Structure and Function studies of the C-terminus of the Human Interferon Gamma

by

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STRUCTURE AND FUNCTION STUDIES OF THE C-TERMINUS OF THE HUMAN INTERFERON GAMMA

BY

ANITA YUK-CHING KWOK

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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Abstract

HuIFN-γ is a multifunctional protein that elicits a number of cellular responses. In the present study, by characterizing the neutralizing monoclonal antibody (mAb), a structure-function study has been performed on the C-terminus of the HuIFN-γ in an attempt to identify any functional domain(s) not previously identified on this pleiotropic molecule. A new antiviral neutralizing mAb MIF3037 which was found to bind to the C-terminus of the HuIFN-γ molecule was identified. The immuno-crossreactivity of this mAb was tested in a competition assay against three previously identified immuno-reactivity groups (E_1, E_1/E_2, E_2) representing two distinct functional epitopes. An additional mAb MIF3125 that was distinct from E_1 but appeared to be partially related to E_2 was also included in this study. Since MIF3037 demonstrated no binding competition with any of the known specificity groups, its epitope must represent a new antiviral epitope. This epitope was named E_3. The failure of MIF3037 to bind to Del-122 which lacked the C-terminal 21 residues allowed the localization of the E_3 to that 21 residue region. This has since been confirmed by specific mapping of E_3 to residue 130-138 (Zu and Jay). They have shown that nuclear localization signal (NLS) like elements are present in both of these epitopes. Furthermore, mAb against E_1 and E_3 neutralize the antiviral activity of HuIFN-γ synergistically. In this study, the Del-122 which lacked the E_3 required 3 times as long to induce an antiviral state (AVS) than HuIFN-γ. Thus having both signals is more efficient in the induction of AVS. This finding is also consistent with the speculation that E_1 and E_3 function as NLS because the efficiency of nuclear translocation is known to be enhanced by multiple NLS on the molecule.

The involvement of the 3 antiviral domains in other IFN-γ functions was also tested. Since none of the mAbs directed against E_1, E_2 and E_3, respectively, was able to neutralize

the antichlamydial activity of HuIFN-γ, the antichlamydial activity must depend on different functional domain of the HuIFN-γ and induced a different cellular pathway. The fact that Del-122 lacks the antichlamydial activity suggests that the functional domain is located within the C-terminus residue 123 - 143. Since MIF3037 which binds to 130 - 138 is unable to inhibit the antichlamydial activity of HuIFN-γ, it is likely not the functional site. The possible locations of the antichlamydial domain are the areas that overlap residue 123 - 129 or 139 - 143.

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List of Abbreviations

aa :	amino acid
AMP :	adenosine 5'-diphosphate
ATP :	adenosine 5'-triphosphate
BSA :	bovine serum albumin
CPE :	cytopathic effect
cpm :	counts per minute
C. trachomatis :	chlamydia trachomatis
CR :	competition ratio: (EU_{50} of test)/(EU_{50} of control)
Del-122 :	deletion variant of recombinant human interferon gamma with 21 residues deleted from the C-terminus
DNA :	deoxyribonucleic acid
dsDNA :	double stranded Deoxyribonucleic acid
E_1 :	antiviral neutralizing eptiope 1
E_2 :	antiviral neutralizing eptiope 2
E_3 :	antiviral neutralizing epitope 3
EB :	elementary body
E. coli :	Escherichia coli
ELISA :	Enzyme linked immunosorbent assay
eIF-2 :	initiation factor 2 for protein synthesis
EMC :	encephalomyocarditis
EMEM :	eagle minimal essential medium
EU :	ELISA units
EU_{50} :	ELISA units of test mAb required to inhibit the binding of 10ng 125-I-mAb probe by 50%
FCS :	fetal calf serum
Fig. :	figure
g :	gram
GAF :	gamma-activating factor
GAS :	gamma-activated site
GBP :	guanylate-binding protein
GDP :	guanosine 5'-diphosphate
GEP :	guanine nucleotide exchange factor
GTP :	guanosine 5'-triphosphate
HBSS :	Hanks balanced salt solution

HLA :	human leukocyte antigen
hr :	hour
HuIFN :	human interferon
IFN :	interferon
IFN-α :	interferon-alpha
IFN-β :	interferon-beta
IFN-γ :	interferon-gamma
IFU/ml :	inclusion forming unit per ml
Ig :	immunoglobulin
IL-1 :	interleukin-1
IL-1R :	interleukin-1 receptor
Iodogen :	1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril
	isopropylthiogalactoside
IRE :	interferon gene regulatory element
ISG :	interferon-α-stimulated gene
ISGF3 :	interferon-stimulated gene factor 3
ISRE :	interferon-stimulated responsive element
M :	molar
mAb :	monoclonal antibody
MAF :	macrophage activating factor
MHC :	major histocompatibility complex
MIF :	monoclonal antibody against human interferon-gamma
ml :	milliliter
mM :	millimolar
min :	minute
muIFN-γ :	murine interferon-gamma
NAV :	neutralization of antiviral titre
NK :	natural killer cells
NLS :	nuclear localization signal
nm :	nanometer
NRDI :	negative regulatory domain 1
OD :	optical density
PBS :	phosphate buffered saline
pfu :	plaque forming unit
PK :	protein kinase
PRDI :	positive regulatory factor
PRDI-BF :	PRDI binding factor
PRDII :	positive regulatory domain 2

rHuIFN-γ :	recombinant human interferon-gamma
RNA :	ribonucleic acid
RAase :	ribonuclease
RB :	reticulate body
RRE :	Rev-responsive element
SDS :	sodium dodecyl sulfate
SDS-PAGE :	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sELISA :	sandwich ELISA
SPG :	sucrose-phosphate-glutamate buffer
sRIA :	competition sandwich radioimmunoassay
SV40 :	simian virus 40
TCA :	trichloroacetic acid
trp :	tryptophan
Tween 20 :	polyoxyethylene-sorbitan monolaurate
^{125}I :	iodine-125
°C :	degrees centigrade
ug :	microgram
ul :	microliter
um :	micrometer

CHAPTER I

INTRODUCTION

Interferon was discovered by Issacs and Lindenmann (1957) during their study on the phenomenon of viral interference. They showed that when heat-inactivated influenza was incubated with embryonic chick cells, a new inhibitor was found in the culture medium that "interfered" with the virus replication and termed the substance interferon. At present, interferon is regarded as a cytokine, an agent used for transmitting regulatory signals pertaining to cell proliferation and differentiation, and as a lymphokine - a substance with immunoregulatory functions. Further studies of this molecule identified a number of other functions. In fact, the biological effects elicited by interferon are very complex. They include anti-viral function (Wheelock, 1965), inhibition of cell proliferation and anti-tumor activity (Clemens, 1985), immunoregulatory functions such as enhanced expression of histocompatibility antigens (Wong et al., 1985), natural killer-cell activity of lymphocytes (Platsoncas, 1984), immunoglobulin production and class switching regulation (Finkelman et al., 1988) and activation of human monocyte cytotoxicity (Nathan et al., 1983).

Based on antigenic specificities, at least three types of interferon have been identified - alpha, beta and gamma. Both IFN-α, primarily produced in leukocytes and IFN-β, predominantly synthesized in fibroblasts are classified as "Type I interferon" whereas IFN-γ, produced by lymphocytes in response to mitogens and specific antigens, is termed as "immune interferon" or "Type II interferon". Table 1 shows the classification and characteristics of different types of human interferon (Joklik, 1990). Among the three species of interferons, IFN-γ is found to be the most potent in antiproliferative and immunoregulatory activities (Rubin & Gupta, 1980, Claeys et al., 1982).

All interferon proteins have precursor polypeptides of 166 amino acid residues and possess N-terminal signal sequences 20 - 23 residues. The sizes of the mature IFN-α, IFN-β

Table 1. Classification and characteristics of human interferon
(Adapted from Joklik, 1990)

Species	IFN-α	IFN-β	IFN-γ
Classified as	Type I	Type I	Type II or Immune
Production in	Leukocytes	Fibroblasts	Lymphocytes
Inducing agent	Viruses dsRNA	Viruses dsRNA	Mitogens Specific antigens
Stability at pH2	Stable	Stable	Acid-labile
Molecular weight	16,500-25,000	20,000	17,000
Isoelectric pt.	5.5-6.6	6.8-7.8	8.6-8.7
Glycosylation	Some	All	All
Length of signal sequence	23	21	23
Primary protein size	166	166	166
Actual protein size	143	145	143
Number of species	>15	1	1
Presence of introns	None	None	3
Chromosome location	9	9	12

and IFN-γ are 143kDal, 145kDal and 143kDal, respectively (Sehgal, 1982; Rinderknecht, 1984). HuIFN-β and HuIFN-γ are glycoproteins (Dorner et al., 1973; Fujisawa & Kawade, 1981) but only some HuIFN-α are glycosylated. Glycosylation is found to be not essential for biological activity (Fujisawa & Kawade, 1981; Havell & Carter, 1981; Knight & Fahey, 1982) which explains why recombinant interferons produced in prokaryotes are as active as natural molecules. There are a number of differences between type I and type II interferons. IFN-γ is distinguished from IFN-α and IFN-β by : i) synthesis in specific cell types and induced by distinct types of agents; ii) inactivated at pH2; iii) little sequence homology; iv) different chromosomal location; v) presence of introns and vi) immunoregulatory and enhanced antiproliferative effects.

1. Induction of interferon

Normal cells do not contain or produce interferon. However, a variety of inducers including double-stranded RNA (dsRNA), virus infection and several metabolic activators and inhibitors are known to induce IFN production by target cells.

The mechanism of how inducers activate the transcription of interferon genes is best examplified by studying the regulation of IFN-β gene expression. The human IFN-β gene expression is induced by virus infections or double stranded RNA such as poly(I).poly(C). Protein synthesis is not required in this induction . A sequence between residues -77 and -37 upstream of the cap site of IFN-β gene, named as the interferon gene regulatory element (IRE), was found to play an important role in transcriptional control (Goodbourn et al., 1985; 1986; Goodbourn and Maniatis, 1988). It consists of three components : two positive regulatory domains (PRDI and PRDII) and a negative regulatory domain (NRDI). The

former has sequence positions between -77 and -55 whereas the latter has sequence position between -63 and -37. Figure 1 shows a simplified diagram of the organisation and sequence positions of the three regulatory domains within the IRE. By means of DNAase I footprinting, Zinn and Maniatis (1986) demonstrated the interaction of some cellular factors with these human β-IFN regulatory regions. They suggest those proteins that bind prior to induction are repressor molecules, whereas another protein that binds after induction is a transcription factor. As a result, the β-IFN gene may be controlled by a negative regulatory mechanism i.e. upon induction, the repressors are dissociated from the DNA binding regions, thereby allowing the transcription factor to bind, which activates transcription. Further studies have indicated that in the normal state, a repressor binds to NRDI and blocks the binding of other transcription factors to the positive regulatory regions. Upon induction, the repressor is removed and two modified proteins PRDI-BFc and PRDII-BF bind to the previously blocked sites PRDI and PRDII, respectively, thus activating transcription. It is likely that the removal of the repressor is due to direct inactivation or displacement by the two modified transcription factors (Keller and Maniatis, 1988). The fact that production of IFN does not require de novo protein synthesis also indicates that the IFN gene is repressed by a labile repressor in normal cell conditions. Furthermore, the induction not only leads to activation of IFN gene transcription, but also the synthesis of biologically active IFNs (Collins, J, 1984; Riordan & Pitha, 1985).

1.1 Induction by virus infection

For most virus infections, synthesis of interferon starts at about 4 hour post-infection, then reaches to peak level when the rate of viral protein synthesis is maximum and gradually

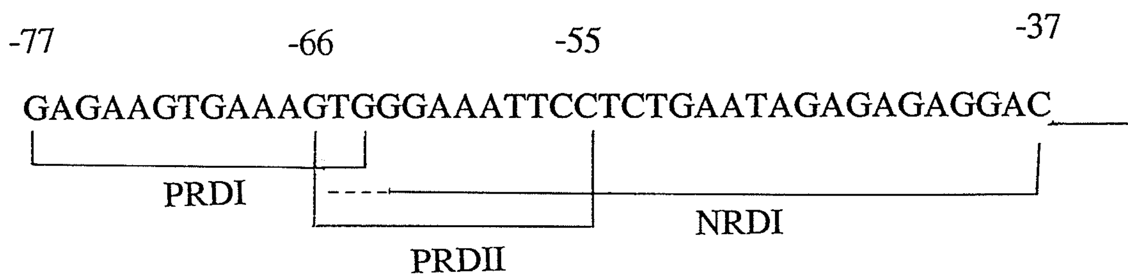

Figure 1. Organisation and sequence positions within the IRE of the human β-IFN (Adapted from Keller and Maniatis, 1988) The sequence between positions -37 and -77 of the human β-interferon gene is shown. The positive regulatory domains (PRDI and PRDII) and the negative regulatory domain (NRDI) are indicated.

declines. Hence the kinetics of interferon production appears to be similar for most types of infections. Induction of interferon is not only limited to those that mount a lytic infection (e.g. reovirus in fish) but also viruses lacking the ability to replicate including inactivated viruses (e.g. heat-inactivated influenza virus). Interferon is induced when the host-cell protein synthesis is inhibited by viral infections. At this stage of infection, the labile interferon repressors are replaced at a reduced level that allows sufficient transcriptional activators to bind to PRDI and PRDII, thereby activating interferon RNA transcription.

1.2 Induction by Double-stranded RNA

Both naturally occurring and synthetic dsRNAs are interferon inducers. Examples are the dsRNAs of reoviruses, the replicative forms of ssRNA-containing animal and bacterial viruses, synthetic polyribonucleotides such as poly rI : poly rC. To be an effective interferon inducer, the substance should have a stable secondary structure, high molecular weight and relatively resistant to ribonuclease (Joklik, 1990). In general, dsRNA is cytotoxic and a potent inhibitor of protein synthesis in many cells. Since the inducibility by poly I:C as well as that by virus infections is governed by the same IRE sequence, it appears that the basis of action of these interferon inducers is the inhibition of synthesis of the labile interferon repressor such that the transcriptional activators to the positive regulatory regions are able to bind. Only type I IFNs are induced by virus infection or dsRNA.

1.3 Induction by metabolic activators and inhibitors

Metabolic activators such as mitogens, lymphokines and specific antigens for committed lymphocytes (Reem et al., 1982) induce the synthesis of IFN-γ. Tumor

promoters such as tetradecanoyl phorbol acetate (TPA), butyrate, dimethylsulfoxide, are metabolic activators that induce both type I and type II interferons production (Stewart II, 1979). Evidence indicates inhibitors of messenger RNA formation (actinomysin D) and protein synthesis (cyclohexamide) are also capable of inducing synthesis of interferon (Ringold et al., 1984).

1.4 Other non-viral inducers

Inteferon can be induced by other cellular infections by a variety of fungi, bacteria, inclusion-forming chlamydia, rickettsiae, protozoa and mycoplasma. Bacterial products such as enterotoxin A, lipopolysaccharide of Brucella abortus and cell wall of mycobacterium tuberculosis have also been found to be inducers of IFN (Stewart II, 1979). All these inducers are cytotoxic and inhibit protein synthesis.

1.5 Priming and Superinduction

It has been reported that cells that have been treated with a low concentration of IFN prior to IFN-induction by virus would respond earlier and synthesize more IFNs than cells not previous treated with IFN. This phenomenon is known as priming (Burke & Issacs, 1958). Another phenomenon related to interferon is known as "superinduction". With the addition of poly IC and the protein synthesis inhibitor cyclohexamide for 5 hours, actinomycin D (inhibitor of mRNA formation) is added and then followed by the reversal of protein synthesis inhibition 1 hour later, there would be approximately 50 times more interferon produced than if cyclohexamide had not been added initially. The possible explanation is as follows : In the first 5 hours, the repressor would be inactivated and

gradually amounts of interferon mRNA rises. After the addition of actinomycin D, part of both the IFN and repressor mRNA would decay. Upon release of the inhibition of protein synthesis, interferon would then be formed predominantly together with greatly reduced amounts of repressor (Sehgal, 1978; Dinter & Hanser, 1987). Studies on the effects of dsRNA [poly(I).poly(C)] on the induction of β-IFN mRNA in the presence or absence of cyclohexamide indicated that superinduction is due to the synergistic effect of dsRNA and inhibitors of protein synthesis (Ringold et al., 1984). A later study by Lebendiker et al. (1987) demonstrated that the expression of the IFN-γ gene could be superinduced by either i) exposure to inhibitors of translation or ii) low doses of γ-irradiation. It has been shown that primary transcription of the IFN-γ gene is not changed when the cells are superinduced by cyclohexamide or γ-irradiation. This finding suggests that the expression of IFN-γ gene is regulated by a labile repressor that acts post-transcriptionally to inhibit the accumulation of mature IFN-γ mRNA sequences.

1.6 Induction of other genes by interferon inducers

Raj and Pitha (1980) demonstrated that in addition to interferon, 23 other proteins were also synthesized during induction by dsRNA - polyI:C. Hence, the expression of these genes is also regulated by mechanisms responsive to poly IC. In a similar way to the induction mechanism of interferon, it is likely that many other genes are controlled by labile repressors and that their transcription can be activated by cellular transcription factors upon the removal of the labile repressors.

1.7 Differential production of different interferon species

The synthesis of the various interferon species is induced to various levels by different inducers in different cells (Bisat et al., 1988; Goren et al., 1986). Bisat et al. (1988) showed that in mouse cells induced with virus infection or dsRNA, the relative levels of α-1, α-4 and α-6 IFN mRNA were different, depending on the cell type. Their results indicate that differential and cell type specific expression of murine α-IFN genes is regulated on the transcriptional level. In addition, interferon gene expression is controlled by labile repressors and cellular transcription factors. As a result, the relative amounts of interferon synthesis would not be the same in different cells.

Under normal conditions, it is known that cells do not contain or produce interferon except in T-lymphoblastoid cells, in which interferon genes are transcribed constitutively (Christofinis et al., 1981). The spontaneous production of IFN seems to involve : i) a modified NRDI region, to which the repressor binds weaker, or; ii) the synthesis of a defective repressor, or; iii) the synthesis of insufficient amounts of repressor (Joklik, 1990). The constitiutive transcription is greatly activated when cellular factors PRDI-BFc and PRDII-BF are modified during induction, thus allowing them to bind to PRDI and PRDII, respectively (Keller and Maniatis, 1988).

2. Interferon genes and regulation

2.1.a Interferon genes

There are at least 15 genes in the human genome for IFN-α, one for IFN-β and one for IFN-γ. All IFN-α genes that have been mapped are located in the short arm of chromosome 9 and lack introns (Owerbach et al., 1981; Trent et al., 1982). The sequence

of the 5'-flanking region of different IFN-α genes is conserved between -64 and -109 nucleotides preceding the cap site and contains all the information necessary for the induced transcription of the IFN-α genes (Weidle & Weissmann, 1983; Ragg & Weissmann, 1983; Ryals et al., 1985). The IFN-β gene which exhibits about 30 % sequence homology to IFN-α genes is also located on chromosome 9 whereas the IFN-γ gene is located on chromosome 12 and exhibits no relatedness to the type I interferon genes. IFN-γ is encoded by a single gene (Derynck et al, 1982) and unlike α- and β- IFN genes, it is interrupted by three introns. The transcription unit is preceded by a promoter region, TATAA box 28 bases upstream and a sequence similar to the CCAAT box 89 bases upstream from the cap site. A polyadenylation signal (AATAAA) is found 16 bases upstream from the 3' end of the mRNA sequences. The flanking regions of the different interferon genes exhibit varying degrees of relatedness. There is at least 75 % similarity among the IFN-α genes but it is quite different for those of IFN-β and IFN-γ (Sehgal, 1982). Although there is no significant homologies of nucleotide sequence for type I and type II IFNs, appropriate alignments suggest certain degree of conservation. Interferon genes have been cloned into various expression vectors such as those in bacteria, yeast and mammals (Kelley et al., 1986; Jay et al., 1984a). Jay et al. (1984a) synthesized a gene for human IFN-γ which was expressed in E. coli and demonstrated biological activity. With the placement of a synthetic ribosome binding site and a T5P25 promoter, recombinant IFN-γ was produced at 16% total cell protein (Jay et al., 1984b).

Apart from human IFN-γ gene, other mammalian IFN-γ genes have also been cloned and sequenced. All known mammalian species have a single IFN-γ gene containing 3 introns in identical positions as the human gene (Weissmann & Weber, 1986). Comparison of their

amino acid sequences indicates that some regions are conserved in all IFN species.

2.1.b Interferon-responsive genes

The various biological responses of IFN are mediated through the transcriptional activation of several IFN-responsive genes (DeMaeyer & DeMaeyer-Guignard, 1988). Table 2 shows a list of IFN-responsive genes and their gene products that are induced by different types of IFN. Those induced by only type I IFNs include ISG15 (Reich et al., 1988), ISG54 (Levy et al., 1986), Mx gene (Horisberger et al., 1983) and 67-kd protein kinase (Lebleu et al., 1976). Those that can be induced by both types of IFNs are guanylate-binding protein (GBP) (Cheng et al., 1983), 2,5-oligo A synthetase (Baglioni & Maroney, 1980) and MHC class I genes (Basham et al., 1982; Fellous et al., 1982). Genes/proteins that are induced by only IFN-γ include MHC class II (Vilcek et al., 1985), Fc receptor for IgG (Guyre et al., 1983), IP10 (Luster & Ravetch, 1987), IP30 (Luster et al., 1988), γ.1 (Fan et al., 1988), IDO (Takikawa et al., 1988), 54-kd cytoskeletal protein (Ulker et al., 1987a), gene encoding endothelial cell differentiation antigen (Duijvestijn et al., 1986) and NO synthase (Karupiah et al., 1993). A gene called 9-27 and its protein product RBP9-27, which is recently found to inhibit HIV-1 expression, is induced by either IFN-α or IFN-γ (Constantoulakis et al., 1993).

Most of these IFN-responsive genes appear to share a common mechanism of transcriptional regulation involving a similar amino acid sequence. Based on several studies, a cis-acting DNA element named interferon-stimulated responsive element (ISRE) has been found in the promoter region of many IFN-inducible genes. The ISRE is essential and sufficient for IFNα/β response (Israel et al., 1986; Reid et al., 1989). It is a conserved regulatory element of all IFN-α-stimulated genes (ISGs).

