BIOLOGICAL ACTIVITY AND THERAPEUTIC APPLICATIONS OF INTRACELLULAR INTERFERON GAMMA AND INTERFERON GAMMA MIMETIC PEPTIDES

By

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As a granddaughter, that has been denied the privilege of celebrating the accomplishments of adulthood with my grandparents, I would like to dedicate this achievement to them as a small way of honoring their lasting influence on my life. My Mom has jokingly told me in the past that I have done some crazy things causing my grandparents to roll in their graves. I know that this accomplishment will put that rolling to rest and allow them to once more brag about their granddaughter among the heavens. To my Grandma, Grandpa, Granny, and Tabo I would like to say, "I miss each of you dearly, and thank you for watching over me."

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BIOLOGICAL ACTIVITY AND THEARAPEUTIC APPLICATIONS OF INTRACELLULAR INTERFERON GAMMA AND INTERFERON GAMMA MIMETIC PEPTIDES

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Interferons are pleiotropic cytokines known to participate in a number of autocrine and paracrine responses including antiviral, antiproliferative, antitumor, MHC regulation, and apoptosis regulation. Gene therapy and mimetic peptides were utilized in this study to examine the biological activities of an intracellular form and lipidated form of interferon gamma (IFN γ). Intracellular IFN γ has been shown to possess biological activity similar to that of extracellularly introduced IFN γ. First, an adenoviral vectoring system was used to express a nonsecreted form of human IFN γ or a non secreted form in which a previously identified nuclear localization sequence (NLS), ^{128}KTGKRKR^{134}, was modified to ^{128}ATGAAAA^{134}. Antiviral activity and MHC class I regulation were observed for cells treated with the vector expressing the nonsecreted wild type form of IFN γ containing a NLS, but these typical responses were not observed for the mutant

IFN γ. More so, human intracellular IFN γ induced biological activity in mouse L cells, which do not recognize extracellularly added human IFN γ. Thus, the biological activity was not due to escaped IFN γ acting at the extracellular receptor domain at the cell surface. Biological function was determined by activation of STAT1α and nuclear translocation of IFN γ, IFNGR1, and STAT1α. Immunoprecipitation of cell extracts with antibody to NPI-1 showed the formation of a complex with IFN γ/IFNGR1/STAT1α. To provide the physiological basis for these effects we show that extracellularly added IFN γ possesses intracellular signaling activation that is NLS dependent as demonstrated in previous studies, and that this activity occurs via the receptor mediated endocytosis of IFN γ. The data are consistent with previous observations that the NLS of extracellularly added IFN γ plays a role in IFN γ signaling. Second, this study examined the biological activity of mimetic versions of human IFN γ (95-134) and murine IFN γ (95−133) when administered to cells by means of the adenoviral vectoring system utilized above. These sequences encompass both the human and murine critical NLS sequences. Biological activity was confirmed by the mimetics' ability to induce both an antiviral state and MHC class I upregulation on human WISH cells, but further characterization is currently underway. Last, this study characterized the biological activity of a synthesized human IFN γ mimetic lipopeptide (95-133L) in which a lipid moiety was added to the amino terminus. It has been shown that lipidated versions of human IFN γ peptide (95-133) have been able to cross the membrane of nonphagocytic cells otherwise unachievable by their nonlipid counterparts. The mimetic was found to induce antiviral activity on mouse L cells and C57BL/6 mice.

CHAPTER 1
INTRODUCTION

Discovery of Interferons

In 1957 British researcher Alick Isaacs and Swiss researcher Jean Lindenmann first observed virus infected chick cells producing an unknown glycoprotein substance that was able to transfer its antiviral capabilities to adjacent cells. The researchers further noted that when one type of virus colonized animal or cultured cells the secreted glycoprotein interfered with the simultaneous colonization of a secondary virus (Isaacs and Lindenmann, 1957). The secreted glycoprotein substance is known today to be part of an inducible cytokine subfamily named "interferons." Terminology for the interferons (IFN) is based upon their cellular origins and stimuli as well as by the receptors to which they bind. There are currently two subtypes of interferons. Type I interferons also known as viral interferons, which include α, β, ω, and τ while γ also known as the immune interferon remains exclusive to the Type II subtype (reviewed in Samuel, 2001; Stark et al., 1998; Boehm et al., 1997). Both IFN α and ω are produced by leukocytes, IFN β is produced by fibroblast cells, and IFN τ is produced by trophoblast cells. Type I interferons can be produced virtually by any cell involved in viral infection, contrastingly Type II IFN γ is produced only by natural killer (NK) cells and T-lymphocytes (Samuel, 2001; Johnson, 1994). There are currently 13 IFN α genes while only one gene has been ascribed to both IFN ω and β. Type I interferons map to the short arm of human chromosome 9 and murine chromosome 4. The single gene of

