"MOLECULAR MECHANISM OF DEFECTIVE TRANSPORT OF VIRAL GLYCOPROTEIN IN INTERFERON TREATED CELLS"

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BIRLA INSTITUTE OF TECHNOLOGY & SCIENCE, PILANI

JANUARY, 1995

"Molecular mechanism of defective transport of viral glycoprotein in Interferon treated cells"

Thesis

Submitted in partial fulfilment of the requirement for the degree of

DOCTOR OF PHILOSOPHY

by

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January, 1995

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ABSTRACT

Interferons (IFNs) have some unusual inhibitory effect on some membrane viruses. These are inhibition of virus assembly, release and budding as seen in most murine leukemia viruses (MLVs), mouse mammary tumor virus (MMTV), Bovine parainfluenza virus (PIV -3), Herpes simplex virus (HSV) and recently Human immunodeficiency virus (HIV); or a small decrease in the production of virus particles together with a remarkable reduction in the infectivity of the released virions, as reported in some infections of MLVs, Vesicular stomatitis virus (VSV), vaccinia, HSV-1 and HIV in lymphocytes and macrophages .

Earlier from this laboratory, it has been reported that IFN treatment (30 I.U./ ml) inhibits (approximately 200-fold) the infectivity of VSV released from mouse L_B cells; however, virus particle-associated RNA, nucleocapsid protein and

viral transcriptase were inhibited less than 10-fold (Maheshwari & Friedman, 1979). These results suggested that IFN treatment induces the production of VSV particles with low infectivity. However, VSV released from IFN treated cells were selectively deficient in viral glycoprotein G and membrane protein. These results certainly would account for the reduced infectivity. Jay et al. (1983) have reported that VSV- G is produced, and is present in the same proportion to other virus proteins in the cytoplasm of both IFN treated and control cells;however, only a very small percentage of the G protein is incorporated into the released virus .

We have investigated the mechanism(s) of IFN- induced inhibition of assembly steps of HSV-1 in mouse cells and HIV-Ada strain in human monocytes. We have also studied the effect of IFN in mouse LMtk$^-$ cells transfected with HSV-1 gD cDNA and L_B cells transfected with VSV-G cDNA. These cells constitutively express the HSV-1gD and VSV -G protein . IFN (10 -100 I.U./ ml) significantly inhibited the infectivity (5-100 fold) of HSV-1; however, no significant difference was observed in the expression of HSV-1 polypeptide in IFN treated and untreated cells. Electron microscopy (EM) studies demonstrated a typical assembly of nucleocapsid of HSV-1 inside the nucleus of IFN treated L_B cells and very few mature virus particles were seen in the cytoplasm and were not apparently able to bud out from the plasma membrane. Immunofluorescence studies showed that most of the HSV-1 gD glycoprotein accumulated intracellularly in IFN-treated cells compared to untreated cells, where most of the gD was localized on the plasma membrane. Double immunofluorescence studies using wheat germ agglutinin (WGA) and subcellular fractionation using Nycodenz gradient showed that IFN inhibited the

transport of HSV-1 gD to the plasma membrane, instead most of the gD accumulated in the trans-Golgi network (TGN). IFN also blocked the transport of HSV-gD and VSV-G in LMtk$^-$ and L_B cells transfected with gD cDNA and G cDNA respectively, in the TGN, as compared to control.

IFN treatment of HIV-Ada infected monocytes showed that the infectivity of virus as measured by reverse transcriptase (RT) activity is inhibited 5-50 fold; however, p24, the major viral protein is inhibited only 2- 4 fold. Immunofluorescence studies showed that gp120 is accumulated intracellularly in IFN -α_{2a} treated HIV infected monocytes. Scanning EM shows fewer HIV-Ada particles at the surface in IFN α_{2a} treated monocytes as compared to the control where numerous virus particles were seen budding out from the plasma membrane of monocytes.

The intracellular pH of the monocytes and L_B cells was measured by laser spectroscopy using SNARF-1, a sensitive probe for quantitative change in the pHi. The intracellular pH was also measured using DAMP, a fluorescent probe, which is taken up by the acidic organelle of the cells. The inhibition in the DAMP distribution in the cytoplasm of the cell demonstrated that IFN raises the intracellular pH(pHi). Immunogold localization of DAMP in L_B demonstrated that IFN caused the alkalinization of TGN. These results suggest that IFN-induced increase in pHi could be responsible for the accumulation of HSV-1 gD, HIV gp120 and VSV-G in the TGN thereby inhibiting the virus replication at the assembly or release of HSV -1 and HIV or production of low infectivity VSV particles deficient in G-protein, which may help in the establishment of latency. This IFN induced increase in the pH of the TGN

may also explain the mechanism (s) of the inhibitory effect of IFN reported on the terminal steps of the replication of MLVs, MMTVs, PIV-3 and vaccinia virus .

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CERTIFICATE

This is to certify that the thesis entitled " **Molecular mechanism of defective transport of viral glycoprotein in Interferon treated cells**" and submitted by Anoop Kumar Singh , ID No . 90PHXF028 for the award of Ph. D. Degree of the Institute , embodies original work done by him under my supervision .

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ACKNOWLEDGMENTS

I am extremely grateful to Dr. Radha K. Maheshwari, my advisor, for his invaluable guidance during the course of this work. I express my heartfelt thanks to him for his constant encouragement, extreme patience, immaculate suggestions and helpful discussions throughout this dissertation. I am thankful to my co-advisor Dr. Gurmel S. Sidhu, who guided me with enthusiasm in every phase of my work. His assistance in Virology, Cell Biology and Microscopy is cherished. I am also very thankful to Dr. Robert M. Friedman, Chairman, Dept. of Pathology, USUHS, Bethesda for his stimulating questions and rational advise on Immunofluorescence microscopy. I would like to thank Dr. S.Venkateswaran, Director, BITS, Pilani, Prof. I.J. Nagrath, Dr. B.M. Mithal, Dr. L. K. Maheshwari, Prof. V.P. Rao and Dr. J.L. Arora for permitting me to work at USUHS, Bethesda, MD for my Ph.D. dissertation. Special thanks are to Drs. John Hay, Chairman, Department of Microbiology, State University of New York, Buffalo and Paul Kinchington, University of Pittsburgh, for providing monoclonal and polyclonal antibody against gD and information over the course of my research.

I am also grateful to Dr. Madhusudana Nambiar, Dept of Microbiology, USUHS for his assistance with the experimentation on electron microscopy. I am grateful to my colleague Ms. Deepa Thaloor for her help and assistance in many experiments. Every one in Dr. Maheshwari's laboratory has helped in someway, notably, Dr. Helen Coon, Dr. Kan Liu and Mr. Jerold W. Sklarsh. I am especially grateful to Dr. P. Prasad, whose help with word processing and computer graphics greatly facilitated my writing and editing. Special thanks to

my friends Sivakumar, Sunil Mehta and Karthik, V. for their help in some way or other during the course of this study.

Without the kindness and support of my family, this research could not have been completed . My parents, uncle and aunt have inspired me on many occasions and have bolstered me with their strength and encouragement . I am also grateful to my brothers Ashok , Ajay and sister in law Sharda for their moral support during the course of my work.

I sincerely thank my wife Sudha for her encouragement and patience.

TABLE OF CONTENTS

INTRODUCTION

Interferon : An Overview

Interferons (IFNs) are inducible proteins or glycoproteins which are known for their antiviral, anti-proliferative, antineoplastic and immunoregulatory properties (Stein et al., 1994; Kubes et al., 1994 and Repetto et al., 1994). There are four different types of IFNs which are grouped in two types, I and II. The type I IFNs include: α (leukocytes α), β (fibroblast), and ω (trophoblast, TP, α_{11}) IFNs and are induced by viral infection. The type II, IFN γ (immune) is induced by mitogenic or antigenic stimulation of T-lymphocytes and natural killer (NK) cells. The human IFN -α family is composed of at least 18 genes. There are six IFN-ω genes and a single IFN -β gene. The genes for three types of IFNs are mapped on the short arm of chromosome 9 and lack introns, whereas IFN -γ is encoded in a single copy gene with three introns located on chromosome 12. The study of the molecular mechanism(s) of IFN induced inhibition of viruses has undergone a dramatic expansion over the last decade. IFNs induce the synthesis of a number of proteins both in vitro and in vivo. The IFN induced 2',5'-A synthetase, 65- 68 KDa protein kinase (PKR), Mx protein and Major histocompatibility antigen (MHC) have been extensively studied and their role has been shown in the inhibition of some virus infections (Sen and Ransohoff, 1993).

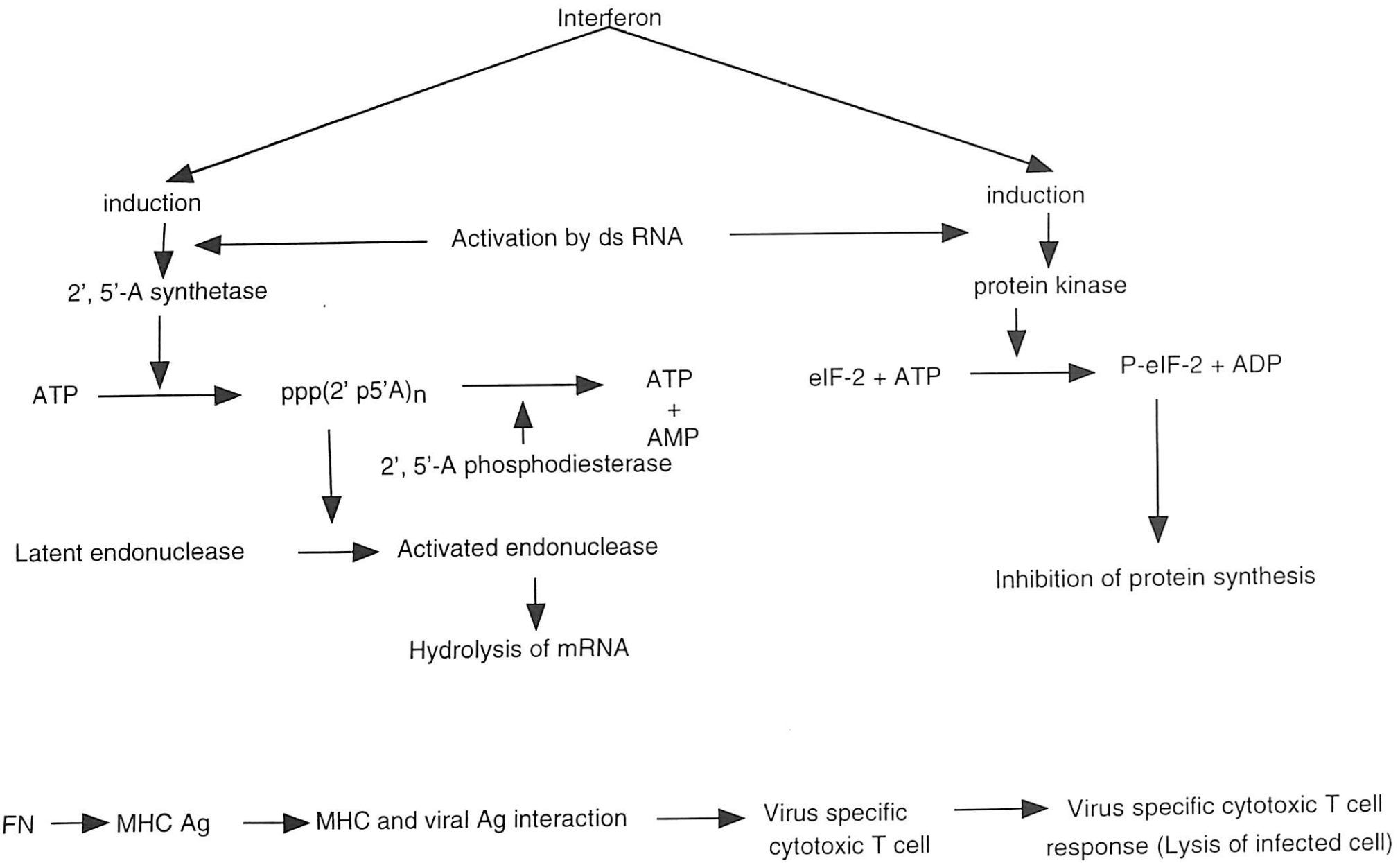
The 2',5'-A synthetase is activated by double stranded RNA (ds RNA) and catalyses the synthesis from ATP of an oligomer of adenylate, linked 2' to 5'. The

trimer and tetramer form in turn activate an endogenous endonuclease (L or F) which cleaves messenger and ribosomal RNA's. 2', 5'-A phosphodiesterase then hydrolyses the 2',5'-A oligomer which reduces the endonuclease activation (Fig 1). The synthesis of PKR is also activated by ds RNA (Fig 1) which gets phosphorylated in presence of ATP and catalyses the phosphorylation of eukaryotic initiation factor-2α (eIF2 α) and consequently blocks further initiation of translation thus inhibiting virus replication (Lee et al., 1993, Lee and Estaban, 1993 and Samuel, 1991).

The IFN induced mouse Mx gene (Pavlovic et al.,1993) encodes a 72-KDa nuclear protein which inhibits the multiplication of influenza virus (Thomas et al., 1992, Garber et al.,1991, Pavlovic et al.,1990 and Staehli et al.,1986) by interfering with the mRNA synthesis of influenza virus (Pavlovic et al.,1992) whereas human Mx A protein is a cytoplasmic 76-KDa protein which inhibits the multiplication of influenza virus. All Mx like proteins from mouse, human, rat and yeast possess the three consensus GTP-binding motifs, indicating that binding to GTP may be an important biochemical activity associated with their biological functions (Melen et al.,1994; Pavlovic et al.,1993; Meier et al.,1990; and Rothman et al.,1990), the role of which remains to be established. Recently, it has been shown that Mx protein could exert antiviral function by diverting virus particles into an intracellular compartment such as lysosome (Sen & Ransohoff, 1993).

Fig. 1: IFN-induced 2'5'-A synthetase mRNA and protein kinase pathway.

A schematic representation of the two antiviral pathways which are activated in IFN treated cells, each leading to the inhibition of translation. The induced enzyme, 2',5'-A synthetase, synthesizes oligomers of adenylate which then activate a nuclease which cleaves mRNA. Whereas, IFN-induced protein kinase phosphorylates eukaryotic initiation factor-2 thereby blocks the initiation of translation and causes subsequent inhibition of protein synthesis. The role of MHC antigen (Ag) in the antiviral activity of IFN has also been shown.


Interferon
induction
Activation by ds RNA
induction
2', 5'-A synthetase
protein kinase
ATP
ppp(2' p5'A)n
ATP
+
AMP
2', 5'-A phosphodiesterase
eIF-2 + ATP
P-eIF-2 + ADP
Latent endonuclease
Activated endonuclease
Inhibition of protein synthesis
Hydrolysis of mRNA
IFN
MHC Ag
MHC and viral Ag interaction
Virus specific
cytotoxic T cell
Virus specific cytotoxic T cell
response (Lysis of infected cell)

IFN-α, -β and -γ are known to induce the expression of class I (HLA-A, B and C) and class II (HLA -D) MHC antigens on different cell types (Samuel,1991). The IFN-induced expression of MHC antigen leads to the antiviral and anti-proliferative action at the cell-cell level, probably by enhancing the antigen specific lytic effect of cytotoxic T- lymphocytes. If the basal level of MHC antigen expression is below the threshold concentration normally required for efficient T-cell response, the IFN induced increase in level of class I and class II MHC antigens increases the efficiency of antigen presentation and therefore leads to a more efficient cellular immune response to viral infection (Fig 1).

Effect of IFN on membrane Viruses

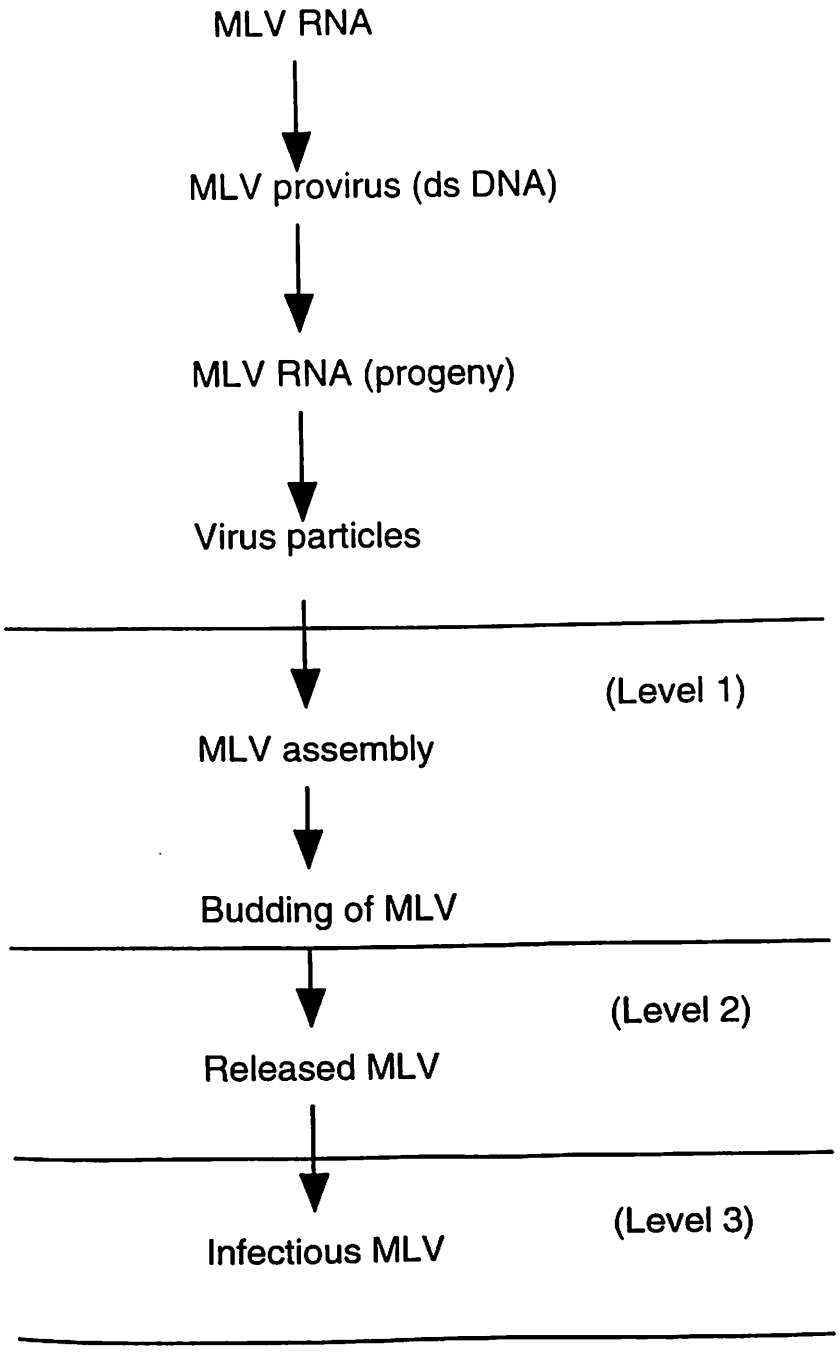
A number of antiviral mechanisms of IFN, such as inhibition of virus directed transcription, methylation of viral RNA, cleavage of CCA terminus of some forms of t-RNA and inhibition of virus directed translation have been extensively studied. RNA tumor virus was the first reported system, where IFN inhibited the virus at the final assembly steps, including inhibition of virus budding and release of non-infectious virus particles deficient in glycoprotein (Fig 2), however, IFN induced inhibition at the terminal step was not limited to only RNA tumor virus, a number of membrane viruses have been shown to be inhibited at terminal steps. A 200-fold reduction in the titer of infectious VSV released from mouse L_B cells treated with IFN (30 I.U. / ml) has been earlier reported from our laboratory; however, virus particle-associated RNA, nucleocapsid protein and viral transcriptase were inhibited less than 10 -fold (Maheshwari and Friedman, 1979). These results suggested that IFN

treatment induces the production of VSV particles of low infectivity. The reduced infectivity of VSV particles released from IFN -treated cells was not due to defective interfering particle production, because only 42S viral RNA was virion associated; in addition, virions produced by IFN treated cells did not interfere with the growth of wild type VSV in BHK or L_B cells. However, VSV released from IFN treated cells were selectively deficient in viral glycoprotein G and membrane protein (Maheshwari & Friedman, 1980; Drebot et al., 1984). These results certainly would account for the reduced infectivity. Jay et al.,(1983) have reported that VSV -G is produced, and is present in the same proportion to other virus proteins, in the cytoplasm of both IFN treated and control cells; however only a very small percentage of the G protein is incorporated into the released virus in the IFN treated cells.

IFN treatment inhibited the release of infectious HSV-1 particles. IFN α and IFN β did not inhibit the synthesis of major nucleocapsid protein but they blocked the release of total extracellular virus particles from IFN- treated cells. The synthesis of glycoproteins gB and gD was drastically reduced/delayed. These results suggest that IFN acts in HSV-1 infected cells by blocking the replication at a late stage in viral morphogenesis and may inhibit the budding process at the nuclear membrane (Chatterjee et al., 1985). Non infective HSV- 1 particles have also been reported in IFN α treated Hela cells without any significant inhibition of HSV protein synthesis (Munoz and Carrasco, 1984). IFN treatment significantly inhibited the release of infectious HSV-1 particles.

Fig. 2: Interferon action on Murine leukemia virus.

The MLV reverse transcriptase forms a proviral ds DNA which acts as a template for progeny virus RNA. The mRNA gets translated to form MLV structural and functional proteins. The MLV RNA and proteins are assembled at the plasma membrane and the new virus buds out from the cell surface. Shown is a representation of IFN induced inhibition of MLV at three different stages. (i) Assembly of virus protein and RNA into particles is inhibited (ii) Budding of the virus particle from the cell surface is inhibited by IFN (iii) In some studies it has been shown that even MLV morphogenesis and release are normal in IFN treated cells but the released viral particles had lower infectivity probably due to alteration in the viral glycoprotein (Friedman, 1981).


MLV RNA
MLV provirus (ds DNA)
MLV RNA (progeny)
Virus particles
(Level 1)
MLV assembly
Budding of MLV
(Level 2)
Released MLV
(Level 3)
Infectious MLV

The main site of inhibition of mouse mammary tumor virus (MMTV) by IFN also seems to be at the final assembly and budding step of virus (Sen and Sarkar, 1980; Yagi et al., 1980) replication cycle. Human IFN-α_{2a} has also been shown to reduce the infectious PIV -3 and inhibit the release of mature virions (Panigrahi et al., 1988). This was later implicated to defective transport and accumulation of HN glycoprotein in the cytoplasm of IFN treated cells (Panigrahi and Mohanty., 1989).