Table 2. List of interferon-inducible genes/proteins

Interferon	Gene/Protein	Reference
Only type I IFN (IFN-α/β)	ISG15	Reich et al., 1988
	ISG54	Levy et al., 1986
	Mx	Horisberger et al., 1983
	67-kd protein kinase	Lebleu et al., 1976
Both type I & II IFNs	Guanylate-binding protein (GBP)	Cheng et al., 1983
	2,5-Oligo A synthetase	Balioni & Maroney, 1980
	MHC class I genes	Basham et al., 1982
		Fellous et al., 1982
		Vilcek et al., 1985
IFN-α or IFN-γ	9-27 gene	Constantoulakis et al., 1993
	RBP9-27 protein	
Only IFN-γ	MHC class II genes	Vilcek et al., 1985
	Fc receptor	Guyre et al., 1983
	IP-10	Luster & Ravetch, 1987
	IP-30	Luster et al., 1988
	γ.1	Fan et al., 1988
	IDO	Takikawa et al., 1988
	54-kd cytoskeletal protein	Ulker et al., 1987a
	Endothelial cell differen-tion antigen	Duijvestijn et al., 1986
	NO synthase	Karupiah et al., 1993

For genes that are responsive to IFN-α/β and IFN-γ such as the GBP gene, ISRE present in GBP gene is sufficient to mediate response to IFN-α. However, for IFN-γ, two overlapping elements in the GBP gene are required for the full response. In addition to ISRE, an overlapping element termed as the γ-activated site (GAS) is found to be a binding site for a cytoplasmic protein, the γ-activated factor (GAF), the binding of which is essential for activation of transcription by IFN-γ (Lew et al., 1991; Decker et al., 1991). On the other hand, sequences similar to ISRE are found in the promoters of genes induced by IFN-γ only (Luster et al., 1985). Recently, Pearse et al. (1991) identified a cis-acting region on the gene encoding $Fc_{\gamma}RI$, the IFN-γ response region (GRR) that is responsible for IFN-γ induction. The GRR region contains elements homologous to those found in MHC class II promoters and GAS in GBP promoter. As a result, they suggest that a common sequence is present in several IFN-γ-inducible genes that is important for transcriptional induction by IFN-γ.

Based on all the above studies, it is likely that IFNs possess different IFN response sequences for type I and II IFNs. The ISRE is essential for the transcriptional activation of IFN-α/β-responsive genes and some IFN-γ-responsive genes. However, for those genes that are only responsive to IFN-γ, it appears that the control of transcriptional activation is regulated through the GAS-like amino acid sequences.

2.2 Interferon gene regulation by IRF-1 and IRF-2

Regulation of transcription in mammalian cells commonly involves the interactions of the regulatory DNA sequences with trans-acting DNA binding proteins. Previous studies showed that transcription of IFN-α and IFN-β was induced by viruses or dsRNA, whereas

for IFN-γ genes, it was induced in T-lymphocytes after mitogenic stimulation (Weissmann & Weber, 1986; Taniguchi et al., 1988). Miyamoto et al. (1988) identified a mouse nuclear factor, termed interferon regulatory factor-1 (IRF-1), in L929 cells, that specifically bound to the upstream regulatory cis-elements of the human IFN-β gene. Their results suggest that PRDI-BF (described by Keller & Maniatis, 1988) corresponds to IRF-1. In addition, IRF-1 is involved in the regulation of other genes such as IFN-α and MHC class II genes. The IRF-1 gene expression is found to be highly induced by Newcastle disease virus in mouse L929 cells. Furthermore, their results show that IRF-1 binds efficiently to repeated hexamer motifs present in IFN genes that function as virus-inducible enhancer elements (Fijita et al., 1988). The fact that IRF-1 binds to the virus inducible cis-elements of the IFN-α and IFN-β promoter, as well as to the interferon response sequence (IRS) of IFN-inducible gene promoters, suggests that it plays an important role in regulating IFN and IFN-inducible gene induction. This hypothesis is further supported by the study on high-level expression of the cloned mouse IRF-1 gene in monkey COS cells (Fujita et al., 1989). It was demonstrated that induction of endogenous type I IFN genes occurred without viral stimulation. As a result, IRF-1 acts as a positive regulatory factor for type I IFN genes. Subsequent studies on IFN gene regulation provided evidence that another DNA binding factor, namely IRF-2, was also involved in gene regulation (Harada et al., 1989). Both genes are inducible by virus and IFN. Interestingly, these two similar trans-acting factors are found to bind to the same cis-acting nucleotide sequences. IRF-1 functions as a transcriptional activator for IFN and IFN-inducible genes but IRF-2 acts as an antagonistic repressor of IRF-1. Consequently, it is suggested that the control of IFN and IFN-inducible gene expression is through competitive interactions of IRF-1 and IRF-2. This IRF-1/IRF-2 regulatory system indicates a product-

mediated feedback mechanism. A later study by the same researchers demonstrated that the expression of both IRF and IFN genes was developmentally regulated in mouse EC cells (Harada et al., 1990). A simplified model for IFN gene regulation is shown in Figure 2. Under normal cell differentiation, IRF-2 acts as repressor and is bound to the promoter of IFN genes. Upon induction by viral infections, the IRF-1 level raises (first signal). It is likely that IRF-1 is modified to achieve a higher DNA binding affinity (second signal) and displaces IRF-2, thereby activating the IFN transcription. However, subsequent cessation of the signals may lead to the opposite effect through IRF-2 binding. This model reflects the interactions of IRF-1 (activator) and IRF-2 (repressor) with the target genes which regulate the IFN system. Furthermore, it explains the IFN gene induction mechanism by viral infections. Previous studies have identified two positive regulated sequence elements PRDI and PRDII as well as a negatively regulated sequence element NRDI within the IFN-β promoter (Goodbourn & Maniatis, 1988). Both IRF-1 and IRF-2 are PRDI-binding factors (Harada et al., 1990) with the latter acting as a repressor and possibly inhibits IRF-1-mediated expression in the absence of virus induction. Upon viral infection, induction of IRF gene expression is stimulated. Since IRF-2 is induced only after induction of IRF-1 (Harada et al., 1989), more IRF-1 bind to PRDI and hence trigger IFN gene activation. The induction process is transient and involves binding competitions of IRF-1 and IRF-2 to the same site.

Apart from being regulators of type I IFN and IFN-inducible genes, other functions have been identified for both IRF-1 and IRF-2. Recently, the human IRF-1 gene was mapped to chromosome 5 by analysis of mouse-human somatic cell hybrids (Itoh et al., 1992) The 5q31.1 region is found to be frequently deleted in patients with leukemia or

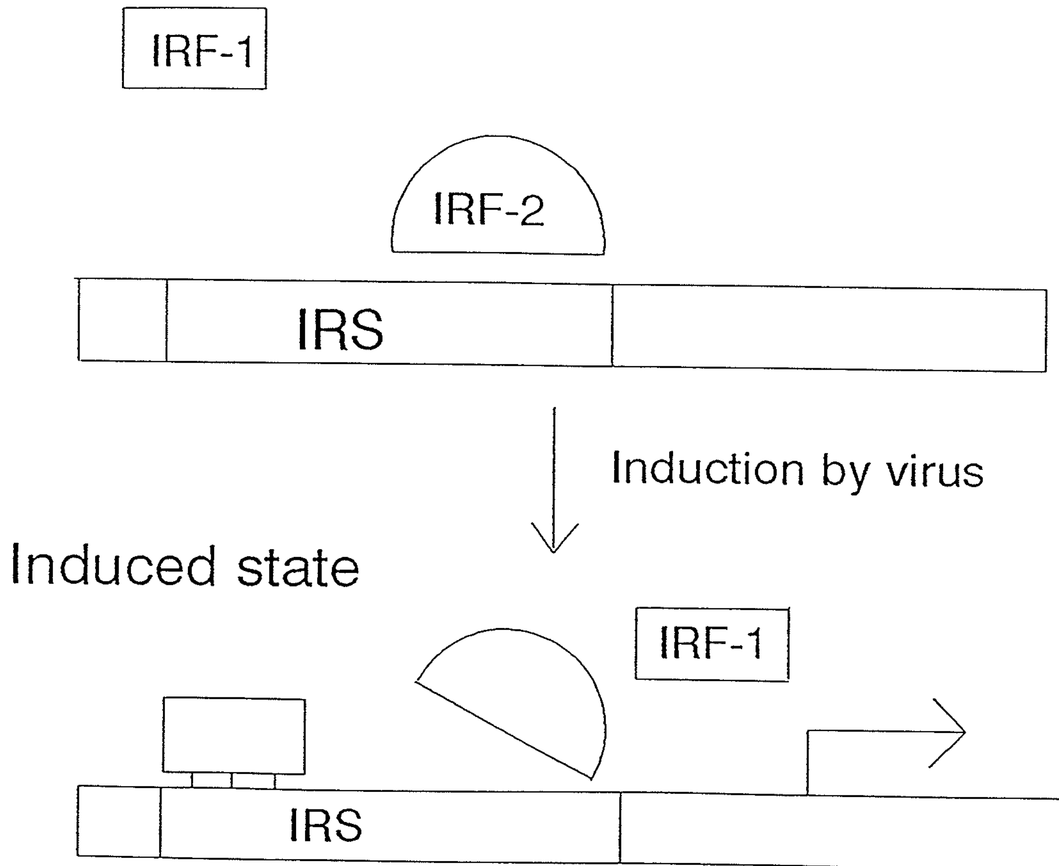

Figure 2. A model for IFN gene regulation through IRF-1 and IRF-2
(Adapted from Keller and Maniatis, 1988; Harada et al., 1990) In normal cell condition, IRF-2 acts as a repressor and is bound to the promoter of IFN gene. Upon induction by viral infections, the IRF-1 level raises and IRF-1 is modified to achieve a higher DNA binding affinity, displacing IRF-2, thereby activating gene transcription

myelodysplastic syndromes (Willman et al., 1993). Furthermore, evidence has shown that IRF-1 and IRF-2 have anti-oncogenic and oncogenic potentials respectively (Harada et al., 1993). Overexpression of IRF-2 gene in NIH3T3 cells enhanced tumorigenicity in nude mice. However, this transformed phenotype can be reversed by concomitant overexpression of the IRF-1 gene. Their observations suggest that cell growth regulation depends on a balance between these two antagonistic transcription factors.

3. Biological activities and mechanism of interferon action

Interferons are secreted proteins with multiple functions against viral, bacterial as well as parasitic infections. They exert various biological activities through the binding to specific cell-surface receptors (Aguet et al., 1988) that triggers the transcription of several genes, from which different protein products synthesized play a crucial role in cellular responses.

3.1 Anti-viral effect

Interferon itself does not actually inhibit virus multiplication but rather induces the synthesis of other proteins that are the effectors of the antiviral state. Studies have shown that the antiviral effect by IFN is due to its interference with the translation of early viral mRNA molecules, leading to inhibition of viral protein synthesis. This basis of antiviral action was first demonstrated by Joklik and Merogan (1966) on vaccinia virus-infected L cells. Subsequent analysis revealed that antiviral mechanism involved different pathways. Two important enzyme systems are found to confer antiviral state when activated by interferon in the presence of either natural or synthetic dsRNA. They are: i) the 2-5-oligoadenylate synthetase-nuclease system and ii) protein kinase system. Another novel antiviral mechanism is genetically determined in mice against influenza viruses (Staeheli &

Haller, 1987). Recently, a new anti-human immunodeficiency virus type I (HIV-1) mechanism has been proposed which involves an RNA binding protein RBP9-27 encoded by the IFN-inducible gene 9-27 (Constantoulakis et al., 1993). Karupiah and his colleagues (1993) also demonstrate that antiviral activity of IFN-γ is due to the IFN-induced nitric oxide synthase.

3.1.a 2,5-Oligo A system

In 1974, a family of small heat-stable inhibitors of protein synthesis was isolated. They are in fact oligomers of the general type 2',5'-pppA(pA)n, with n ranges from 1 to 15 and n = 2 being the predominant homolog. This family of oligonucleotides, designated as 2,5-oligo A, is synthesized by oligoisoadenylate synthetase enzyme (Ratner et al., 1977; St. Laurent et al., 1983), which is induced by interferon (Hovanessian & Kerr, 1979; Yang et al., 1981). In the presence of dsRNA, this enzyme will catalyze the polymerization of ATP into oligo A chains having monophosphate linkages at the 2',5' hydroxy of successive ribose moieties. The 2'5'-oligo A formed then activates a preexisting ribonuclease, RNase L, which is capable of cleaving viral and cellular RNA (Slattery et al., 1979; Nilsen et al., 1981). The 2',5'-phosphodiester bond in 2,5-oligo A is resistant to normal nucleases. A particular phosphodiesterase cleaves these oligonucleotides to yield ATP and AMP. The 2,5-oligo A synthetase can be induced by both types of IFN, with type I having a higher level of inducibility.

3.1.b 67-kd protein kinase system

Type I IFNs also induce the synthesis and activation of a protein kinase (Gupta et al., 1982). This 67-kd protein kinase, sometimes referred to as P1, is normally inactive. During virus infection or in the presence of dsRNA, it autophosphorylates and also phosphorylates

the small subunit of protein synthesis initiation factor eIF-2 (or eIF2-α). The initiation factor eIF-2 binds GDP and guanine nucleotide exchange factor (GEF) that facilitates GDP and GTP exchange, with subsequent formation of a complex which is the initial reaction in protein synthesis. With the α-subunit being phosphorylated, eIF-2 does not function in the GDP/GTP exchange reaction, thereby fails to initiate protein synthesis (Levin et al., 1976). Further study by Koromilas et al. (1992) demonstrate that dsRNA-protein kinase is crucial in the control of cell growth and functions as a suppressor of cell proliferation and tumorigenesis.

3.1.c Mx gene

Lindenmann and his associates (1963) observed that almost all but one inbred strain of A2G mice were highly susceptible to influenza virus. They suggested that the resistance in A2G mice was specified by a gene. This gene, later named as Mx gene was found to locate on mouse chromosome 16 and on human chromosome 21 (Reeves et al., 1988). It encodes a protein which accumulates in the nucleus of mouse cells (Noteborn et al., 1987) but in the cytoplasm of human cells (Staeheli & Haller, 1985). Transcription of this gene is inducible by type I but not type II interferon (Staeheli et al., 1984). By means of chromosomal DNA analysis, Hug et al. (1988) showed that the wild-type Mx1 gene which encoded Mx protein contained 14 exons spreading over 55 kilobases of DNA. Mutations in Mx1 gene in influenza virus-susceptible mouse strains result in inhibition of Mx protein synthesis. Further study demonstrates that Mx consists of more than one interferon-responsive transcription unit since a second Mx gene (Mx2) has been identified (Staeheli & Sutcliffe, 1988). Comparison of the primary structures of Mx1 and Mx2 mRNA indicated more than 90% homology at the amino termini. In fact, there are other Mx-related proteins

and mRNAs found in several species. For instance, two Mx-related genes have been identified in humans (Aebi et al., 1987) and two distinct Mx-related proteins are found in bovines (Horisberger, 1988). Evidence has shown that the Mx protein inhibits primary transcription of influenza virus genes but the mechanism of antiviral action of Mx proteins remains unknown (Krug et al., 1985).

3.1.d RBP9-27 RNA binding protein

The effect of IFN against HIV-1 is well-documented (Poli et al., 1989; Meltzer et al., 1990). It was shown that IFN-α was the antiviral agent that suppressed HIV expression in chronically infected cell lines. Recently, Constantoulakis et al. (1993) has found that a RNA binding protein which is encoded by the IFN-inducible 9-27 gene inhibits HIV-1 expression. The genome of HIV is very complex. Apart from the gag, pol and env genes, it encodes several other regulatory proteins, two of these, called Tat and Rev, are necessary for virus replication (Sodroski et al., 1985; Feinberg et al., 1986). Results indicated that a cis-acting Rev responsive element (RRE) interacts with the HIV-1 Rev protein. RRE, which consists of 210 nucleotides and locates in the env coding region of HIV-1, has a complex secondary structure that provides the right conformation for Rev to bind and function (Olsen et al., 1990; Malim et al., 1989). Malim et al. (1990) reported the interaction of HIV-1 Rev trans-activator and its cis-acting RNA target sequence, RRE, was essential for the expression of HIV-1 structural proteins, and hence viral replication. As demonstrated by Constantoulakis and his associates (1993), a gene 9-27, inducible by IFN-α and IFN-γ, encoded a RNA binding protein, RBP9-27, which was found to bind to the RRE of HIV-1 and inhibited viral expression. Their results suggested an IFN-induced antiviral mechanism through the induction of RBP9-27. In the presence of IFN-α or IFN-γ, the gene 9-27 is induced with

subsequent synthesis of RBP9-27 protein. The induction of this cellular factor RBP9-27 results in the inhibition of HIV-1 expression due to its binding with RRE of HIV-1 that interferes with the Rev-RRE interaction, thereby antagonizes Rev function for viral expression. Further study on RBP9-27 may shed some light to the mechanism of IFN action during HIV infection.

3.1.e Nitric oxide synthase

Karupiah et al. (1993) demonstrated the correlation of antiviral activity of IFN-γ with nitric oxide (NO) production. Upon exposure to inhibitors of NO synthase, viral replication was restored in IFN-γ treated macrophages. However, epithelial cells with no detectable NO synthesis showed inhibition of viral replication when transfected with a cDNA encoded with inducible NO synthase or treated with organic compounds that generate NO. All these observations suggest the induction of antiviral effect of IFN-γ is due to the induced NO synthase.

3.2 Inhibition of cell proliferation

Interferon inhibits cell growth in a variety of cell types, with different sensitivities. It regulates primary cells, established cells, normal cells and tumor cells (Creasey et al., 1983; Pfeffer et al., 1979). In general, both type I and II IFN have the ability to inhibit cell growth with IFN-γ having more potent effect than the former. IFN exerts the anti-proliferative effect at all stages of the cell cycle. It has been shown that IFN inhibits or reduces RNA synthesis and proteins that are essential for initiating DNA synthesis during postmitotic period (G_1). As a result, cells continue their cycle at a slower rate or enter the resting phase (G_0) (Bordens & Ball, 1981). In addition, Kirkpatrick (1984) demonstrated that

the IFN-mediated antiproliferative effect was reversible once IFN was withdrawn.

The anti-proliferative effects of interferon involve different modes of action. As reported by Ozaki et al. (1988), the enzyme indolamine 2,3-dioxygenase (IDO) was involved in anti-proliferation. IDO is found in various mammalian tissues and is induced either by IFN treatment in normal and malignant tissues or by viral infection. IDO catalyzes the formation of N-gotmyl-L-kynmenine from L-tryptophan which plays a role in short term anti-proliferative effects of IFN-γ. The addition of tryptophan to growth medium reverses the anti-proliferative effect of IFN-γ on KB cells in a dose and time dependent manner. However, cells do not respond to tryptophan supplement after prolonged treatment with IFN-γ.

The ability of IFN to modulate cell growth and differentiation may be one of the factors contributing to the anti-tumor effect (Fisher et al., 1983). Several lines of evidence indicate that some IFN-inducible proteins may have tumor-suppressor activities. Previous findings suggest that IRF-1 has antiproliferative effects in vivo and in vitro (Abdollahi et al., 1991; Yamada et al., 1991). Recently, Harada and associates (1993) demonstrated that IRF-1 and IRF-2 had anti-oncogenic and oncogenic potentials, respectively. They found that overexpression of IRF-2 gene in NIH3T3 cells led to enhanced tumorigenicity in nude mice. Hence, the control of cell growth and tumorigenicity was likely due to the elevated expression of IRF-2. However, by increasing the expression of IRF-1, the transformed phenotype displayed by NIH3T3 cells with overexpression of IRF-2 was reversed. Their results indicate that IRF-1 functions to suppress tumor growth. They suggest that cell growth regulation in fact depends on a balance between IRF-1 and IRF-2. The anti-oncogenic

function of IRF-1 gene is further supported by the location of human IRF-1 gene, which has been mapped to chromosome 5q31.1, a region frequently deleted in patients with leukemia or myelodysplastic syndromes (Willman et al., 1993). In addition, some of the IFN-inducible genes and their gene products are also found to involve in the inhibition of cell proliferation and tumor suppression. Examples are the dsRNA-dependent protein kinase (Koromilas et al., 1992) and RNaseL (Zhou et al., 1993), which down regulate protein synthesis in target cells upon induction by IFN.

3.3 Immunomodulatory function

IFN has been shown to play a vital role in modulating the function of the immune system. Among the three types of IFNs, IFN-γ is the most potent and some of the immunoregulatory effects are mediated exclusively by IFN-γ. IFN-γ therefore is an important regulator of lymphokine productions in immune responses. IFNs enhance the expression of class I MHC (Israel et al., 1986) and class II MHC antigen (Steeg et al., 1982). In mouse macrophages, IFN-γ was found to induce Ia-antigens through a secondary factor (Walker et al., 1984) whereas in human cells, IFN-γ induced antigens encoded by HLA-DR and HLA-SB. Apart from this, it also enhances the expression of the high affinity Fc receptor for monomeric IgG in myelo-monocytic cells (Perussia et al., 1983). IFN-γ is likely a macrophage-activating factor (MAF) since it induces the cytotoxicity of human monocytes against tumor target cells (Le et al., 1983). Evidence has shown that IFN-γ stimulates the maturation of B cells to immunoglobulin secretion as well as enhances the expression of B cell and endothelial cell differentiation (Sidman et al., 1984; Sonnenfeld et al., 1978; Leibson et al., 1984). On the contrary, they have inhibitory effect on melanocyte

hormone-stimulated melanogenesis (Fisher et al., 1981) and the differentiation of fibroblasts into adipocytes (Keay & Grossberg, 1980).

3.4 Effect on structural components of the cell

Several lines of evidence indicate that IFNs have the ability to induce the synthesis of certain cytoskeletal components. Pfeffer et al. (1980) demonstrated that IFNs enhanced the organisation of microfilaments and the redistribution of cell-surface fibronectin. Their results suggest that IFN triggers a pathway in fibroblasts that involves alterations in the plasma membrane and the actin-containing microfibrofilaments. Studies of human Hela-S_3 carcinoma cells showed that HuIFN-β inhibited cell proliferation in suspension cultures (Wang et al., 1981). They also found that the number of intermediate-type junctions and submembranous microfilaments was increased. It was then hypothesized that in both tumor and normal cells, HuIFN-β was likely to induce a coordinated response in the cell membrane and its associated cytoskeletal proteins. Pfeffer et al. (1987) have shown that IFN-α binds to specific high-affinity receptors on the cell surfaces of human Daudi lymphoblastoid cells. The IFN-receptor complexes which are insoluble in Triton X-100 accumulate in IFN-α-sensitive but not IFN-α-resistant cells. Moreover, the accumulation in IFN-sensitive cells is inhibited with the addition of cytochalasin B, indicating that the Triton-insoluble complexes are coupled to the cytoskeleton. In IFN-sensitive cells, binding appears to increase receptor affinity for IFN as well as coupling of some receptors to the cytoskeletal matrix. Hence, the association of cytoskeletal matrix and IFN-receptor complexes may play an important role in mediating the anti-proliferative effect of IFN-α in Daudi cells.

Ulker et al. (1987a) reported that a 54-kd protein was induced by HuIFN-γ in human

amnion U cells. It was shown that anti-p54 polyclonal antibody cross-reacted with anti-intermediate filament antibody and the intermediate filament components were also induced by IFN-γ. Based on the biochemical and immunologic properties, p54 appears to be a cytoskeleton-associated polypeptide. It is highly likely that the p54 protein is an IFN-induced cytoskeletal component. Study on the kinetics of induction of this p54 protein and the antiviral state (using vesicular stomatitis virus) indicate that synthesis of p54 preceded the induction of antiviral state - AVS (Ulker & Samuel, 1987b). The rate of induced-p54 accumulation decayed significantly within 24 hr following the removal of IFN-γ with a half-life of about 3 days. Similarly, the AVS decayed within 3-4 days after the removal of IFN-γ. They hypothesize that p54 - possibly a cytoskeleton-associated polypeptide, is involved in the antiviral mechanism of action of IFN-γ.