Table 1. Overview of the interferons

Interferon	Type	Cellular Source	Biological Activities[a]
Alpha (α)	I	leukocytes	antiviral, antiproliferative, antitumor, MHC regulation, apoptosis regulation
Omega (ω)	I	leukocytes	antiviral, antiproliferative, antitumor, MHC regulation
Beta (β)	I	fibroblasts	antiviral, antiproliferative, antitumor, MHC regulation, apoptosis regulation
Tau (τ)	I	trophoblasts	antiviral, antiproliferative, pregnancy signal
Gamma (γ)	II	T-lymphocytes NK cells	antiviral, antiproliferative, antitumor, MHC regulation, apoptosis regulation

[a]reviewed in Samuel, 2001; Stark et al., 1998

Type II IFN γ is located on the long arm of human chromosome 12 and murine chromosome 10 (Samuel, 2001). An overview of the interferons is outlined in Table 1.

Biological Activity of Interferons

Interferons are pleiotropic cytokines known to participate in a number of autocrine and paracrine responses. Research into the network of biological activities and additional IFN-regulated genes is ongoing. The antiviral responses of interferons were the first recognized and subsequently have become the best-characterized responses. Various IFN-initiated mechanisms have evolved to attack the susceptible viral replication pathway (reviewed in Samuel, 2001; Stark et al., 1998; Boehm et al., 1997). Encephalomyocarditis (EMC) virus is an example of the viruses susceptible to the antiviral mechanisms of the dsRNA-dependent protein kinase (PKR) pathway (Samuel , 2001; Stark et al., 1998). Double stranded RNA binding to inactive PKR results in its autophosphorylation and activation. Activated PKR then induces the serine phosphorylation and inactivation of protein synthesis initiation factor, eIF2α. The inactivation of eIF2α results in the inhibition of protein synthesis and transcriptional control by the virus (Samuel, 2001; Stark et al., 1998). Another antiviral mechanism, the 2-5A multienzyme pathway, activates 2', 5'- oligoadenylate synthetase and RNase L, which prompts viral RNA cleavage (Samuel, 2001; Stark et al., 1998). Negative strand RNA virus, vesicular stomatitis virus (VSV) of the *Rhabdoviridae* genus, is susceptible to transcriptional inhibition accomplished by the IFN-inducible GTPases known as the Mx proteins. Binding of GTP to MxA proteins induces the proteins to form tight oligomeric complexes that are thought to inhibit the viral polymerases by steric hindrance (Samuel , 2001; Stark et al., 1998). Lastly, the deamination of adenosine has been identified as a

posttranscriptional RNA modification that results in adenosine to inosine hypermutation editing (Samuel, 2001; Stark et al., 1998). The extensive effects of this editing significantly alter the biological processes of the affected viruses. These various inhibitory pathways of the antiviral response often culminate in two additional IFN-regulated events, antiproliferation and apoptosis. The latter two events are crucial in the host's ability to exert direct inhibitory effects on tumor or malignant cancer cells as well as to efficiently rid the host of these afflicted cells by activating cytotoxic effector cells. In addition to the antiviral implications of interferons, both Type I and Type II interferons participate in a myriad of immunoregulatory responses aiding in communication between the cellular and humoral effector limbs of the immune response. The effective communication and organization mounts an efficient host-defense against attack. Although both types of interferons are involved, IFN γ is key in this aspect of biological activity while IFN α/β's responsibilities are more focused on elevating responses that offer the host adaptive immune response mechanisms to fend off viral infection. General biological activities of IFN γ are outlined in Table 2.