Esteban et al., (1984) have shown that IFN treatment induces the production of defective vaccinia virus particles with decreased phosphorylation of core polypeptide and glycoprotein content. Their adsorption, penetration and uncoating was shown to be of lesser extent as compared to vaccinia virus in untreated cells. Ho et al., (1985) have observed that IFN-α inhibits the HIV-infection of peripheral blood lymphocytes. Immunofluorescence data revealed that IFN-α treatment reduced the percentage of cells infected with HIV. In an another study, IFN treatment after PMA stimulation of infected U1 and ACH-2 cells did not reduce the percentage of cells expressing HIV specific protein as seen by fluorescence microscopy. IFN treatment did not suppress the profile of expressed HIV proteins, however, the production or release of the virion was inhibited (Poli et al., 1989). Recently, Hansen et al., (1992) have shown that IFN has minimal effect on the synthesis of HIV DNA, RNA and protein. The virions released from IFN treated cells were 100 -1000 fold less infectious than an equal number of virions from control cells, suggesting that IFN induces defect in the assembly of gp 120 onto mature viral particles that accounts for significant loss of virion infectivity.

These studies clearly suggest that IFN inhibits the replication of many membrane viruses at a late stage in the replication cycle. Although the antiviral activity of IFN can be expressed in several ways, out of which two appears to be more significant. These are, inhibition of virus assembly, release and budding or a small decrease in the virus particle production together with a marked reduction in the infectivity of the released virions (Table 1).

Inhibitory Effect	Viruses
Inhibition of virus assembly, release and budding	Murine leukemia virus Mouse mammary tumor virus Herpes simplex virus -1 Bovine parainfluenza virus Human immunodeficiency virus-1
Combination of a small reduction in the production of virus particles, and a large reduction in the infectivity of progeny virus.	Murine leukemia virus Vesicular stomatitis virus Herpes simplex virus-1 Human immunodeficiency virus-1 Vaccinia virus

Table 1: IFN induced inhibition of enveloped viruses

Structure and replication of viruses

Viruses have been classified based on their unique structure, size, genome and presence or absence of envelope. The replication cycle of DNA and RNA viruses have several events in common. They attach to a specific receptor on the plasma membrane, penetrate into cells, uncoat themselves (enveloped viruses) and then release their nucleic acid to a specific intracellular location, during which a complex series of biosynthetic events occur. In order to study the antiviral activity of IFN against various viruses reported here, it is important to review various steps of replication cycle of HSV-1, VSV and HIV-1.

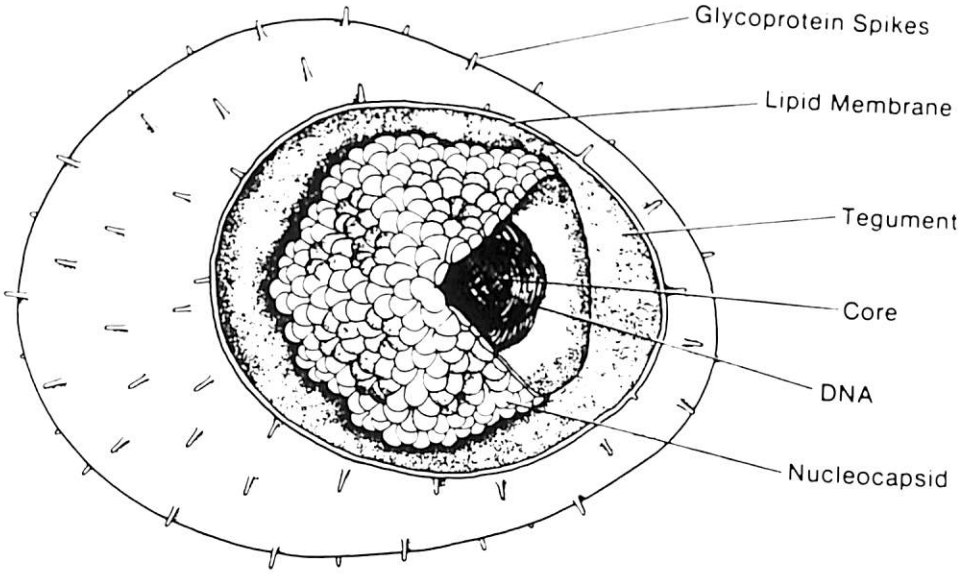
Herpes simplex virus-1

HSV-1 is a member of the family Herpetovirida, genus herpesvirus (Lycke et al., 1989) and is approximately 180- 200 nm in diameter. The herpes virion consists of four structural elements. (a) An electron opaque core. (b) An icosadelta hedral capsid that encloses the core. (c) An electron dense asymmetrically distributed material abutting the capsid and designated as tegument. (d) An outer membrane or envelope which surrounds the capsid and tegument (Fig. 3).

HSV-1 has a linear ds DNA genome with a molecular weight of 10^8 and contains approximately 70 open reading frames (MeGeoch et al., 1985). HSV DNA consists of two covalently linked components designated as L (long) and S (short) comprising approximately 82 % and 18 % of genome respectively.

Fig. 3: Structural model of the herpes virus.

A cross sectional diagram of herpes virus showing the individual components. (Adapted from Nermut and Steven, 1987).


Glycoprotein Spikes
Lipid Membrane
Tegument
Core
DNA
Nucleocapsid

Virus infection begins with the attachment of the virus to a cellular receptor and then it penetrates the cell by fusion of the viral envelope with the membrane (Spear et al 1985., Campadelli et al., 1988; Deluca et al., 1982; Fuller et al., 1987 and Para et al., 1980). Transcription of HSV is mediated by the host protein either with the help of virion protein or alone, thereafter it is mediated by protein which is made after infection but before the initiation of viral DNA synthesis and lastly transcription is coupled to the initiation of DNA synthesis. These three steps of transcription which result in the production of mRNA leads to the synthesis of three different polypeptides alpha, beta and gamma (Roziman et al., 1974). Mature HSV-1 contains a phosphoprotein called VP16 which activates the expression of the alpha gene (Post et al., 1981; Campbell et al., 1985).

Five alpha proteins which have been identified namely ICP-0,ICP-4, ICP-22, ICP-27 and ICP-47. ICP-0, ICP-4 and ICP-22 are known to activate HSV-1 beta and gamma gene expression (Mavromara et al., 1986; Gelman and Silverstein, 1985; Godowski et al., 1986 and Deluca et al., 1985). Beta proteins are mostly non structural proteins and are essentially involved in DNA replication (Wu et al, 1988). The gamma protein includes mostly viral structural polypeptide. These structural and non structural proteins are transported to the nucleus where the nucleocapsids are assembled. Nucleocapsids associate with region of the inner nuclear lamella which is modified by viral glycoproteins, and bud into perinuclear space (Nii et al., 1988). Virus particle buds from the nuclear membrane and is then transported to the Golgi complex before exocytosis. The complete cycle of viral replication occurs within 15 hr following infection. The genome of HSV -1 encodes information for at

least nine antigenically distinct glycoproteins designated as gB, gC, gD, gE, gG, gH, gI, gK and gL (Bauck et al.,1979; Eberle et al.,1980; Gompels et al.,1986; Hutchinson et al 1991; Longnecker et al.,1987; Marsden et al., 1984 and Roziman et al., 1984). The entry of HSV takes place after a cascade of events that occur at the cell surface (McClain and Fuller, 1994; Fuller and Lee, 1992). The glycoproteins gB, gD and gH have been shown to be essential for viral infectivity (Cai et al.,1987, 1988; Campadelli et al., 1988; Desai et al., 1988; Fuller et al.,1987, 1989; and Little et al.,1981; Ligas et al., 1988).

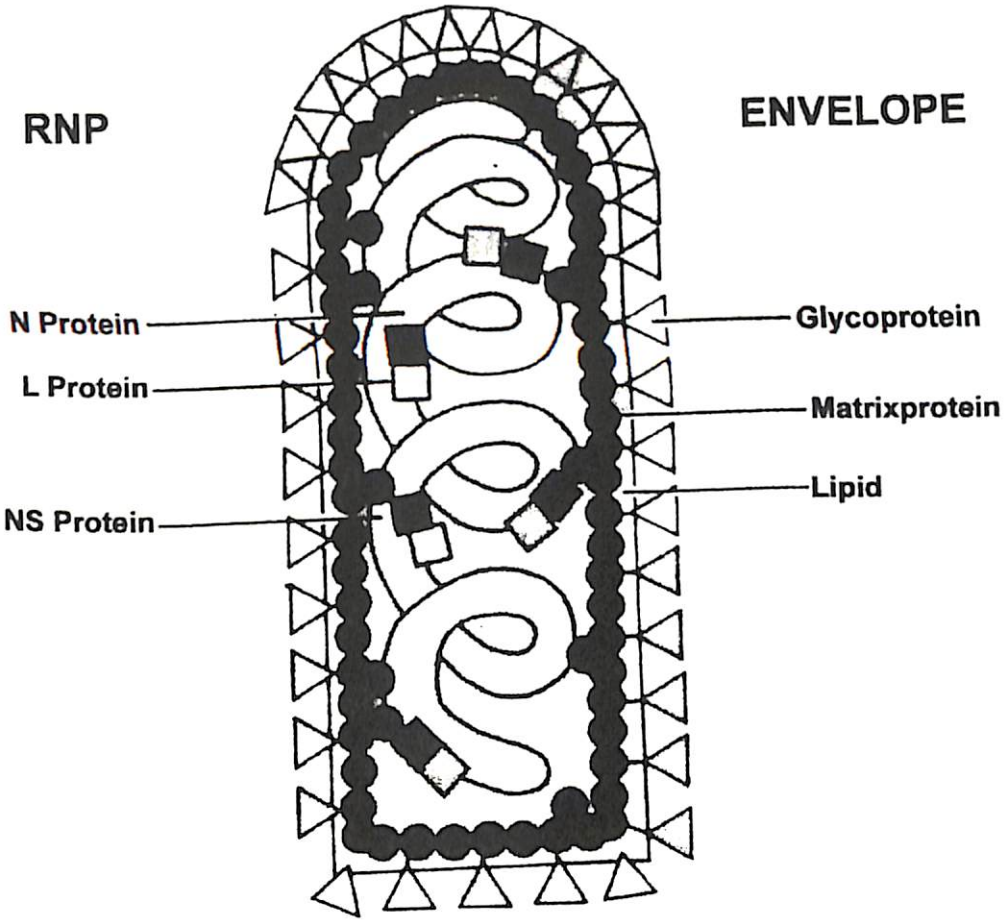
Vesicular stomatitis Virus :

VSV is about 180 nm in length and 65-70 nm in width, bullet shaped RNA virus (Fig.4). There are three viral proteins associated with RNA to form the nucleocapsid: large nucleocapsid (L), nucleocapsid (N) and smaller nucleocapsid (NS), whereas, lipid envelope consist of two viral proteins: the matrix (M) and glycoprotein (G) (Wagner et al., 1975). The M protein is found along the internal surface of the envelope while G protein forms spikes extending outward from the surface of the virus particle (Rose et al., 1980; Zakowski et al., 1980). The infection begins with the binding of G protein to glycolipid and phospholipid receptors on the cell surface. Penetration into the cell requires a low surrounding pH. (Maltin et al., 1982). The virus enters into the cell through coated pits and moves to coated vesicles and then to large collecting vacuoles and finally to secondary lysosomes. This fusion of VSV virion membrane with the vacuolar membrane requires low pH which is provided by a proton pump mechanism. The enzymology of the VSV transcriptase reaction is dependent on the three structural proteins, the N protein

which complexes with the RNA genome to form a template, as well as L and NS proteins which collectively comprise the polymerase (Emerson et al., 1987). This N-protein-RNA (RNP) core gives rise to a leader RNA and five messenger RNAs, all of which are capped and polyadenylated either by one or two possible mechanisms. A single positive strand RNA with a length equivalent to template RNA is endonucleolytically cleaved at specific sites to produce individual RNAs. Alternatively, transcription terminates at the 3' end of leader RNA and re-initiates to synthesize mRNA. These successive termination and reinitiation events at specific sites lead to the formation of mRNA for N, NS, M, G and L proteins. Once transcribed, capped and polyadenylated, viral mRNAs are translated into five structural proteins N, NS, M, G and L. The viral protein synthesis is required for the replication of genomic RNAs (Davis et al., 1982). N protein is necessary for initiating the process of RNA replication. The L-NS complex acts as RNA polymerase for transcription. Nucleocapsid possessing positive stranded RNA then acts as a template for L- NS mediated synthesis of progeny genomic RNA which is again encapsidated with NS, N, L as they are synthesized. The latter nucleocapsid serves as template for further secondary transcription to yield additional mRNA. The progeny RNAs and NS, L and N proteins form nucleocapsids which bud through the cell membrane containing G and M proteins to form bullet shaped virus particles.

Fig. 4: Structure of Vesicular stomatitis virus

Schematic diagram of VSV showing Large nucleocapsid (L) and Nucleocapsid (N) whereas lipid envelope consists of matrix protein M and glycoprotein G.

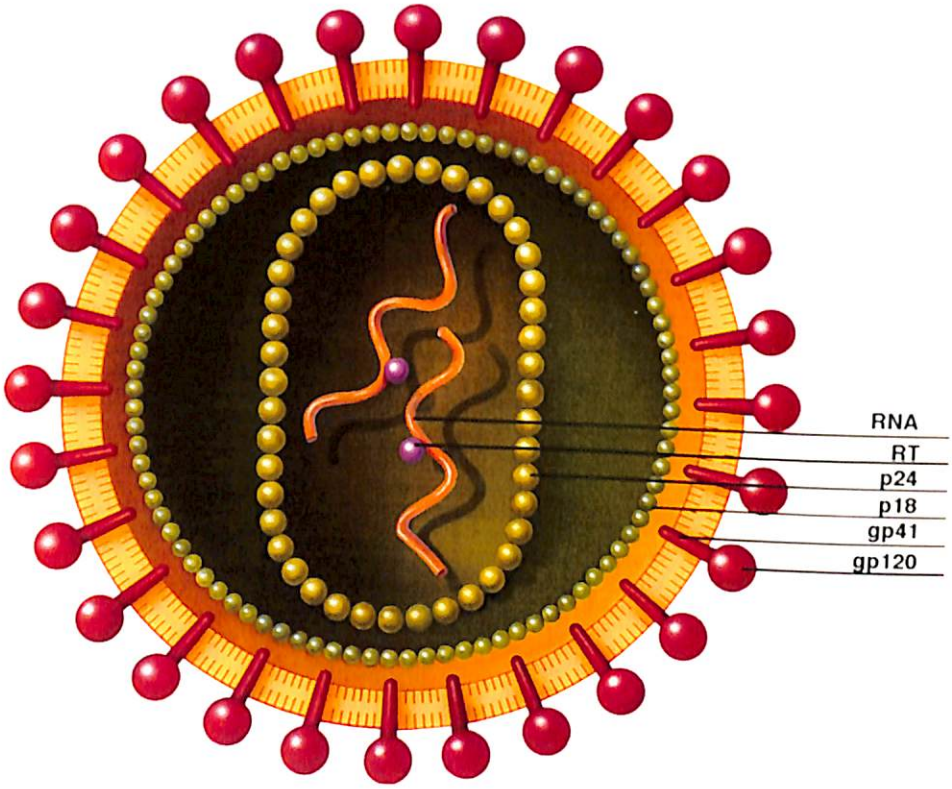

RNP
ENVELOPE
N Protein
L Protein
NS Protein
Glycoprotein
Matrixprotein
Lipid

Human immunodeficiency virus -1 (HIV-1)

The HIV is the causative agent of AIDS, and belongs to a unique family, Retroviridae, a group of small enveloped, positive strand RNA viruses (Fig.5). Its replication is initiated by binding of virus to the surface of CD_4^+ T cells or monocytes, or cells expressing CD_4+ molecules via viral surface protein gp 120 with the CD_4^+ molecule. Once virus enters into the target cell, a ds DNA copy of the HIV RNA genome is synthesized in the presence of reverse transcriptase (RT). The viral DNA is transported to the nucleus along with the viral gag protein and viral integrase. Integrase catalyzes a cleavage and ligation reaction in which the viral DNA genome gets integrated into the host DNA. Integrated viral DNA acts as a template for transcription of viral RNA, where it is regulated by viral as well as cellular factors (Cullen, 1991; Gaynor, 1992 ;Steffy and Wong-staal 1991; Subbramanian and Cohen, 1994). The early gene expression produces small multiple spliced RNAs that encode the various viral regulatory molecules. Late gene expression involves the production of the larger RNAs that encode viral structural proteins, enzymatic activities and synthesis of new viral genomic RNA. Translation of HIV mRNA results in synthesis of HIV regulatory molecule, the structural proteins and enzymes. The HIV envelope protein gets inserted into the host cell membrane whereas viral capsid proteins and enzymes undergo proteolytic processing by the viral protease. New viral particles are assembled and packaged at the adjacent cell membrane. These particles bud out from the cell and acquire their lipid envelope.

Fig. 5 : Structure of Human immunodeficiency virus -1

Schematic cross-section of virus particle, revealing its different components. gp 120 is the major extracellular envelope glycoprotein. gp41 is the transmembrane envelope glycoprotein, p24 is the major core antigen, p18 is the myrisitilated gag protein.



Transport and Processing of viral glycoprotein

The intracellular transport, location and functional significance of various steps in the synthesis and processing of membrane bound and secreted glycoproteins is an important aspect in the assembly of the various subcellular membranes and the organelles that these membrane define. Viral glycoproteins are believed to be synthesized and found at an earlier stage of maturation in the rough ER and outer nuclear membranes. HSV glycoproteins are transported into the inner nuclear membrane via the pore complexes which join the inner and outer nuclear membrane (Harris et al., 1978). HSV-1 glycoproteins are transported from perinuclear space to Golgi where asparagine linked (N -linked) oligosaccharides are changed from high mannose form (immature form) to the complex form (mature form) (Johnson et al., 1983; Serafini et al., 1981 and Wenske et al., 1982) and serine linked (O -linked) oligosaccharides are incorporated to the glycoprotein (Oloffson et al.,1981 and Johnson et al., 1982). It has been shown that processing of the HSV glycoproteins from immature to mature form in the Golgi complex is associated with a discrete shift in the mobility of the polypeptide on SDS-PAGE (Cohen et al., 1980; Eberle et al., 1980 and Johnson et al., 1983). The virions moves from the Golgi apparatus to the cell surface. Johnson et al (1983) have shown that when cells are treated with ionophore monensin, virions containing partially processed glycoprotein accumulate in distended cytoplasmic vacuoles. Other glycoproteins which are not associated with the surface of the virion also come up to the cell surface and are probably transported in the same way. The intracellular transport and processing of HSV -1 glycoproteins differs in various ways

as compared to VSV-G.

G-protein is synthesized on the membrane bound ribosomes of rough endoplasmic reticulum (Grubman et al.,1975 and Morrison et al.,1975), where two N -linked oligosaccarides are added (Etchison et al., 1974 and Li et al., 1978). It has been reported that G is transported to the cell surface through clathrin coated vesicles (Rothman et al., 1980), where N-linked oligosaccharide is processed (Bretz et al., 1980; Roth et al., 1982; Tabas et al., 1979 and Tabas et al., 1978). The protein then moves from Golgi to plasma membrane where it is used in the envelopment of viral nucleocapsid which is assembled in the cytoplasm (Knipe et al.,1977, 1977). Previously it has been shown that HSV-1 glycoproteins are transported to the Golgi apparatus and are processed much slowly (Cohen et al., 1980, Johnson et al., 1983 and Zezulak et al 1983) as compared to VSV -G which can be detected in Golgi within 5-10 min and reach to the cell surface in 20 min (Bergman et al., 1983 and Knipe et al., 1977). The slow intracellular transport of HSV glycoproteins could be due to accumulation of these viral glycoproteins in the nuclear membrane. It is also possible that they are transported specifically to the nuclear membrane, where they participate in the envelopment of viral glycoprotein to the Golgi complex and cell surface.

The HIV genome encodes envelope glycoproteins which project from the membrane surface of mature particles. The envelope protein of HIV is translated as a 88 KDa precursor protein (Allan et al., 1985). This protein moves from ER to Golgi apparatus and gets glycosylated to form the precursor gp160. This precursor gp160 is cleaved in the Golgi network to gp 120 and gp 41(Willey et al., 1988 a) in

the presence of a furin like enzyme (Morikawa et al., 1993). Most of the uncleaved gp160 moves to lysosome, where it is degraded, while gp 120 is transported to the cell surface. It has been shown that cleavage of gp 160 is necessary for the production of infectious virus particles (Dash et al., 1994).

The molecular mechanism of IFN-induced inhibition at the terminal steps of enveloped virus replication is not yet completely elucidated. Earlier studies from our laboratory (Singh et al.,1988) have shown that IFN treatment inhibited the transport of VSV- G from Golgi complex to the plasma membrane. The failure of G to be transported to plasma membrane in the IFN treated cell probably leads to the formation of G-deficient virus particles and therefore, less infectious virus particles, which is consistent with the known morphogenesis of VSV and the role of G- protein in the process. Subcellular fractionation studies using sucrose and Nycodenz gradient have shown that most of the VSV-G from the IFN treated cells accumulates in the TGN (Maheshwari et al., 1991).

The data reviewed here focuses on the IFN induced inhibition of HSV-1 in mouse L_B cells and HIV -Ada in human monocytes at the assembly step. The results suggest that physiological doses of IFN–β and IFN -α_{2a} inhibited the infectivity of HSV -1 (5-100 fold) and HIV -RT activity (5- 50 fold) without significant inhibition in the synthesis of viral protein. IFN caused an accumulation of HSV-1 gD in mouse L_B cells and gp 120 in human monocytes intracellularly. We have also demonstrated that IFN blocked the transport of HSV -1 gD in LMtk$^-$ cells transfected with gD cDNA and VSV-G in L_B cells transfected with G cDNA. Double

immunofluorescence studies demonstrated the gD was co-localized with the WGA indicating that IFN blocked the transport of gD within TGN. The subcellular fractionation studies using Nycodenz gradient have shown that most of the gD from IFN treated cells accumulates in the TGN.

Several studies have suggested the role of intracellular pH in membrane trafficking in mammalian cells (Laurie and Robins, 1991). IFN s have been shown to induce cytoplasmic alkalinization as demonstrated by SNARF-1, a sensitive pH (pHi) indicator. IFN inhibited the transport of HSV-1 gD, HIV-1 gp 120 and VSV-G protein and the alkalinization of the cytosol was associated with TGN, indicating that pH change may be responsible for the block in the transport.

MATERIALS AND METHODS

Cell lines, interferon and virus

A subclone 11, derived from the mouse L_B cell line (originally obtained from D.C. Burke, University of Warwick, Coventry, U.K.) by a single cell cloning method (Singh et al., 1988) was maintained in Eagle's MEM (EMEM) supplemented with 10% fetal bovine serum. Mouse LMtk$^-$ cells (Originally obtained from Dr. P.G.Spear, North Western University, Chicago, IL) containing gD cDNA under control of the human metallothionein promoter II expresses gD constitutively and were maintained in Dulbeco's MEM (DMEM) supplemented with 10% fetal bovine serum. Recombinant murine IFN-β and human IFN α_{2a} were obtained from Torray Industries, Japan and F-Hoffmann La-Roche, Nutely, NJ, USA respectively.