3.5 Inhibition of chlamydial growth

Chlamydiae are small obligate intracellular energy-dependent bacteria with three known species : C. trachomatis, C. psittaci and C. pneumoniae (Moulder, 1991). They share a common developmental cycle that makes up of two phases - the elementary body (EB) which is the extracellular infectious form and the reticulate body (RB) which is the intracellular non-infectious form. In the process of infection, the EBs are internalized and remain in a membrane bound inclusion and later become transformed into RBs. Upon completion of the replicative cycle, the RBs are transformed into EBs that are released into the extracellular fluid to infect other cells (de la Maza & Peterson, 1982).

Both type I and II interferons are known to inhibit chlamydial multiplication (Lengyel, 1982; Ozanne & Pearce, 1980). Several mechanisms for IFN-γ induced antichlamydial

activity have been proposed. In vivo, phagocytes are activated by IFN-γ to destroy the ingested bacteria. In macrophages, IFN-γ stimulates the synthesis of microbial reactive metabolites of molecular oxygen (Nathan et al., 1983) as well as other anti-microbial factors with oxygen-independent mechanisms (Rothermel et al., 1986). Another antichlamydial mechanism of IFN-γ is by inducing the production of indoleamine-2-3-deoxygenase (IDO) that degrades tryptophan (trp) to kynurenine. As a result, chlamydia is inhibited because of trp starvation. However, trp degradation is not found in untreated cells. As expected from such mode of action, chlamydial inhibition is reversed by the addition of trp to the culture (Byrne et al., 1986). The concentration of IFN-γ required to reduce infectious yield depends on the concentration of trp added. For example, the inhibition of C. trachomatis biovar LGV growth in IFN-γ treated HEp-2 cells was completely reversed by adding trp 24 hour post-infection, but the reversion was only partial if trp was added 2 days post infection (Shemer et al., 1987). Addition of trp 3 days post-infection to both C. psittaci (Byrne et al., 1989a) and C. trachomatis (Byrne et al., 1988) infection on host cells treated with IFN-γ failed to restore chlamydial growth. It seems therefore, that prolonged deprivation of trp caused irreversible inhibition of chlamydial growth.

Evidence also indicates that IFN-γ mediated cytotoxicity may play a role in inhibiting chlamydial multiplication. When mouse fibroblast cultures (L cells) were infected with C. psittaci 6BC, IFN-γ added to the host cells would induce a cytolytic response depending on the inoculum size of the 6BC, the concentration of IFN-γ, and the time of IFN-γ exposure. No cytotoxicity was observed when cells were treated with 50ug/ml choramphenicol (prevents the differentiation of infectious EBs to RBs) but if choramphenicol was added 18 hr post-infection, cytotoxic activity was detected. Addition of 100U/ml penicillin (interferes with

chlamydial division but not metabolic activity) did not abolish the cytotoxic effect but only reduced it to lower level. Furthermore, cytoxicity was inhibited by adding 0.2ug/ml of cyclohexamide (inhibitor of protein synthesis) without affecting chlamydial growth. However, a delayed addition of cyclohexamide (12 hr postinfection and IFN-γ treatment), cytotoxicity was restored. As a result, IFN-γ-mediated cytotoxicity also depends on de novo host cell protein synthesis. Based on these observations, IFN-γ serves as a cytolytic cytokine which exhibits specificity for host cells only after cell infection. (Byrne et al., 1988; Byrne et al., 1989b).

3.6 Cell surface receptors

The main function of a cell surface receptor is to bind effectors and transduce signals through ligand binding and/or internalization from the effectors to the cells. There are different types of receptors - cell surface (membrane) receptors and intracellular receptors. The demonstration that interferons have no biologic activity within the cells when they are synthesized but rather they must first be secreted and then readsorbed by cells (Vengris et al., 1985) indicate that the activity of interferon is exerted at the plasma cell membrane level when it binds to specific cell surface receptor. (Friedman, 1979; Kohn, 1978). Two IFN receptors (Branca & Baglioni, 1981; Orchansky et al., 1984), one for type I IFN and the other for type II IFN have been identified. The former has its gene located on chromosome 21 (Fournier et al., 1985; Razinddin et al., 1984) whereas the gene for IFN-γ receptor is found on chromosome 6 (Rashidbaigi et al., 1986). The type I IFN receptor interacts with various IFN-α species with different affinities (Aguet et al., 1984). There is a correlation of the biologic activities of different IFN species with their receptor binding affinities (Aguet

et al., 1984; Uze' et al., 1985). Tumor cells are shown to possess more IFN-γ receptors than normal cells (Uecer et al., 1986). For the type II or IFN-γ receptor, purification and cloning have been performed (Aguet et al., 1987; 1988; Calderon et al., 1988). Significant results have been gathered to indicate functionally active human or murine IFN-γ receptors are comprised of at least two components : the IFN-γ receptor itself and an accessory component encoded on human chromosome 21 or murine chromosome 16 (Jung et al., 1987; Jung et al., 1990; Hibino et al., 1991). The species-specific interaction between the receptor and the accessory component occurs within the receptor's extracellular domain (Hibino et al., 1992; Hibino et al., 1991). Farrar and associates (1991) demonstrated that the intracellular domain of the IFN-γ receptor was crucial to form a functionally active receptor. Their experiments were performed in L cells or SCC16-5 cells, with the absence or presence of a single copy of human chromosome 21, respectively. When full-length HuIFN-γ receptors were transfected to L cells, the receptors showed ability to bind and internalize HuIFN-γ but did not respond to it. On the contrary, SCC16-5 cells transfected with HuIFN-γ receptors bound and responded to HuIFN-γ. However, a non-functional receptor was formed when SCC16-5 cells were transfected with mutant IFN-γ receptor lacking all but 3 a.a. of the intracellular domain. By the use of a set of overlapping deletion mutants, two regions have been identified for functional activity : i) the membrane-proximal 48 a.a. required for both receptor-mediated ligand internalization and functional activity, and ii) the 39 a.a. at the C-terminus required for biologic response induction. They further identified a functional domain in the C-terminus of the IFN-γ receptor which was essential and specific for biological activity (Farrar et al., 1992). They have demonstrated that 3 a.a. in this region are specifically essential for receptor activity - Tyr-440, Asp-441, and His-444. Based on

their observations, the hydroxyl side chain of Tyr-440 is hypothesized to play a crucial role in initiating the biologic responses of IFN-γ.

When interferon is internalized, it would bind rapidly to the nuclear membrane (Kushnaryov et al., 1986) and that the internalized interferon can be degraded rapidly (Anderson et al., 1983). Interferon-receptor complexes are subject to diffusion and aggregation on the cell surface, followed by endocytosis (Aguet et al., 1982; Evans & Secher, 1984). Although the mechanism of different biologic effects of interferon is still unclear, internalization may trigger nuclear events that finally lead to biologic activities, such as the establishment of the anti-viral state (Joklik, 1990).

3.7 Signal transduction

Signal transduction mechanism of cell surface receptors involves the binding of a specific ligand to its cell surface receptor that triggers a number of intracellular signals. These signals regulate gene transcription and protein products synthesized would elicit different cellular responses. Lew et al. (1989) demonstrated that transcriptional activation of GBP by IFN-α was rapid, transient and cyclohexamide resistant. However, activation by IFN-γ was slower, sustained and delayed by cyclohexamide. Upon withdrawal of IFN-α, the transcription decayed rapidly whereas removal of IFN-γ resulted in a continual transcription. Furthermore, addition of various kinase inhibitors would block the transcriptional response to IFN-γ but not IFN-α. Their results suggest that the signal transduction pathway of IFN-α and IFN-γ is significantly different and a novel kinase may be involved in gene activation by IFN-γ. However, it appears that not any of the known second messengers are involved in both pathways.

It is well-documented that IFN-α binds to its specific cell surface receptor and activates transcription of IFN-α-stimulated genes (ISGs). The ISRE, a conserved regulatory element present in all ISGs, is found to activate gene transcription through the binding of a transcriptional factor called interferon-stimulated gene factor-3 (ISGF3). In fact, ISRE mediates transcriptional response not only to IFN-α, but also to IFN-β and for some genes (such as GBP) that respond to IFN-γ (Reid et al., 1989; Lew et al., 1989). ISGF3 is the primary transcriptional activator for IFN-α-induced genes (Kessler et al., 1990). This transcriptional factor is a complex consisting of 4 proteins (113, 91, 84 and 48kd). In response to IFN-α (or IFN-β) binding to the receptor, the 113, 91 and 84 kd polypeptides in the cytoplasm are phosphorylated. In the phosphorylated state, these three polypeptides form one subunit of ISGF3 (ISGF3α). The other subunit is the 48kd protein, which binds to the ISRE with a low affinity. Since the synthesis of this protein can be induced by IFN-γ, it is called ISGF3γ. In the phosphorylated state, ISGF3α associates with cytoplasmic ISGF3γ to form the mature ISGF3 and this complex is then translocated to the nucleus. The binding of ISGF3 to the ISRE activates gene transcription (Dale et al., 1989; Levy et al., 1989). However, the signaling pathway of IFN-γ is quite different. Upon IFN-γ binding to its specific cell surface receptor, the 91kd cytoplasmic protein is phosphorylated, which then enters the nucleus and binds the GAS-like sequence of IFN-γ-stimulated genes for transcriptional activation. Studies indicate that the 91kd protein may serve different functions in the transcriptional activation by IFN-α and IFN-γ. In cells treated with IFN-α, the p91 is phosphorylated and form the high affinity DNA binding complex ISGF3 for ISRE binding, it does not contact DNA itself but rather the ISGF3γ directly binds to the cis-acting DNA ISRE (Lee et al., 1990). However, in cells treated with IFN-γ, the p91 phosphorylated

protein binds to another DNA element (other than ISRE) - the GAS for IFN-γ-mediated transcription (Shuai et al., 1992). Molecular cloning of the genes encoding different polypeptides of the ISGF3α subunit allows the identification of a structure called SH2 domain which is commonly found in proteins that are targets for tyrosine kinases (Fu et al., 1992; Schindler et al., 1992a). A cDNA for a non-receptor tyrosine kinase, TYK2 has been isolated (Velazquez et al., 1992). It is tempting to speculate that the interferon receptor may interact directly with TYK2 protein without the need for a second messenger. The TYK2 protein would then be activated in response to interferon and in turn phosphorylated ISGF3α (Whiteside & Goodbourn, 1993). Figure 3 shows one of the well-established pathways for signal transduction by IFN-α and IFN-γ.

3.8 Nuclear localization

Previous studies on the requirements for nuclear translocation of proteins indicate that a small basic sequence, named nuclear localization signal (NLS), is sufficient (Dingwall et al., 1982). There are two criteria of being an NLS : i) fusion to a non-nuclear protein will direct the protein to the nucleus, and ii) deletion or mutation of a normally nuclear protein will cause it accumulate in the cytoplasm (Silver, 1991). Later studies show that different proteins have different a.a. sequences that are found to be nuclear targeting. For instance, SV40 large T antigen has an NLS consisting of a contiguous basic a.a. whereas nucleoplasmin has an interdependent bipartite motif (Kalderon et al., 1984a; Robbins et al., 1991). Other studies on the process of nuclear import indicate that at least two steps are involved : i) rapid binding at the nuclear envelope surface, and ii) slow translocation through the nuclear pores (Richardson et al., 1988). The whole process is temperature and energy-

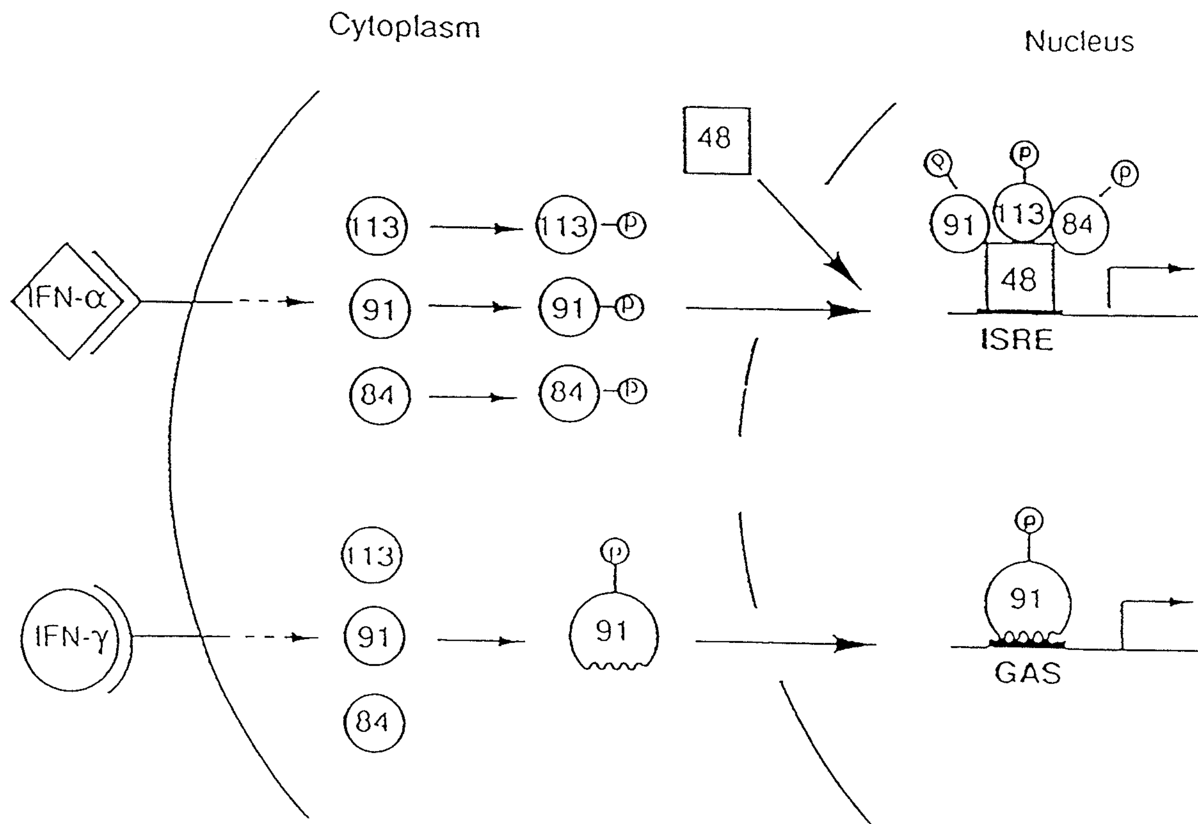

Figure 3. Model for signal transduction of IFN-α and IFN-γ. (Adapted from Shuai et al., 1992 & Schindler et al., 1992) Upon binding to its specific receptor, IFN-α triggers the phosphorylation of three cytoplasmic proteins, p113, p91 and p84 to form a complex ISGF3α. Together with another cytoplasmic protein p48, it forms a ISGF3 complex and enters into the nucleus. Binding of ISGF3 to ISRE of IFN-α-stimulated genes will activate gene transcription. For IFN-γ, binding to its specific receptor will lead to the phosphorylation of p91, which enters into the nucleus and directly binds to the GAS of the IFN-γ-responsive genes to trigger gene activation.

dependent. The binding step is signal-sequence specific and requires NLS receptors either as soluble recognition factors or components of the nuclear pore complex (Silver, 1991). The second step - translocation, which is ATP-dependent, involves the transport of the nuclear pore complex from the nuclear envelope into the nucleus (Finlay et al., 1987; Richardson et al., 1988). In the cold or in the absence of ATP, the import substrates would accumulate at the cytoplasmic side of the nulcear envelope without transport. Addition of lectin or wheat germ agglutinin (WGA) will block translocation but not the binding process, hence giving a similar effect as that of temperature and ATP.

Curtis et al. (1990) has found that the cytokine interleukin-1 (IL-1) binds to its cell surface receptor to form IL-1R complex that is ultimately translocated to the nucleus. It was demonstrated that in murine cell line EL-4, after internalization for 4 h, IL-1 was still bound to its receptor without degradation. Internalized IL-1R was shown by electron microscope autoradiograph that it accumulated in purified nuclei. To determine whether nuclear receptors exist, ^{125}I-IL-1α binding to isolated nuclei from cells not exposed to IL-1 was examined. It was found that no nuclear receptors for IL-1 was detected in the nuclei of cells before internalization. This suggested that the cell surface receptor was translocated to the nucleus only in the presence of IL-1. It is likely that the receptor may play a role in the regulation of gene transcription. A new mechanism is therefore proposed which is different from the well-known signaling pathway of several typical hormones such as insulin, which activates a second messenger - tyrosine kinase upon receptor binding (Yarden and Ullrich, 1988). Interestingly, HuIFN-γ (another cytokine) has been found to contain a NLS-like element (Zu and Jay, 1991). It appears that if the NLS functions in nuclear translocation, then the HuIFN-γ molecule and/or its complex with the receptor is likely to play a role in

the nucleus to effect transcription regulation that results in various biological responses.

4. Structure of IFN-γ

The three-dimensional X-ray crystal structure of recombinant HuIFN-γ has been reported by Ealick et al.(1991) using multiple isomorphous replacement (MIR) techniques. IFN-γ forms a dimer in solution. Each monomer contains 6 α helixes (named A to F), constituting 62% of the molecule, but with no β sheet. The length of the helices ranges from 9 to 21 residues. The dimeric structure of HuIFN-γ is stablized by the intertwining of helices across the subunit interface with several intersubunit interactions. Although the 3-D structure of IFN-γ is not common among many globular proteins, the intertwining pattern of its subunits is similar to the Trp repressor, which is also comprised of α helices and exists as a dimer (Schevitz et al., 1985). Previous studies showed that mature HuIFN-γ molecule had 2 glycosylation sites at residues 25 to 27 (between helices A and B) and residues 97 to 99 (at the end of helix E), respectively (Rinderknecht et al., 1984). Both of these sites are exposed on the surface of the IFN-γ molecule. In addition to N-linked glycosylation, the native HuIFN-γ is heterogeneous at the COOH-terminus due to different degrees of C-terminal processing. As the removal of up to 13 amino acid residues from the C-terminus does not affect conformation (Arakawa et al., 1986) and the absence of carbohydrate does not significantly change the conformation of HuIFN-γ, the dimeric structure and high α-helical content are important characteristics of the native HuIFN-γ. Since antibodies directed to both the N-terminus (Magazine et al., 1988) and the C-terminus (Seelig et al., 1988) can neutralize biological activities, and the N-terminal helix of one subunit is required to maintain the cleft that accommodates the C-terminal helix of the other subunit, Ealick et al. (1991)

hypothesize that the receptor-binding region requires the intact dimer structure with the N-terminus of one subunit and the C-terminus of the other. In addition, it appears that the N-terminus is critical for maintaining the overall structure of the dimeric molecule.

Comparison of amino acid sequences for different mammalian IFN-γ species shows a considerable amount of homology. Figure 4 compares the a.a. sequences of IFN-γ of human (Tanaka et al., 1983), bovine (Gray & Goeddel, 1987), mouse (Gray & Goeddel, 1983), rat (Dijkema et al., 1985) and sheep (McInnes et al., 1990). Three of the most highly conserved regions in the sequences are on helices C and F (most buried helices in the dimer) and at the beginning of the C-terminal tail (Earlick et al., 1991).

5. Structure-function studies

The knowledge of the IFN structure and location of the functional domain would provide a better understanding of how molecules interact to elicit cellular responses. The a.a. sequence of HuIFN-γ has been deduced by DNA sequencing of the cDNA (Grey et al., 1982; Devos et al., 1982) as well as direct sequencing of the natural protein (Rinderknecht et al., 1984). However, the exact location of the active site(s) remains unidentified.

5.1 Use of monoclonal antibody

One approach to characterize and localize the general location of the active site(s) is by the analysis and epitope localization of neutralizing monoclonal antibody (mAb). A number of laboratories have developed neutralizing mAbs to study and identify the functional epitopes on HuIFN-γ (Le et al., 1984; Meager et al., 1984; Kelder et al., 1986). Alfa and Jay (1988) reported three epitope specificity groups E_1, E_1/E_2 and E_2. Based on their cross-reactivities, two distinct antiviral neutralizing epitopes E_1 and E_2 of the HuIFN-γ molecule

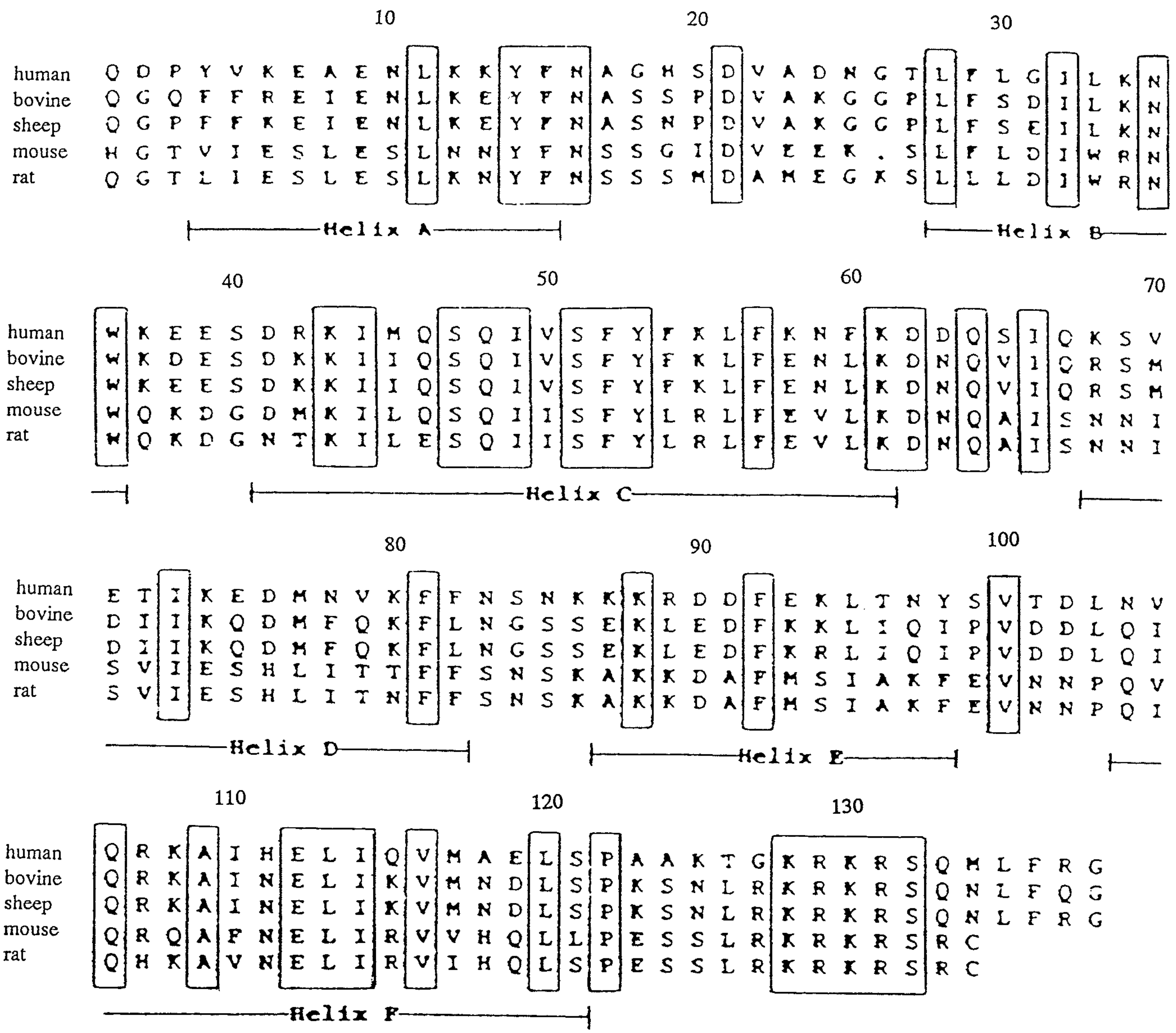

Figure 4. Amino acid sequences of IFN-γ derived from human (Tanaka et al., 1983), bovine (Gray & Greddel, 1987), sheep (McInnes et al., 1990), mouse (Gray & Goeddel, 1983) and rat (Dijkema et al., 1985).

were identified. Although different research groups have raised mAbs to different forms of HuIFN-γ, including synthetic peptides as well as natural and recombinant HuIFN-γ whole molecules that had been purified by different methods for structure-function studies, there is, however, no correlation of the epitope specificities between the mAbs developed in different laboratories, and it is not clear if there are other neutralizing epitopes on this pleiotropic molecule that are not represented in the epitope typing scheme proposed by Alfa and Jay. Yang (1991) analysed the immuno cross-reactivities of seventeen mAbs from six laboratories and found no additional reactivity group that is not already represented in the classification scheme of Alfa and Jay (Table 3). As a result, all HuIFN-γ neutralizing mAbs remain classified into three immuno-reactive groups representing at least two distinct functional epitopes.