Table 2. General biological activities of IFN γ[a]

$CD4^+$ T cell differentiation
Macrophage activation
Regulation of MHC class I and class II antigen presentation
Regulation of Ig heavy-chain switching

[a]reviewed in Samuel, 2001; Stark et al., 1998

As stated earlier, it is beneficial for the host immune response to maintain a level of modulation between both the cellular and humoral responses, and IFN γ with the help of

interleukins (ILs), IL-12 and IL-4, dictate the differentiation of $CD4^+$ naïve T cells. IFN γ facilitates the production of T helper 1 (Th1) cells by increasing the synthesis of IL-12 as well as regulating the expression of the β2 receptor subunit of IL-12 on naïve $CD4^+$ T cells in order to maintain their ability to respond to IL-12. IFN γ plays a similar role for lymphocytes by increasing their expression of IL-2 receptors (Boehm et al., 1997; Johnson and Farrar, 1983). These acts, in addition to inhibiting the production of IL-4 and Th2 cell proliferation, allow IFN γ to simultaneously modulate T helper cell mediated immunity and T helper antibody mediated immunity (Boehm et al., 1997; Stark et al., 1998). The IFN γ-induced respiratory burst in macrophages and neutrophils is another facet of cellular immunity that is regulated by IFN γ. In humans, the transfer of one electron to oxygen by NADPH oxidase forms a superoxide anion, which has the potential to evolve into other toxic oxygen compounds like hydrogen peroxide and hydroxyl radicals that will be instrumental in killing the macrophages' contents. A similar respiratory burst in murine macrophages involves a reactive nitrogen intermediate nitric oxide (NO), produced by inducible nitric oxide synthase (iNOS) that also functions to kill the cellular contents. Both $CD4^+$ and $CD8^+$ T cell responses are enhanced by IFN γ's ability to upregulate MHC class I and class II molecules respectively (Samuel, 2001; Stark et al., 1998; Boehm et al., 1997). The upregulation of MHC class II molecules and thereby the enhancement of antigen presentation is unique to IFN γ as Type I interferons have only been shown to upregulate MHC class I for activation of cytotoxic T-lymphocytes (CTLs). Moreover, by regulating the expression of specific proteins required for the production of antigenic peptides, IFN γ has an affect on the amount and variance of the antigenic peptides presented by MHC class I and class II. Further

involvement of IFN γ extends into the production of antibodies by B cells and Ig heavy-chain switching responses of humoral immunity, which allows for more specific and effective action against targeted microbial and viral antigens.

Therapeutic Applications of Interferons

Today interferons are being used in the patient care field as biological response modifiers, and have even been approved for administration to patients with various diseases. The characteristic antiviral, immunomodulatory, antiproliferative, and apoptotic responses of interferons make them obvious candidates for treatment of various virus infections and malignancies. Since the earliest approval of IFN α, for treatment of Hairy Cell leukemia in 1986, interferons have gained rapid recognition by the Food and Drug Administration (FDA) as potential therapeutic drugs (reviewed in Samuel, 2001; Johnson et al., 1994; Door, 1993). The following year saw the addition of papillomavirus induced genital warts and AIDS related Kaposi's sarcoma to the list of those diseases treatable by IFN α (Samuel, 2001; Johnson et al., 1994; Door, 1993). IFN γ was the second interferon to gain approval by the FDA as a treatment for the hereditary immune disorder chronic granulomatous disease (Samuel, 2001; Johnson et al., 1994). IFN γ is used in this case to boost the ability of the inhabited macrophages to destroy their intracellular contents with toxic compounds produced during respiratory bursts. Next it was discovered in 1991 that a six-month course of IFN α was sufficient to eliminate symptoms of chronic hepatitis C, and continual treatment was determined to fend off the unfortunate occurrence of relapse. Many biotechnology and pharmaceutical companies began manufacturing isotypes of IFN α for use with hepatitis C patients, and shortly after the FDA also approved these same drugs for their antiviral affects on chronic hepatitis B

(Samuel, 2001; Johnson et al., 1994; Door, 1993). IFN β did not reach FDA approval until 1993 when it became the first drug approved therapy for the inhibition of autoimmune degradation of the myelin sheath, which is implicated in the onset of relapsing forms of multiple sclerosis (Johnson et al., 1994). There are many new applications for interferon immunotherapy seeking FDA approval, among those include parasitic Leishmaniasis (Johnson et al., 1994), metastatic melanoma (Hollis et al., 2003), cutaneous T-cell lymphoma (Echchakir et al., 2000), and various diseases of the lung (Antoniou et al., 2003).