The HSV-1(F) (obtained from J. Hay, SUNY, Buffalo, NY) was plaque purified and passaged at multiplicities of 0.01 in Vero cells. The HSV-1 stock was assayed by determining its cytopathic effect (CPE) in Vero cells; the titer was 2x 10^9 $TCID_{50}$/ cell.

Reagents

Gold conjugated goat anti-mouse IgG was obtained from Cappel; 3-(2,4-dinitroanilino-3'-amino-N-methyldipropylamine (DAMP) and monoclonal mouse anti-dinitrophenyl (DNP) IgG were kindly provided by Dr. R.G.W. Anderson, University of Texas, Dallas, TX, USA; (5-N-2,3-dihydroxypropyl acetamide)-2,4,6-

triiodo-N, N'-bis (2,3-dihydroxypropyl) isophthalamide (Nycodenz) was procured from Nycomed Pharma, Oslo, Norway. Endo-β acetyl glucosaminidase H (Endo H) and neuraminidase were obtained from Sigma Chemical Co., St. Louis, MO, USA. The plasmids pSVGL and pSV_2-neo were a generous gift from Dr. John. K Rose, The Salk Institute, San Diego, CA, USA. Fluorescein isothiocynate (FITC) - conjugated goat antimouse IgG and Rhodamine-conjugated goat antimouse IgG were obtained from Cappel, West Chester, PA, USA. ^{35}S-labelled methionine was obtained from ICN Biomedical Inc. Irvine, CA, USA. ^{32}P labelled ATP and ^{35}S-Protein A were obtained from Amersham International, UK. The enhancer used for the protein gels was purchased from Dupont, Boston, MA, USA. Protein assay reagent kit and the protein standards were obtained from Pierce at Rockford, IL, USA. The methionine free media, Eagle's Minimum Essential Media (EMEM), Dulbeco's Minimum Essential Media (DMEM) and RPMI-1640 were obtained from Flow laboratories, Irvine, Scotland, GIBCO and BRL, Gaithersburg, MD, USA respectively.

Assay for antiviral activity

The antiviral activity of IFN was assayed as described previously (Maheshwari et al., 1994). Briefly, L_B cells were grown to semiconfluency in EMEM with 10% FBS in 96 well plates and treated in triplicates with IFN (0-100 I.U./ml) for 16 hr; IFN was then removed and the cells were washed and infected with HSV-1 for 1 hr at a multiplicity of 0.1 or 0.5 $TCID_{50}$/ cell. Unadsorbed virus was removed by washing and the cells were replenished with fresh medium with or without

chloroquine (CHL10 μM). After 24 hr, plates were examined forCPE, sonicated, and assayed for infectivity. Supernatant from replicate wells were pooled and virus yield determined. Data were plotted as inhibition of virus yield (log_{10}).

Plaque assay

L_B cells were plated in a six well plate and treated with various doses of IFN for 16 hr. Cells were then washed and infected with the virus at 0.5 $TCID_{50}$ per cell. After 1 hr of virus adsorption, the unadsorbed virus was removed and cells were replenished with fresh media with or without CHL (10μM). Cells were removed after 24 hr post infection and samples were centrifuged for 10-15 min at 1500 rpm. The pellets were then resuspended in the supernatant and samples were sonicated. The plaque assay was performed in 6-well plate using 10 fold dilution. After about 48 hr, plates were checked for plaques. The number of plaques were counted and plates were then fixed with 10% buffered formalin and stained with 0.5 % crystal violet stain.

Virus Growth

L_B cells were grown on petri plates and treated with various concentration of IFN (0- 250 I.U./ml) for 16 hr. IFN was removed, cells were washed and infected with a multiplicity of 1 $TCID_{50}$/ cell of HSV-1 for 1 hr. Unadsorbed virus was then removed and fresh medium was added. Eight hr postinfection, the cells were lysed with RIPA buffer. Lysates were dotblotted onto a nitrocellulose hybond-ECL

(Enhanced chemiluminescence) membrane and dried. Non-specific binding was blocked using 5% dried milk and 0.1% Bovine Serum Albumin (BSA). ECL detection of viral proteins was then performed with rabbit anti HSV-1 polyclonal antibody as primary antibody using manufacturer's protocol (Amersham, International PLC, Buckinghamshire, England, U.K.). Briefly, the nitrocellulose blot was incubated in 5 % non fat dried milk in Phosphate buffered saline (PBS) with 0.15% Tween 20 to prevent non specific binding and were then reacted with primary antibody raised against HSV-1 in rabbit for 2 hr. Unbound antibodies were washed and the blot was incubated with horse radish peroxidase-conjugate (HRP, 1:800 dilution) for 1 hr. The bound antibodies were detected by reacting the blots with ECL detection reagent (supplied with the kit) for 1 min. The blot was exposed to Hybond -ECL film for 2 min and were processed in a Kodak processor.

Metabolic Labelling of L_B Cells infected with HSV-1

L_B cells were plated in 6-well plates and treated with IFN for 16 hr. Cells were then washed and infected with the virus at a $TCID_{50}$ of 0.5 per cell. After 1 hr of virus adsorption, the unadsorbed virus was removed and cells were replenished with fresh medium with or without CHL. Four hr post infection (p.i), cells were labelled with ^{35}S-methionine for 3 hr in methionine free media supplemented with 2% dialyzed serum. The cells were washed with PBS and lysed in 50mM Tris, 150mM NaCl, 0.5% SDS, 1%Nonidet P-40. Equal amount of radioactivity were loaded on

a 10% SDS -PAGE gel.The gel was treated with an enhancer (En^3 Hance; New England Nuclear, Boston, MA), dried and autoradiographed using a Kodak XLR film.

Transmission Electron microscopy

L_B cells were plated in a 6- well plate at a confluency of 60% and treated with or without IFN (100 I.U./ml) for 16 hr. Cells were then washed and infected with HSV-1 at a $TCID_{50}$ of 0.5 per cell.The plates were rocked gently for 1 hr and the unadsorbed virus was removed. Four hr post infection, the cells were fixed in 2% glutaraldehyde at room temperature for 1hr. There after, cells were washed extensively with Cacodylate buffer and post fixed for one and half hr in buffered 2% osmium tetroxide solution. Cells were then washed and dehydrated in a graded series of ethanol and embedded in Epon -plastic resin. Thin sections were cut perpendicular to the plane of the cell monolayer using an ultra microtome. The cut sections were stained with uranylacetate, and analyzed with a JEOL transmission electron microscope.

Localization of HSV-1 gD

L_B cells were grown on coverslips to 70% confluency and treated with IFN (0-100 I.U./ml) for 16 hr. Cells were then washed and infected with HSV-1 at a multiplicity of 0.5 $TCID_{50}$ / cell for 1 hr. Unadsorbed virus was removed, fresh medium added and the cells incubated for an additional 8 hr. For the visualization of the gD on the surface, the living cells were incubated with anti gD polyclonal

antibody at 4°C to prevent the endocytosis of the antibody. Cells were then washed and incubated with FITC- conjugated goat anti-rabbit IgG. For intracellular localization, cells were fixed in chilled acetone. The cells were then incubated with 50 μl of anti gD monoclonal antibody for 30 min. After washing extensively with 1X PBS containing 0.1% BSA, they were incubated with 50 μl of FITC-conjugated goat antimouse IgG for 30 min, washed and mounted on glass slides for viewing under a Zeiss fluorescence microscope.

Similarly LMtk$^-$ cells were treated with IFN for 12 hr and then cells were replenished with fresh media containing IFN and/or CHL (10, 100 μM) and were processed for indirect immunofluorescence in permeablized and unpermeablized cells as described above.

Co-localization of HSV-1 gD protein and Golgi-complex by double immunofluorescence

To determine whether gD remained associated with Golgi-complex, double immunofluorescence was used to co-localize the gD and WGA. WGA binds to the Golgi-complex inside the cell. LMtk$^-$ cells were grown on coverslips and treated with IFN (500 I.U./ml) for 16 hr. For co-localization of gD with WGA, the cells were first incubated with 0.5 mg/ml WGA for 60 min on ice to saturate surface WGA binding sites. After washing with PBS, the cells were fixed for 10 min in chilled acetone, permeabilized using 0.1% saponin, and were incubated with rhodamine-WGA for

30 min. The cells were then incubated with anti-gD monoclonal antibody and FITC-conjugated goat anti-mouse IgG.

Western Immuno blot analysis of gD protein in LMtk- cells

LMtk$^-$ cells were plated in six well plates and treated with or without IFN for 16 hr. Cells were then washed twice with PBS and were lysed in 50mM Tris, 150 mM NaCl, 0.5% SDS, 1% Nonidet P-40, leupeptin, aprotonin and Phenyl Methyl Sulfonyl Fluoride (PMSF1mg/ml). Cells were dissolved in SDS sample buffer and subjected to electrophoresis on a 10% SDS-Polyacrylamide gel and proteins were transferred to a nitrocellulose membrane by electroblotting. After protein transfer the nitrocellulose blots were soaked in blocking solution (5% non fat dried milk and 0.2 % BSA) to prevent non specific binding and then incubated with monoclonal antibody against gD protein for 16 hr at room temperature. After extensive washing with wash buffer. (Tris HCl 50mM, NaCl 150mM, EDTA 5mM, Tween 0.05%), bound antibody was detected by reacting the blots with ^{35}S-Protein A for 1 hr at 37° C followed by autoradiography.

Biochemical localization of HSV-1 gD in LMtk$^-$ cells

LMtk$^-$ cells were treated with or without IFN (100 I.U./ml) for 16 hr and were washed once with methionine free EMEM. Cells were then incubated for 1 hr in the methionine free EMEM and then labelled with ^{35}S-methionine (100μCi / ml). After a 45 min pulse, cells were washed with EMEM containing 10

fold excess unlabelled methionine and 20 μg/ml cycloheximide and then incubated in the same medium for another 4 hr. Cells were pelleted by centrifugation and then resuspended in buffer containing 8% sucrose, 0.05M EDTA and tricine. Cells suspension was passed through a 22 gauge needle 30 times and then centrifuged to pellet the nuclei. A 11.2 ml (10-30 % w/v) continuous gradient of Nycodenz in 17 ml tubes was prepared by diffusion method. The gradient was kept at 4°C for 16 hr. The post nuclear supernatant was centrifuged at 3000g for 10 min to get mitochondrial pellet.The post heavy mitochondrial supernatant was centrifuged at 20,000 g for 30 min to get light mitochondrial pellet. The supernatant was recentrifuged at 100,000g for 30 min. The light mitochondrial fraction was resuspended in 4 ml of 40% Nycodenz and was underlayered at each gradient and centrifuged at 54,000g for 1.5 hr and then 1.0 ml of fractions were collected with a needle. Each fractions were analyzed for β-N- acetyl glucosaminidase, galactosyl transferase and subjected to 10 % SDS-PAGE (Laemmli., 1970) for the localization of gD. The protein gels were fixed (1% acetic acid, 30% methanol) for 45 min and were treated with an enhancer (En^3Hance; New England Nuclear, Boston, MA), dried and autoradiographed using Kodak XLR films.

Protein estimation

Protein estimation was carried out using Pierce protein estimation kit as recommended by manufacturer. Briefly, 190 μl of 50 parts of reagent A and 1 part of reagent B was taken for each sample in a 96 -well plate. 10 μl of sample lysate

fraction was added and incubated at 37° C for 30 min. Different concentrations of albumin standard (1 mg/ml, 0.5 mg / ml, 0.25 mg /ml and 0.125 mg/ml) were also taken along with the test samples. The absorbance was measured at 540 nm against a blank. Total protein in each fraction was calculated and plotted against the fractions number.

Assay for UDP-galactose-glycoprotein-galactosyltransferase

An assay mixture containing sodium cacodylate (6.0 μM), Magnesium chloride (0.2μM), Manganese chloride (1.2μM), dithiothreitol (1.0 μM), UDP- P [1-^{3}H] galactose (0.1 μ mol), Feutin (0.5 mg treated with sulfuric acid), 5% Triton X100 and 20 μl of samples were taken in a centrifuge tube. 10 μl of UDP- D [1-^{3}H]-galactose (12.9 Ci/m mol, 0.05 mCi /0.5 ml) was taken and made to 100 μl for the direct use in a single assay mixture. The assay mixture was incubated for 30 min at 30°C and the reaction was terminated by placing the mixture in boiling water for 90 sec. Protein bound radioactivity was determined by TCA precipitation. Briefly, the samples were spotted on 0.45 mm pore size millipore filters and were then soaked in ice cold 10 % TCA for 45 min. After extensive washing with 5% TCA at room temperature, the filters were gently boiled for 15 min in 5% TCA. Filters were washed with 5% TCA at room temperature and then with 95% ethanol. The filters were air dried and were then dissolved in Ready Solv HP (Beckman, Fullerton, CA, USA), and radioactivity was counted in Scintillation counter LS 6000TA (Beckman, Fullerton, CA, USA).

Assay for N- acetyl glucosaminidase

To determine the activity of N-acetyl glucosaminidase, An assay mixture of 2 volumes of sodium citrate buffer containing 2 mg/ml albumin (pH -4.5), 1 volume of 7.5 mM N-acetyl -p-nitrophenyl glucosaminidase and 1volume of sample was taken in a tube. Assay mixture was incubated for 60 min at 37^0 C and then the color was developed with K_2CO_3. The absorbance was read at 420 nm against the blank and compared with the p-nitrophenol standard curve.

Intracellular distribution of DAMP in LMtk$^-$ cells

LMtk$^-$cells were grown on coverslips until 50% confluent and treated with IFN (100 I.U./ ml). DAMP was localized as described previously (Anderson and Pathak, 1985). Briefly, cells were incubated with DAMP (30 µM) for 30 min at 37°C and then fixed for 15 min at room temperature in 3% (w/v) paraformaldehyde. Cells were permeabilized with Triton X-100, treated with 50 µl monoclonal mouse anti-DNP IgG(50µg/ml) and incubated for 60 min. After washing extensively, they were incubated with 50 µl FITC-conjugated rabbit anti-mouse IgG for 60 min, washed and mounted on glass slides for viewing under a Zeiss fluorescence microscope to localize DAMP.

Isolation and culture of monocytes

Monopacks were received from Blood Bank, NIH and monocytes were recovered from peripheral blood mononuclear cells (PBMC) of HIV and hepatitis B-seronegative donors after leukopheresis by Ficoll-hypaque gradient centrifugation. Monocytes were then isolated by an adherence method. The purity of the monocytes was checked by flow cytometry using a monocyte marker CD-14 and leukocyte marker CD-45; 99% of the adherent cells were monocytes. Monocytes were cultured as adherent cell monolayers in RPMI-1640 supplemented with 10% heat-inactivated AB human serum (Irvine Scientific, Santa Ana, CA), 50 µg/ml gentamicin, and 100 I.U./ml recombinant human GM-CSF (a generous gift from the Cetus Corp. Emeryville, CA).

HIV infection of Monocytes, RT activity and p24 Antigen assay

HIV-Ada, a monocytotropic strain of HIV-1 was obtained from AIDS Research and Reference Program, NIAID, NIH, Bethesda. Monocytes ($3x10^6$ cells) were infected with HIV-Ada (300,000 CPM of RT) for 5 hr, unadsorbed virus was removed, and the cells were refed with fresh medium containing 100 I.U./ml units of human GM-CSF. IFN (0-100 units) was added after 7 days of HIV-Ada infection. Culture supernatants were harvested at 20 days and tested for the presence of HIV-RT and p24 activity. RT assays were performed in a final volume of 200 µl containing 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 5 mM dithiotheritol, 5 mg of Poly (γA)-oligo (dT) per ml, 83 µg/ml of dATP and 50 µCi ^3H TTP. Incubation was

carried out at 37°C for 1 hr, and the reaction was terminated by adding 10 µl tRNA and 3 ml ice cold 10% trichloracetic acid (TCA) containing 0.2% sodium pyrophosphate. The acid insoluble material was collected on glass filters, washed, and radioactivity determined in a scintillation counter.

The p24 antigen was determined by an antigen capture ELISA as per manufacturer's (Dupont, MA) protocol. Briefly, HIV -1 p24 core antigen that was released upon the lysis of virus in the sample were captured by a highly specific monoclonal antibody to HIV-1 p24 core antigen immobilized to microplate. The captured antigen was complexed with biotinylated polyclonal antibody to HIV-1 p24 core antigen and probed with streptavidin -HRP conjugate. This complex was detected by incubation with Orthophenylenediamine-HCl (OPD) which produced a yellow color proportional to the amount of HIV core antigen captured. Each absorbance was calibrated against the absorbance of HIV-1 p24 antigen standard curve.

Effect of IFN on HIV -Ada infected monocytes

Monocytes were grown on chambers and were infected with HIV-Ada for 5 hr., unadsorbed virus was removed and cells were replenished with fresh medium containing 100 I.U./ml of GM-CSF. IFN was added after 7 days of HIV-Ada infection. Monocytes were visualized under the microscope and then were fixed with 1% paraformaldehyde and 4% glutaraldehyde in 0.1 M cacodylate buffer (pH -7.4) for 3 hr. The samples were washed osmicated, dehydrated in graded

series of ethanol, freeze dried, sputter coated with hummer -5 (Technica) and viewed under a JEOL scanning electron microscope.

Localization of HIV-1 gp120

For the localization of gp120, monocytes were infected with HIV-Ada, and treated with IFN (0-100 I.U./ml) 7 days after infection. Monocytes were fixed in chilled acetone after 10 days of IFN-treatment. Indirect immunofluorescence was done using monoclonal antibody to gp120 (F105; catalog # 857, obtained from AIDS Research and Reference Program, NIAID, NIH) and FITC-conjugated goat anti-mouse IgG. Samples were viewed and photographed using a Zeiss photomicroscope III with a 63 x oil immersion lens.

Intracellular pH of monocytes

pHi was measured with the help of laser spectroscopy (Baanett et. al.,1990) using carboxyl -seminapthorhedofluor -1 (SNARF) a sensitive indicator. Cells were loaded with SNARF-1 and excited with 514 nm argon laser. The fluorescence was observed as detected by detector 1 (636 nm) and detector 2 (587 nm). The change in ratio (636/ 587 nm) in IFN treated and untreated cells were compared. This channel ratio was calibrated to pH gradient. Briefly, cells were washed with RPMI 1640 medium containing 25 mM HEPES without serum. Cells were incubated for 30 min with 10 μM SNARF-1 and unloaded probe was removed by washing 2 times with the RPMI 1640 medium containing 25 mM HEPES, and then cells were

scanned every 30 sec with a scanning argon laser microscope. At the indicated times, pH of the mixture containing high K^+ and 10 mM nigericin was changed to 6.8, 7.4, 7.7, 8.0 and 8.5. Nigericin equilibrated extracellular pH (pH0) = pHi. The fluorescence intensity as a function of time was recorded by detector 1(636 nm) and detector 2 (587 nm) during the addition of buffer with different pH during the time course. The fluorescence ratio was digitized into the channel ratio and then channel ratio was converted to pHi using the calibration curve. Fluorescence emission spectra from cells exposed to SNARF-1 (excited at 514 nm) were pH sensitive, with protonated and unprotonated emission maximal at 587 and 636 nm respectively. The change in the ratio of the emission intensity of these two peaks reflected the alteration in pHi.

Monocytes were treated with IFN as indicated, washed twice with albumin free RPMI 1640 media containing 25 mM HEPES and 10 ml of 1mM SNARF-1 was added in 1 ml of medium to give a final concentration of 10 mM SNARF -1. The dishes were incubated at 37°C for 40 min in a CO_2 incubator. The pHi was determined from titration curves generated using the proton ionophore nigericin as described.

Localization of DAMP in the Golgi-complex by Gold Labelling

L_B cells at 70% confluency were treated with IFN (0-30 I.U./ml) for 16 hr. Cells were then further incubated for 1 hr with DAMP at 37°C and were fixed in a fixative containing 1% glutaraldehyde, 1% paraformaldehyde, 0.2% picric acid, 0.5

mM calcium chloride, 0.1 M phosphate buffer, pH 7.4 for 2 h, followed by uranyl acetate treatment and dehydration in acetone. Samples were embedded in fresh LR-Gold resin plus benzoin methyl ether (initiator) in Beem capsule and were polymerized with U.V. light. Immunogold staining was performed on silver thin section on 400-mesh nickel grids at room temperature. Grids were hydrated for 5 min in TBS, blocked in 5% normal goat serum for 10 min in TBS, incubated for 1 hr with primary anti-DNP Ab (1:10), washed and then incubated in gold conjugated goat anti-mouse IgG (10 nm, Zymed) for 1 hr, washed in TBS, fixed for 5 min in 2% glutaraldehyde, osmicated for 15 min in 2% osmium tetroxide, and stained in lead citrate for 5 min and then viewed under a JEOL electron microscope.

Endo-β acetylglucosaminidase H (endo H) and Neuraminidase Digestion

Control and IFN-treated VSV-infected L_B cells were incubated for 5 hr at 37°C after virus adsorption. Cells were washed once with methionine free media, incubated for 1 hr in the same medium and then labelled with [^{35}S] methionine (50 μCi/ml) for 20 min. Cells were washed with EMEM containing a 10 fold excess of unlabelled methionine and 20 μg/ml cycloheximide, and then incubated in the same medium for another 2 hr. Cells were then subjected to subcellular fractionation on sucrose and Nycodenz gradients. Nycodenz fractions rich in both G protein and TGN marker were pooled and immunoprecipitated using protein-A-sepharose and monoclonal anti-G antibodies as described previously (Maheshwari et al, 1991). Samples were resuspended in 50μl of 0.1M sodium phosphate buffer, pH 6.1, 0.1% Triton X-100, 0.03% SDS and 20mM EDTA and divided into two equal sized aliquots

and 0.5 mU endo H was added to one of the aliquots. Similarly 5 mU of neuraminidase was added and all the samples with or without endo H or neuraminidase were kept at 37°C for 16 h, after which an equal volume of 2X sample buffer containing 2% SDS was added. Samples were run on 10% SDS-PAGE and gels were dried and autoradiographed.

Transfection of L_B Cells

Mouse cells were grown in EMEM containing 10% Fetal Bovine serum to late log phase. Plasmid pSVGL containing G cDNA and pSV_2-neo containing a selectable marker gene (neomycin) were ethanol precipitated and mixed in the ratio of 10:1 respectively. Cells were trypsinized using 0.05% trypsin containing 0.53 mM EDTA, and washed two times with Hepes buffered saline (HeBS). 10 µg mixed DNA (pSVGL + pSV_2-neo) was added to the cells ($5x10^6$) in 0.7 ml of cell suspension and kept in ice for 5 min. Cells were then placed in the cuvette in the electroporation apparatus and were shocked at 340 volts and 960 µF capacitance and kept in ice for 10 min. The cells were then plated in normal medium for overnight, washed with serum free medium and grown in 10% EMEM containing neomycin (G418, 1 mg/ml) for 10-14 days. Using cylinders, various colonies were picked up and grown in complete medium containing G418. Cells from each colony were tested for the expression of G cDNA and G protein.