5.2 Multiple functional sites

In the analysis of 21 neutralizing mAbs developed in our laboratory, none of the mAbs had the ability to inhibit HuIFN-γ binding to its receptor (Alfa and Jay, unpublished data). It seems likely that the receptor binding domain is separated from the antiviral effector site(s) as neutralization of activity by the mAbs tested did not depend on the inhibition of HuIFN-γ to bind to the cell surface receptor. This is also consistent with the observations reported by Fish et al. (1988) that changes in 2 residues associated with IFN-γ's β turns led to a reduction in antiviral and antiproliferative activities without affecting IFN-receptor interaction. Alfa and Jay (1988) hypothesized that HuIFN-γ contained more than one active site. One domain may be involved in affinity binding to the cell surface receptor to allow another domain to interact with some cellular molecules for signal transduction. The surface

Table 3. **Epitope Assignment of Anti-HuIFN-γ mAb**

EPITOPE	E1	E1/E2	E2	E2'
MAb Name	3009	3094	3055	3125
69B.J	0.014	0.001	-	-
113B.J	0.153	0.011	-	-
220A12.J	0.292	0.010	-	-
73A.J	0.483	0.634	-	-
3009	1.000	0.050	-	-
N1B42	1.553	0.037	3.160	-
B133.3	51.231	1.093	19.239	-
3094	-	1.000	-	-
B1	-	0.012	-	-
3055	-	0.610	1.000	34.901
B133.1	-	0.468	5.962	5.574
KM48	-	0.339	1.317	2.888
3125	-	47.500	0.201	1.000
5J	-	-	0.275	0.301
B3	-	-	0.686	0.473
4SB3	-	-	1.230	0.351
KM61	-	-	38.152	-
KM45	-	-	-	-

receptor may function to modify the conformation of the effector site or to bind HuIFN-γ so that the IFN-γ molecule is brought close to the signal transduction molecule to form a complex for transducing signals.

It would appear that the functionally active human or murine IFN-γ receptors consists of at least two components : the IFN-γ receptor itself and an accessory component encoded on human chromosome 21 or murine chromosome 16 (Jung et al., 1987). The fact that human chromosome 21 and mouse chromosome 16 are required to complement the respective IFN-γ binding component suggests that the accessory component must also be species specific. This conclusion is based on the fact that the transfection of the receptor alone in heterologous cells is not sufficient for response to IFN-γ. The corresponding accessory component is required to complement the receptor. This observation is consistent with the conclusion that receptor binding and effector domains on the HuIFN-γ are separated.

Many findings suggested that the various biologic activities of IFNs might be dissociated (Pestka et al., 1987). Ziai and his associates (1986) demonstrated that mAbs directed against HuIFN-γ had neutralizing activity on the antiviral, antiproliferative functions as well as reducing the expression of human lymphocyte class I and II antigens, but had no effect on the binding of IFN-γ to melanoma cells and its ability to decrease the expression of a high molecular weight-melanoma associated antigen. All these observations suggest that distinct functional domains exist on IFN-γ which are responsible for the various effector functions. The fact that there is only one high affinity receptor molecule for HuIFN-γ also implies that to activate the many different functional activities, there must be multiple effector sites.

5.3 Structure-function study of the C-terminus of HuIFN-γ

Previous studies have shown that one of the most highly conserved regions on different mammalian IFN-γ species is a short basic amino acid sequence at the beginning of the C-terminus (Ealick et al., 1991). Furthermore, studies on deletion mutants with various amino acid residues truncated from the C-terminal tail resulted in a drastic loss of biological activity. For instance, Leinikki et al. (1987) studied the relative sensitivity of the C-terminus of HuIFN-γ to enzyme digestion using pronase and the arginine-specific proteases. Digestion of the IFN-γ molecule resulted in a recombinant IFN-γ fragment that lacked 11 C-terminal amino acids. Data indicate that this IFN-γ fragment has 1000- to 2000- fold reduction in receptor-binding capacity and 10-fold and 50-fold decrease in antiviral and macrophage-activating activities, respectively. Structural analysis revealed that the cleavage occurred between residues 129 and 130 of IFN-γ. Their results suggest that the C-terminal region of HuIFN-γ significantly contributes to the formation of the receptor-binding site of the molecule. This was the first report on the importance of the C-terminal region for the binding of IFN-γ to its specific cellular receptor. It appears that either the C-terminus contains some active sites that interact with the cellular IFN-γ receptors, or it essentially contributes to the allosteric formation of the binding sites in other locations of the molecule. The crucial role of the C-terminus of IFN-γ in eliciting biological responses is also suggested by Seelig et al. (1987). By producing a neutralizing antibody (47N3-6) directed against the full length rHuIFN-γ, they demonstrated that 47N3-6 could bind to the whole rHuIFN-γ molecule and a truncated variant lacking the first 3 a.a. residues but it did not recognize another variant lacking 15 a.a.residues from the C-terminus of rHuIFN-γ molecule. However, this mAb was found to bind to a synthetic oligopeptide (15 a.a. residues) that

corresponded to the C-terminal region 132-146 a.a. residues of the HuIFN-γ. Results indicated that 47N3-6 could bind to a fragment of 7 a.a. corresponding to residues 132-137 of rHuIFN-γ but only weakly bound to fragment corresponding to residues 138-146. The specific activities of the variant (with C-terminus truncated) were 3- and 60- fold lower than the full length IFN-γ's antiviral and antiproliferative activities, respectively. All their observations suggest that at least a portion of the C-terminal region (in particular, 132-137 a.a. residues) may be an essential domain required for the expression of biological activity. In addition, de la Maza et al. (1987) demonstrated the antichlamydial, antiviral and antiproliferative activities of HuIFN-γ were dependent on the integrity of the C-terminus of the IFN-γ molecule. These findings indicate the essential involvement of the C-terminal tail of HuIFN-γ in the induction of biologic activities.

At present, little is known about the structure-function relationships that exist within the IFN-γ molecule. Several studies provide contrasting evidence on the importance of the C-terminus. Rose (et al., 1983) demonstrated that a rHuIFN-γ variant with deletions from the C-terminus at position 131 retained full antiviral activity. Furthermore, Kung et al. (1986) also reported that no significant loss of activity could be observed for IFN-γ truncated variant even up to 15 a.a. resiudes removed from the C-terminus. By means of gene modifications, a C-terminal deletion variant Del-122 (with 21 a.a. residues deleted) was constructed in this laboratory (Luk et al., 1990). Functional analysis of Del-122 showed that this molecule was 2- and 3- fold reduction in antiviral and antiproliferative specific activity, respectively than the full length HuIFN-γ molecule. These observations suggest that the C-terminus is not essential for functional activity.

A large panel of mAbs that bind to HuIFN-γ has been developed in this laboratory.

In an attempt to identify a mAb that neutralizes receptor binding and to determine if there is any additional antiviral neutralizing epitope(s) not previously identified, mAbs were screened for neutralizing activity. Thus far, no neutralizing receptor binding mAb has been identified. However, a mAb, MIF3037, which is of IgM type, was found to neutralize antiviral activity of HuIFN-γ. To test if the epitope recognized by MIF3037 represents a separate domain different from the previously identified epitopes (Alfa and Jay, 1988), a binding competition assay was performed. In the present study, we reported the identification of a distinct functional epitope E_3. Results on binding studies indicated that the general location of E_3 was within the 21 a.a. residues of the C-terminal region. Further functional analysis of this domain was carried out and the involvement and importance of the C-terminus in different biological activities was also discussed in this study.

CHAPTER II

IDENTIFICATION OF THE ANTIVIRAL NEUTRALIZING EPITOPE E_3

INTRODUCTION

Human interferon-gamma (HuIFN-γ) is a cytokine that elicits multiple cellular responses. Localizing the active site(s) is an important step in characterizing the molecular interaction of HuIFN-γ that effects the various cellular functions. However, the location of the active site(s) has not been identified. One approach to study the structure-function relationship is to characterize the neutralizing mAb on the function of the molecule and to identify the neutralizing epitopes on the molecule. These type of study makes the assumption that neutralization is caused by steric hinderance at the functional domain of the target molecule as a result of the mAb binding to its epitope. Alternatively, neutralization may result from a conformational change induced by the binding of the mAb which disrupts the functional structure at a distant location. Fortunately, the latter case occurs rarely. Since antibodies generally bind to only 4 - 10 amino acid residues, a neutralizing epitope must be at or adjacent to the functional site in order for the bound immunoglobulin to interfere with the functional domain. Thus the localization of the epitope will help identify the functional domain.

Many researchers have developed neutralizing mAbs to study and identify the functional epitopes (Ziai, et al., 1986; Oda, et al., 1986; Alfa, et al., 1987). The most systematic approach to identify the different immuno-reactivity groups of the anti-HuIFN-γ mAbs is that of Alfa and Jay (1988), which found, within their panel of 21 antiviral neutralizing mAbs, three immuno-reactive groups : E_1, E_2 and E_1/E_2. MAbs of the E_1 group do not compete with those of the E_2 group for HuIFN-γ binding, indicating that the E_1 and E_2 epitopes are separate domains on the HuIFN-γ important for the antiviral function. MAbs of the E_1/E_2 group compete with some of the mAbs of the E_1 and/or E_2 groups and may bind

to regions of the HuIFN-γ that partially overlap the E_1 and E_2 epitopes. However, none of the known epitopes was found to be associated with receptor binding function. In the present study, the identification of MIF3037 which represents a new reactivity group E_3 is described. The E_3 epitope represents a distinct functional epitope on the HuIFN-γ.

MATERIALS AND METHODS

Monoclonal Antibodies

The production and characterization of the anti-rHuIFN-γ mAb MIF3009, MIF3094, MIF3055 and MIF3125 were as previously described (Alfa, et al., 1987; Alfa & Jay, 1988). These mAbs were prepared as ascites fluids by injecting hybridoma cells into BALB/c mice that had been primed 7 days earlier with an intraperitoneal injection of 0.5 ml pristane. The mAbs were affinity purified from the ascites fluid on Protein A-Sepharose as outlined by the manufacturer (Pharmacia, Baie d'Urfe, Canada). Briefly, the different preparations of ascites fluids obtained from mice were centrifuged at 500x g for 10 min to remove cell debris. ProteinA - Sepharose CL-4B column was equilibrated with 50 ml phosphate buffered saline (PBS; 0.136M NaCl, 0.003M KCl, 0.008M Na_2HPO_4, 0.001M KH_2PO_4, pH7.4). Two ml of ascites fluid was loaded onto the column. After 1 hr, the column was washed with 100 ml PBS. Then the adsorbed mAb was eluted with acetic acid in saline (0.58% v/v glacial acetic acid in 0.85% w/v NaCl) and collected into a beaker containing 10 ml of 1M Tris, pH8.0 with continuous stirring. The purified immunoglobulin was dialysed at 4°C containing 0.02% sodium azide and later concentrated by ammonium sulfate (BRL Life Technologies, Inc., Gaithersburg, MD, USA) precipitation to 50% saturation at 4°C. After centrifugation at 1000x g for 15 min, the precipitate was redissolved in 2 ml of $T_{50}N_{30}$ buffer (50mM Tris-HCl pH8.0, 30mM NaCl). The purity of each mAb was then evaluated by SDS-PAGE on a 10% gel (Fig. 5). MIF3037, which is an IgM, was similarly produced in BALB/c mice and used as ascites fluid without further purification. The name, source description and references of each of these mAbs are summarized in Table 4. These mAbs were stored at -20°C until use.

ELISA

To test whether the new mAb MIF3037 binds to the carboxyl terminus of the HuIFN-γ molecule, a direct ELISA was performed to measure the reactivity of MIF3037 to the full-length rHuIFN-γ molecule and a deletion variant Del122 (with 21 amino acid residues truncated from the whole rHuIFN-γ molecule) developed by Luk et al.(1990). Briefly, 50 μl/well rHuIFN-γ and Del122 (dilution from stock to have 1000U/ml in 50mM carbonate buffer, pH9.4) were added into an ELISA plate and was allowed to be adsorbed to the well in an overnight incubation at 4°C. The subsequent nonspecific binding to the ELISA wells was reduced by incubating 3% BSA in PBS at 37°C for 2 hr. MIF3037 was serially diluted in 1% BSA in PBS added to the coated ELISA tray and incubated at 37°C for 2 hr. The wells were washed three times with 100μl PBS containing 0.02% Tween 20 (T20-PBS). Peroxidase-conjugated goat anti-mouse IgG, IgM, IgA (Cedarlane Laboratories, Hornby, Canada) was diluted 1/3000 with T20-PBS containing 1% BSA and 50μl was dispensed into each well. The plate was incubated at 37°C for 1 hr and then washed five times with T20-PBS. Colour development was achieved by incubation with freshly prepared 2,2'-azino-bis (3-ethylbenzthiazolinesulphonic acid) (ABTS; Sigma Chemical Co., St Louis, MO) at 0.015% (w/v) in 100mM citrate buffer, pH8.0, containing 0.03% (v/v) H_2O_2. The reaction was allowed to proceed at room temperature for 15 minutes in the dark and then 50μl of 10% (w/v) SDS was added to stop the reaction. Absorbance values were determined using an ELISA reader (Model EL308, Bio-Tek Instruments Inc, Burlington, VT) equipped with a 405ηm filter. The end-point was taken as the reciprocal of the dilution in the last well that produced an absorbance value of $>$ 0.150 which was three times greater than the average background value (0.05).

The ability of the mAb to bind HuIFN-γ was tested using sandwich ELISA (Alfa et al., 1987). ELISA plates were coated with purified rabbit anti-HuIFN-γ immunoglobulins at 0.5μg/well. The plates wete incubated overnight at 4°C and the residual binding sites in the wells were blocked by incubation with 3% (w/v) bovine serum albumin (BSA) in PBS. HuIFN-γ diluted with 1% BSA in PBS was added to the ELISA plates and incubated at 37°C for 2 hr and then washed with PBS. The mAbs were serially diluted 1/2 in BSA-PBS [1%(w/v) BSA in PBS] and then 50μl was transferred to the appropriate well in the ELISA tray and incubated at 37°C for 1 hr. After washing with T20-PBS, the reactivity of mAbs to IFN molecules were detected using the same reagents and methods as described above.

Antiviral neutralization

The antiviral neutralization (NAV) assay was carried out as described by Alfa and Jay (1988). Briefly, the tested mAb was serially diluted in a 96-well culture plate that contained monolayer cultures of A_{549} (human lung carcinoma) cells at 4 x 10^4 cells/well. A constant amount of HuIFN-γ, 4 working units/ml (10U/ml), was added. After incubation at 37°C for 18 hr, each culture was challenged with encephalomyocarditis (EMC) virus at a multiplicity of 5 $TCID_{50}$/cell for 24 hr. The monolayers were fixed and stained with 0.25% crystal violet, 20mM Tris-HCl, pH7.5, 0.9%(w/v) NaCl in 20% (v/v) methanol and the excess dye was washed out with tap water. Methanol (100μl) was added to each well to extract the dye that was adsorbed by the viable cells. The absorbance was determined with an ELISA reader equipped with a 590 ηm filter. The NAV titre was defined as the reciprocal of the dilution that could neutralize 1 reference unit of HuIFN-γ. Thus, the NAV

titre was obtained by taking the reciprocal of the dilution at 50% protection against the virus challenge and multiplying by the concentration of the HuIFN-γ in the assay (10 U/ml). The activity of HuIFN-γ was standardized against the US National Institutes of Health Reference HuIFN-γ, Gg23-901-530.

^{125}I-labelling of mAb

Purified immunoglobulin was labelled with carrier-free Na^{125}I using the method of Fraker and Speck (1978). Briefly, glass test tubes were coated with Iodogen (Pierce Chemical Co., Rockford, IL) by dispensing 50μl of 2% Iodogen in chloroform (w/v) into each tube and allowing the chloroform to evaporate completely. The purified immunoglobulin (50 μg in 50 μl PBS) and 0.5 mCi of carrier-free Na^{125}I were added to the Iodogen-coated tube. The reaction was allowed to proceed for 15 min and then the reaction mixture was transferred to a separate tube. The Iodogen-coated reaction tube was rinsed with 50 μl PBS which was then pooled with the reaction mixture. The free Na^{125}I was separated from bound [^{125}I]immunoglobulin by column chromatography on Sephadex G-100 fine in a 0.4 x 25 cm column. Labelled immunoglobulin prepared in this manner had > 95% precipitability in 10% TCA.

Competition sandwich radioimmunoassay (sRIA)

The competition sRIA of Alfa and Jay (1988) was previously described. However, EU was used as the unit for quantitation rather than mg of mAb. Briefly, 96-well ELISA trays were coated with the immunoglobulin fraction of a rabbit anti-(SDS-denatured HuIFN-γ) (PIF3003) at 100 μg/ml in carbonate buffer) at 4°C overnight. The residual binding sites

in the wells were blocked by adding 100 μl of 3% BSA-PBS/well and incubating at 37°C for 2 hr. A limiting amount of HuIFN-γ (8 x 10^4 IU/50 μl of 1% BSA-PBS) was added to each well and incubated at 37°C for 2 hr, and then washed with T20-PBS. Each tested mAb was serially diluted 1/5 in 1% BSA-PBS in a separate 96-well trays and then a constant amount of [^{125}I]mAb (200,000 - 400,000 c.p.m.) was added to each well. A 50 μl aliquot of each dilution was transferred to the appropriate wells of the HuIFN-γ-coated tray. The ELISA plate was incubated overnight at 4°C and then washed five times with PBS. The wells were allowed to air dry and individual wells were cut out with a hot-wire. The radioactivity in each well was determined using an LKB gamma-counter. All experiments were performed in duplicate. The concentration ratio of captured antigen (HuIFN-γ) and [^{125}I]mAb (probe) were optimized to produce 50% inhibition of binding when an equimolar amount of the homologous mAb was added. MAbs MIF3009, MIF3055, MIF3094 and MIF3125 were used as the radiolabelled probes in the competition analysis. The end-point was determined as the number of EU of mAb required to inhibit the binding of the radiolabelled probe by 50%.

RESULTS

Five mAbs produced in this laboratory were studied (Table 4). The relative concentration of each of the mAb was determined based on their reactivities with excess HuIFN-γ in a standard ELISA and expressed as ELISA U/ml (EU/ml). One EU/ml was defined as the reciprocal of the maximum dilution that produced a positive reaction of 0.15 A_{405} (three times background at < 0.05 A_{405}) in a standard sELISA. All mAbs were found to have no reactivity with extracts of the E.coli cell that harbour the same expression vector which lacked the HuIFN-γ coding sequence. The NAV titre was also determined and expressed as the reciprocal of the dilution that could neutralize 1 U of HuIFN-γ (NAV/ml)(Table 5)

Previous study has allowed the classification of the mAbs into distinct (non-cross-reacting) groups E_1 and E_2 as well as an E_1/E_2 group that cross-reacts with either or both E_1 and E_2 (Alfa & Jay, 1988). A mAb, MIF3037, directed against the HuIFN-γ was recently developed and found to be of the IgM type. Its dose-dependent binding to captured HuIFN-γ in a sELISA was shown in Fig.6. A typical dose-response binding of the MIF3037 to the ELISA plate was observed. Similar HuIFN-γ binding curves for MIF3009 of the E_1 epitope group, MIF3055 of the E_2 group and MIF3094 of the E_1/E_2 group were also demonstrated (Fig. 6). The ability of MIF3037 to recognize Del-122 which lacked the C-terminal 21 amino acid residues was tested. As shown in Figure 7, MIF3037 was able to bind to HuIFN-γ in a typical sELISA but failed to recognize Del-122.

The HuIFN-γ neutralization activity of MIF3037 was tested in an antiviral neutralization assay. In order to enhance its sensitivity, the amount of HuIFN-γ in the assay was kept at a minimum (4 working units/ml). The neutralization activity is reflected in the

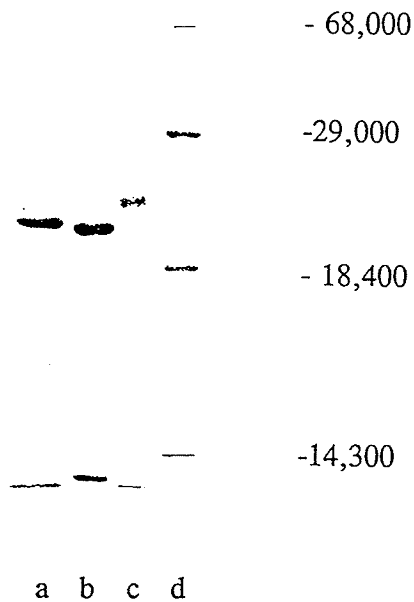

Figure 5. 10% SDS polyacrylamide gel of purified mAbs
Equivalent amounts (0.6ug) of mAbs against HuIFN-γ : MIF3009 (lane a), MIF3055 (lane b), MIF3094 (lane c) and molecular weight markers (lane d) were separately resolved by SDS-PAGE in 10% gel. The markers (from top to bottom) were : bovine serum albumin (68,000), carbonic anhydrase (29,000), β-lactoglobulin (18,400) and lysozyme (14,300), respectively.