Although these applications are quite impressive, interferon therapy has its drawbacks in some patient populations. Various studies have tracked patient side effects following the drugs administration both orally and intravenously and have reported flu-like symptoms and unexplainable cases of depression have been reported (Johnson et al., 1994; Steinmann et al., 1993). To a lesser extent some primary care physicians are starting to fear the less common but more severe life threatening adverse effects of interferon therapy that include but are not limited to autoimmune diseases, acute renal failure, and thrombotic-thrombocytopenic purpura (Steinmann et al., 1993). The complications are not surprising given the crucial homeostasis of autoimmunity and self-tolerance easily disrupted by administration of exogenous interferon. Until solutions to these side effects are reached, precautions are being taken by lowering the dosage of the drugs to nontoxic levels.

Future of Interferon Therapy

With an ever-increasing number of patients in need of interferon therapy there is undoubtedly a high demand for a more efficient, patient friendly interferon. Current

research is addressing the previously mentioned toxic side effects endured by patients. One solution gaining support is interferon gene therapy. Interferon delivered by the gene therapy modality would result in the transcription of interferon-inducible genes that control antiviral, antiproliferative, and immunomodulating properties. Unlike the short serum half-life of intravenous or orally administered interferon proteins, gene therapy tends to yield a more stable and sustained level of interferon (Ahmed et al., 2001). Adenoviral vector delivery of IFN α2b, a form of IFN α that is FDA approved, has been shown to maintain high levels for up to two weeks in mouse models (Ahmed et al., 2001). In addition, interferon therapy delivered by adenovirus (Ahmed et al., 1999; Zhang et al., 1996), retrovirus (Mecchia et al., 2000;Tuting et al., 1997), and nonviral vectors (Horton et al., 1999; Coleman et al., 1998) have been utilized to achieve a localized distribution, thereby successfully avoiding the toxic side effects incurred by the broad distribution of the interferon protein therapies. Gene therapy delivered by these types of vectors offers scientists the added ability to exploit their capacity to infect various types of cells as well as nondividing cells. Moreover, scientist may regulate the levels of expression by placing the interferon genes under a predetermined promoter control.

Researchers have taken the idea of compartmentalizing the interferon delivery one step further by utilizing nonsecretable or intracellular forms of these interferons in conjunction with the vector delivery systems. The rationale for the idea is based on surprising findings of intracellular cytokines (Will et al., 1996, Rutherford et al., 1996; Szente et al., 1994) and interleukins (Roth et al., 1995; Dunbar et al., 1989) capable of inducing the same biological activities as their secreted counterparts. For example,

intracellular IL-6 has been reported to activate platelet-derived growth factor (PDGF)-induced cell growth typical of secreted IL-6, but this response is completed without ligand binding (Roth et al., 1995). Initial studies conducted with an intracellular IFN γ peptide have also proven to elicit the same biological responses of its secreted form (Szente et al., 1994). Subsequent studies uncovered that greater than 90% homology in the cytoplasmic region of human and murine IFNGR1 sequences that bind both human and murine IFN γ ligand allow the intracellular receptor domain to be species nonspecific (Szente et al., 1994). This finding was surprising due to the fact that the initial trials with exogenously added IFN γ to the IFN γ extracellular domain of the receptor complex was species specific (Bach et al., 1997; Szente et al., 1994). The mechanism and implications behind this intracellular activation are discussed in greater detail at a later point in this study.

Recently, Type I IFN τ, initially labeled as a pregnancy recognition hormone in ruminants, has gained attention for its ability to be administered at doses described to be toxic for all other interferons (Soos and Johnson, 1999). Although IFN τ's role is not well understood in humans, in other mammals and mammalian cell culture it has been demonstrated to inhibit reverse transcriptase, tumor cell proliferation, and possess antiviral capabilities like other Type I interferons (Soos and Johnson, 1999). The implications of these characteristics are promising and scientists hope to parlay the positive results into human models after further testing.