Expression of G cDNA and G protein

Immunofluorescence staining for G protein in transfected cells of various clones were carried out using anti VSV-G monoclonal antibody (kindly provided by Heinz Arnheiter, NIH, Bethesda, MD, USA) followed by rhodamine goat anti-mouse IgG, (Maheshwari et al., 1994). Samples were viewed and photographed using a Zeiss photomicroscope III with a 63x oil immersion lens. Clones showing high immunofluorescence for the G protein were selected for the expression of G cDNA. DNA was prepared from non-transfected and transfected (clone 1-3) cells (Sambrooket al.,1989). Briefly, cells were washed with 1 X PBS and lysed with extraction buffer (10 mM Tris.Cl pH-8.0, 0.1 M EDTA, 20 μg/ml RNAse and 0.5% SDS) and then treated with Proteinase K (final conc. 100 μg/ml). The suspension of lysed cells was placed at 50 ^{0}C for 3 hr. the solution was extracted with phenol equilibrated with 0.5 M Tris. Cl (pH-8) for 3 times and then DNA was dialyzed at 4^{0}C against 4 liters of a solution of 50 mM Tris.Cl (pH -8), 10 mM EDTA (pH -8). The DNA was blotted to nytran filters. pSVGL plasmid was digested with Xho-I and run on 1% low melting agarose. G cDNA insert was electroeluted and labelled with ^{32}P using α-^{32}P-ATP and Klenow enzyme. The labelled probe was purified using a spin column of Sephadex G-50 (Pharmacia). The probe was hybridized to the blotted DNA and exposed to X-ray film.

Localization of G protein by Immunofluorescence

pSVGL transfected cloned cells were treated with IFN (100 I.U./ml) for 16 hr. The untreated and IFN-treated cells were washed with PBS and fixed in chilled acetone. Immunofluorescence staining was performed using anti VSV-G monoclonal antibody followed by FITC-conjugated goat antimouse IgG. Cells were viewed under a Zeiss fluorescence microscope.

RESULTS

HERPES SIMPLEX VIRUS-1

Effect of IFN on HSV-1 infectivity

The supernatant obtained after sonication from the IFN treated (0-100 I.U./ml) and HSV-1 infected cells, were assayed for infectivity. IFN (10-100I.U./ml) significantly inhibited the infectivity of HSV-1 (5-100 fold) (Table 2) in L_B cells. The inhibition of the infectivity of HSV-1 was dependent on the dose of IFN and multiplicity of virus used.

Synergistic effect of IFN and CHL on the infectivity of HSV -1

The plaque assay was performed on samples treated with IFN (0-30 I.U./ ml). The results indicate that IFN treatment reduced the number of plaques formed in a dose dependent manner (Fig.6). However, the number of plaques formed was less when cells were treated with both IFN and CHL together compared to either drug alone, indicating that subeffective doses of IFN and CHL, when given together enhance the antiviral effect.

We have also assayed the combined effect of IFN and CHL by cytopathic effect. The supernatant obtained from L_B cells infected with HSV-1 and treated with or without IFN (0-100 I.U./ml) and/or CHL (10μM). It is evident from the Table 3 that IFN and CHL enhance the antiviral activity of HSV-1 as compared to either drug.

Table 2: Effect of IFN on the infectivity of HSV-1

L_B cells were treated with IFN (0-100 I.U./ml) for 16 hr and infected with HSV-1 at a multiplicity of 0.1 or 0.5 $TCID_{50}$ /cell. Unadsorbed virus was removed and the cells were replenished with fresh medium. After 24 hr, plates were examined for cytopathic effect (CPE), sonicated, and assayed for infectivity.

Treatment IFN (units/ml)	HSV -1 Inhibition (log_{10})	
	0.1 $TCID_{50}$/cell	0.5 $TCID_{50}$/cell
0	--	--
10	1.02	0.33
30	1.50	0.80
100	2.00	1.00

Fig.6: Synergistic effect of CHL and IFN on plaque formation

The plaque assay was performed on IFN treated L_B cells infected with HSV-1 at a multiplicity of 0.5 $TCID_{50}$/cell with or without CHL. The ten fold serial diluted virus samples were checked for their ability to form the plaques.

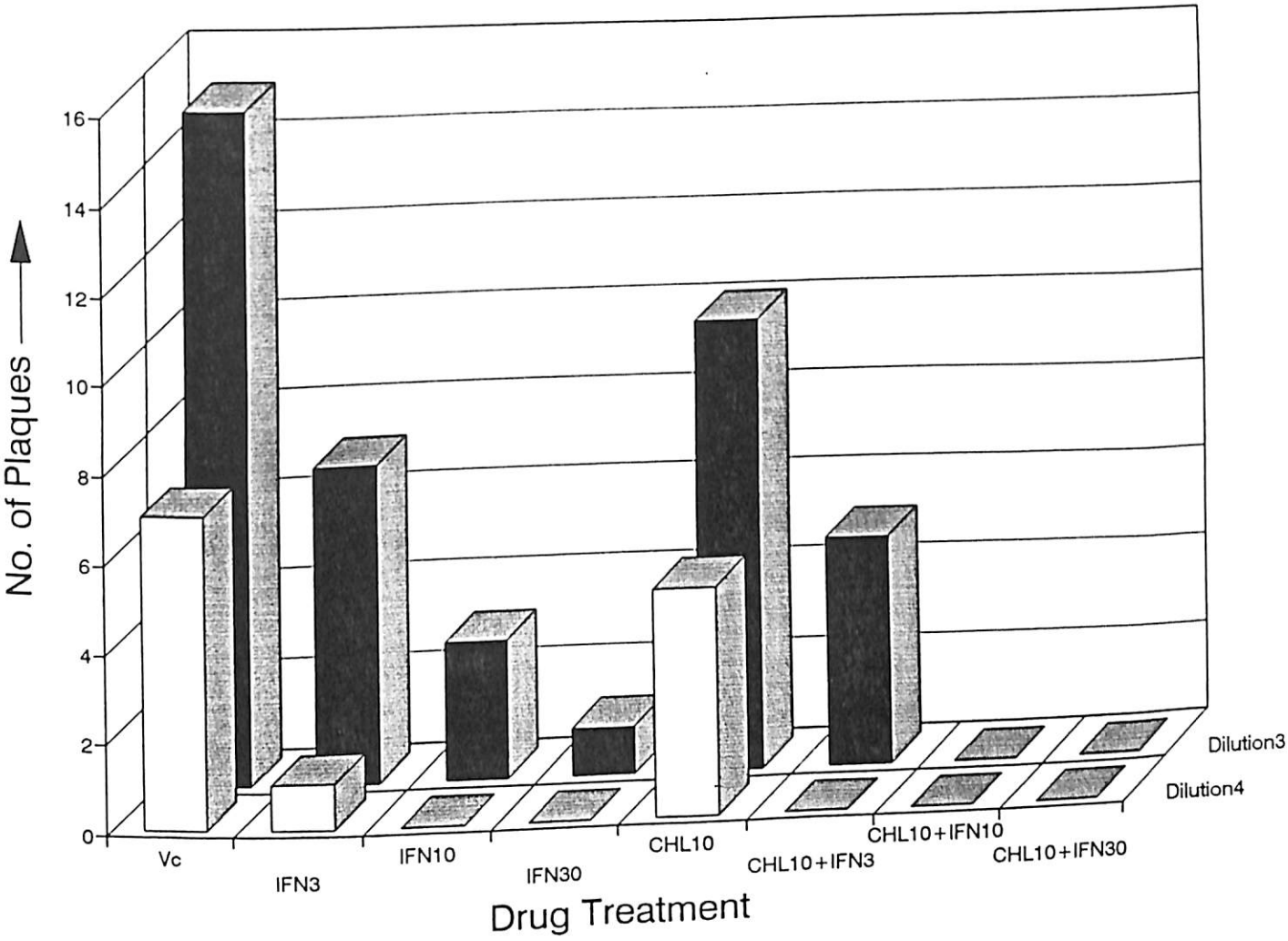

No. of Plaques
16
14
12
10
8
6
4
2
0
Vc
IFN3
IFN10
IFN30
CHL10
CHL10+IFN3
CHL10+IFN10
CHL10+IFN30
Drug Treatment
Dilution3
Dilution4

Table: 3 Effect of IFN and CHL on the infectivity of HSV-1.

Cells were treated with IFN for 16 hr and infected with HSV-1 at a multiplicity of 0.5 $TCID_{50}$ /cell. Unadsorbed virus was removed and the cells were replenished with fresh medium with or without CHL. After 24 hr, plates were examined for CPE, sonicated and assayed for infectivity.

Treatment IFN (units/ml)	HSV-1 Inhibition (log_{10})	
	without CHL	with CHL (10 μM)
0	--	0.33
20	0.33	1.33
100	1.00	1.83

Effect of IFN on HSV-1 Growth

The dot blot showed that there was no apparent decrease in the viral protein in the IFN treated cells as compared to control (Fig. 7) suggesting that IFN did not inhibit the HSV-1 growth.

Effect of IFN on the synthesis of virus specific proteins

The results show no significant difference in the inhibition of viral proteins in IFN and/or CHL treated cells as compared to control (Fig. 8). The autoradiogram indicates the faint band of ICP4 which is the major transcription regulatory and a very early protein. The synthesis of VP5 (a major nucleocapsid protein), ICP6 (A subunit of ribonucleotide reductase), ICP8 (major DNA binding protein) and viral specific proteins seem to be minimally affected in IFN and/or CHL treated cells compared to the control.

Effect of IFN on the assembly of HSV-1 Nucleocapsid

HSV -1 specific proteins did not appear to be inhibited in the IFN treated cells, thus ruling out the possibility that the virus replication is inhibited at the penetration or any early stage before viral DNA synthesis. We, therefore determined the effect of IFN treated cells on the assembly of HSV-1 nucleocapsid in L_B cells. No significant difference was seen in intranuclear virus particles in treated and untreated group (Fig. 9). However, IFN treated cells clearly show fewer virus particles in the extracellular space suggesting that the assembly of HSV-1 is blocked by IFN at the late stages in HSV-1 morphogenesis (Fig. 9).

Fig. 7: Viral growth in the presence of IFN.

L_B cells were treated with IFN (0-250 I.U./ml) and were infected with HSV-1. Cellular lysates were prepared after 8 hr post infection, dot blotted and virus was detected with anti HSV-1 rabbit polyclonal antibody using ECL detection method.

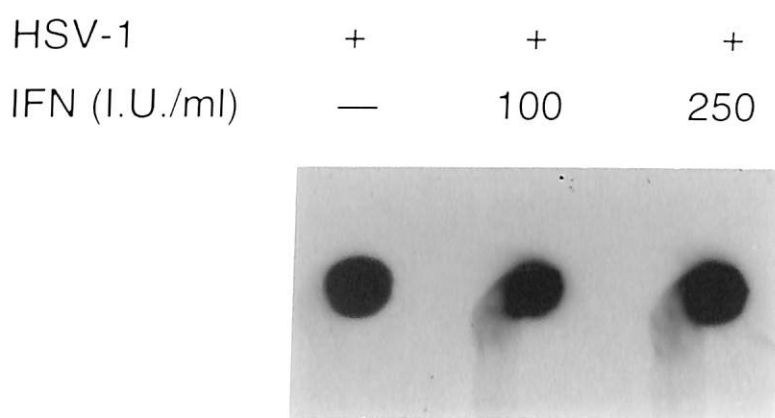

HSV-1 + + +
IFN (I.U./ml) — 100 250

Fig. 8: Early synthesis of HSV-1 specific protein in IFN and /or CHL treated cells. L_B cells were treated as described earlier and then were infected with HSV-1 and metabolically labelled with ^{35}S methionine for 3 hr. The cells were lysed and equal amount of radioactivity was run on 10 % SDS-PAGE and gels were autoradiographed using Kodak X-ray film.

Lane 1-8 in the autoradiogram are cell control, virus control, IFN 20, IFN 100, IFN 500, CHL10, CHL10 + IFN 20 and CHL 40 respectively.

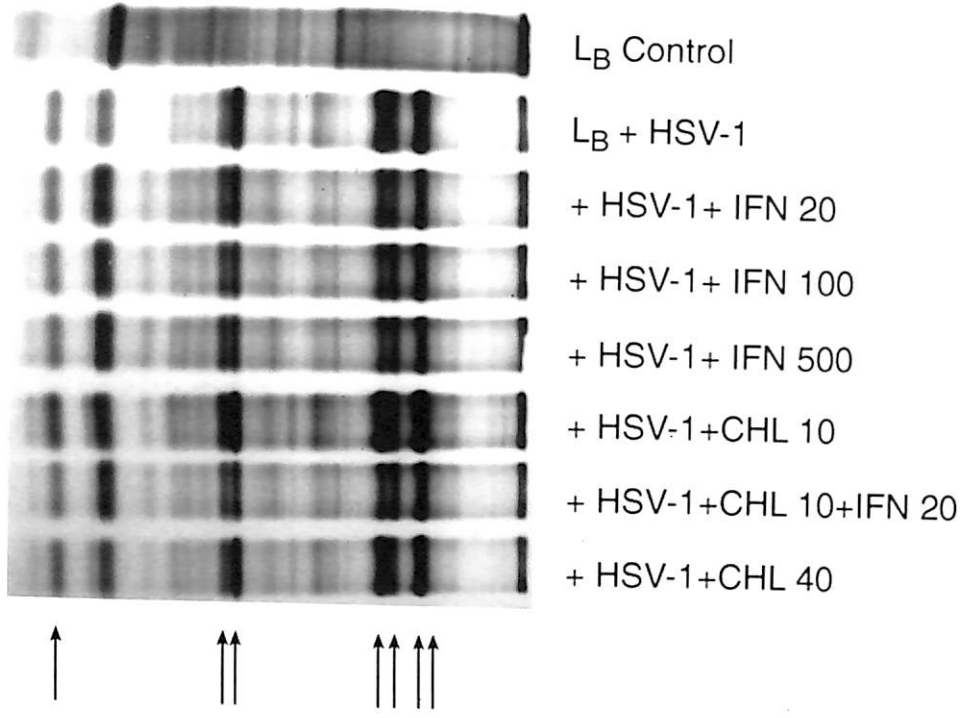
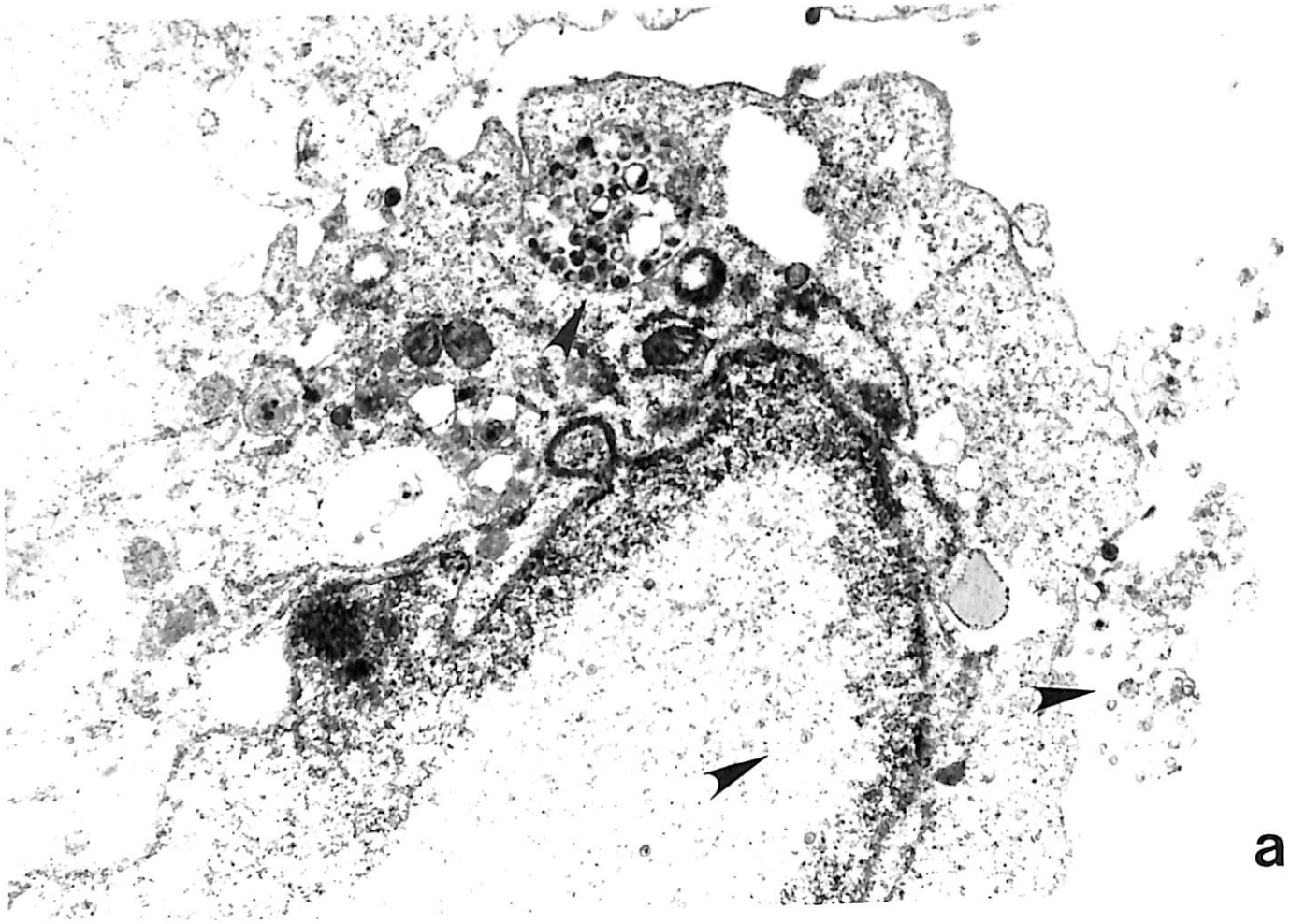
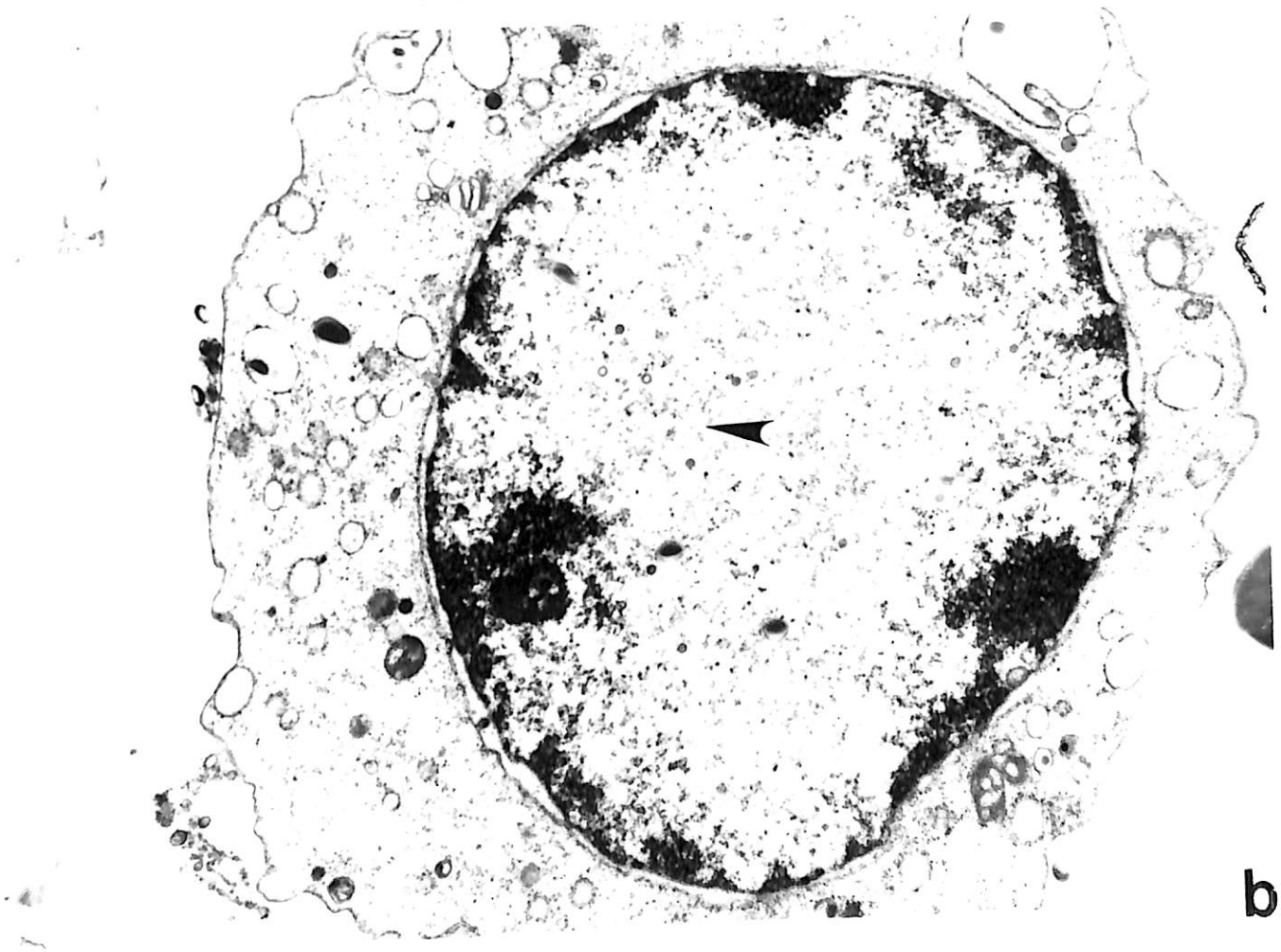

L_B Control
L_B + HSV-1
+ HSV-1+ IFN 20
+ HSV-1+ IFN 100
+ HSV-1+ IFN 500
+ HSV-1+CHL 10
+ HSV-1+CHL 10+IFN 20
+ HSV-1+CHL 40

Fig.9: IFN-induced inhibition of HSV-1 in mouse L_B cells.

The cells were treated with IFN for 16 hr and then infected with HSV-1 at 0.5 $TCID_{50}$/cell. The cells were fixed in 2% glutaraldehyde and were post fixed in 2% osmium tetroxide and 0.5% uranyl acetate. Sections were cut and viewed under a JEOL microscope.

A) Control, arrow indicating assembled virus particles inside the nucleus and large number of virus particles in the extracellular space (magnification x54,000). B) IFN 100, most of the virus particles are seen in the nucleus whereas very few can be seen in the extracellular space (magnification x 54,000).


a


b

Localization of gD in Unpermeablized cells

The localization of the gD glycoprotein on the cell surface was initially compared in unpermeabilized living cells. Untreated L_B cells infected with HSV-1 showed a bright immunofluorescence capping pattern (Fig.10 a) which reflects the presence of cell surface associated gD protein where as in IFN-treated cells gD glycoprotein could not be detected on the cell surface (Fig.10 b). Similarly, untreated LMtk$^-$ cells showed a high expression of gD on the cell surface (Fig.10 c), whereas in IFN treated cells, there was significantly less gD on the surface (Fig.10 d).