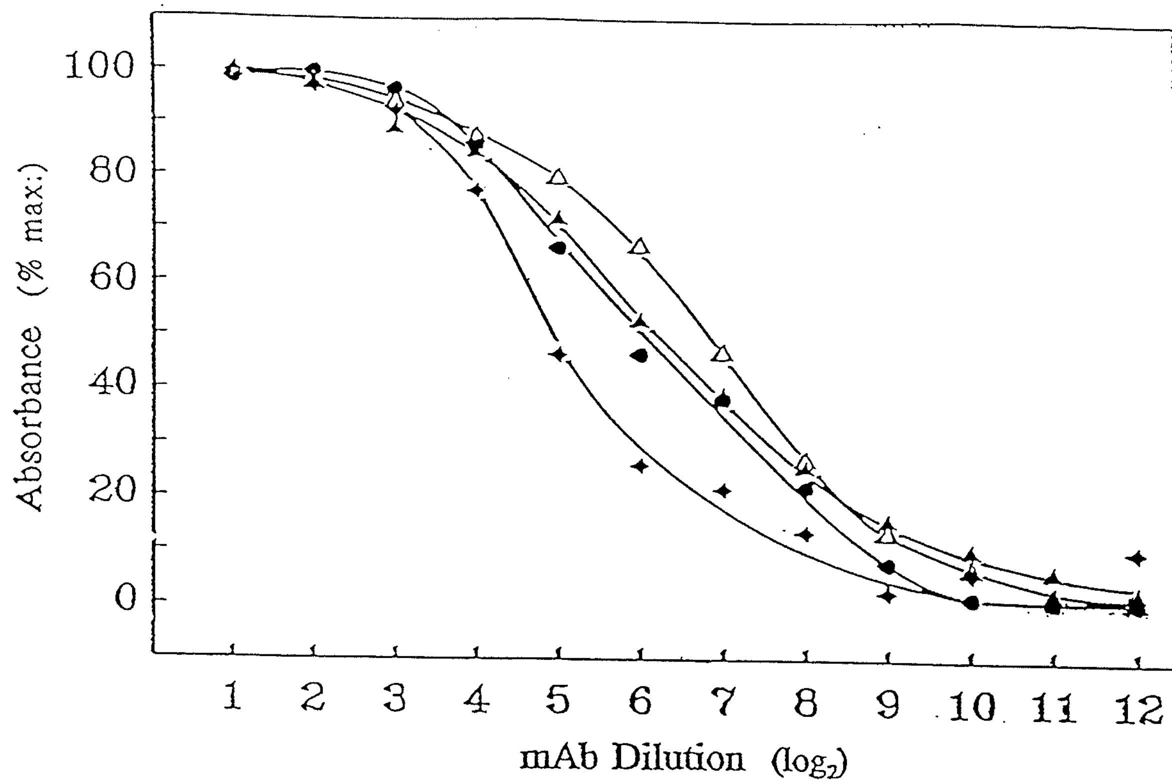

Figure 6 HuIFN-γ binding activities of mAbs in a sELISA. HuIFN-γ antigen for the sELISA was captured onto the ELISA plate with rabbit anti-HuIFN-γ immunoglobulin which has been adsorbed to the plastic plate. mAbs MIF3009 (▲), MIF3055 (●), MIF3094 (✦) or 3037 (△) were serial 2-fold diluted into ELISA plates and 50 μl was transferred to the corresponding wells in the HuIFN-γ plate. The amount of mAb bound to the HuIFN-γ in the plate was detected by peroxidase conjugated goat anti-mouse IgA, G and M. Results are expressed as percentage of maximum for each mAb.

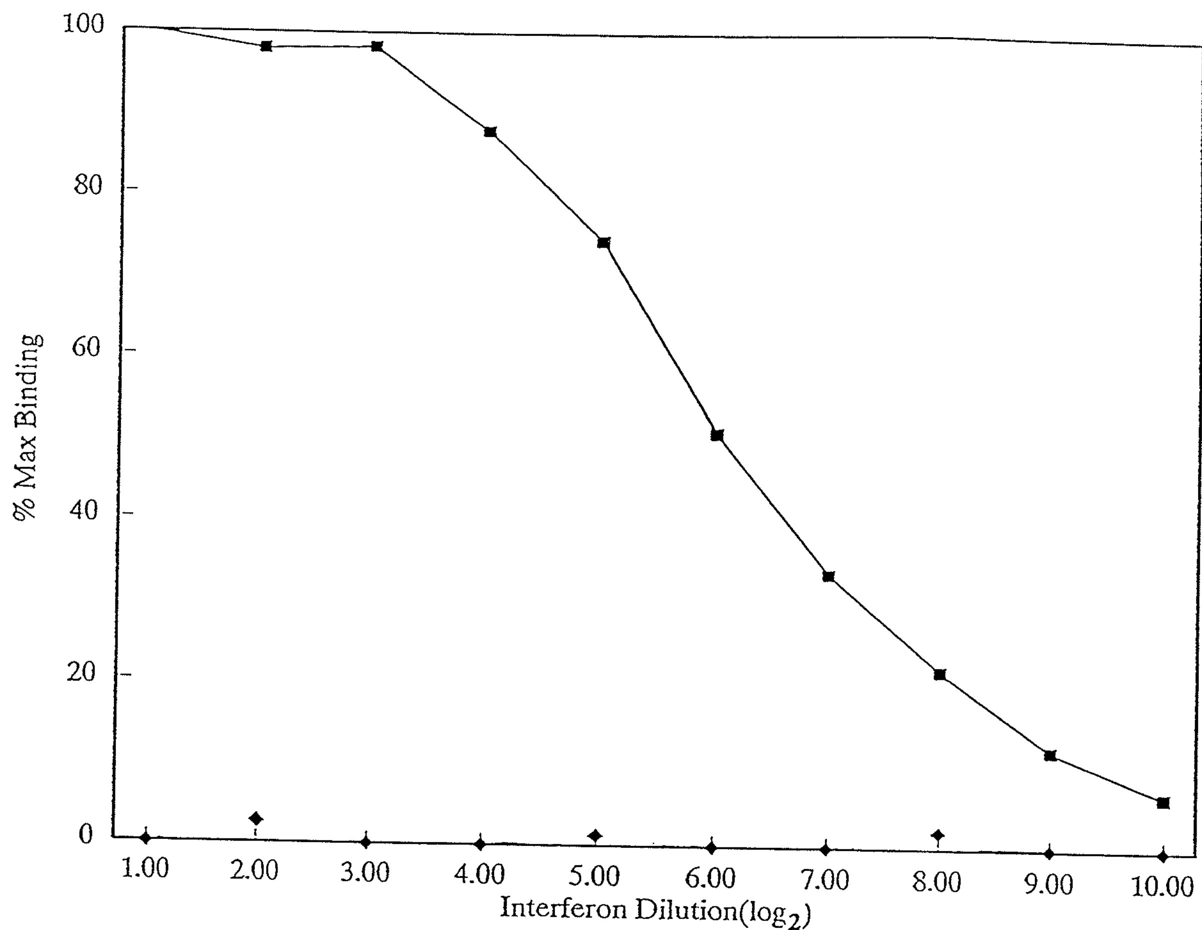

Figure 7. Binding activities of MIF3037 on HuIFN-γ and Del122 in ELISA.
MIF3037 was serial 2-fold diluted into successive wells in a ELISA plate and 50μl was transferred to the corresponding wells in an ELISA plate which was previously coated with equivalent amounts of either HuIFN-γ (■) or Del122 (◆) antigen. The amount of mAb bound to the HuIFN-γ and Del122 was detected by peroxidase conjugated goat anti-mouse IgA, G and M and subsequent colour development with ABTS substrate. Results were expressed as a percentage of maximum MIF3037 binding.

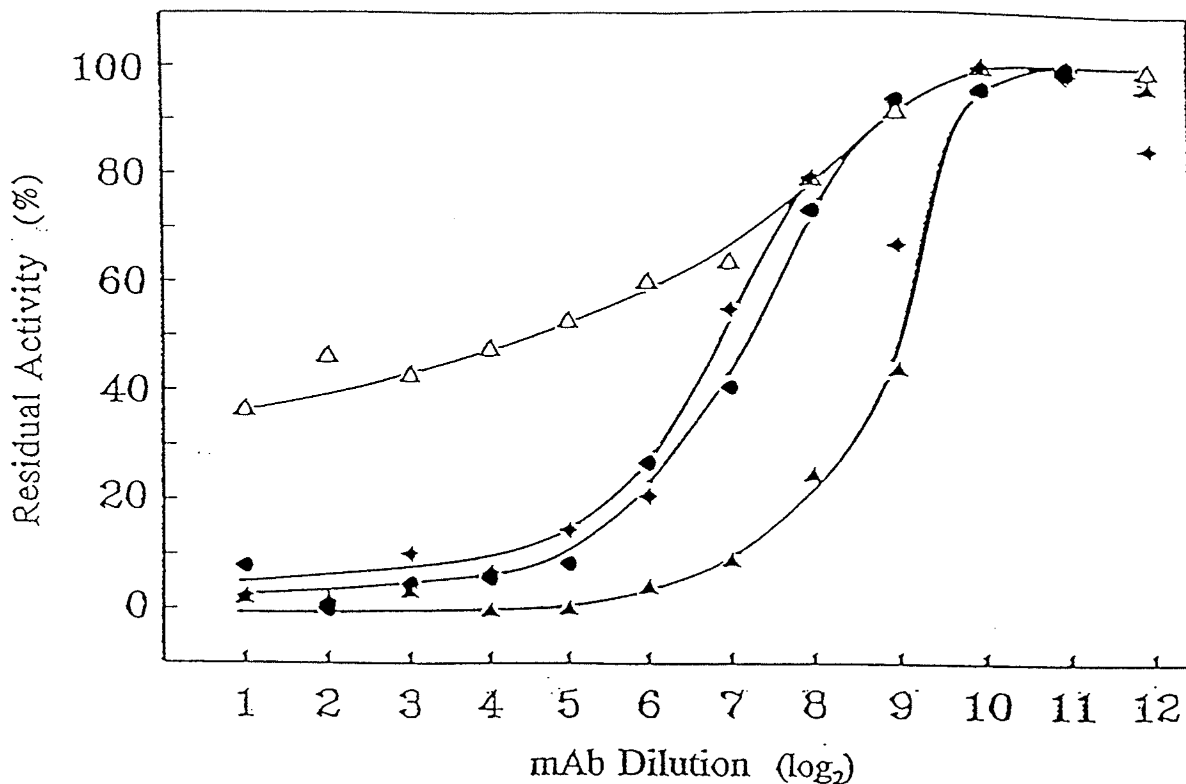

Figure 8. Neutralization of Antiviral Activities by mAbs. MAbs MIF3009 (▲), MIF3055(●), MIF3094(✦) or MIF3037 (△) were serial 2-fold diluted into micro-titre cultures of A549 human lung carcinoma cells. HuIFN-γ was then added to each well to a final concentration of 4 working units/ml. After incubation for 18 hr at 37° C, the cultures were challenged with 5 $TCID_{50}$ of EMC virus for 24 hr. Viable cells were then detected by staining with crystal violet and determined spectrophotometrically at 590nm. Average absorbance of cultures that had not been treated with HuIFN-γ ($<0.1A_{590}$) served as the background in each assay. Results are expressed as a percentage of the antiviral activity remaining compared to that of similarly treated and EMC virus challenged cultures in the absence of any mAb (100%).

Table 4. Monoclonal antibodies evaluated

Mab Name	Immunizing Antigen	Ig Subtype	Antiviral Neutralization	Sample Prep.	Research Group
MIF3009	rHuIFN-γ	IgG_1	Good Neutralizing	Purified Ig	Alfa and Jay
MIF3094	rHuIFN-γ	IgG_1	Good Neutralizing	Purified Ig	Alfa and Jay
MIF3055	rHuIFN-γ	IgG_1	Good Neutralizing	Purified Ig	Alfa and Jay
MIF3125	rHuIFN-γ	IgG_1	Good Neutralizing	Purified Ig	Alfa and Jay
MIF3037	rHuIFN-γ	IgM	Neutralizing	Ascites fluid	Unpublished

Table 5. Characterization of mAbs

Mab Name	NAV/ml	EU/ml	NAV/EU
MIF3009	4.19×10^7	1.02×10^6	41.10
MIF3094	3.28×10^4	6.40×10^4	0.51
MIF3055	1.05×10^7	6.40×10^5	16.40
MIF3125	1.31×10^7	1.60×10^4	81.90
MIF3037	1.23×10^4	1.18×10^4	1.04

Table 6. Competition ratio of anti-HuIFN-γ mAbs

	E1	E1/E2	E2	
Mab Name	MIF3009*	MIF3094*	MIF3055*	MIF3125*
MIF3009	**1.000**	1.367	–	–
MIF3094	-	**1.000**	-	-
MIF3055	-	2.237	**1.000**	12.800
MIF3125	-	-	0.548	**1.000**
MIF3037	-	-	-	-

*[^{125}I-labelled mAbs; (-) denotes no detectable competition at CR > 100; maximum CR obtained for MIF3037 in competition with [^{125}I]-labelled MIF3009, MIF3055 and MIF3094 was 32,988, 5874, and 2233 respectively.
MAb MIF3009, 3055, 3094 and 3125 were serial diluted and mixed with a constant amount of [^{125}I]-labelled probes. Each mixture was then transferred into the corresponding wells of another ELISA plate containing a constant and limiting amounts of captured HuIFN-γ. After overnight incubation, the radioactivity in each well was determined using an LKB gamma-counter. The end-point was determined as the number of EU of mAb required to inhibit the binding of the labelled probe by 50% (EU_{50}). CR is the ratio of the EU_{50} of tested mAb relative to the EU_{50} of the homologous mAb.

amount of crystal violet adsorbed by the remaining viable cells at the end of the challenge period. Typical dose-response neutralization of the HuIFN-γ by MIF3009, MIF3055 and MIF3094 was observed (Fig. 8). A similar result was obtained using MIF3037, although the NAV titre was lower and 100% neutralization was not achieved at the maximum mAb concentration attainable in these experiments (Fig. 8). The result, however, clearly demonstrated that MIF3037 is a neutralizing mAb.

In order to determine if the epitope recognized by MIF3037 is distinct from previously identified epitopes (Alfa et al., 1987; Alfa & Jay, 1988), MIF3037 was analysed in a competition assay using a modified sRIA. Three mAbs, MIF3009, 3005 and 3094 representing the three previously characterized epitope specificity groups, E_1, E_2 and E_1/E_2, respectively, were selected for the present study. Additionally, mAb MIF3125, that was distinct from E_1 but appeared to be partially related to E_2, was also included in this study. Each of the four mAbs, MIF3009, 3094, 3055 and 3125, was purified and radiolabelled with ^{125}I to a specific activity of approximately 10mCi/mg and approximately 2 mol of ^{125}I/mol of immunoglobulin. Subsequent analysis on a 10% reducing SDS-PAGE showed that only two polypeptide bands corresponding to the H and L Ig chains were present in each of the purified mAbs (Fig. 5). Previous analysis indicated that ^{125}I-labelling of these mAbs to this level did not affect the specificity or binding capacity of these mAbs to the HuIFN-γ (Alfa and Jay, 1988). Each of the unlabelled mAbs was tested by sRIA against each of the four radiolabelled mAb probes. The expected immunocomplex is shown as follows :

(ELISA plate)-(rabbit anti-HuIFN-γ)-(HuIFN-γ)-([^{125}I]mAb)

Since the amount of HuIFN-γ is constant and limiting, the presence of an unlabelled mAb that can compete with [^{125}I]mAb will proportionally reduce the binding of the [^{125}I]mAb to

the immobilized HuIFN-γ . The amount of test mAb, expressed in ELISA units, required to inhibit the binding of the [^{125}I]mAb probe by 50% (EU_{50}), was taken as the end-point. Unlabelled homologous mAb (same mAb as the probe) was included as a reference control in each set of competition experiments and the EU required to inhibit the binding of the ^{125}I-labelled probe by 50% was determined. Theoretically, an equivalent amount of unlabelled mAb would inhibit 50% of the binding of similar amount of the homologous [^{125}I]mAb. In order to control for any variability in different ^{125}I-mAb preparations and to allow comparison of the binding efficiency of different mAbs, the EU_{50} of each mAb was normalized to that of the homologous mAb control which was taken as 1.00. The results of these competition assays are presented in Table 6 as competition ratios of : CR = (EU_{50} of test)/(EU_{50} of homologous control).

All homologous mAb controls have CR ratios of 1.00. MAbs that bind the epitope better than the probe have a CR < 1.00, whereas those that bind weaker than the probe have a CR > 1.00. Where CR > 100, the mAb is considered non-competitive with the [^{125}I]mAb probe and therefore does not bind to the same epitope. This is shown as " - " in Table 6 to simplify the table of results and enhance comprehension of the table. The results showed that MIF3009 (E_1 group) did not compete with MIF3055 (E_2 group). MIF3125, with a CR of 0.548 against [^{125}I]MIF3055, clearly falls into the E_2 group and not a separate group of its own. However, MIF3037, which is a neutralizing mAb, did not compete with any of the ^{125}I-labelled mAb probes, even at an excess of 2233, 5874 and 32,988 times that of MIF3094, MIF3055 and MIF3009, respectively. MIF3037, therefore, must bind to a completely separate epitope.

DISCUSSION

Functional analysis of neutralizing mAbs is a powerful approach for initial analysis of structural-function relationships of macromolecules. Several research groups have developed HuIFN-γ-neutralizing mAbs for this purpose (Le et al., 1984; Kelder et al., 1986). In particular, Alfa et al. (1987) developed a panel of 21 antiviral-neutralizing mAbs and identified three immunoreactivity groups : E_1, E_2 and E_1/E_2 groups. Based on their immune cross-reactivities patterns, two distinct neutralizing epitopes E_1 and E_2 were reported (Alfa and Jay, 1988). C. Yang (1991) applied the epitope specificity classification to 14 neutralizing mAb provided by 5 other laboratories and found that each of these mAb falls into one of the three reactivity groups without ambiguity.

In an earlier study (Alfa and Jay, 1988), MIF3125 binding to HuIFN-γ was found to be not affected by mAb of the E_1 group and it was inhibited only weakly (at 35 - 300 molar excess) by mAb of E_2 and E_1/E_2 groups. It was suggested that the MIF3125 epitope may be related but not necessarily identical to the E_2 domain and it was provisionally assigned as E_2' pending further studies. The weak inhibition may be a result of MIF3125 having a very strong affinity for the E_2 domain or MIF3125 recognizing a separate eptiope which is near to the E_2 domain.

In the present HuIFN-γ competitive binding study, the comparison of the efficiency of competition was based on ELISA unit, which represents the amount of mAb bound rather than the total mAb input, to minimize the effects due to differences in affinity. Thus, the result reflects the functional involvement of that epitope and is less affected by the differences in the binding capacity of the individual mAb. The larger the NAV/EU ratio, the more effective is the neutralizing ability. Using this approach, the results in the current study

generally confirmed the relative neutralizing activity among these mAbs as previously reported (Alfa and Jay, 1988). Each of the reference mAbs, MIF3009, 3055 and 3094, gave a reactivity pattern which characterized it as members of the E_1, E_2 and E_1/E_2 groups, respectively (Table 5). The competition pattern of MIF3125, however, was not significantly distinct from that of MIF3055 of the E_2 group (CR=12.8). Based on this result, it is clear that MIF3125 recognized the E_2 epitope. This work was carried out in parallel with C. Yang (1991). Although this discussion is based only on data obtain by myself and reported here, the conclusion is the same as that found by Yang.

It is interesting to note that none of the mAbs generated by 6 laboratories was able to inhibit HuIFN-γ binding to the cell surface receptor. Thus, 35 neutralizing mAbs developed by 6 different laboratories using various methods of HuIFN-γ purification, immunization and hybridoma screening have identified only two functional epitopes and apparently none of these is associated with receptor binding function. In a continued effort to identify the different functional epitopes of the HuIFN-γ to locate the different functional domains, additional mAbs previously produced in the laboratory were screened. One mAb, MIF3037, was found to have antiviral neutralizing activity (Fig. 8). Other characteristics of MIF3037 were summarized in Table 5. The epitope specificity of MIF3037 was therefore examined by competitive binding to HuIFN-γ. MIF3037 did not demonstrate competition for HuIFN-γ binding with any of the reference mAbs. MIF3037, therefore, must recognize an epitope which is distinct from the E_1 and E_2 epitopes. Since MIF3037 neutralizes the antiviral activity of HuIFN-γ, the region recognized by MIF3037 must be a neutralizing epitope. This new functional epitope is named E_3. Thus, there are three neutralizing epitopes, E_1, E_2 and E_3, identified on the HuIFN-γ, resulting in four epitope specificity

groups : E_1, E_2, E_1/E_2 and E_3.

Functional neutralization by specific antibody binding can, on rare occasions, result from conformational changes that affect a distant functional site, rather than direct steric hindrance at the active site. However, with the exception of the MIF3037 for the E_3 epitope, because there are large numbers of different mAbs isolated in different laboratories which fall into distinct reactivity groups, it is highly unlikely that any of the E_1 and E_2 epitopes represents neutralization by conformational changes. Steric hindrance at the functional site is likely to be the mechanism for the neutralization. The epitopes E_1 and E_2, therefore, must either be the functional site, or very close to the functional site, that is necessary for the induction of the antiviral activity. This confidence cannot be fully extended to the E_3 epitope at this time. However, the fact that MIF3037 did not interfere with the binding of MIF3055, which recognizes only native (functional) HuIFN-γ, suggests that the conformation of the HuIFN-γ is maintained after the binding of MIF3037.

None of the neutralizing mAbs thus far identified was able to inhibit the attachment of the HuIFN-γ molecule to the cell surface receptor. Indeed, our preliminary data also indicated that none of the neutralizing epitopes identified is essential for the receptor binding function. We hypothesize that the receptor binding function and the signal transduction functions are separate on the HuIFN-γ molecule and that the epitopes E_1, E_2 and E_3, separately or jointly, form the effector structure(s) on the HuIFN-γ molecule that is essential for signal transduction either across the membrane or other intracellular signalling mechanisms that ultimately effect the appropriate switch in gene expression that establishes the antiviral state.

At present, only epitope E_1 has been precisely located on the HuIFN-γ (residues 84 -

94) and the amino acid sequence at the E_1 domain resembles the nuclear localization (targeting) signal (NLS) (Zu & Jay, 1991) of many nuclear proteins(Kalderon et al., 1984a; 1984b). The failure of MIF3037 to bind to Del122 which lacks the C-terminal 21 residues (Fig. 7) suggested that the E_3 epitope is located in the C-terminus. This has since been confirmed by Zu and Jay (personal communication) by specific mapping of the E_3 epitope to residue #130-138.

Previous study in this laboratory showed that a recombinant deletion variant of HuIFN-γ (Del-122) with 21 a.a. residues deleted from the C-terminus is biologically active (Luk et al., 1990). Del-122 remains 30% of antiviral activity of that of the full length of HuIFN-γ. As a result, the C-terminus is hypothesized to be non-essential for functional activity. In the present study, an antiviral neutralizing epitope E_3 is identified within the C-terminal region. It seems contrasting that functional domain exists in the C-terminus but is not essential for antiviral activity. Based on the neutralizing pattern, MIF3037 demonstrates neutralizing activity but even at very high concentration, the mAb is unable to neutralize the activity to the baseline. It is likely that E_3 contributes to the biological activity but must function with some other epitopes in order to be non-essential and yet functional. E_3 is therefore appeared to be a functional-enhancing domain since the absence of this domain does not significantly affect HuIFN-γ in antiviral activity but rather the presence of E_3 increases the efficiency of the molecule to function.

In conclusion, based on the results presented here, a new antiviral epitope E_3 on the C-terminus of the HuIFN-γ has been identified by the neutralizing mAb MIF3037. E_3 is non-essential but rather a functional-enhancing domain as the HuIFN-γ molecule lacking E_3 remains functional with lower activity. The epitope specificity typing scheme for HuIFN-γ

neutralizing mAbs must also be expanded to include a total of four immunoreactive groups : E_1, E_2, E_1/E_2 and E_3 groups. This classification scheme not only serves as a mAb epitope reference, it also provides a means to correlate results from different investigators as well as allows understanding of results obtained with different neutralizing mAbs.