Mechanism of Action of Interferon Gamma

As of ten years ago, the once elusive pathway of interferon signaling activity had been elucidated and agreed upon as a whole by the interferon community. Indeed,

interferons utilize different receptors and may originate from different cell types to initiate the signal, but the signaling pathways exhibit overlapping and involvement of the same signaling elements to elicit their responses. Unlike most other signaling pathways, both Type I and II interferon signaling pathways lack the typical secondary messengers and instead rely on the reactions of two unique protein families. Not only are these pathways direct, but they are fast as well, the interferon receptor mediated response achieves protein translation in 15 minutes (Levy et al., 2002). Both the protein tyrosine kinases termed Janus kinases (JAK1 and JAK2) and the signal transducing and activators of transcription (STAT1) found involved with the interferon pathways are members of even larger protein families. There are currently seven members of the STAT family and four members of the Janus kinase family utilized by interferons as well as other cytokines and growth factors for signaling via the JAK/STAT pathways (Levy et al., 2002; Johnson et al., 2001; Samuel, 2001). Janus kinase enzymes function to phosphorylate specific tyrosine residues found at the intracellular carboxyl terminal region of the interferon receptors thereby readying the docking site for the binding of the STAT's Src-homology-2 (SH2) domain and subsequent activation (Levy et al., 2002; Johnson et al., 2001). The activated STATs bear the responsibility of entering the nucleus and binding to a distinct set of genes under promoter control to activate the transcription and translation of proteins.

As summarized in Figure 1 the mechanism of IFN γ receptor signal transduction progresses as follows. The activated 34 kDa noncovalent homodimer, IFN γ, is thought to bind to its respective cell surface receptors found mainly outside the lymphoid system (Samuel et al., 2001). From this vantage the ligand activates an intricate cascade of

molecular interactions, however, its physical involvement ends here. It is then up to the remaining constituents to continue conveying the signal. The receptor itself is a two component system of two interferon gamma receptor-1 (IFNGR1) chains pre-associated with a Janus kinase, JAK1, and two IFNGR2 chains pre-associated with their own Janus kinase, JAK2 (Bach et al., 1997). Binding of the IFN γ ligand to the two IFNGR1 chains results in their dimerization and subsequent association with the adjacent IFNGR2 chains (Subramaniam et al., 2000; Bach et al., 1997). When the two IFNGR1 chains and two IFNGR2 chains complex, they come together in a way that allows for their preassociated JAK1 and JAK2 to transactivate (Stark et al., 1998). Next, the JAKs phosphorylate the two important tyrosine 440 residues found on the IFNGR1 chains, and this action readies the IFNGR1 chains for dual STAT1 docking (Stark et al., 1998; Bach et al., 1997). As both cytosolically latent STAT1 proteins bind they are immediately brought into proximity with and phosphorylated by the receptor bound JAKs at tyrosine residues 701 (Stark et al., 1998; Bach et al., 1997). The activated tyrosine phosphorylated STAT1α proteins (~180 kDa dimer) are then released, and come quickly together to form a homodimer complex via reciprocal phosphotyrosine-SH2 interactions near the carboxyl terminus of the protein (Levy, 2002; Samuel, 2001; Stark et al., 1998). Activated STAT1α then translocates to the nucleus via GTPase activity of the Ran/Importin