Intracellular Localization of HSV-1 gD

In IFN-treated L_B cells (Fig.11 B,C) most of the HSV-1gD protein accumulated intracellularly compared to controls where most of the gD was localized on the cell surface (Fig.11 A). We also studied the effect of IFN on intracellular accumulation of gD protein in transfected LMtk$^-$ cells. In the untreated control cells, most of the gD protein was expressed on the surface (Fig. 12 B), whereas in IFN-treated cells most of the gD protein was localized intracellularly (Fig.12 C,D). There was no fluorescence in the cells incubated with FITC-conjugated goat antimouse IgG (Fig. 12 A), ruling out nonspecific binding of the antibody.

Localization of HSV-1 gD in CHL and IFN treated cells

The earlier data suggested that IFN and CHL given together enhances the inhibition of HSV-1 infectivity as compared to the control or either drug given alone. Therefore, we have studied if CHL and IFN might act synergistically on the accumulation of gD.

Subeffective doses of CHL or IFN do not block the transport of gD protein in LMtk$^-$ cells (Fig.13 B,C) however, intracellular accumulation of gD was seen in cells treated in combination with these subeffective doses of CHL and IFN (Fig. 13D), suggesting that IFN and CHL have acted synergistically in blocking the transport of gD intracellularly (Fig.13 D).

Fig. 10: Immunofluorescence for gD on the surface of the living cell.

Indirect immunofluorescence was performed on the living cell using anti gD polyclonal antibody raised in rabbit at 4°C followed by FITC-conjugated goat anti rabbit IgG.

(a) L_B- virus infected, untreated cells, (b) L_B-virus infected, IFN (100 I.U./ml) treated cells, (c) LMtk$^-$ control cells, (d) LMtk$^-$ IFN (500 I.U./ml) treated cells.

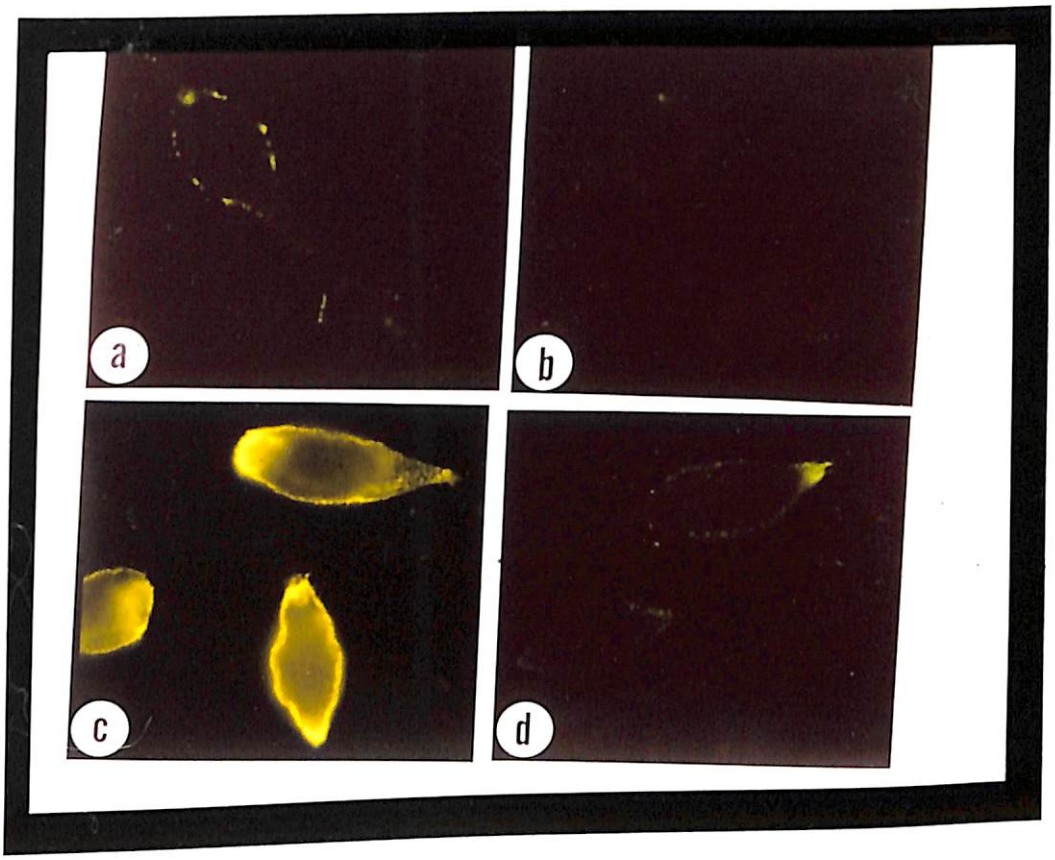

a
b
c
d

Fig. 11: Localization of HSV-1 gD in HSV-1 infected L_B cells

L_B cells were treated with IFN for 16 hr and then infected with HSV-1 at 0.5 $TCID_{50}$ /cell. Unadsorbed virus was removed and cells were incubated in fresh medium for 8 hr. Cells were then fixed and HSV-1 gD was localized by indirect immunofluorescence.

(A-C) : A- untreated; B-treated with IFN 30 I.U./ml; and C-treated with IFN 100 I.U./ml.

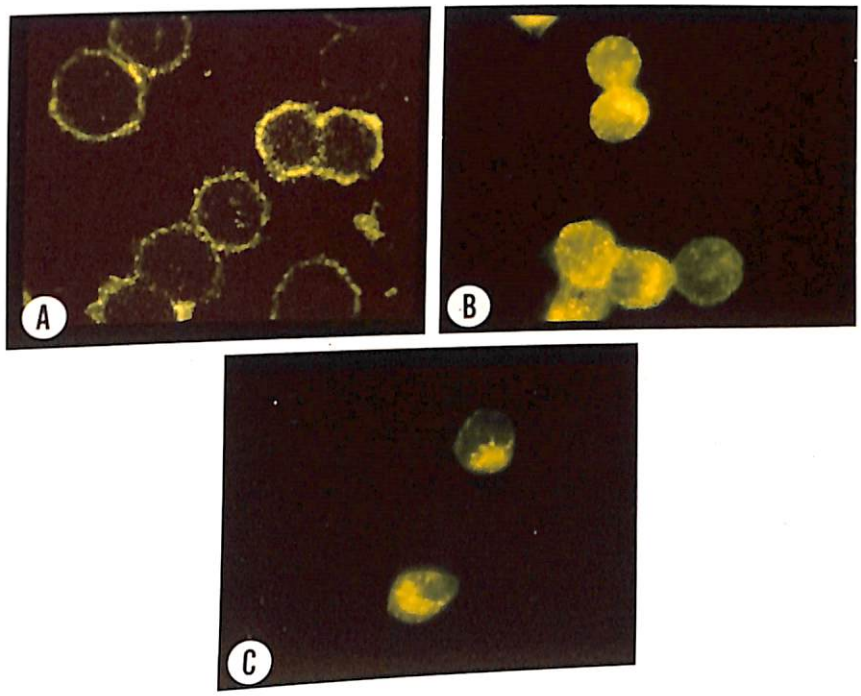

A
B
C

Fig. 12: Localization of HSV-1 gD in LMtk$^-$ cells

LMtk$^-$ cells were treated with IFN for 16 hr and then fixed in chilled acetone for 10 min. The HSV -gD was localized by indirect immunofluorescence using an anti-gD monoclonal antibody followed by FITC-conjugated goat antimouse IgG.

(A-D): A- negative control; B-untreated; C- IFN (30 I.U./ml) and D- IFN (100 I.U./ml).

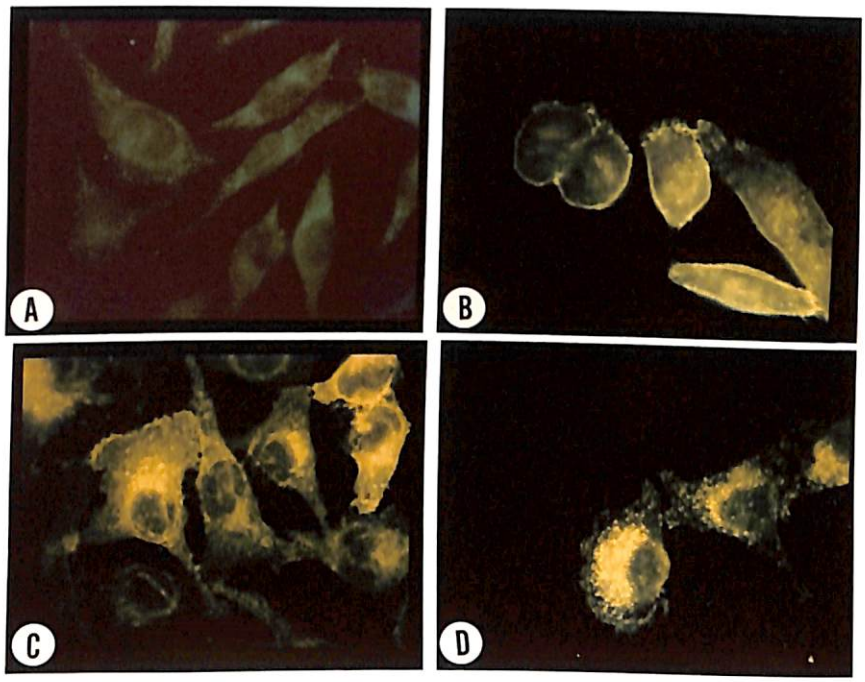
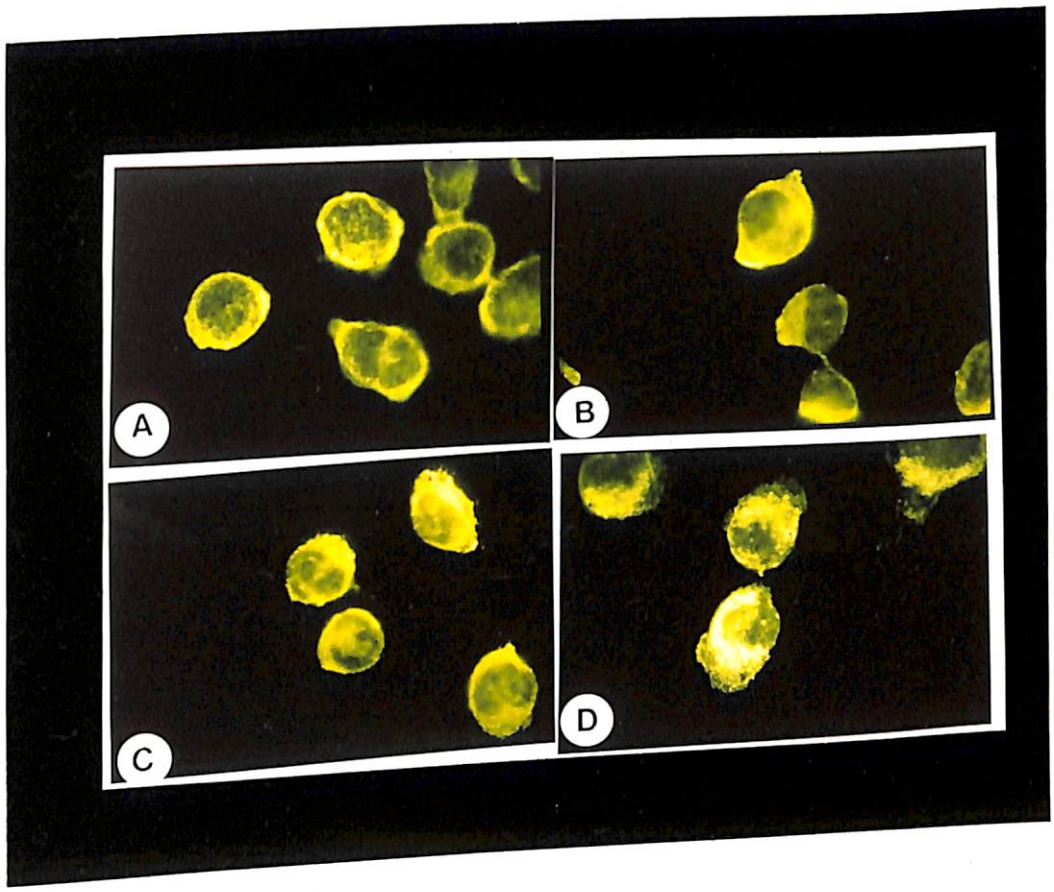

A
B
C
D

Fig. 13 : Localization of HSV-1 gD in the presence of low doses of CHL or IFN

LMtk$^-$ cells treated with IFN/CHL or both were fixed in chilled acetone. An indirect immunofluorescence was performed using anti gD monoclonal antibody followed by FITC-conjugated goat antimouse IgG.

(A-B) : A- Untreated cells; B- treated with IFN 10 I.U./ ml; (C-D) : C- CHL 10μM; D-CHL 10μM + IFN 10 I.U./ml


A
B
C
D

Localization of gD in the TGN

WGA binds to TGN, and was therefore, used to determine whether gD remains associated with Golgi-complex using double immunofluorescence. In fixed, untreated LMtk$^-$ cells most of the gD can be localized on the plasma membrane (Fig.14 a); there was perinuclear and intracellular localization by WGA (Fig. 14 b) for Golgi staining. There was clearly a co-localization of gD protein with WGA in the cytoplasmic structures in IFN-treated, transfected LMtk$^-$ (Fig. 14 c, d) as compared to untreated cells (Fig. 14 a, b), where bulk of the gD protein appeared to be localized on the plasma membrane suggesting that IFN blocked gD protein transport within TGN. There was no immunofluorescence in the cells incubated with FITC-conjugated goat antimouse IgG (Fig.14 e), ruling out non-specific binding.

Intracellular accumulation of gD glycoprotein in IFN treated LMtk$^-$ cells

LMtk$^-$ cells were treated with or without IFN for 16 hr and the cell lysates were subjected to SDS-PAGE and were then transferred to nitrocellulose paper. The blot was incubated with rabbit anti gD serum and probed with ^{35}S -Protein-A. For the quantitation of gD protein, the autoradiogram was scanned using densitometer (Fig. 16). The results indicate that there was 3.89 fold more accumulation of gD protein in IFN treated cells compared to control (Fig. 15). In the next experiment, LMtk$^-$ cells were labelled with ^{35}S- methionine and the cell organelles were separated.

Biochemical Localization of gD in the Golgi membrane

The effectiveness of Nycodenz density gradient centrifugation for the purification of Golgi membranes was assessed by comparing the Trans Golgi marker across the gradient markers for the other subcellular compartments. The results show the Golgi marker (fraction # 2,3 and 4) were well separated from the marker for the lysosome (Fig.17). Most of the Golgi membrane was found at 10-17.5 %(Fraction number 3) interface. In IFN treated cells most of the gD accumulated in the Golgi membrane as compared to the control cells, where no significant expression of the gD protein was seen in the Golgi membrane fractions. These experiments were carried out in gD transfected LMtk$^-$ cells which constitutively express the gD protein, therefore, gD is always present in small quantity at TGN fractions obtained from the untreated cells, however, the gD within the TGN of IFN treated cells was present several fold more as compared to the control (Fig.18).

Determination of the intracellular distribution of DAMP

DAMP, a basic congener of DNP, accumulates in intracellular acidic compartments, such as the vesicles and cisternae associated with the TGN. The distribution of DAMP was determined by fluorescence microscopy. DAMP was localized in large perinuclear vacuoles throughout the cytoplasm of untreated cells (Fig. 19 A), whereas, in IFN treated cells, inhibition of DAMP into these vacuoles was seen (Fig.19 B), suggesting that IFN caused an increase in pH of the intracellular compartments.

Fig.14: Co-localization of gD protein and Golgi-complex in LMtk$^-$ cells by double immunofluorescence.

The cells were incubated with 0.5 mg/ml WGA for 60 min on ice and then were incubated with rhodamine-WGA for 30 min. After washing, cells were fixed and incubated with anti gD monoclonal antibody followed by FITC-conjugated goat antimouse IgG.

Lanes: (a, b)-untreated cells; (c, d)-IFN (500 I.U./ml)-treated cells; (e) cells incubated with only goat FITC-conjugated mouse IgG (secondary antibody); (a-c) localization of gD; (b-d) same cells showing the localization of WGA.

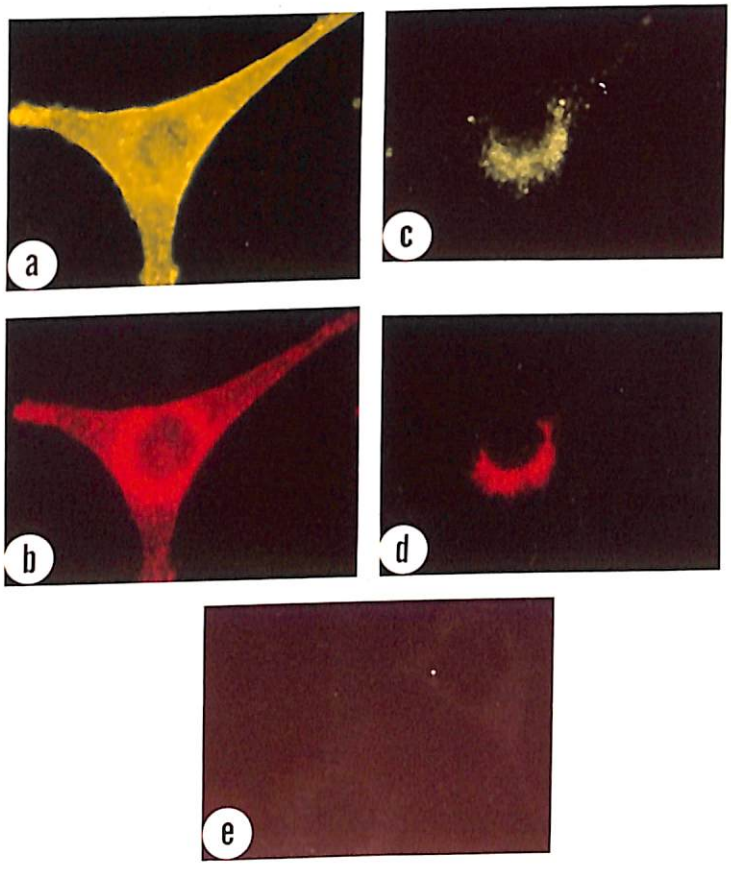

a
c
b
d
e

Fig.15: Effect of IFN on the intracellular accumulation of HSV -1 gD protein in mouse LMtk$^-$ cells.

LMtk$^-$ cells were treated with or without IFN for 16 hr. The cellular lysates were run on 10% SDS-PAGE. The proteins were transferred on to nitrocellulose membrane and incubated with anti gD-monoclonal antibody. The bound antibody was detected by reacting the blot with ^{35}S Protein-A followed by autoradiography.

Lanes: 1- non transfected cell; 2-Untreated; 3- IFN 50 I.U./ml.

Non
Transfected
Cells
LMtk- Control
LMtk- + IFN 50

Fig.16: Quantitation of synthesis of gD protein in IFN treated LMtk$^-$ cells

The autoradiogram was scanned using a densitometer (Hoefer Scientific Ins., San Francisco, GS300 transmittance reflectance scanning densitometer) and area under the curve was calculated.

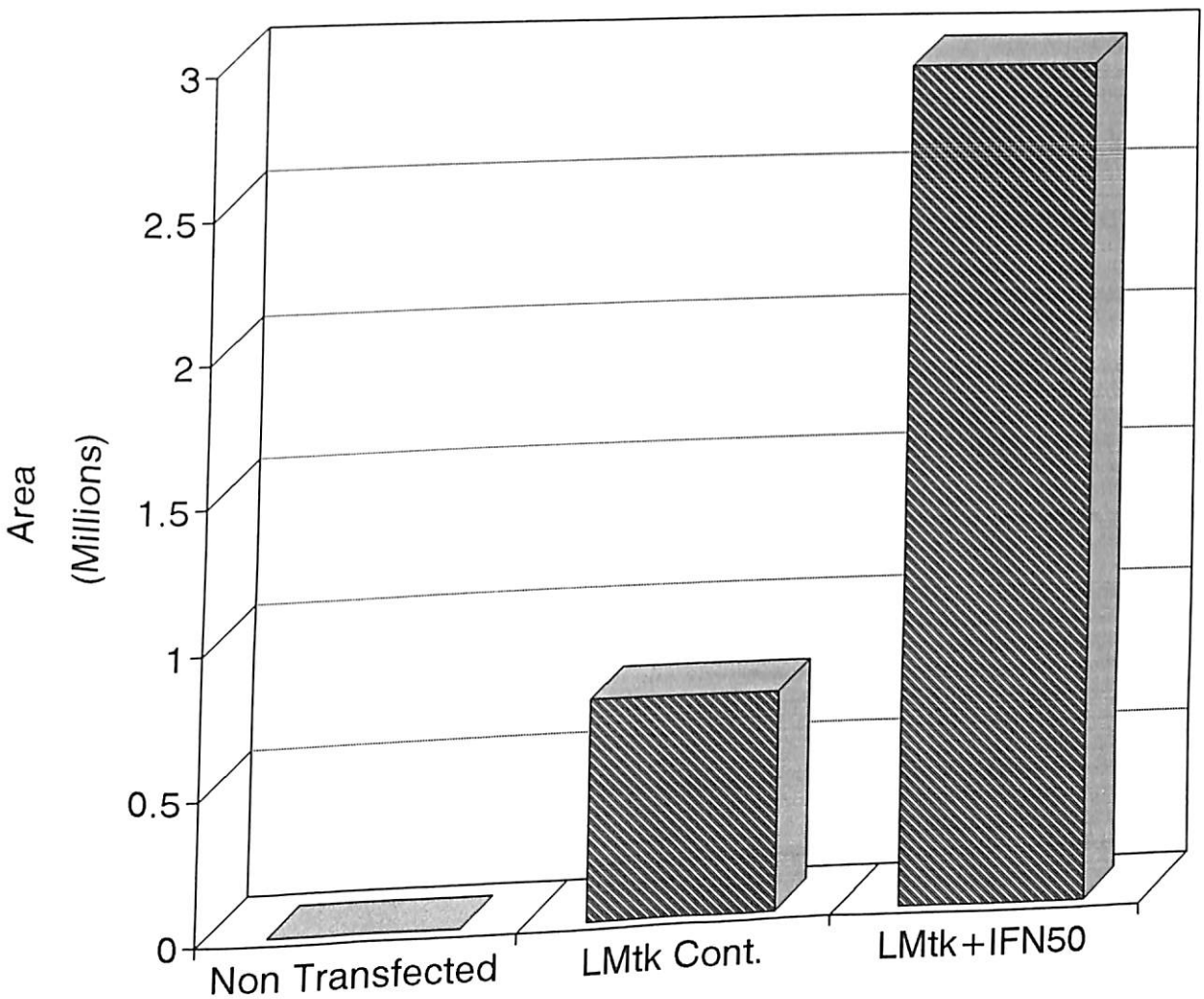

Area
(Millions)
3
2.5
2
1.5
1
0.5
0
Non Transfected
LMtk Cont.
LMtk+IFN50

Fig. 17: Distribution of subcellular markers in the Nycodenz preparative gradient

Cells were treated and labelled as described in the materials and methods. Crude Golgi membranes were pelleted by centrifugation of the light mitochondrial fraction for 90 min at 52,000 rpm. Fractions were collected and distribution of UDP -galactosyl -glycoprotein transferase and N- acetyl glucosaminidase was compared with that of protein. Fraction 1 is from the top of the gradient.