CHAPTER III

EFFECTS OF EPITOPES E_1 AND E_3 ON THE KINETICS OF ANTIVIRAL STATE DEVELOPMENT

INTRODUCTION

As shown in Chapter II, eptiope E_3 is neutralizing and it appears to reside within the C-terminal 21 residue because MIF3037 was not able to bind to Del-122. X. Zu in this laboratory has recently applied the synthetic peptide mapping method to identify epitope E_3 and to locate it to residue 130 to 138. This domain resides within the C-terminal deleted fragment that was deleted in Del-122 and therefore confirmed the mapping result discussed above. As pointed out previously, epitope E_3 is an antiviral enhancement domain that itself is not essential as demonstrated by the antiviral activity of Del-122 (Luk et al., 1990). It is interesting to note that located at the N-terminal junction of the E_3 epitope is a sequence of 4 basic residues : K^{129}-R-K-R which resembles another basic element : K^{86}-K-K-R found within the neutralizing epitope E_1 (Zu and Jay, 1991). Both of these elements are basic in nature and satisfy all the characteristics of nuclear localization signals (NLS).

The fact that E_3 is a functional enhancement epitope suggests that E_3 may cooperate with another functional domain. In this laboratory, Zu has demonstrated that E_3 functions synergistically with E_1 but has no effect on E_2 (personal communication). However, it remains unknown whether the cooperation would result in an increase in the rate of antiviral development or an increase in the level of the antiviral activity.

Dworetzky et al. (1988) demonstrated that the rate of microinjected protein-coated gold particle uptake increased as a function of the number of SV40 T antigen NLSs per gold particle. These observations suggested that multiple NLSs increases the efficiency of nuclear translocation. Furthermore, studies on nucleoplasmin by Dingwall and his associates found that the number of signals on the molecule affects the rate of localization greatly but the ultimate level of localization is not very different (Dingwall, et al., 1982). Whether the level

of localization would be affected by different signals is still unclear.

A deletion variant of HuIFN-γ, Del-122, which lacks the E_3 domain has been constructed in this laboratory (Luk et al., 1990). The antiviral activity was only 3 to 10 fold lower than the full length rHuIFN-γ molecule. In this study, the rate of induction of antiviral state (AVS) in response to equivalent activity of HuIFN-γ and Del-122 are compared. Since E_1 and E_3 are found to act synergistically in antiviral activity (Zu and Jay, personal communication), having more epitopes (signals) would be reflected by either an increase in the rate of biological induction or the level of the biological activity. Based on the cooperative function of E_1 and E_3, we hypothesize that E_3 will increase the rate of antiviral development. Similarly, based on the effect of multiple signals on nuclear transport, we further postulate that if both E_1 and E_3 epitopes on the HuIFN-γ function as NLS, then the same antiviral functional units of the full-length HuIFN-γ which carries 2 NLS signals (E_1 and E_3) should be able to induce the antiviral state faster than similar number of units of Del122 that carries only 1 signal (E_1).

MATERIALS AND METHODS

Extraction and purification of rHuIFN-γ and Del-122

The construction and cloning of the HuIFN-γ coding sequence (Jay, et al., 1984b) and its efficient expression by plasmid vector pJP_1R_3 in Escherichia coli (Jay, et al., 1984a) have previously been described. The recombinant proteins in this study were expressed by a modified plasmid vector $pJP_{14}R_9$ and were isolated from the transfected E. coli host LE392. The deletion variant Del-122 was developed by Luk et al. (1990). Individual clones were grown in 2 ml terrific broth (TC; 1.2%Tryptone, 2.4% Yeast extract, 0.4% glycerol, 0.17M KH_2PO_4, 0.72M K_2HPO_4) containing 20 μg/ml tetracycline (Sigma Chemical Corp., St. Louis, MO, USA) for 4 hr and later transferred to 500 ml TC broth with shaking in the dark overnight at 37°C. Cells were harvested by centrifugation at 5000 x g in a Sorvall GSA rotor for 10 min at 4°C. It was then washed twice with an equal volume of TN buffer pH8.0 at 4°C. The final cell pellet was resuspended in 10 ml of TN buffer and then disrupted by passing the cells twice through a Carver press (Fred.S. Carver Inc., NJ) held at -20°C. The cell debris was removed by centrifugation in a Sorvall SS34 rotor at 10,000 x g for 60 min at 4°C. The supernatant was collected for affinity column purification. Briefly, the column was prepared by coupling purified anti-HuIFN-γ mAb MIF3052 (Alfa & Jay, 1988) to cyanogen bromide activated Sepharose 4B (Pharmacia Fine Chenicals, AB, Montreal, Quebec, Canada) according to the methods of the manufacturer. Before loading onto the affinity column, supernatants of the cell lysates were adjusted to 50 mM Tris, 500 mM NaCl at the same pH8.0. The adsorbed rHuIFN-γ or Del-122 was then eluted with 50 mM diethylamine hydrochloride, pH11.2. Each 1 ml fraction was collected into tubes containing 0.1 ml of 2M Tris-HCl (pH7.5) for neutralization. The purified proteins were stored at 4°C

until further use.

Estimation of protein concentration

Protein concentration of purified rHuIFN-γ or Del-122 was estimated by the method of Schaffuer and Weissmann (1973) using AmidoBlack (Sigma) adsorption microassay. Briefly, the sample was precipitated with 10% trichloroacetic acid (TCA; Fisher) in the presence of 1% SDS, vaccum filtration was performed using a Millipore membrane (0.22μm; Millipore Corp., Bedford, MS, USA) and staining was done with 0.1% Amidoschwarz 10B in methanol : glacial acetic acid : distilled water (45:10:45 vol%). The protein-dye complex was eluted by immersing filters in tubes containing 2 ml eluent solution (25mM NaOH, 0.05%v/v aqueous ethanol) at room temperature for 10 min with frequent vortexing. Its absorbance was determined at 630ηm on a spectrophotometer (Shimadzu UV-160; Shimadzu Corp., Kyoto, Japan). Tubes containing 0, 0.3, 1.0, 2.0, 3.0 μg of BSA were run in paralled to provide the standard curve . The resulting standard curve was used to calculate the protein content of unknown samples.

Antiviral Assay

A standard virus-induced cytopathic effect assay was used to titrate the antiviral activity of rHuIFN-γ and Del122. Briefly, HuIFN-γ or Del122 was serially diluted in a 96-well tissue-culture tray (Corning, Medford, MA) containing 4 x 10^4 A_{549} cells/well. After 18 hr incubation at 37°C, 5%CO_2, the cells were challenged with EMC virus at a multiplicity of 5$TCID_{50}$/cell. After a further 24 hr incubation at 37°C, 5%CO_2, the cells were stained with 0.25%(w/v) crystal violet, 20mM Tris-HCl, pH7.5, 0.9% (w/v) NaCl in 20% (v/v)

methanol for 10 min. The monolayers were washed and the bound dye was eluted from each of the stained cultures with 100 μl of methanol. Absorbance was determined using an ELISA plate reader with 590 ηm filter. The titre was determined as the reciprocal of the dilution that produced 50% CPE when compared to the untreated cell control.

EMC replication cycle

Encephalomyocarditis (EMC) is an RNA-containing virus that belongs to the picornavirus family. EMC is sensitive to the antiviral activity of HuIFN-γ (Alfa & Jay, 1988). A time-course experiment was performed to determine its replication cycle. Firstly, A_{549} cells were seeded in T-25 tissue-culture flask in RPMI medium (GibcoBRL, Burlington, Ont, Canada) with 10% fetal calf serum (FCS). The monolayer of cells were infected with EMC at 2pfu/cell for 30 min. Culture was washed with PBS and replaced with 5 ml fresh, completed RPMI medium. At various times post-infection, 0.1 ml aliquots were collected and virus yield of each aliquot was determined by serial diluting each aliquot onto A_{549} cells in a 96-well tissue culture plate and stained after 48 hr of incubation at 37°C, 5%CO_2. The virus titre was determined as the reciprocal of the dilution that produced CPE.

Pulse-chase experiment

To compare the rate of antiviral state development (AVS) of rHuIFN-γ and Del-122, a pulse-chase experiment was set up as follows : A_{549} cells were treated with a high dose of 2,000U/ml rHuIFN-γ for 2 hr at 2, 4, 6 ,8, 10 hr prior to EMC infection. The cells were washed and chased with fresh RPMI medium. Then, the cells were challenged with EMC at 2$TCID_{50}$/cell for 7 hr. Virus yield in each culture was determined. Results were

expressed as a percentage of the virus yield of the untreated control. A similar procedure was performed with Del-122 and results were compared.

RESULTS

In this study, the kinetics of antiviral state developement of HuIFN-γ and Del-122 were compared to determine whether having the functional enhancement domain E_3 would significantly increase the efficiency of HuIFN-γ in developing antiviral activity. Both the full length rHuIFN-γ and truncated variant Del-122 were purified by immuno-affinity column chromatography. Purified fractions were assayed for protein concentration and antiviral activity. The specific activity of rHuIFN-γ was 1.36×10^8 U/mg and 5 times greater than that of Del122, which had a specific activity of 2.56×10^7 U/mg (Table 7). These data were consistent with previous findings that Del-122 had a 3 to 10 fold lower specific activity than the rHuIFN-γ molecule (Luk et al., 1990). In order to check whether similar amounts of antiviral units of rHuIFN-γ and Del-122 produce a similar activity curve in a standard antiviral assay, 128U/ml of each purified preparation were serially diluted into microtitre cultures of A_{549} cells and assayed for their antiviral titres. As shown in Fig. 9, the activity curve was essentially identical.

EMC virus was chosen for studying the rate of AVS development for IFN because of its sensitivity and its short replication cycle. Virus yield at each time point post-infection was determined by quantifying the virus yield with a serial dilution on A549 cell cultures for 48 hr and the viral titre was taken as the reciprocal of the dilution that produced CPE. Figure 10 demonstrates the accumulation of EMC as a function of hours post-infection in a standard EMC infection. One replication cycle was found to be 7 hr (Fig. 10). This replication cycle was remarkably reproducible in repetitive experiments. Seven hours infection time was selected as optimal condition.

In the HuIFN-γ pulse-chase experiment, an equivalent amount of antiviral units of

Table 7. Specific Activity of rHuIFN-γ and Del-122

Protein Name	Antiviral Activity (U/ml)	Concentration (μg/ml)	Specific Activity (U/mg)
rHuIFN-γ	1.36×10^6	10.0	1.36×10^8
Del122	6.40×10^4	2.5	2.56×10^7

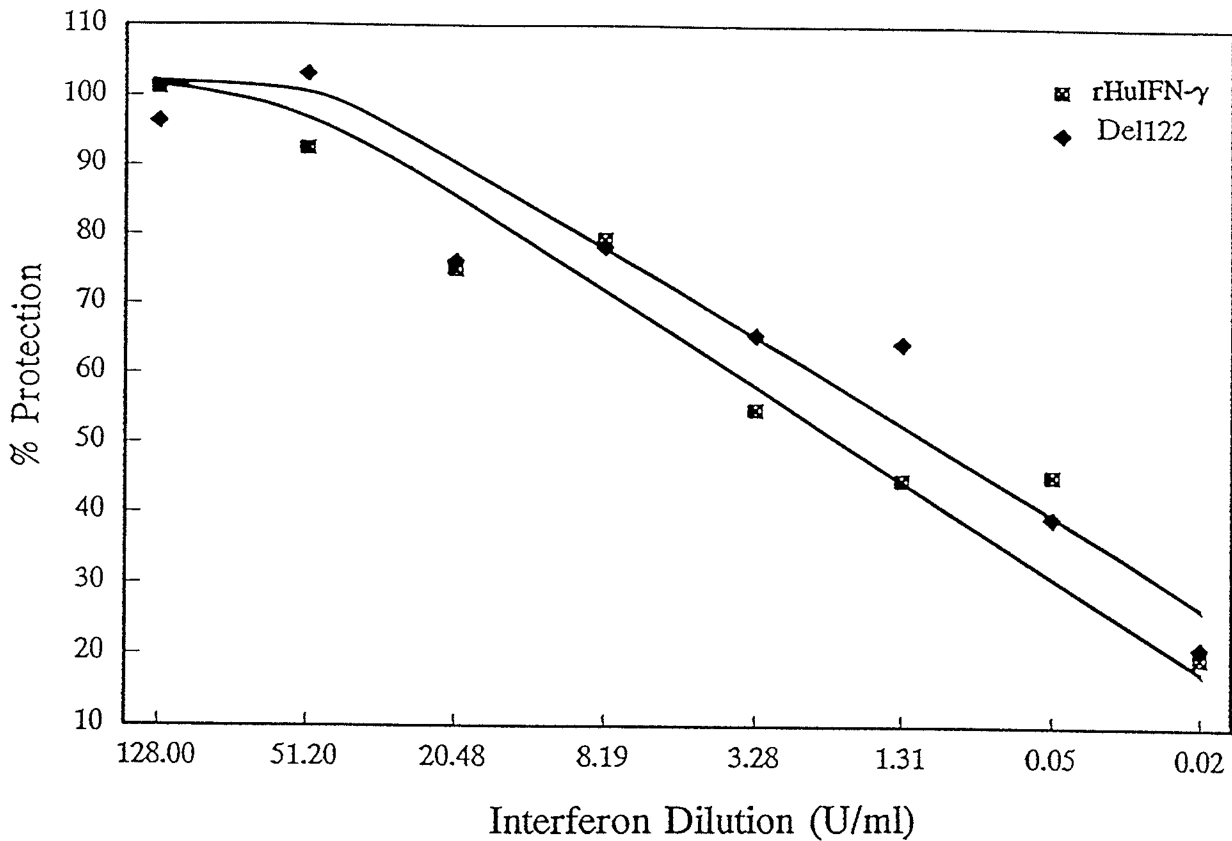

Figure 9 Antiviral activities of purified rHuIFN-γ and Del122. Purified rHuIFN-γ or Del122 at 128 U/ml was serially diluted onto A_{549} cell cultures seeded at 4 x 10^4 cells/well. The cells were challenged with EMC virus at a multiplicity of 5 $TCID_{50}$/cell after 18 h of incubation at 37°C, 5% CO_2. The plate was stained with crystal violet solution after a further 24 h incubation. Absorbance was determined using an ELISA reader with a 590ηm filter. Results were expressed as a percentage of cell protection.

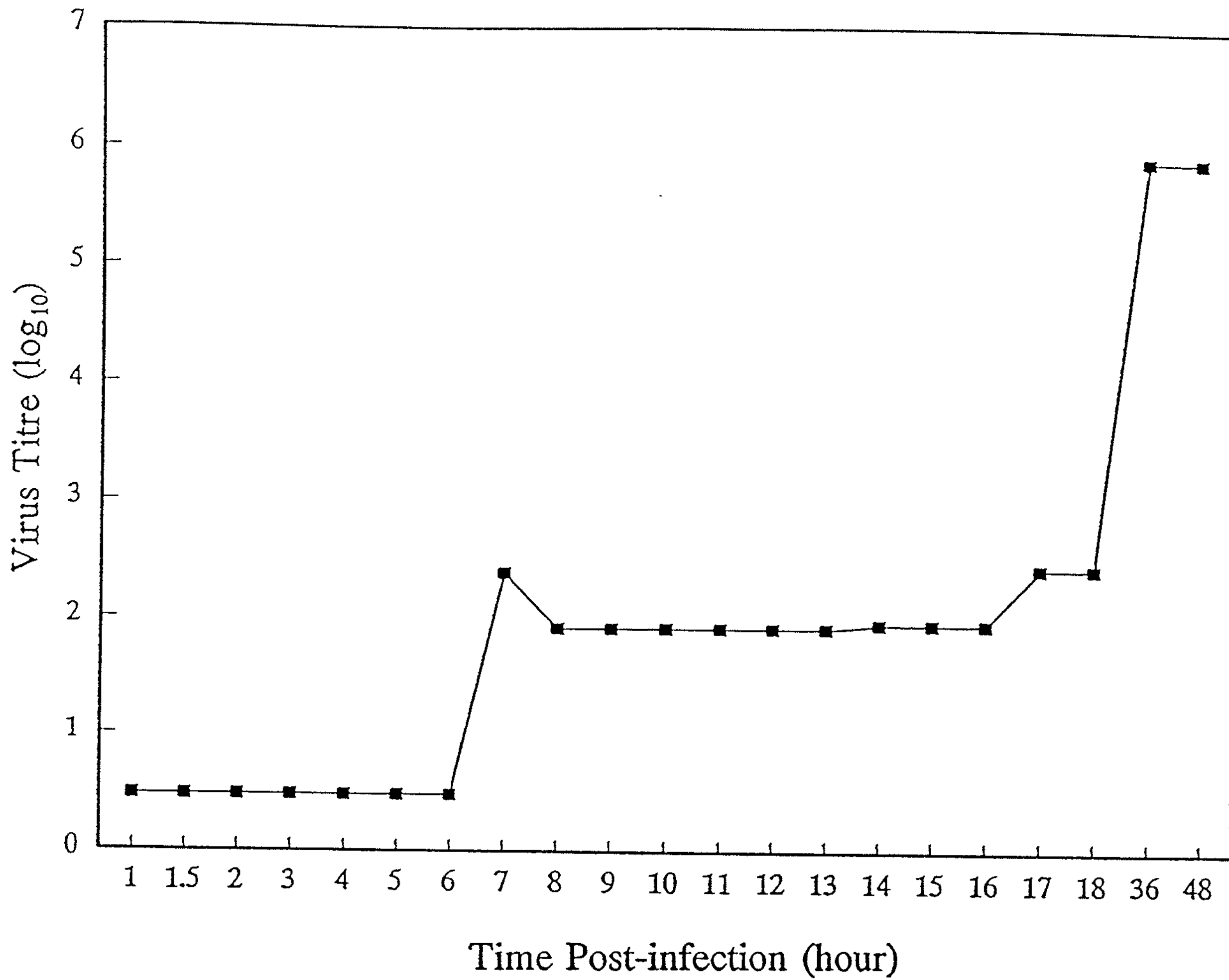

Figure 10 EMC virus replication cycle. A monolayer of A_{549} cells seeded in a 25 cm^2 tissue culture flask was infected with EMC virus at 2pfu/cell for 30 min. The culture was washed with PBS and replaced with fresh completed RPMI medium. At various times post-infection, 0.1 ml aliquots were collected and the virus yield was determined by serial diluting each aliquot onto A_{549} cells in 96-well plate and stained after 48 h.p.i. The virus titre was obtained as the reciprocal of the maximum dilution that produced CPE.

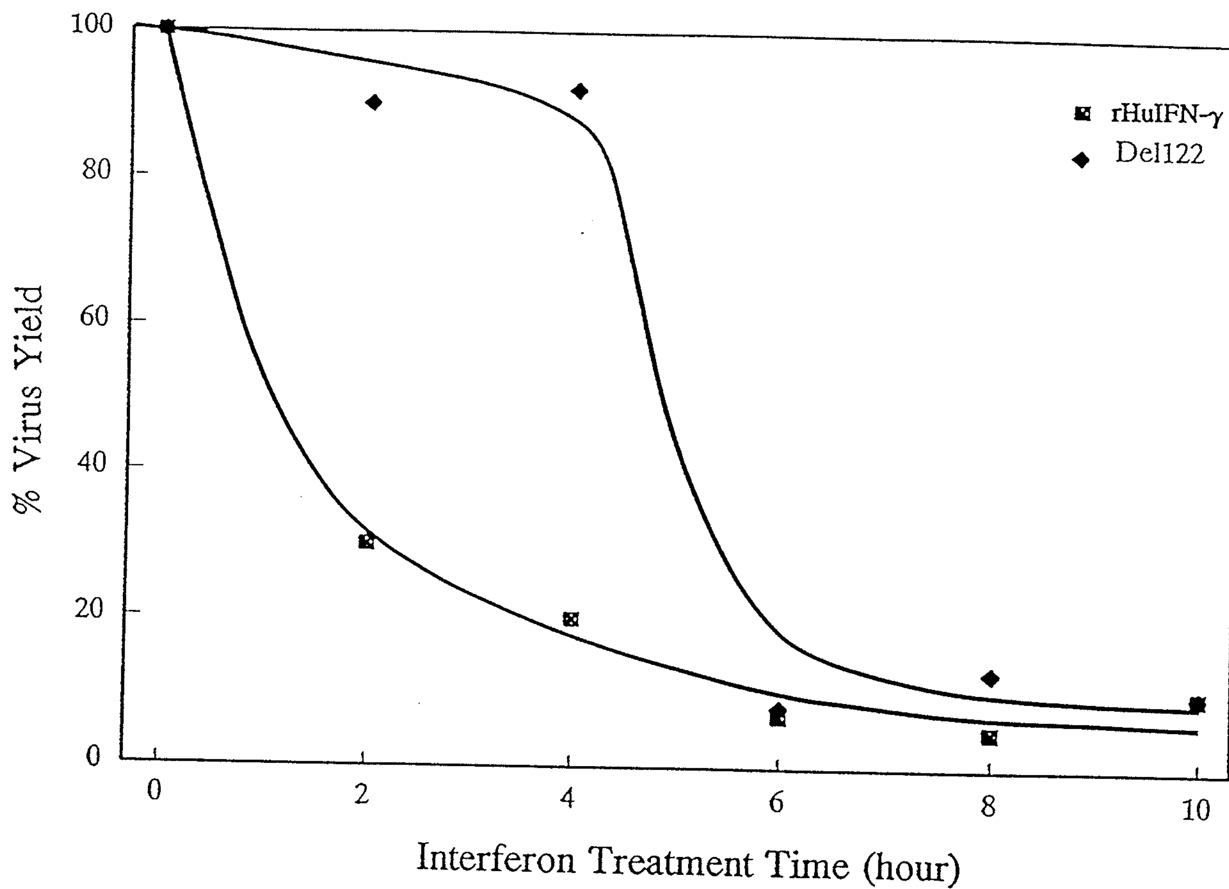

Figure 11 Effects of rHuIFN-γ and Del122 on the rate of antiviral state development. Cultures of A_{549} cells were pulse treated with rHuIFN-γ or Del122 (2,000 U/ml) for 2 h, washed and then chased for 2, 4, 6, 8 h prior to infection with EMC at 2 $TCID_{50}$/cell. At 7 h.p.i., the cultures supernatants were collected and the virus yield was determined as described in Figure 10 legend. Results were expressed as a percentage of the virus yield of the untreated culture control.

HuIFN-γ and Del-122 was used so that the latter has greater molar concentration to account for any variablity of these two molecules in binding. The experiment was performed in duplicate and the results were averaged and were plotted in Fig. 11 as a percentage of the virus yield as a function of time. The time shown in the graph represents 2 hr treatment plus chase before EMC challenge. The percentage virus yield of IFN-treated cells relative to the untreated control reflects the level of AVS development. Results indicated a significant difference in the rate of AVS developement. At 50% reduction in virus yield, rHuIFN-γ required only 1-1/4 hr but no inhibition of EMC was observed in cells treated with Del122 at that time point. At a later time of 5 hr post-treatment, cells treated with Del122 attained the same level of AVS development at 50% virus reduction. However, at 6 hr post-IFN treatment, complete AVS development could be achieved by both interferon proteins. This suggested that although the full length rHuIFN-γ developed AVS in a faster rate, both molecules will ultimately induce an AVS to full extent. These results indicate that the rHuIFN-γ molecule having an additional functional domain E_3 induces an antiviral state at a faster rate than Del-122, but that eventually both molecules produce the same antiviral level. Based on these observations, it is consistent with our hypothesis that E_1 and E_3 may function as NLS.

