence of integrated HBV or SV40 genomes had no significant effect on the antiviral and antiproliferative effects of IFN and generally these cell lines were as sensitive to IFN as their controls. This indicates that this type of persistent infection does not significantly modify the overall IFN mechanism in these cells. It is interesting to note that in the hepatoma cell lines in particular the two main IFN enzyme pathways are induced to a variable extent. In none was the protein kinase activated while there was variable activation of the E enzyme. The activation of these two enzyme pathways has been regarded as the main mechanism involved in the antiviral effect of IFN (Torrence, 1982), but the lack of correlation between the IFN-mediated antiviral state and the two enzyme pathways has been previously reported (Verhaegen et al., 1980; Hovanessian et al., 1981; Meurs et al., 1981). Cell lines which are sensitive to the antiviral effect of IFN

but do not stimulate both enzyme pathways have been reported (Meurs et al., 1981; Holmes and Gupta, 1982; Sen et al., 1983). The significance of the results of these workers and those obtained in this thesis is that the IFN mediated antiviral effect is not always dependent in the activation of the E enzyme or the protein kinase pathways. The intracellular events leading to the development of the antiviral response may vary not only from one cell system to the other (Hovanessian et al., 1981; Meurs et al., 1981), but also from virus to virus (Samuel and Knutson, 1981; Tomita et al., 1982b). The involvement of another pathway in providing an IFN-antiviral effect can then be suggested.

In contrast to the two DNA virus persistent infections, the presence of RNA viruses such as SSPE or Sendai viruses altered dramatically the sensitivity of a cell line to IFN. This change was evident within a few passages in the development of persistence, and was characterised by a significant decrease in the anti-proliferative and antiviral effects of IFN. This shows that in this type of persistent infection, the presence of the RNA virus somehow alters the cell's response to IFN. As the E enzyme was induced to a slight extent in both cell lines this would indicate that IFN had some minimal action. It is possible that in these persistent infections the presence of the virus affects the initial action of IFN, feasible propositions being a reduction in the number of IFN receptors on the cell membrane or a block in some messages after IFN's entry into the cell. This would therefore render the cell insensitive to IFN.

Further investigation into the exact mechanism of this

phenomenon is important as abolition of IFN's antiviral effect, one of the major defence mechanisms of the host, may be important in the establishment of persistent infections caused by RNA viruses such as measles. Measles virus has been implicated not only as the cause of SSPE but also multiple sclerosis and Paget's diseases (Rebel et al., 1980; Haase et al., 1981; Basle et al., 1985). A decreased sensitivity to IFN may be a common theme in the development of these three diseases and also of other chronic degenerative diseases of unknown etiology.

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