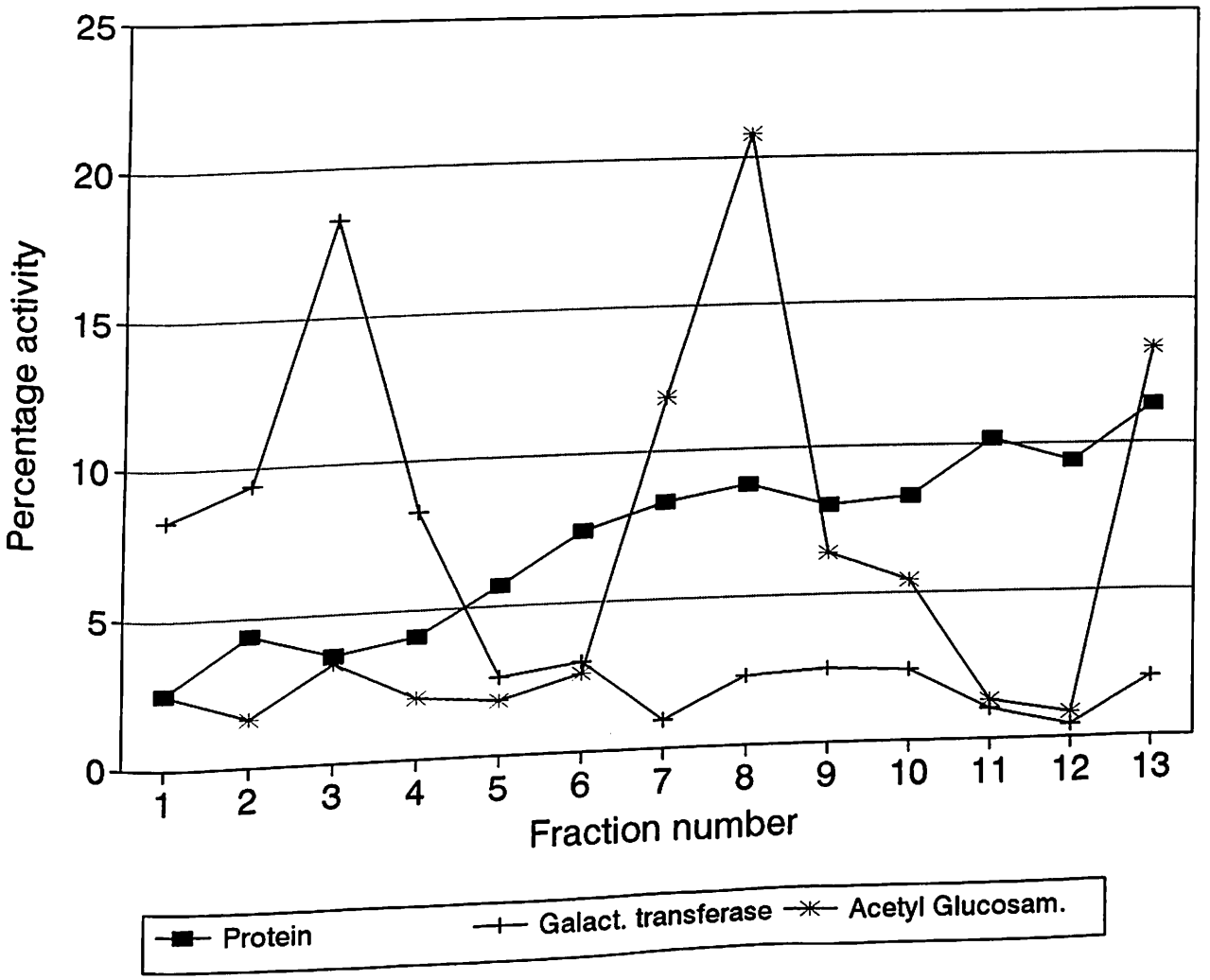

25
20
15
10
5
0
Percentage activity
1
2
3
4
5
6
7
8
9
10
11
12
13
Fraction number
Protein
Galact. transferase
Acetyl Glucosam.

Fig. 18: Distribution of HSV-1 gD in Nycodenz gradient

LMtk$^-$ cells were treated with or without IFN and were fractionated on Nycodenz gradient. Fractions (as indicated) obtained from the Nycodenz gradient were subjected to 10 % SDS-PAGE and gels were autoradiographed using Kodak XLR films. Fraction number 1 is from the top of the gradient.

(A) Control cells (B) Cells treated with IFN-100 I.U./ml.

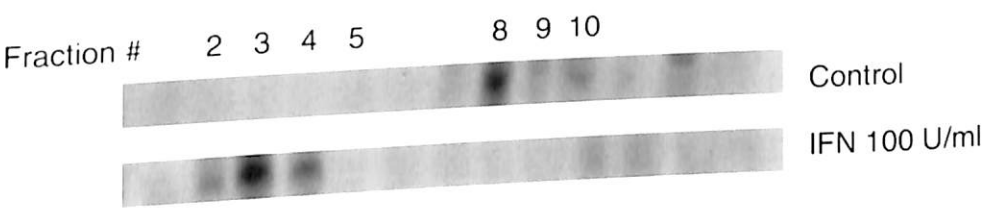
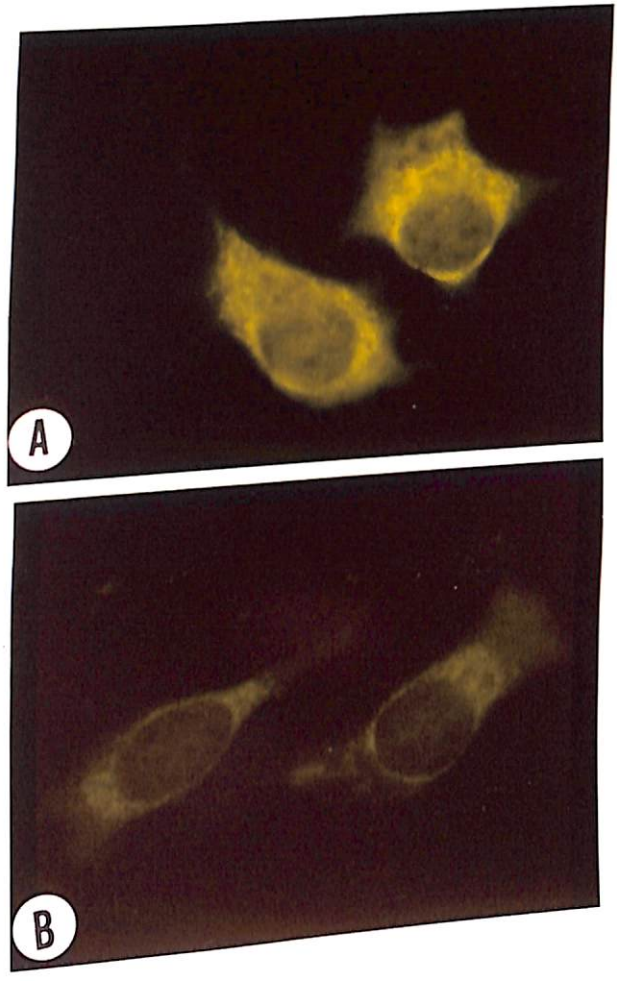

Fraction #
2
3
4
5
8
9
10
Control
IFN 100 U/ml

Fig. 19: Intracellular distribution of DAMP in LMtk$^-$ cells.

IFN treated or untreated cells were incubated for 1 hr with DAMP at 37° C. Cells were washed and fixed in 3.7% paraformaldehyde. An indirect immuno fluorescence was performed using mouse anti DNP and FITC-conjugated goat antimouse IgG.

(A) Untreated cells; (B) cells treated with IFN (100 I.U./ml) for 16 hr.


A
B

HUMAN IMMUNODEFICIENCY VIRUS -1

Effect of IFN on HIV-Ada

Table 1 shows that RT activity was significantly decreased (5-50 fold) with an increase of IFN dose as compared to controls, however, p24 antigen was inhibited only 1-4 fold (Table 4). These results indicate a significant decrease in viral infectivity without a large decrease in virus protein synthesis.

Scanning Electron microscopy of HIV-Ada infected monocytes

The light microscopy data suggest that monocytes treated with low concentration of IFN show a dose dependent reversal of the cytopathic change associated with HIV infection. IFN 30 and 100 U/ml show formation of lesser number of multinucleated giant cells as compared to control (Fig. 20). The electron micrographs (Fig. 21) show the presence of significant number of virus particles budding from the surface as compared to IFN treated monocytes where fewer virus particle were seen budding out indicating IFN inhibited the HIV assembly thereby fewer virus particle were formed.

Localization of gp 120

In IFN-treated, infected monocytes most of the HIV gp120 accumulated intracellularly compared to untreated monocytes, where most of the gp120 could be localized on the cell surface (Fig. 22).

Table 4 Effect of IFN α on human monocytes infected with HIV-Ada

Monocytes were infected with HIV- Ada (300,000 cpm of RT/3x10^6cells) for 5 hr, unadsorbed virus was removed and cells were replenished with fresh medium. IFN was added after 7 days of HIV-Ada infection. Culture supernatants were harvested at 20 th day and HIV RT was quantitated.

p24 antigen was determined by antigen capture ELISA using manufacturer's protocol.

Treatment IFN (units/ml)	HIV-ADA Inhibition			
	RT Activity		p24 Antigen	
	cpm/ml	Percentage inhibition	(mg/ml)	Percentage inhibition
0	427634	---	43	---
10	69655	84	41	5
30	26992	94	24	44
100	8302	98	11	74

Fig. 20: Light micrograph of IFN treated and HIV -Ada infected human monocytes. Monocytes were infected with HIV- Ada for 5 hr and treated with IFN (0-100 I.U./ml) 7 days after infection. Infected and uninfected monocytes were visualized and photographed. Each treatment has low and high magnification power pictures.

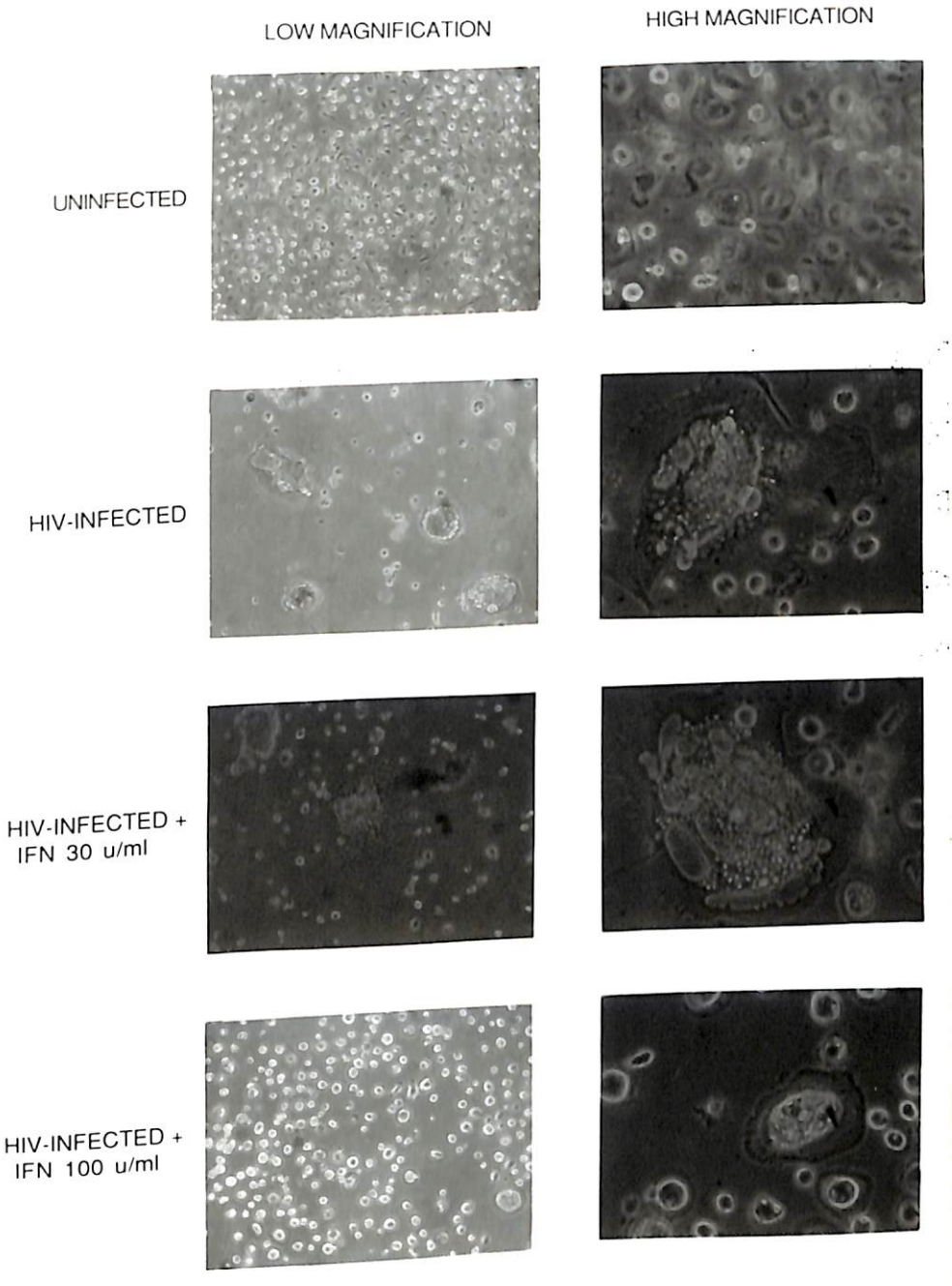

LOW MAGNIFICATION
HIGH MAGNIFICATION
UNINFECTED
HIV-INFECTED
HIV-INFECTED + IFN 30 u/ml
HIV-INFECTED + IFN 100 u/ml

Fig.21: Scanning electron micrograph of IFN treated and HIV-Ada infected human monocytes.

Monocytes were infected with HIV-Ada for 5 hr and treated with IFN (0-100 I.U./ml) 7 days after the infection. The cells were fixed, osmicated, dehydrated in ethanol and processed for scanning electron microscopy.

(A) HIV-Ada infected monocytes; (B) IFN (100 I.U./ml) treated and HIV-Ada infected human monocytes and (C) normal monocytes.

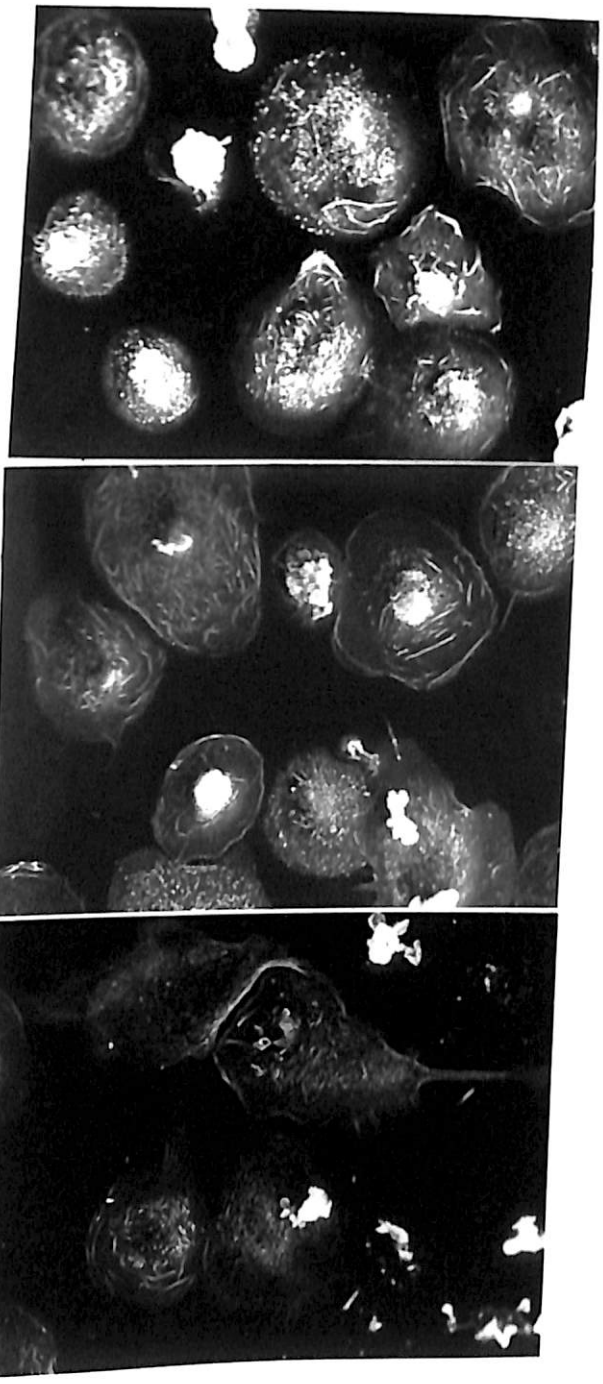
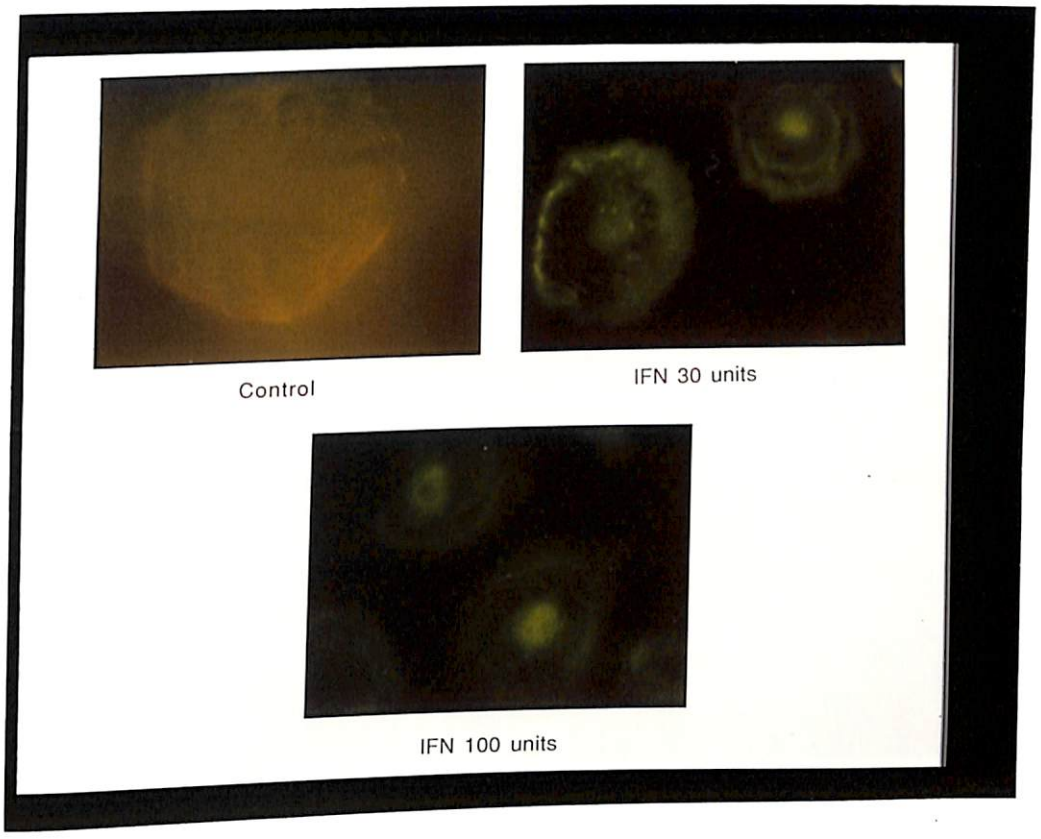

HIV-INFECTED
HIV-INFECTED +
IFN 100 u/ml
UNINFECTED

Fig. 22: Intracellular expression of HIV gp 120

Monocytes were infected with HIV -Ada for 5 hr and treated with IFN (0-100 I.U./ml) 7 days after infection. They were fixed in chilled acetone after 10 days of IFN treatment and were processed for indirect immunofluorescence. Top panel : untreated control , IFN 30 I.U./ml and bottom panel : IFN (100 I.U./ml)


Control
IFN 30 units
IFN 100 units

Calibration of pHi by ratio imaging and Intracellular pHi of Monocytes

The calibration (Fig.23) shows the fluorescence intensity as a function of time as recorded by detector I and detector II during addition of buffers of different pH during the time course. The fluorescence ratio was digitized into channel ratio and was calibrated to pHi using a calibration curve (Fig. 24).

We have investigated the effect of IFN on the intracellular pH in monocytes. Data show that IFN treatment caused an increase in pHi (0.3-0.35 units) compared to untreated monocytes (Fig. 25).

Fig. 23: Fluorescence in detector I and detector II

The fluorescence was detected by detector I and detector II. The shift in fluorescence indicates a change in ratio in IFN treated (upper panel) cells as compared to control cells (lower panel).