DISCUSSION

Over the past few years, a lot of research effort has been focused on transportation of proteins into the nucleus. The first demonstration that nuclear import required a short stretch of amino acids was demonstrated by Dingwall et al. (1982) in the studies of nuclear translocation of nucleoplasmin. Their results showed that nucleoplasmin, without its C-terminal tail, would not enter the nucleus when introduced into the cytoplasm by microinjection, but remained in the nucleus when directly injected into the nucleus. In addition, they found that the C-terminal peptide alone was sufficient for nuclear translocation. Their studies indicate that the C-terminal portion of nucleoplasmin is crucial for passage of the complete protein into the nucleus.

Further studies on protein transport showed that specific NLSs reside in the transported protein (Landford & Butel, 1984; Kalderon, et al., 1984). Robbins et al. (1991) reported that nucleoplasmin NLS consisted of two interdependent regions of basic amino acids. The nuclear localization failed only when both domains were mutated i.e. mutations in either alone had no effect on nuclear localization activity. Although most NLSs are rich in basic amino acids, not all of them are of the prototypic NLS of SV40 T antigen (Pro-lys-lys-lys-arg-lys-val) (Silver, 1988). Subsequent analysis indicate that some proteins contain more than one functionally redundant NLS. For example, nuclear localization of polyoma large T antigen is eliminated completely only when both of its non-identical NLSs are mutated (Richardson et al., 1986). Evidence has also shown that the position of the NLS and its context in the transported protein are crucial (Roberts, et al., 1987; Nelson & Silver, 1989). For instance, when SV40 T antigen NLS was placed in the buried hydrophobic domain of pyruvate kinase, it would become a nonnuclear protein, implying that NLS must

be exposed on the surface of the protein for interaction with the other proteins to be imported. Based on the observations that both functional epitopes E_1 and E_3 which contain NLS-like elements (Zu and Jay, personal communication) are exposed on the surface of HuIFN-γ, it is highly likely that these two antiviral domains in fact have a further function involving nuclear transport.

In order to determine whether E_3 functions cooperatively with E_1 to increase the rate of AVS development, the effect of E_1 and E_3 on the kinetics of development of an AVS in A_{459} cells was therefore studied. It is known that nuclear transport is a slow process and the rate of transport not only depends on the sequence of NLS but also the number of signals present. Based on the effect of multiple signals on nuclear transport, results obtained in this kinetic study on antiviral state development may also reflect if E_1 and E_3 function as NLS since the efficiency of nuclear translocation is dependent on the number of signals present.

In this study, to eliminate the possibility of a difference in binding affinity of rHuIFN-γ and Del-122, an equivalent concentrations of antiviral units of both proteins were added in this study. As a result, even though Del-122 lacking the C-terminus (or E_3 domain) may be slower in binding than rHuIFN-γ, the higher molar concentration of Del-122 would offset such a difference. At time zero of interferon treatment, virus yield was taken as 100% i.e. no AVS was developed. However, after approximately 1-1/4 hr, a 50% reduction in virus yield was observed for those cells treated with rHuIFN-γ treatment whereas no EMC inhibition (i.e. no AVS development) was demonstratable in cells treated with Del122 at the same time point. With the increase in time, cells with Del122 treatment demonstrated the same extent of EMC inhibition as that treated with rHuIFN-γ. Hence, the rate of AVS development in response to Del-122 was significantly slower although the eventual level of

AVS (virus resistance) was similar at 6 hr post treatment. Full-length rHuIFN-γ was at least 3x faster than Del-122 in developing antiviral state. It appears that the more NLS that the peptide carries, the greater is its efficiency in developing AVS, thus affecting the rate of AVS development. These results are consistent with our hypothesis that both E_1 and E_3 functional epitopes on rHuIFN-γ function as NLS based on the effect of multiple signals. However, the current study was only an indirect measure and how E_1 and E_3 contribute to the nuclear import function is still not clear at this time.

In previous studies, much effort has been focused on the pathways linking signal transduction and nuclear events. Although a significant amount is known about the ISGF3 transcription activiation pathway, nothing is known about how the binding of HuIFN-γ to the cell surface receptor leads to the signal transduction from the surface receptor to the nucleus. It may involve several steps and often many common intermediates (Whiteside & Goodbourn, 1993). Since much evidence has shown interferons combine with specific receptors and exert their various biologic effects at the plasma cell membrane level (Vengris, et al., 1975; Friedman, 1979), the molecular basis of these biologic activities involves the induction of transcription of various genes by interferon, and the proteins produced by these genes effect a number of cellular responses. One of the well-established signaling pathway of IFN-γ is the phosphorylation of the 91kd cytoplasmic protein which enters the nucleus and binds to the GAS-like sequence of IFN-γ-responsive genes to activate transcription. This suggested pathway does not involve the nuclear translocation of the IFN-γ. However, one cannot exclude the possibility that there may be different pathways of IFN-γ action and that HuIFN-molecule having NLS-like elements appears to play a role in nuclear transport. Curtis et al (1990) demonstrated that IL-1 binds to its receptor to form a complex and is translocated to

the nucleus. The internalization of IL-1 and its receptor is correlated with signal transduction. Since a cytoplasmic deletion mutant of IL-1R which loses the ability to mediate IL-1 signal transduction (Curtis et al., 1989) is internalized in response to IL-1 (Curtis, unpublished data), it appears that receptor internalization alone is not sufficient for signal transduction. A model is suggested that the IL-1 receptor may be targeted by IL-1 to the nucleus. After nuclear localization, the IL-1R complex may participate directly or indirectly as part of the mechanism that regulates IL-1-induced gene transcription. It is interesting to note that three consecutive sequences are highly conserved in the mouse and human IL-1 receptor that contain a number of basic residues. These may function together as a complete nulcear localization signal (Dingwall & Laskey, 1986; Dingwall et al., 1987).

Recently, IFN-γ in the mouse system has been localized in the nucleus (Bader & Wietzerbin, 1992). These results further demonstrate that IFN-γ may itself be involved in the nuclear translocation function. Comparison of the a.a. sequences of different mammalian IFN-γ shows that the NLS-like element within E_3 of HuIFN-γ (K-R-K-R) is highly conserved. The fact the muIFN-γ contains the highly conserved NLS sequence and enters the nucleus indicates the NLS is likely to function in nuclear translocation. Since HuIFN-γ has 2 NLS-like elements, we speculate that the HuIFN-γ itself may be involved in nuclear transport, thereby representing a different signaling pathway. Results in our present study are consistent with E_1 and E_3 being NLS and may suggest a pathway linking the transport from the membrane to the nucleus by HuIFN-γ similar to the proposed pathway of IL-1. With the binding of IFN-γ to its specific cell surface receptor, an IFN-receptor complex is formed in the cytoplasm. Both E_1 and E_3 domains (contain NLS) on HuIFN-γ may direct the interferon-receptor complex to the nucleus and subsequently, multiple nuclear events such

as transcriptional DNA interactions are triggered. As a result, gene transcription is activated.

Based on the results in this study, our newly identified antiviral epitope E_3, as well as the previously identified epitope E_1, function cooperatively to increase the rate of induction of antiviral state. These results are consistent with our hypothesis that both E_1 and E_3 may function as NLS in nuclear import. At present, it is still unknown if NLS-like E_1 and E_3 really participate in nuclear function since there is no direct evidence on nuclear localization of HuIFN-γ. Subsequent studies on nuclear localization of HuIFN-γ and functional analysis on creating mutations in either or both domain(s) are necessary to provide a better understanding in the mechanism of HuIFN-γ action.

CHAPTER IV

THE C-TERMINUS OF HuIFN-γ IS ESSENTIAL FOR ANTI-CHLAMYDIAL ACTIVITY

INTRODUCTION

Chlamydiae are obligate intracellular pathogens that are the most frequently encountered sexually transmitted disease in human (Schachter, 1983). Numerous studies indicated IFN-γ inhibits the growth and infectivity of different chlamydia strains (Byrne, 1982; Kazar, 1971). However, the degree of inhibition depends on various factors such as cell lines and chlamydia species. Previous work have shown that antimicrobial activity can be induced by IFN-γ in several host-pathogen systems (Byrne & Turco, 1988; Murray, 1988). It seems unlikely that there is a single mechanism of IFN-γ-mediated antimicrobial activity induced in host cells against the intracellular microbes. In fact, several lines of evidence suggested that there are different pathways that IFN-γ induces inhibition on chlamydial growth. These pathways include tryptophan degradation by induced IDO (Byrne et al., 1986), production of microbial reactive metabolites (Nathan et al., 1983; Rothdermel et al., 1986), activation of human macrophages and IFN-γ-mediated cytotoxicity (Byrne, et al., 1988; Byrne, et al., 1989b). Recently, Mayer et al. (1993) demonstrated the induction of synthesis of nitric oxide by IFN-γ inhibits chlamydial proliferation in McCoy cells. Their results showed that the antichlamydial effect was directly related to the amount of nitric oxide, and the synthesis of which was IFN-γ dose dependent. Furthermore, the antichlamydial activity mediated by IFN-γ could be blocked by N-guanidino monomethyl L-arginine (MLA), an inhibitor of nitric oxide synthesis. Indeed, HuIFN-γ induces many cellular responses and some of these do not appear to be induced by the same pathway. For example, not all IFN-γ responsive genes have GAS or respond to the phosphorylated p91. This implies that there may be different signal transduction pathways to allow different biological responses to be differentially regulated.

In previous studies, eptiopes E_1, E_2 and E_3 have been characterized by neutralizing mAbs as antiviral neutralizing epitopes. In the present study, the ability of these mAbs to neutralize antichlamydial activity was tested. In addition, the antichlamydial, antiviral and antiproliferative activity of full length rHuIFN-γ and Del-122 are compared with an attempt to determine the importance of the C-terminus in anti-chlamydial activity. We demonstrate that the C-terminus is crucial for antichlamydial activity but none of these antiviral neutralizing epitopes are involved in the antichlamydial function of HuIFN-γ. An antichlamydial domain may reside in either residue 123 - 129 or 139 - 143 within the C-terminal region.

MATERIALS AND METHODS

Preparation of L2 Elementary Body

A stock of C. trachomatis serovar L2 (L2) was obtained from Dr. Grant McClarty. Stock cultures were prepared by the method described by Caldwell et al. (1981). Briefly, a monolayer of Hela cells was infected at high titre with L2 for 2 hr at 37°C, 5%CO_2. The cells were washed and replaced with EMEM medium (Eagle minimal essential medium with 10% fetal calf serum) and allowed to grow for 48 hr at 37°C, 5%CO_2. The cells were harvested and sonicated for 3s. Cell debris was removed by centrifugation at 1000 x g for 10 min. EBs of L2 in the supernatant were further concentrated by centrifugation at 15000 x g for 30 min. The resultant pellet was washed with HBSS (Hanks balanced salt solution) and suspended in sucrose-phosphate-glutamate (SPG) buffer (0.18M sucrose, 3mM potassium phosphate, 3.6mM sodium phosphate, 5mM glutamic acid, pH7.4). The stock was stored at -70°C until use.

Inclusion Assay for chlamydia

To quantitate the infectivity of L2 stock, A_{549} cells were seeded at 10^5 cells/well in 96-well plate overnight at 37°C, 5%CO_2. The monolayer was washed with phosphate buffered saline (PBS) and L2 was serially diluted onto the plate. After incubation for 2 hr, 37°C, the inoculum was removed and cells were washed with PBS. Fresh medium was added and cells were incubated for 48 hr at 37°C. Then, the monolayers were washed with PBS. Fixation was done by adding 100ul/well absolute methanol for 20 min at room temperature. The cells were washed with PBS. Mab 4D10 was diluted 1 : 500 in 2% BSA in PBS, 50ul/well and incubated for 70 min at 37°C, 5% CO_2. The plate was washed with

PBS-Tween 20 for 10 min per wash three times on a rocking platform. Peroxidase-conjugated goat anti-mouse (1:2,000 in 2% BSA-PBS; 50 μl/ml) added and incubated for 45 min at 37°C, 5%CO_2. After washing with PBS-Tween 20 three times, peroxidase substrate 4-chloro-1-naphthol (1%(w/v)in methanol, 0.01%(v/v)hydrogen peroxide) was added at 100 ul/well. The plate was emptied and washed with 200 μl/well PBS and 100 μl/well PBS with 0.02% sodium azide was added for storage at 4°C. All experiments were performed in triplicate and the titre was expressed in IFU/ml.

Anti-chlamydial Assay

IFN-induced anti-chlamydial activity was assayed using a modified inclusion assay. A_{549} cells were seeded in 96-well plate and treated with equivalent amounts (ηg/ml or antiviral units/ml) of rHuIFN-γ and Del122 for 24 hr. The cultures were infected with L2 at a titre that would result in 10% - 20% of the cells in the control culture (no IFN treatment) developing inclusions 48 hr post-infection. At 2 hr post-infection, the innoculum was removed and the cultures were processed as described in the inclusion assay to visualize the inclusions for counting. The experiments were repeated three times, and the number of inclusions in IFN-γ or Del122 - treated monolayers was expressed as a percentage of the number of inclusions present in the control monolayers.

Antiviral Assay

The assay involved the measurement of the inhibition of cytopathic effect of encephalomyocarditis virus challenge on A_{549} cells. The method has been described previously (Alfa & Jay, 1988).

Antiproliferative Assay

A_{549} cells were seeded at 10^4 cells/well and incubated for 24 hr at 37°C. rHuIFN-γ and Del122 were serially diluted onto the plate. Cells were allowed to grow for 3 days until the control culture (no treatment) almost reached confluency. The cells were washed, fixed and stained with crystal violet solution (0.25% in 20mM Tris-HCl, pH7.5, 0.9% (w/v) NaCl, 20% (v/v) methanol). The excess dye was washed away with tap water. Methanol (100 ul/well) was added to extract the dye and the absorbance was determined with an ELISA reader fitted with a 590 nm filter. Results were expressed as % of control cultures (without IFN).

Antichlamydial Neutralization Assay

In order to test whether mAbs neutralizing antiviral activity have neutralizing effect on chlamydial activity, 3 mAbs, MIF3009, MIF3125 and MIF3037 which neutralize epitopes E_1, E_2 and E_3, respectively were chosen for this study. A_{549} cells were seeded at a density of 4 x 10^4 cells/well in 96-well tissue culture plate. A mixture of 3,000U/ml rHuIFN-γ and serial dilution of the 3 mAbs in RPMI medium were prepared and stored at 4°C for 2 hr. The mixture of rHuIFN-γ and mAbs were added correspondingly onto the culture plate and incubated for 24 hr at 37°C, 5% CO_2. After washing with 100ul/well PBS, the monolayers were infected with L2 for 2 hr at 37°C, 5% CO_2. Inoculum was removed and fresh mixture of HuIFN-γ and mAbs were added onto the plate and incubated for 46 hr. Inclusion staining was performed as previously described. All experiments were done in duplicate and results were expressed as % L2 infection present in control monolayer that had rHuIFN-γ treatment only.

RESULTS

In order to determine the effect of HuIFN-γ in the antichlamydial activity, an immunocytochemical method to quantitate cytoplasmic inclusion body (reticulate body) of C. trachomatis (L2) was developed and calibrated. Cytoplasmic inclusion bodies were examined under 100X magnification and the number of inclusions per microscopic field in 4 random fields were counted and averaged. Data were plotted as shown in Figure 12. Results were expressed as inclusion forming units (IFU)/ml and the titre of L2 stock was calculated at 2.19×10^8 IFU/ml. Since the number of inclusions relative to the L2 titre is not linear over the entire range, all L2 titres were evaluated by infecting cultures with 5-fold serial dilutions and only cultures that provided 30 to 200 inclusions per field was used for quantitation.

In order to test whether any of the antiviral epitopes are involved in the anti-chlamydial activity, attempts were made to neutralize the antichlamydial activity with mAb MIF3009 (E_1), MIF3125 (E_2) and MIF3037 (E_3). As shown in Fig 13, none of the mAbs MIF3009, 3125 and 3037 at 2.62×10^6 NAV/ml, 8.19×10^5 NAV/ml, and 7.69×10^2 NAV/ml, respectively demonstrated any antichlamydial neutralizing activity.

In an effort to find if the C-terminus is involved in the antichlamydial activity, the ability of Del-122 to inhibit L2 infection was compared with that of rHuIFN-γ. Each rHuIFN-γ and Del-122 preparation was adjusted to 500ηg/ml and was serially diluted 1/5 in a microtitre plate containing A_{549} cells and incubated for 24 hr. As shown in Figure 14, 20 ηg/ml of rHuIFN-γ was required to inhibit L2 by 50%. However, Del122 at the highest concentration of 500 ηg/ml did not demonstrate any anti-chlamydial activity.

We have determined that the antiviral specific activity of rHuIFN-γ (1.36×10^8U/mg) to be 5 times that of Del122 (2.56×10^7U/mg). In order to avoid variations as a result of

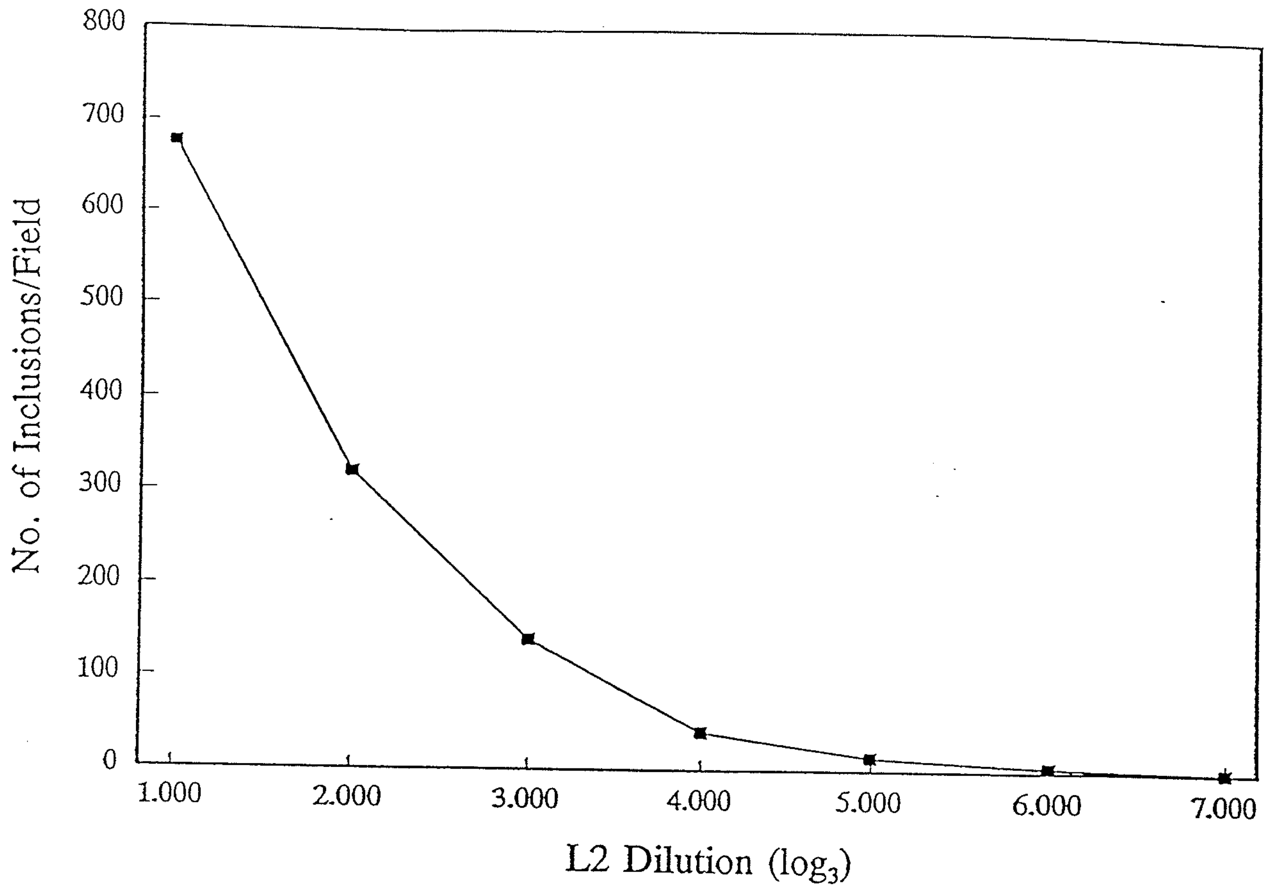

Figure 12 Inclusion assay for C. trachomatis L2. L2 was serially diluted 1:3 onto confluent A_{549} cell monolayers in a 96-well plate. After 2 h of incubation at 37°C, 5% CO_2, the inoculum was removed and the cultures were washed with PBS. Fresh medium were added and the cultures were incubated for a further 48 h. The cultures were washed with PBS and fixed with methanol for 20 min at room temperature. Inclusions were detected by incubation with Mab 4D10 (1:500) for 70 min, followed by peroxidase conjugated goat anti-mouse antibody (1:2000) for 45 min. Peroxidase substrate 4-chloro-1-naphthol was added for colour development. Inclusions were counted and results were expressed as number of inclusions/field.

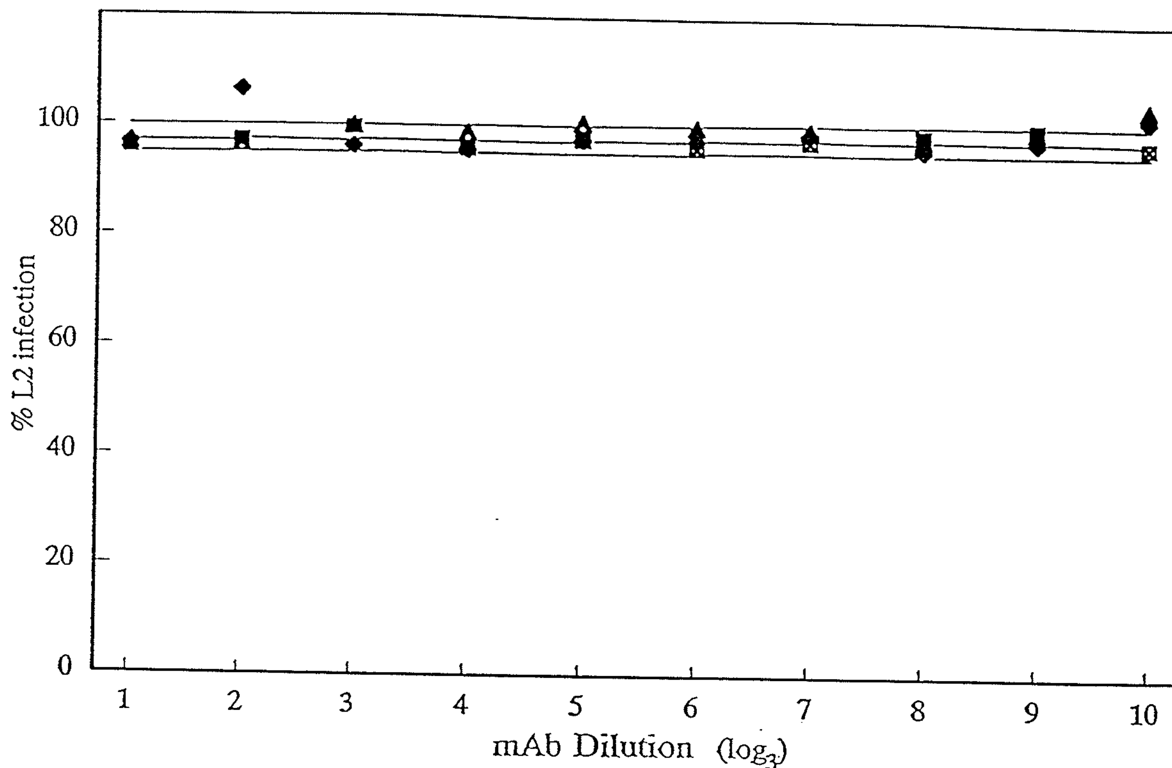

Figure 13. Antichlamydial Neutralization Activity of MIF3009, MIF3125, and MIF3037. A549 cells seeded at a density of 4 x 10^4 cells/well in a 96-well tissue culture plate. A mixture of rHuIFN-γ and 1:3 serial dilution of MIF3009 (■), MIF3125 (◆) and MIF3037 (▲) were added onto the culture plate and incubated for 24 hr, 37°C, 5% CO_2. After washing with 100ul/well PBS, the monolayers were infected with L2 for 2 hr. Inoculum was removed and fresh mixture of HuIFN-γ and mAbs were added onto the plate and incubated for 46 hr. Inclusion staining was performed as described previously in Fig. 12 legend. Results were expressed as % L2 infection present in control monolayer that had rHuIFN-γ treatment only.