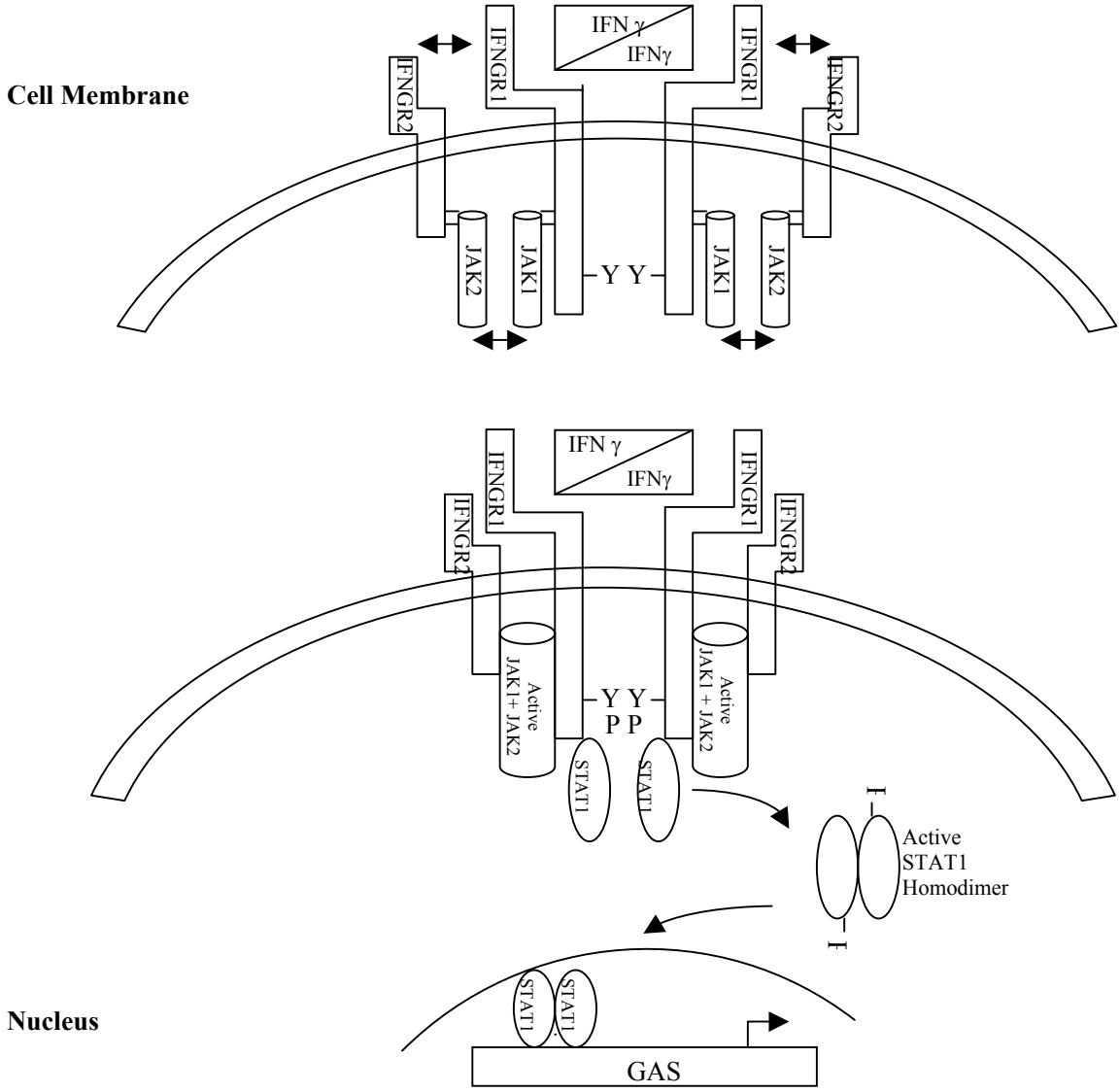


Figure 1. Signaling pathway of Type II IFN γ. IFN γ binds as a homodimer to IFNGR1 chains (also know as the alpha chains). The ligand binding causes formation of the receptor complex resulting in phosphorylation and activation of preassociated JAK 1 and JAK2. JAK1 activation readies a site for cytosolically latent STAT1 docking. STATs will release and dimerize for translocation to the nucleus via the Ran/importin pathway. STATs will bind to specific promoter elements called GAS elements to initiate transcription of IFN γ inducible genes.

mechanism (Stark et al., 1998; Bach et al., 1997). It follows that, the nuclear STAT1α homodimer binds to gamma-activated sequence (GAS) elements of IFN γ-inducible genes that will then be actively transcribed (Stark et al., 1998; Decker et al., 1997).