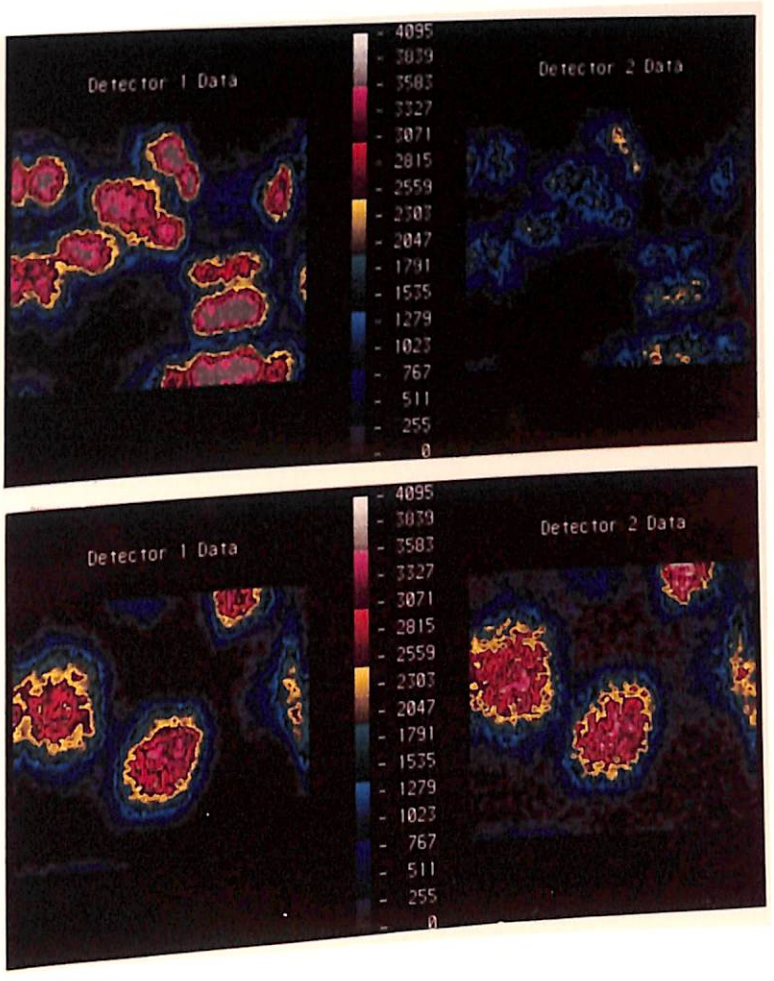

Detector 1 Data
Detector 2 Data
4095
3839
3583
3327
3071
2815
2559
2303
2047
1791
1535
1279
1023
767
511
255
0
Detector 1 Data
Detector 2 Data
4095
3839
3583
3327
3071
2815
2559
2303
2047
1791
1535
1279
1023
767
511
255
0

Fig. 24: Calibration of pHi

Top inset figure, shows fluorescence intensity as a function of time as recorded by detector I (636 nm) and detector II (587nm) during addition of buffer with different pH during the time course. The fluorescence ratio was digitizedinto the channel ratio and then channel ratio was converted to pHi using the calibration curve.

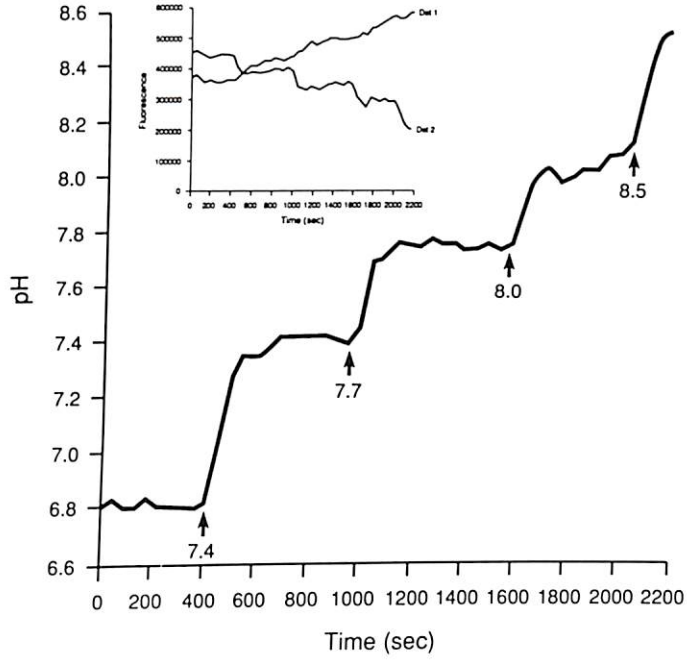
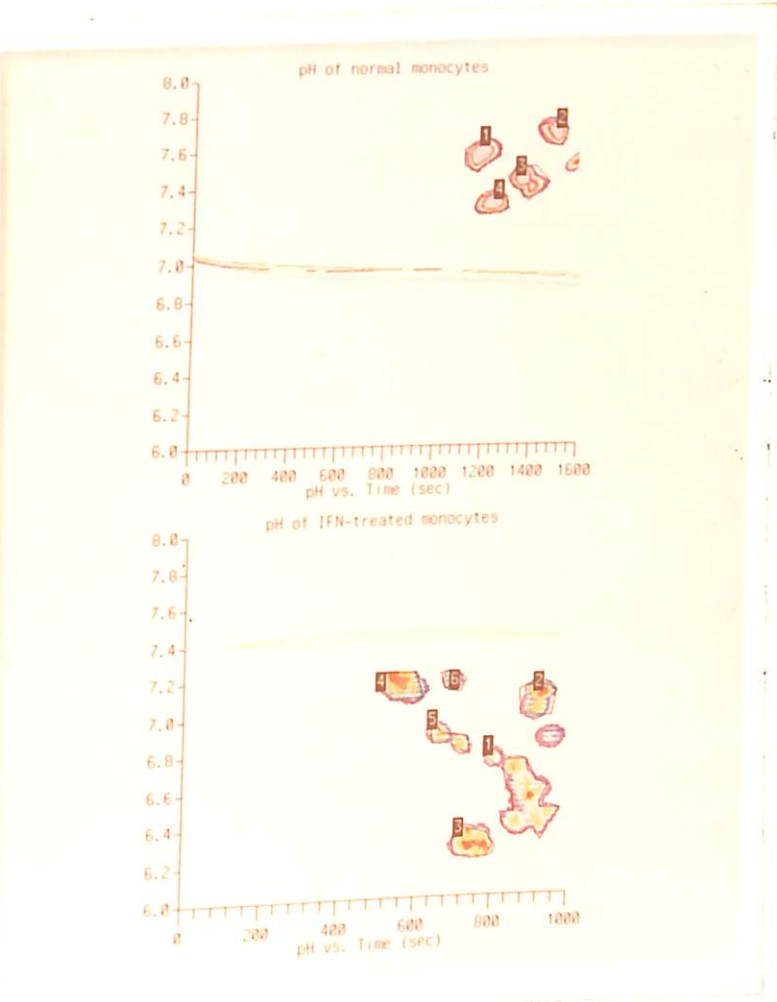

pH
8.6
8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
0 200 400 600 800 1000 1200 1400 1600 1800 2000 2200
Time (sec)
7.4
7.7
8.0
8.5
Fluorescence
Time (sec)

Fig. 25: Effect of IFN on the pHi of monocytes.

Monocytes were treated with IFN for 16 hr. and washed twice with albumin free medium and 10 ml of 1 mM SNARF-1 was loaded within 60 min and unloaded SNARF-1 was removed by washing. Intracellular fluorescence was measured simultaneously at 636 and 587 nm on Meridian Instrument (ACAS 470). The change in emission intensity of these two peaks reflected the alteration in pH.

Top panel; pHi of untreated monocytes, bottom panel : pHi of IFN treated monocytes.The areas selected on right hand side of each panel represent the monocytes which were scanned and the fluorescent ratio averaged to measure the pHi.


pH of normal monocytes
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
6.4
6.2
6.0
0
200
400
600
800
1000
1200
1400
1600
pH vs. Time (sec)
pH of IFN-treated monocytes
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
6.4
6.2
6.0
0
200
400
600
800
1000
pH vs. Time (sec)

VESICULAR STOMATITIS VIRUS

Distribution of DAMP by Gold-Labelling using Electron Microscopy

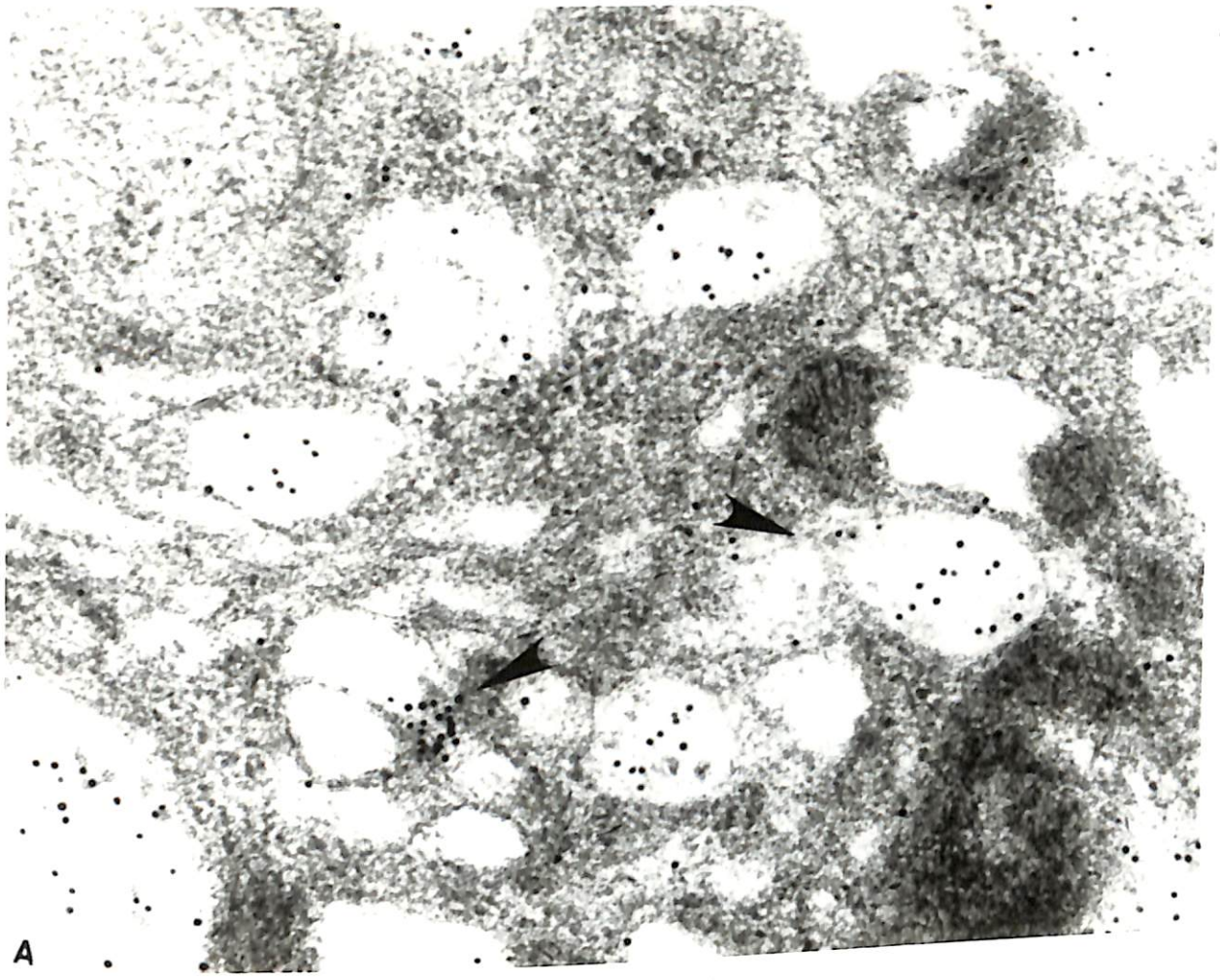
DAMP, a basic congener of DNP, accumulates in acidic intracellular compartments, such as vesicles and cisternae associated with the TGN. To determine the pH of the subcellular organelles, we used DAMP localization by gold labelling. The significant presence of gold particles demonstrated the labelling of the Golgi-complex for DAMP in untreated cells (Fig. 26 A). However, in IFN-treated cells, very little or no labelling of TGN (Fig.26 B) was observed by gold, demonstrating that IFN caused the alkalinization of TGN.

Sensitivity to endo H or Neuraminidase

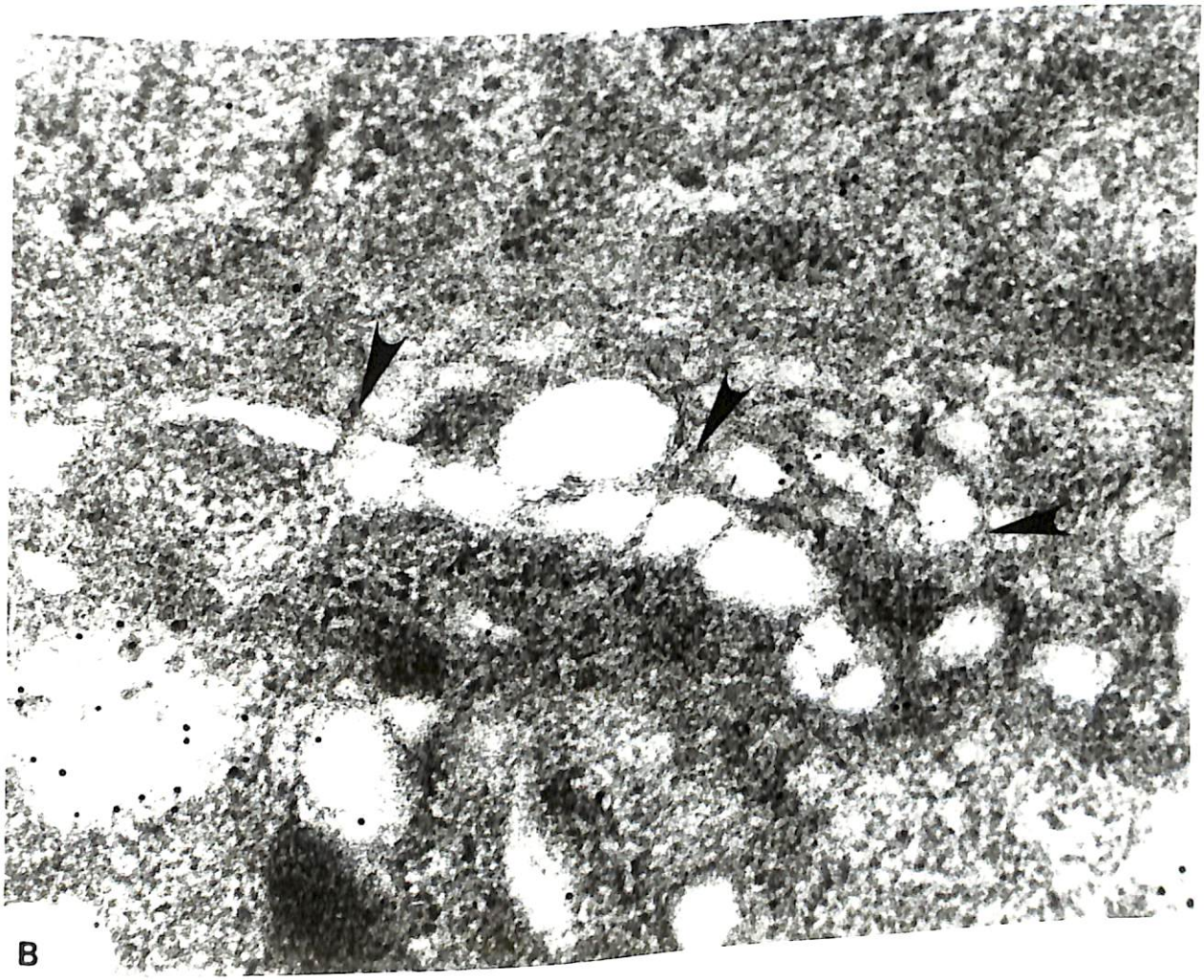
G protein obtained by immunoprecipitation from TGN-enriched fractions from a Nycodenz gradient was subjected to endo H digestion. There was no change in the mobility of G protein after endo H digestion (Fig. 27), demonstrating that the G protein was endo H resistant. Furthermore, the G protein was digested with neuraminidase to examine sialylation of the proteins in TGN and to rule out that endo H resistant peptide may not be associated with medial-Golgi. The mobility of G protein digested with neuraminidase was increased (Fig.27), showing that the G protein was neuraminidase sensitive and was fully sialylated, confirming that G protein was indeed TGN associated.

Fig.26: Localization of DAMP in the Golgi-complex by Gold labelling

IFN-treated (30 I.U./ml) or untreated L_B cells were incubated for 1 hr with DAMP (30 μM) at 37°C. Cells were carefully washed with PBS and fixed with 2% glutaraldehyde. Immunogold staining was performed by incubating the cells with anti-DNP-IgG, washed with cacodylate buffer and then incubated with gold conjugated goat antimouse IgG. Cells were postfixed in 1.5% Osmium tetr-oxide and 0.5% uranyl acetate. Sections were cut and viewed using a JOEL electron microscope. The presence of gold particles demonstrated the labelling of the Golgi-complex for DAMP. (A) Untreated L_B cells (E/M, magnification x 54,000). (B) IFN-treated cells (E/M, magnification x 54,000).



A

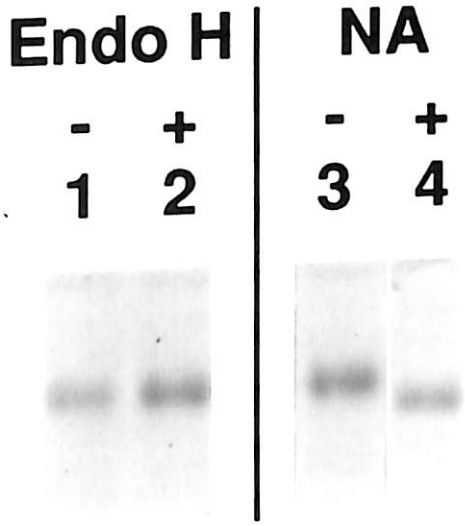


B

Fig.27: Sensitivity of VSV-G to endo H or neuraminidase

TGN enriched fractions obtained from IFN (100 I.U./ml) treated and VSV-infected L_B cells were pooled. VSV-G was immunoprecipitated using a monoclonal antibody to G-protein. G was incubated in the presence and absence of endo H or neuraminidase at 37°C for 20 hr. Samples were run on SDS-PAGE and gels were autoradiographed using Kodak X-ray film.

Lanes 1 and 2 demonstrate that G was endo H resistant. Lanes 3 and 4 showed that the decrease in the mobility was due to sialic acid addition.


Endo H
NA
-
+
-
+
1
2
3
4

Expression of G cDNA in Genomic DNA of Transfected Cells

The immunofluorescence assay showed different levels of expression for G protein in three clones, therefore, these clones were examined for the presence of G cDNA. Genomic DNA was prepared from untransfected and transfected L_B cells and blotted to Nytran filter. The Nytran filter was hybridized to a ^{32}P-labelled G protein cDNA probe. Figure 28 shows that the three clones showed hybridization to the probe, however, the probe hybridized strongly to clone 2 compared to clone 1 and clone 3. Clone 2 was also found to express maximum G protein and therefore, was used in our further studies. Clone 2 cells were grown in 10% EMEM and stocks were frozen in liquid nitrogen.

IFN Blocks the Transport of G protein in Transfected Cells

Transfected cells were thawed and grown in medium containing G418 upto 6 passages. The immunofluorescence studies show that most of the cells express VSV-G protein, indicating that these cells were stably transfected. However, its expression is decreased with the increase number of passages.

Cells were treated with IFN (100 I.U./ml) for 16 hr and the G protein was localized by immunofluorescence. Most of the G protein was localized on the surface of the cells in transfected cells (Fig. 29 B) while in IFN - treated cells (Fig. 29 C) the G was accumulated intracellularly.

Fig.28: Expression of G cDNA in L_B cells

L_B cells were transfected with pSVGL containing cDNA encoding the G protein and pSV2-neo plasmid (10:1) using electroporation method. Transfected cells were grown for 10-14 days in media containing G418 (1 mg/ml) and a single cell from each colony was picked and grown again in G418 containing media. Several clones were screened for the expression of G protein using immunofluorescence. These clones showing the immunofluorescence for G protein were selected and DNA was prepared and blotted to nytran filter. The filter was hybridized to ^{32}P-labelled G cDNA probe prepared using Klenow enzyme. Untransfected L_B cells served as a negative control.

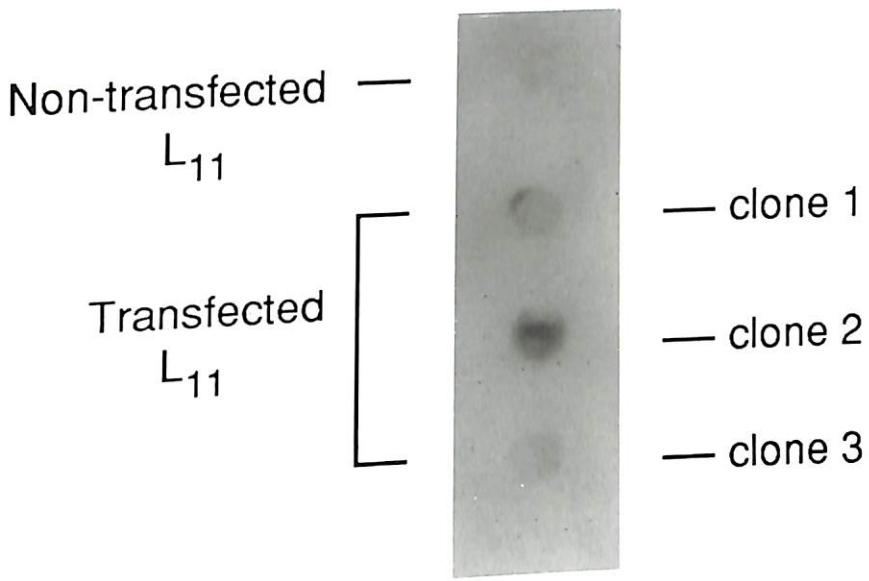
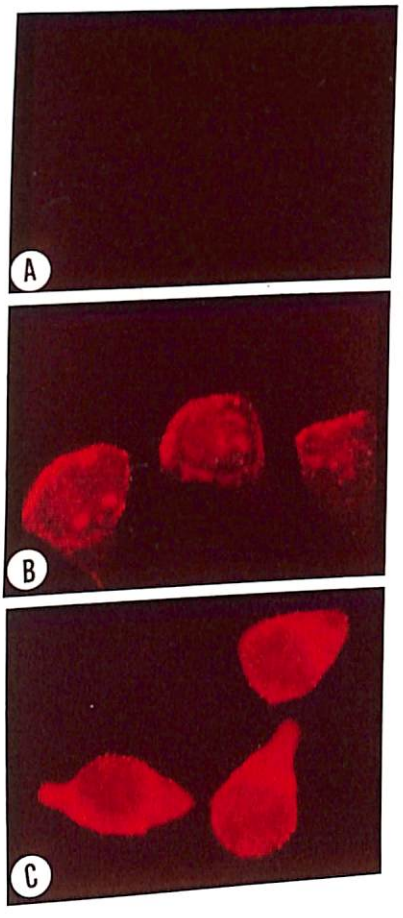

Non-transfected
L_{11}
Transfected
L_{11}
clone 1
clone 2
clone 3

Fig. 29: Block of transport of VSV-G by IFN in G cDNA transfected cells.

Transfected L_B cells were grown on coverslips to 70% confluency and were treated with IFN (100 I.U./ml). After 24 hr cells were fixed in chilled acetone. Indirect immunofluorescence was performed using anti-VSV-G monoclonal antibody (1° Ab) followed by rhodamine conjugated goat antimouse IgG (2° Ab).

A) Untreated cells were incubated with only 2° Ab to check its non-specific binding to the cells; B) Untreated-; C) IFN treated cells.