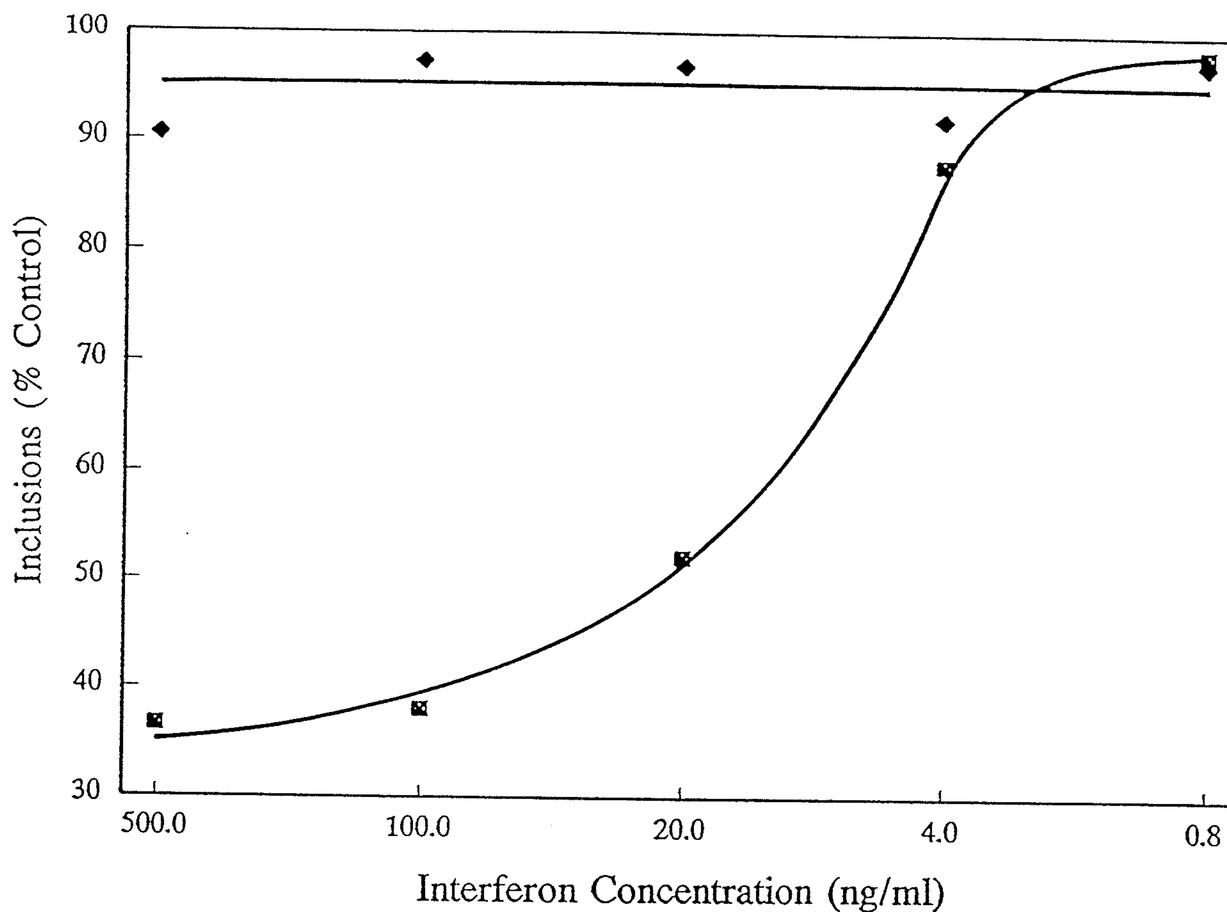

Figure 14 Comparison of anti-chlamydial activities of rHuIFN-γ & Del122. Confluent A_{549} cultures were treated with equivalent serial dilutions (starting at 500ηg/ml) of rHuIFN-γ (■) or Del122 (◆), for 24 h and infected with L2 that was adjusted to produce inclusion bodies in 10% - 20% of the untreated cultures at 48 h.p.i. The cultures were processed as described in Figure 12 legend to visualize the inclusions. Results were expressed as a percentage of the number of inclusions of treated cultures relative to the untreated control.

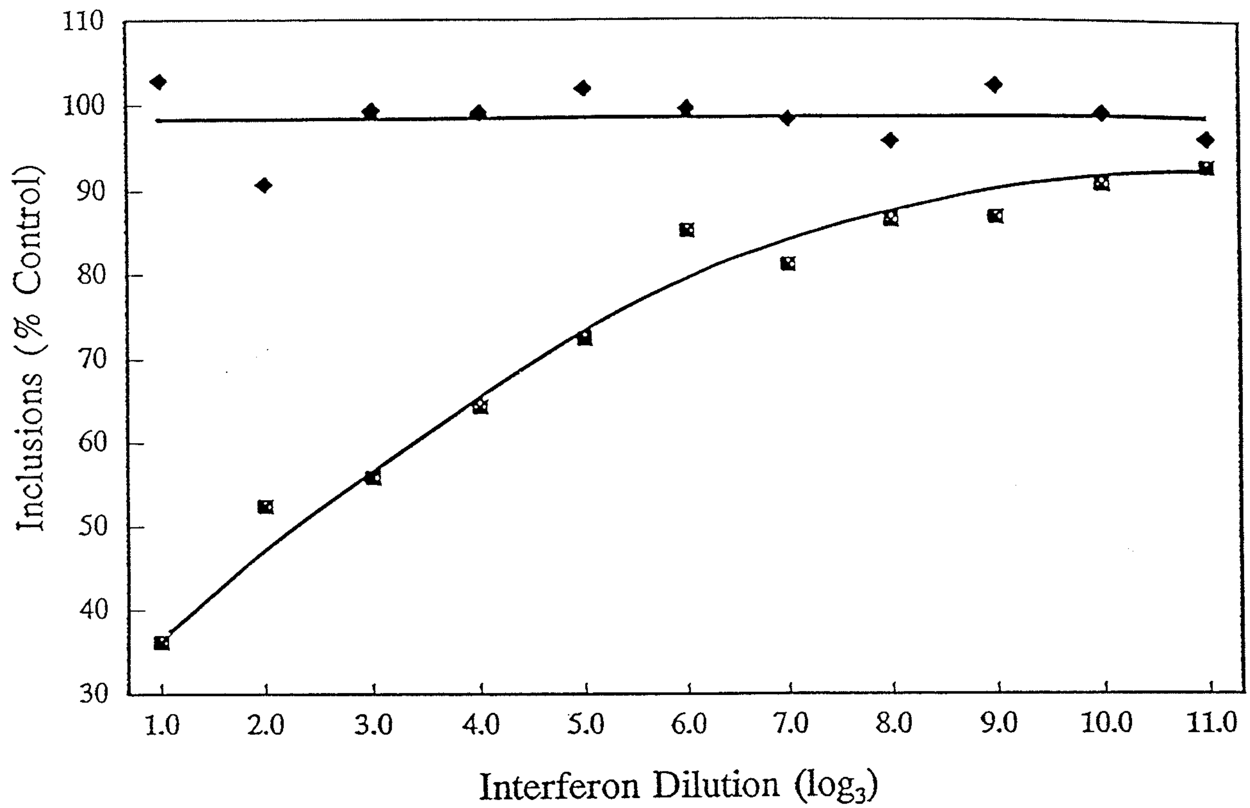

Figure 15 Comparison of anti-chlamydial activities of rHuIFN-γ & Del122. Confluent A_{549} cultures were treated with equivalent serial dilutions (starting at 30,000 U/ml) of rHuIFN-γ (■) or Del122 (◆) for 24 h. Cultures were infected with L2 and the plates were processed as described in Figure12legend. Results were expressed as a percentage of the number of inclusions of treated cultures relative to the untreated control.

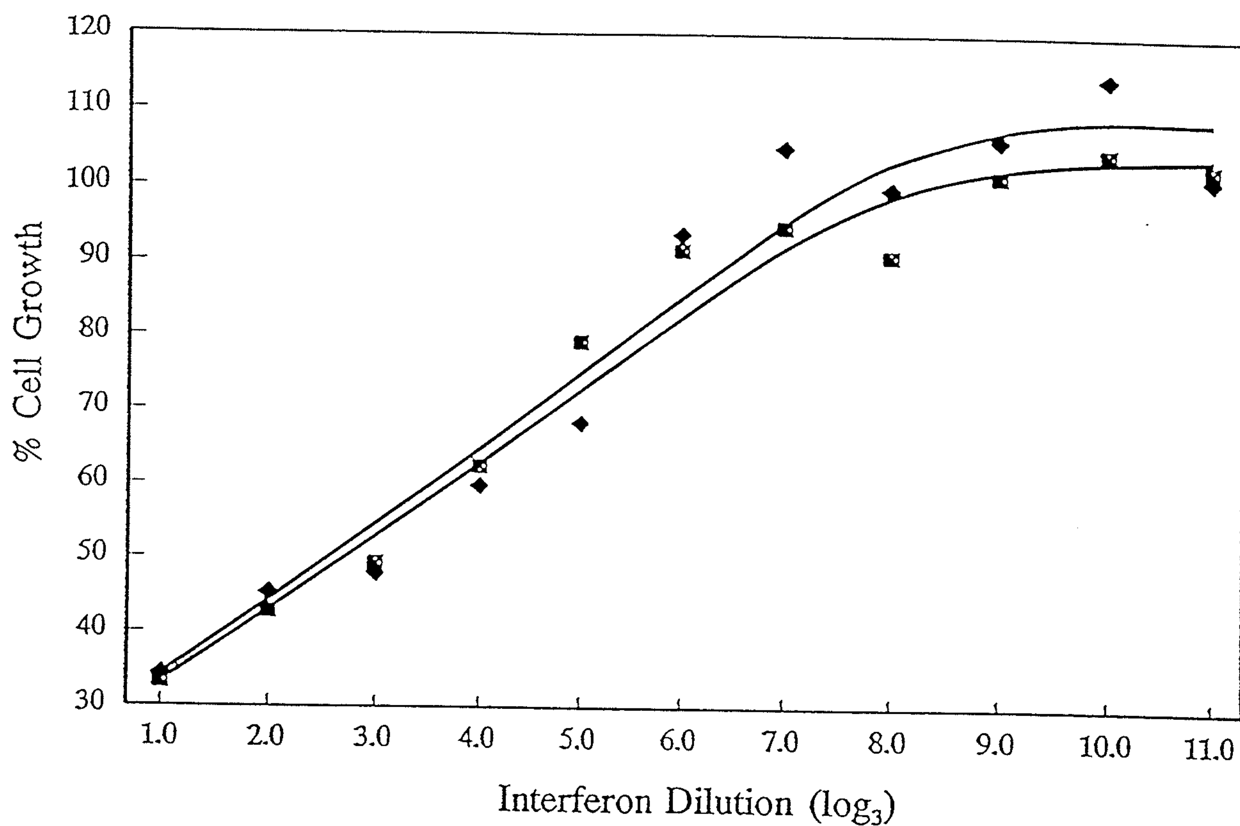

Figure 16 Comparison of anti-proliferative activities of rHuIFN-γ and Del122. A_{549} cells seeded at 10^4 cells/well in a 96-well microtitre plate were incubated overnignt at 37°C, 5% CO_2. Equivalent amounts of rHuIFN-γ (■) or Del122 (◆) at 20,000U/ml were serially diluted 1:3 into the culture wells. Cells were fixed and stained with 0.25% (w/v) crystal violet in 20mM Tris-HCl, pH7.5, 0.9% (w/v) NaCl, 20% (v/v) methanol on day 3, then washed in tap water. The retained dye was extracted with 100 μl methanol and quantitated with an ELISA reader fitted with a 590ηm filter. Results were expressed as percentage of cell growth in tested culture relative to untreated control.

differences in overall specific activity, we compared the anti-chlamydial activity using equivalent antiviral activity as the basis of comparison. As shown in Figure 15, the 50% inhibition end-point was achieved with rHuIFN-γ at 3,000 U/ml, but no anti-chlamydial activity was detected at 30,000 U/ml Del-122. Indeed, significant inhibition (20%) was already observed with 300 U/ml rHuIFN-γ.

In order to determine if the C-terminus also affects other HuIFN-γ activities, we compared the anti-proliferative activities of rHuIFN-γ and Del-122. In this experiment, an equivalent antiviral units of rHuIFN-γ and Del-122 was serially diluted into microtitre well cultures of A_{549}. A characteristic sigmoid curve was obtained with either molecule (Figure 16). The anti-proliferative activity curves of rHuIFN-γ and Del-122 were similar when compared at equivalent antiviral unit concentration. Thus the effect of the loss of the C-terminus on the anti-proliferative activity was not different from the effect on the antiviral activity.

DISCUSSION

The effect of IFN-γ on chlamydial multiplication has been reported by several investigators using different cell lines (Shemer & Savor, 1985; Rothermel, 1983). Shemer and Savor demonstrated that human carcinoma cells treated with HuIFN-γ prior to infection resulted in a great reduction of chlamydia trachomatis (L2/434/Bu) infectious particle yield. Compared with the untreated controls, infected cell cultures pre-treated with IFN-γ have fewer infected cells, fewer chlamydiae per inclusion and less infectious yields. Their study using electron microscopy further showed that interferon did not affect chalmydial conversion to reticulate bodies but had effect on the extent of maturation to elementary bodies.

Previous studies on IFN-induced inhibition of C. trachomatis by de la Maza et al. (1985) indicated that the antichlamydial effect by particular IFN subtypes IFN-αA, IFN-αD and their hybirds in Hela cells is dissociated from antiviral and antiproliferative effects but there is a correlation among the antichlamydial, antiviral and antiproliferative activities of IFN subtypes in McCoy cells. Their observations suggest that these biological effects may be mediated by different mechanisms. In a mouse fibroblast cell line (McCoy cells), the effect of recombinant murine IFN-γ on the growth of C. trachomatis was examined (de Le Maza et al., 1985) The antichlamydial activity of muIFN-γ was very potent even at very low IFN concentration. This same IFN concentration resulted in significant inhibition of EMC virus yield (antiviral activity) but had minimal effect on antiproliferative activity. Their results showed that murine IFN-γ activates different pathways different from those induced by IFN-α and -β since antichlamydial activity of IFN-α/β in the same cell system was significantly lower than the antiviral activity (de la Maza et al., 1984; Stewart, 1979). They further demonstrated that antichlamydial activity in McCoy cells did not depend on

tryptophan concentration. Since HuIFN-γ-induced anti-toxoplasma activity was dependent upon trp concentration (Pfefferkorn, 1984), they suggested that there are different IFN-γ-mediated pathways in these two intracellular pathogens although they share common entry mechanisms and intracellular replication sites.

Whether HuIFN-γ makes use of similar pathway to induce antiviral and antichlamydial activities was unknown. Since we have defined three antiviral neutralizing epitopes, it was possible to determine whether these epitopes were also involved in antichlamydial activity. Results demonstrated that none of the mAbs from each of the 3 epitopes was able to neutralize the antichlamydial activity of HuIFN-γ. Thus, molecular domain(s) essential for the induction of antichlamydial activity must be independent from those important for the induction of antiviral response.

In order to determine whether the C-terminal region is essential for the antichlamydial activity, we have compared the antichlamydial activity of rHuIFN-γ and of Del-122. The latter lacked the C-terminal 21 residues. While rHuIFN-γ at 20ηg/ml was able to inhibit L2 by 50%, Del-122 did not demonstrate any antichlamydial activity at 500ηg/ml. Since Del122 has a specific antiviral activity 5 times lower than the full-length molecule (rHuIFN-γ), the function comparisons were made on basis of equivalent antiviral units. The rHuIFN-γ was able to achieve a 50% inhibition of chlamydial growth at 3,000 U/ml. However, Del122 demonstrated no detectable anti-chlamydial activity even at 30,000 U/ml, which was the maximum concentration achieved in the experiment. A level of 20% inhibition of chlamydial growth was achieved with only 300 U/ml rHuIFN-γ. This is compared to Del-122 which did not demonstrate a hint of anti-chlamydial activity even at 100 times that concentration (30,000 U/ml). Taking 20% inhibition as the endpoint, the antichlamydial activity of Del-

122 must be at least 100 times lower than that of the rHuIFN-γ.

Based on the findings of de la Maza et al. (1985) that there is no association between antichlamydial and antiproliferative effects of muIFN-γ in the mouse cell lines, attempts were made in this study to compare the antichlamydial and antiproliferative effects of HuIFN-γ in human cell lines. As a result, the antiproliferative activity of rHuIFN-γ and Del-122 was compared. It was observed that the antiproliferative activity curve of Del-122 was not different from that of the HuIFN-γ when the IFN concentrations were normalized to equivalent antiviral activity units. Since Del-122 did not demonstrate a loss of antiproliferative activity, the loss must be specific to the antichlamydial activity. The antichlamydial domain, and therefore the induction pathway, must be separated from those of the antiviral and antiproliferative activities.

On the basis of this result, the C-terminus must play an important role in the anti-chlamydial activity. Thus, there may exist within the C-terminal 21 residues (residues 123 - 143) a functional domain for the anti-chlamydial function. Since the E_3 domain, when neutralized by MIF3037, did not affect the antichlamydial activity, residues 130 - 138 must not be essential for the induction of this activity. The likely location of this antichlamydial domain must be either 123 - 129 or 139 - 143. Further study is required on the localization of the functional domain on HuIFN-γ in the inhibition of chlamydia. However, the fact that the C-terminus is essential for antichlamydial but not antiviral or antiproliferative effects indicates that there is a different structural requirement for these activities. It appears that the mechanism or pathway of IFN-γ action on these biological effects must be different.

CHAPTER V

CONCLUSION

HuIFN-γ is a secretory protein which exhibits various biological activities through the binding to its specific cell surface receptor to activate transcription of different responsive genes. One of the well-established signaling pathway of IFN-γ is the nuclear translocation of cytoplasmic p91 protein which upon phosphorylation will enter into the nucleus and binds to the cis-acting DNA sequence (GAS-like) upstream in the promoter region of most IFN-responsive genes (Shuai et al., 1992). However, this is likely not the only pathway as it cannot account for the pathway for those genes without the GAS-like sequence.

Comparison of the amino acid sequences of all known mammalian IFN-γ species shows certain conserved regions. One of the highly conserved regions is a few basic a.a. residues within the C-terminal region (Ealick et al., 1991). Over the past few years, much effort were focused on analyzing the structure-function relationship of this pleiotropic molecule. Some research groups studied the C-terminus by creating deletion mutants to determine how the C-terminal tail affects the function of the molecule. Contrasting evidence are provided by different researchers on the importance of the C-terminus. While Luk et al. (1990) demonstrated up to 21 a.a. residues deleted from the C-terminus of HuIFN-γ retains biological activity, Leinikke et al.(1987) and Arakawa et al. (1986) reported a substantial reduction in antiviral activity of their truncated variants. However, it should be noted that the latter groups used either enzymatic cleavage or denatured and then renatured molecules for their investigation, whereas Luk et al. (1990) maintained the deletion variant Del-122 in its native form throughout the purification procedure. Results of the in vitro transcription-translation studies showed that Del-122 was important for in vitro renaturation since the denaturation and subsequent renaturation of HuIFN-γ resulted in only 1-log reduction in antiviral activity, but a similar treatment of Del-122 resulted in more than a 2.5 log reduction

in activity. It is still unclear if the crucial role of the C-terminus on refolding in vitro may explain the failure of some research groups to detect biological activities. The fact the Del-122, without the C-terminal 21 a. a. residues, is biologically active indicated that the C-terminal region is non-essential for biological functions.

Apart from studying deletion variants, the structure and function relationships of HuIFN-γ were studied by screening monoclonal antibodies that neutralize biological activities of HuIFN-γ (Wang et al., 1984; Ziai et al., 1986). The antigenic epitopes recognised by neutralizing mAbs are likely at or very closely related to the active sites of HuIFN-γ. Thus far, mAbs from different laboratories are grouped into 3 immuno-reactivity groups representing 2 distinct functional epitopes (Alfa and Jay, 1988; Yang, 1991).

In the present study, a new antiviral neutralizing mAb against HuIFN-γ (MIF3037) has been identified. Here we report a new antiviral epitope E_3, that has no binding competition with any of the known specificity groups. Hence, the epitope typing scheme of HuIFN-γ has to be expanded to include 4 specificity groups : E_1, E_1/E_2, E_2 and E_3 representing 3 distinct functional epitopes. In addition, our data indicate that the general location of this epitope is within the C-terminal 21 residue region. This puzzling result immediately leads to the question on the involvement of the C-terminus in functional activity because it is found to be non-essential (Luk et al., 1990) and yet in this study, a functional domain is identified within this region. The observation that MIF3037 is neutralizing but does not have the ability to completely neutralize the antiviral activity of HuIFN-γ suggests that it is only a functional enhancing domain, which appears to cooperate with some other epitopes to give a full function. We evaluated further the ability of E_3 to function together with other epitopes in a cooperative fashion. Based on the observations of the kinetics of

antiviral state development, E_3 increases the rate of antiviral state induction and functions cooperatively with the previously identified eptiope E_1. These results are also consistent with our hypothesis that E_1 and E_3 act as NLS since the efficiency of nuclear translocation is enhanced by having more signals. Hence, a common domain of HuIFN-γ molecule may be responsible for different biological activities.

In order to test if the same domain confers different activities, the antichlamydial neutralization using the 3 antiviral neutralizing mAbs directed against E_1, E_2, and E_3 respectively, was studied. Since no antichlamydial neutralizing activity was detected, the antichlamydial activity must depend on a different domain of the HuIFN-γ and thereby a pathway different from that of the antiviral activity. As the loss of the C-terminus significantly reduces antichlamydial activity but not antiviral and antiproliferative activity, it indicates that a functional domain exists within the C-terminus residue 123 - 143. Since MIF3037 (binds to residue 130 - 138) did not neutralize antichlamydial activity of HuIFN-γ, the area that overlaps residue 130 - 138 is possibly not the functional domain.

The present study serves the purpose of identifying a functional enhancing epitope E_3 within the C-terminal region that explains the long contradictory arguments on the importance of the C-terminus of HuIFN-γ in eliciting biological activities. Furthermore, results suggest that HuIFN-γ exerts various biological functions through different pathways.

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