A
B
C

DISCUSSION

Over the years, a number of antiviral mechanism (s) of IFN have been described, which result in the inhibition of virus directed transcription, methylation of viral RNA, cleavage of CCA terminus of some forms of t-RNA, or the inhibition of virus translation. The IFN mediated block of virus replication occurs at multiple levels and it is at least partially determined by the sensitivity of the cell type, size of virus inoculum, dosage and the time of the treatment of IFN. The inhibition of replication of RNA tumor viruses by IFN was the first reported system in which virus protein synthesis was not the target of IFN action. Instead, IFN treatment seems to inhibit or interfere with the various steps in the final assembly step (Friedman and Pitha, 1984). However, IFN-induced inhibition of the terminal steps in virus replication was not limited to RNA tumor virus. The inhibition of the replication of a variety of membrane viruses which are inhibited by IFN at a late stage in the virus replication cycle can be explained in two major ways: inhibition of virus assembly, release and budding or the combination of a small decrease in the production of virus particles and a much larger reduction in the infectivity of the released virions.

Earlier studies with VSV from our laboratory have shown that IFN treatment inhibited the viral infectivity by 200 fold but the virus particle production was inhibited only 3-5 fold. The reduced infectivity was not due to the fact that defective virus particles were formed but because, the virus released from the IFN treated cells were deficient in glycoprotein G and membrane protein M (Maheshwari et al., 1980;

Drebrot et al., 1984). Following the report on selective inhibition of G-protein in VSV, it was shown that MLV produced from IFN-treated TB cells (Friedman et al., 1980) was also markedly deficient in glycoprotein (gp 69/71). Qualitative and quantitative studies from our laboratory have shown that IFN treatment inhibits the transport of VSV-G from Golgi-complex to the plasma membrane (Singh et al., 1988). Recent studies using sucrose and Nycodenz gradient subcellular fractionation gradient have shown that most of the VSV-G from IFN treated cells accumulates in TGN enriched fractions (Maheshwari et al., 1991).

We were interested to look at this inhibitory effect of IFN on the growth of other membrane associated viruses. Therefore, in the present study, we have investigated the molecular mechanism (s) of IFN-induced inhibition of assembly steps of HSV-1 and HIV-Ada replication.

It has been reported that IFN-α has no significant effect on the HSV-1 polypeptide synthesis or on assembly, but the IFN treated cells released non infectious virus particles (Munoz and Carrasco., 1984). Whereas in another study, cloned human IFN α_{2a} and IFN -β blocked the HSV-1 replication at a late stage of virus replication cycle specifically at the level of virus core exit from the nucleus. Further, no extracellular virus particles were released although assembly of nucleocapsids inside the IFN treated cell was seen by electron microscopy. The synthesis of gD, gB and gE proteins was shown to be drastically reduced in IFN treated monkey kidney cells, human fibroblast and neuroblastoma cells; however, no significant inhibitory effect on the synthesis of major nucleocapsid protein was

seen (Chatterjee et al., 1985, 1989, 1990). The inhibition of immediate early (α) polypeptide biosynthesis and a subsequent reduction of early (β) and late (γ) gene product has been reported in IFN treated cells but the amount of IFN used for the experiment was very high (1000 I.U./ml) (Gloger and Panet, 1984). In contrast, we show here that physiological doses of IFN block the HSV-1 at a late stage(s) of virus replication cycle. The IFN treated cells do not show major difference in the virus protein profile as compared to untreated cells. The presence of ICP4, ICP6 and other virus specific proteins indicates that virus was not inhibited at penetration or any other early stage before viral DNA synthesis. IFN (10-100 I.U./ml) treatment inhibited the infectivity of HSV-1(5-100 fold) without any significant inhibition in the synthesis of virus specific protein in IFN treated cells. Lesser number of plaques were formed in IFN treated cells as compared to control which goes consistent with the viral infectivity data. The electron microscopic studies showed the presence of assembled HSV core inside the nuclei of both the IFN treated and untreated cells but numerous extracellular particles were seen in the untreated cells indicating that IFN has probably blocked the release of assembled virions. Earlier studies have shown that the extracellular particles released from IFN treated neuroblastoma cells lacked gD protein which was implicated to the structural changes in the gD molecule in IFN treated cells (Chatterjee et al., 1990).

IFN -α has also been shown to inhibit HIV -1 replication (Ho et al., 1985) and as a result has been used clinically for the treatment of HIV infection. The trials of exogenous IFN -α in the early HIV disease showed a significant reduction in the

level of p24 antigen in the plasma of the treated subjects, and fewer AIDS - associated opportunistic infections (Lane et al., 1988; Edlin et al., 1992). The mechanism(s) of the anti-HIV activity of IFN is not fully understood but a number of studies have suggested that IFN inhibits the HIV replication at a number of levels from the early step of HIV replication (Kornbluth et al., 1989) to proviral DNA formation or integration (Shirazi and Pitha, 1992, 1993). In other studies it has been shown to inhibit HIV replication by inhibiting translation (Edery et al., 1989; Sengupta et al., 1990) and formation of certain NF-κB dimers which bind to the rev responsive element and thereby block rev function (Popik and Pitha, 1992). Inhibition of release of RT and viral antigen into the culture supernatant by IFN after phorbol ester stimulation of U1(Promonocytic) and ACH-2 (T-lymphocytic) cell lines which are chronically infected with HIV -1 and constitutively express low level of virus has also been reported. Further IFN -α inhibited the production or release of whole HIV virion as compared to control but no effect was observed in cell associated viral protein (Poli et al., 1989). Infection of monocytic U937 cells with the HIV-LAV isolate resulted in the establishment of two types of chronically infected cells, producing infectious virus (Coccia et al., 1992). In the first, 2-3 fold higher levels of p24 were found in cells treated with IFN-α due to defect in virus release; in the second, IFN -α treatment resulted in the specific reduction of HIV -1 proteins without any apparent reduction in cellular proteins. Recently, it has been reported that IFN-α treatment caused a defect in the incorporation of gp120 into mature virus particle in T cells; this could account for less infectivity, but there was only 3 fold inhibition of

HIV in lymphocytes treated with IFN (Hansen et al., 1992). Previous studies reported a dramatic inhibition of HIV replication in monocytes treated with IFN-α (more than 500 I.U./ml) either at the time of virus challenge or several days before HIV infection (Gendelman et al.,1990). In contrast, in our studies physiological doses of IFN -α (10 -100 I.U. /ml) added 7 days after HIV infection of monocytes, did not significantly inhibit HIV protein synthesis as demonstrated by the level of p24; however, RT activity was decreased 5- 50 fold within 20 days indicating an inhibition in the production of infectious virus particles. Light and scanning EM studies also indicate that IFN inhibits the formation of multinucleated giant cells and the HIV assembly leading to a drastic inhibition of particle formation.

Biochemical and immunocytochemical observations have shown that both apically and basolaterally bound newly synthesized glycoproteins traverse the same intracellular pathway from endoplasmic reticulum (ER) to the Golgi complex. Protein from rough ER travel in a vectorial fashion through cis, medial and TGN for post translational processing and are sorted in specialized secretory vesicles which fuse with the cell surface. Movement of proteins between these compartments occurs by the budding and fusion of transporting vesicles (Rothman, 1994; Rothman and Orci, 1992). The vesicle budding (carrier of newly synthesized protein) from TGN is driven by a coordinated assembly of cytoplasmic components and membrane subunits such as ADP- ribosylation factor (ARF), GTP, coatmer protein and acyl CoA. The assembly of the coat is initiated by activation of ARF (or closely

related proteins) by GTP binding. ARF is required for the budding of secretory vesicles and constitutive transport vesicles from the TGN (Fig.30). The bud pinches off in a process involving periplasmic fusion requires the addition of acyl CoA and is stimulated by ATP and moves to the plasma membrane. In the absence of acyl-CoA, coated bud accumulates. Thus the Golgi complex plays an important role in protein sorting and trafficking to the cell surface. In case of HSV-1 the glycoprotein processing may be important for its assembly, budding and expression of the protein on the infected cell surface (Chatterjee et al., 1989; Gompets and Minsion, 1986).

Several studies have shown that BFA blocked the transport of HSV-1 glycoproteins and prevented the migration of viral particles from the nuclear membrane to the cell surface (Cheung et al., 1991; Chatterjee et al., 1992). Similar effects of BFA have been shown on the transport of VSV-G protein from ER to Golgi complex (Domes et al., 1989; Misumi et al., 1986 Takatsuki et al., 1985). HIV -1 gp 160 has also been shown to bind CD_4 with high affinity in the ER thereby preventing the delivery of CD_4 to the plasma membrane (Kawamura et al., 1989; Crise et al., 1990; Jabbar and Nayak, 1990; Bour et al., 1991; Willey et al., 1992 a). The viral proteins expressed in cDNA transfected cells in the absence of the other viral component or viral infection are being recognized, segregated and delivered to the plasma membrane by an endogenous cellular sorting apparatus. Recently penv AU3 plasmid containing a DNA fragment of the tat rev and env genes of HIV -1 was transfected into the Hela and Jurkat cells. The stable transformants

express gp160, which is fully glycosylated, cleaved to gp120 and gp 40, and transported to the plasma membrane(Gama Sosa et al., 1989).

In the present study, we have used HSV-1 gD cDNA transfected LMtk$^-$ and VSV-G G cDNA transfected L_B cells to study the block in the transport of gD and G protein by IFN in a virus free cell system. Fluorescence and biochemical studies indicate that the gD transfected LMtk$^-$ cells constitutively express gD protein whereas expression of VSV-G protein was transient and lasted for 4-5 passages. In this study inhibition of virus growth does not seem to be due to an inhibition of protein synthesis since virus protein synthesis is marginally inhibited. We, therefore, studied the effect of IFN on the transport of HSV-1 gD and HIV -Ada gp120 glycoproteins, by indirect immunofluorescence. HSV-1 gD protein accumulated intracellularly as compared to control cells where most of the protein was localized on the cell surface. IFN caused a similar block in the transport of gD in LMtk$^-$ and VSV- G in pSVGL transfected cells in the absence of the viral component. However, higher doses of IFN were required for the accumulation of gD in the transfected LMtk$^-$ or pSVGL cells as compared to L_B cells infected with HSV -1, but the overall effect in both the cases was found to be same. In IFN treated, infected monocytes most of HIV gp120 accumulated intracellularly, as compared to untreated monocytes, where most of the gp120 could be localized on the cell surface. Thus the effect of IFN on HIV-1 was similar to that on HSV-1 and other viruses. Earlier studies have indicated that IFN inhibits the transport of VSV-G from Golgi complex to the plasma membrane and most of the G protein was associated with TGN (Singh et al., 1988) These observations were supported by those of

Panigrahi and Mohanty (1989) showing that IFN caused defective transport of hemagglutinin-neuraminidase glycoprotein of bovine PIV-3 in bovine turbinate cells. Recently Hansen et al, (1992) have shown that there is a marked accumulation of HIV glycoprotein gp120 in IFN α (500 I.U./ml) treated T- cells as compared to untreated cells leading to production of virions deficient in gp120. WGA bound to the Golgi complex was used to determine whether gD remained associated with Golgi complex. HSV-1gD protein was co-localized with WGA in the cytoplasmic structures in IFN treated LMtk$^-$ cells as compared to untreated cells, where bulk of the gD appeared to be localized on the plasma membrane. The co-localization of gD and WGA expression suggested that IFN blocked the transport of gD protein transport within the Golgi complex. There was 3.89 fold more accumulation of gD protein in IFN treated cells. However, when the different organelles were separated and analyzed for the expression of gD protein, the difference was much more. The gD could be seen localized in Golgi membrane fractions when treated with IFN whereas there was no significant gD protein in the Golgi fraction of control cells. Earlier IFN has been shown to block the transport of VSV-G in the Golgi complex in VSV infected Hela cells (Maheshwari et al., 1991). The Nycodenz fractions obtained from earlier studies when subjected to endo H digestion has shown no change in the mobility of G, but when G was digested with neuraminidase, there was an increased mobility of G-protein, thereby confirming the addition of sialic acid to the protein in TGN.

Endocytosis which involves the delivery of an extracellular macromolecule to a different intracellular site and exocytosis which involves transporting newly synthesized protein out of the cell use a series of vesicles. These vesicles undergo various changes during the transport and contain membrane receptors. The luminal of these vesicles are often maintained at an acidic pH. Several studies have confirmed that endosome, lysosome and Golgi complex are normally acidic and have a pH of 4.5-6.5 (Tycho and Maxfield, 1982; Galloway et al., 1983). This pH is maintained by an ATP dependent proton pump (Yamashiro et al., 1983; Stone et al., 1983; Schneider et al., 1981). Any alteration or disruption of this pH gradient interferes with the normal functions of vesicles (Basu et al., 1981). Primary amines such as ammonium chloride (NH_4Cl), CHL, dansyl cadaverine (DCA) and methylamine are weak bases and acidotropic agents (Seglen, 1983; Ohkuma and Poole, 1978); and interrupt recycling of receptors by inhibiting the intracellular transport of secretory and membrane proteins to the plasma membrane by raising the pH of acidic Golgi bodies, (Seglan, 1983; Maltin, 1986). NH_4Cl has been shown to inhibit the multiplication of HSV -1 at a late stage of virus infection (Helenius et al., 1980). CHL has been shown to inhibit the multiplication of Semliki forest virus and HSV (Koyama and Uchida, 1989). The expression of VSV -G at the cell surface during final stages of G assembly is prevented in CHL treated cells (Dille and Johnson, 1982). Earlier Maheshwari et al., (1991) have shown that IFN and primary amines raise the intracellular pH in Hela and L_B cells. In the present study using SNARF -1, we have demonstrated that IFN raises the pH of monocytes. We also used a fluorescent probe DAMP which is a basic congener of dinitro phenol (DNP)

that concentrates in acidic compartments. These studies carried out on LMtk$^-$ cells clearly indicate that IFN treatment reduced the localization of this compound, suggesting that IFN raises the pH of LMtk$^-$ cells.

The Golgi apparatus plays an important role in exocytic pathway. This pathway is altered by monesin, an ionophore that disrupts the proton pump gradient (Ledger and Tanzer, 1984; Tartakoff, 1983; Boss et al., 1984). The presence of proton pump has been reported in the Golgi complex (Zhang and Schneider, 1983; Glickman et al., 1983) isolated from rat liver. Pierre Cosson et al., (1989) have indicated that acidification of the cytosol to a pH lesser than 6.8 inhibits the reversible membrane transport in exocytic and endocytic pathway. Another study has demonstrated that in Madin-Darby Canine Kidney (MDCK) cells transfected with a recombinant chicken lysozyme gene at a lower cytoplasmic pH of 5.8 the secretion of gp80 was retarded and the transport of lysozyme was completely inhibited in ER (Pilansky and Chaudia-Koch-Brandt, 1992). In the present study, we used EM and gold labelled DNP-IgG to localize DAMP distribution in TGN, in IFN treated or untreated cells as previous reports show TGN to be the most acidic compartment in the cell (Anderson and Pathak, 1985). The presence of gold particles in the TGN of the untreated cell showed TGN to be an acidic compartment, whereas, there was not very significant labelling in the TGN as compared to other organelles in IFN treated cells, indicating that IFN -caused the alkalinization of theTGN. Earlier studies involving the kinetics have reported that treatment of cells with IFN for 9-16 hr is required to give an optimal increase in pHi which correlates with the anti VSV activity of IFN (Maheshwari et al., 1991). Protein kinase C (PKC)

is a known activator of Na^+/H^+ antiporter system (Pouyssegur et al., 1988) and there are reports that human IFNs activate the translocation of PKC to the plasma membrane (Pfeffer & Reich, 1989). Maheshwari et al., (1991) have shown an increase in Na^+ in cells treated with IFN and have suggested the possible role of Na^+/H^+ antiporter system in this pH change. Similar effect has been shown for IFN -γ in murine macrophage, where rapid influx of Na^+ was shown to be initiated by exchange of Na^+ and H^+ by means of N^+/H^+ antiporter system.

A variety of ligands bind to the cell surface and receptors are internalized by receptor mediated endocytosis (Brown et al., 1983; Helinius et al., 1983; Pastan and Willingham, 1983) at a neutral pH. Transferrin, a major iron transport protein is internalized by receptor mediated endocytosis (Hemmaplardh and Morgan, 1977; Octave et al., 1981; Karin and Mintz, 1981). It has been demonstrated that binding of iron atom to transferrin is pH dependent and it occurs at a pH lesser than 5. Weak bases have also been shown to inhibit the intracellular transport and facilitate accumulation of iron (Morgan, 1981; Octave et al., 1982). Using specific inhibitors of the vacuolar proton pump, it has been recently shown that endosome acidification is necessary to maintain rapid recycling of intracellular receptors back to the plasma membrane (Johnson et al., 1993). These findings were supported with another observation indicating that inactivation of proton pump leads to the accumulation of TGN 38 / 41 (Chapman & Munro, 1994; Reaves & Bantiny, 1994).

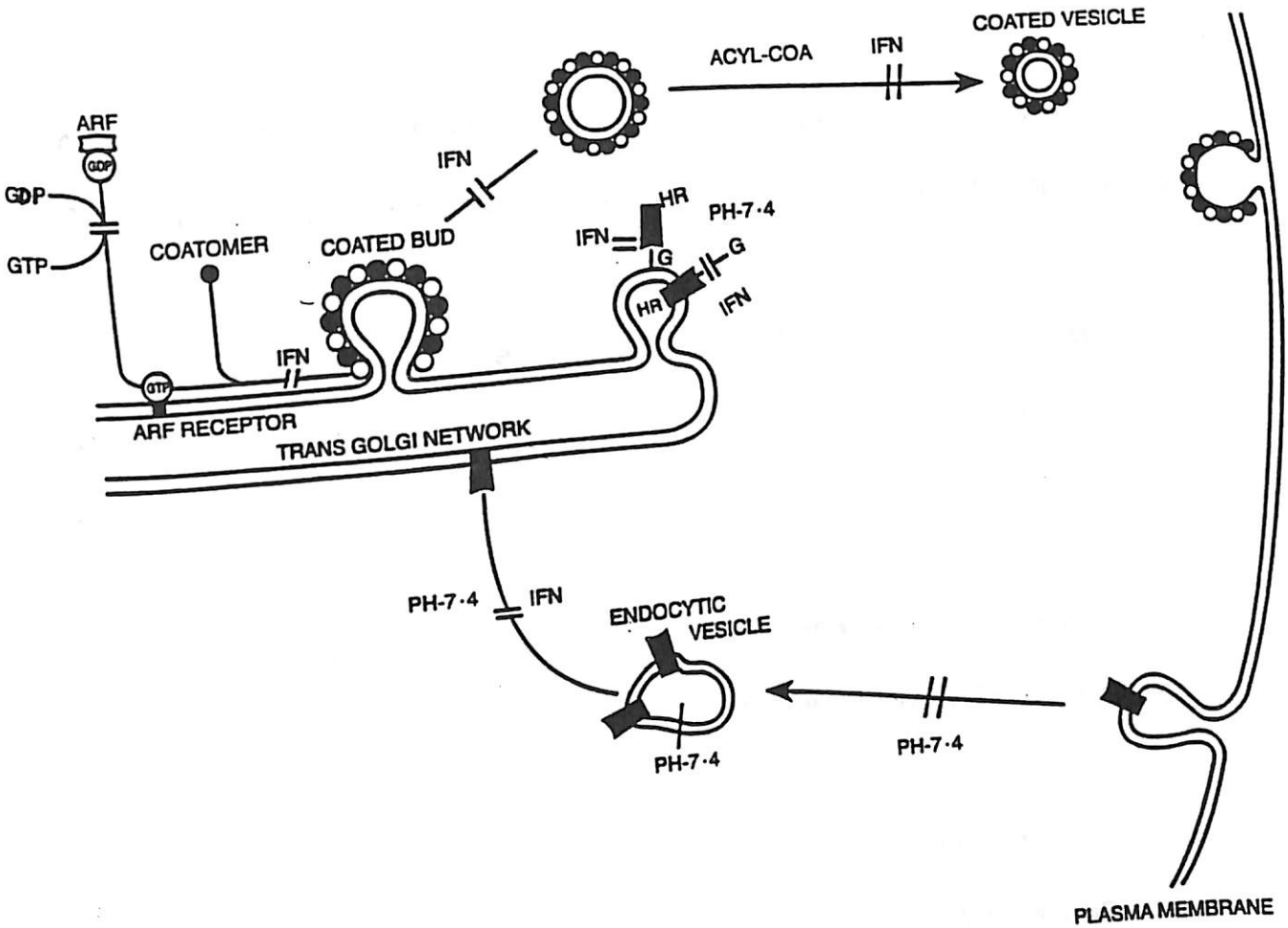
These studies suggest that an acidic environment could be important for the transport of HSV-1 gD, VSV-G and HIV -1 gp120. If the association of ligand and

receptor is prevented by IFN due to increase in pH then (i) The receptor- ligand complex might not reach the targeted vesicles, (ii) The receptor-ligand complex may be trapped in the targeting vesicles, thus effectively interrupting receptor recycling or (iii) the receptors that are normally degraded rather then recycled could be protected against the degradation. IFN may, therefore have acted in these cells by raising the pH in the TGN, thus inhibiting the transport of HSV-1 gD, VSV-G and HIV-1 gp120 to the plasma membrane. This might lead either to the inhibition of virus assembly or release as shown with HSV-1 and HIV -1 or to the production of VSV particles of low infectivity deficient in G protein. This IFN induced increase in pHi of the TGN may also explain the inhibitory effect of IFN reported on the terminal steps of other enveloped viruses including MLVs, MMTVs, PIV-3 and vaccinia virus. This mechanism of IFN action may help in the establishment of latency during virus infection.

The work presented here was designed to investigate the mechanism of IFN induced inhibition of HSV-1, HIV-1, and VSV. I hope that the data emerging from this work will be of use in the pursuit of a better understanding of the transport and trafficking of viral glycoproteins. The role of V-ATPases and ligand-receptor interaction upon IFN treatment might reveal some unanswered secret that drives the imagination of all of us.

Fig. 30: A schematic model of the proposed mechanism of the block in the transport.

Proteins are transported from ER to cis, medial, TGN via transport vesicles. We hypothesize that IFN can interfere with any of the factors important in the secretory pathway of proteins thereby accumulating protein in TGN. The luminal of these vesicles and TGN, which are normally acidic becomes basic when treated with IFN thereby interrupting the normal pH gradient. We suggest that the defective acidification of the vacuolar ATPase of TGN including endosome and surrounding vesicles could possibly lead to the following(s) (i) The receptor-ligand might not reach the targeted vesicles (ii) receptor-ligand may be trapped in the targeting vesicles thus effectively interrupting receptor recycling (iii) receptor usually degraded rather than recycled could be protected against such degradation. This might explain the block of transport at the TGN and subsequent reduction in the release of infectious virus particles.


COATED VESICLE
ACYL-COA
IFN
ARF
GDP
GDP
GTP
IFN
COATOMER
COATED BUD
IFN
HR
PH-7·4
G
G
HR
IFN
IFN
GTP
ARF RECEPTOR
TRANS GOLGI NETWORK
PH-7·4
IFN
ENDOCYTIC
VESICLE
PH-7·4
PH-7·4
PLASMA MEMBRANE

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