Experimental Rationale

However, the established pathway outlined above fails to incorporate recent findings concerning the events of the Type II IFN γ signaling pathway. For the last ten years or more the Johnson lab has collected evidence in regards to the interferon signaling pathway, the implications of which, will challenge the current receptor signal transduction dogma. As outlined above the conventional view of the cytokine receptor's role is to translate the extracellular signal of ligand binding into a cytosolic signal transmitted with the help of intracellular signaling components like kinases and transcription factors to ultimately regulate gene expression. The plausible alternative suggests that there may be additional modes by which signals reach the nucleus (Subramaniam et al., 1999; Jans and Hassan, 1998). There is initial evidence of various internalized ligands, either in complex with their respective receptors and signaling components or alone, being sufficient to transmit that extracellular signal to the nucleus and activate transcription (Subramaniam et al., 2001; Jans and Hassan, 1998). Jans has compiled an impressive list of ligands and receptors that are found colocalizing to the nucleus with the help of the ligand's putative nuclear localization sequence (NLS) to which this method of signal transduction may apply: insulin, nerve growth factor, EGF, PDGF, GH, gonadotropin, and various interleukins (Lin et al., 2001; Subramaniam et al., 2001; Jans et al., 1998). A classic NLS is a short polybasic amino acid sequence

sufficient to direct its protein to the nucleus, and distinctive from shuttle sequences in that they are not cleaved during transport (Jans et al., 1998).

The inspiration for exploring a plausible alternative to the signaling mechanism drew upon a well-known physiological characteristic of eukaryotic cells. The fact that eukaryotes compartmentalize their DNA within a nucleus, but carry out protein synthesis in the cytoplasm necessitates a means of transport across the nuclear envelope for proper cellular functioning (Jans and Hassan, 1998). In other words, transcribed mRNA must cross the nuclear envelope in order to initiate protein synthesis at the ribosome, while enzymes responsible for transcription, gene regulation, and DNA replication likewise must possess the ability to cross the nuclear envelope (Jans and Hassan, 1998). The nuclear pore complexes (NPCs) of the nuclear envelope make this transport possible with one important regulating factor; molecules larger than 40-45 kDa must contain a NLS while smaller molecules are allowed to pass through the nuclear sieve by means of passive diffusion (Jans and Hassan, 1998). The Ran/importin pathway regulates the nuclear transport of proteins across the NPC. The importin-α subunit of the pathway binds upon recognition of the protein's NLS. Then the importin-β subunit is responsible for the binding of the importin-α/ protein complex to the Ran GTPase found in the cytosol, which energizes the transport across the NPC (Subramaniam et al., 2001). Previous models (see Figure 1) have stated that the 180-kDa STAT1α homodimer crosses into the nucleus via the Ran/importin pathway to activate transcription (reviewed in Samuel, 2001; Stark et al., 1998; Boehm et al., 1997). Yet, STAT1α is a 180-kDa homodimer that clearly demonstrates the need for an NLS in order to cross the nuclear pore complex. Evidence that STAT1α lacks a functional NLS was initially demonstrated

by means of mutational analysis using importin-α homolog nucleoprotein interactor-1 (NPI-1) to test for the presence of an NLS (Sekimoto et al., 1997). The mutational studies failed to demonstrate a sequence in STAT1α that would bind the NPI-1, and this fact suggests that the STAT1α requires another NLS-containing peptide to facilitate its entry into the nucleus (Sekimoto et al., 1997). As a result of previous work on the C-terminal domain sequence of the IFN γ ligand which is encompassed by murine IFN γ residues 95-133 and human IFN γ residues 95-134 our lab has shown that this prototypical NLS is in fact accomplishing the chaperoning function for nuclear transport of STAT1α (Subramaniam, 1999). Interestingly, studies with the NLS-containing murine IFN γ agonist/mimetic peptide 95-133, human IFN γ agonist/mimetic peptide 95-134, and the IFNGR1 chain Johnson's lab has established that IFN γ's NLS is also responsible for the nuclear transport of an entire transcription complex composed of the IFN γ, IFNGR1, STAT1α, and NPI-1 (reviewed in Subramaniam, 2001).

The first phase of this study proposes that intracellular IFN γ's NLS plays the same chaperoning role of extracellularly added IFN γ when administered to cells by means of an adenoviral vector system. The hypothesis was tested by developing experiments, which assessed the effects of intracellular IFN γ on the biological activity of murine and human cell lines as compared to an intracellular IFN γ peptide that has been engineered with a mutated NLS. The second phase of this study proposes that mimetic versions of human IFN γ (95-134) and murine IFN γ (95-133) when administered to cells by means of an adenoviral vector system yield the same biological activity as wild type IFN γ. Experimentation on the biological activity induced by the mimetic peptides was

used to test this hypothesis. The last phase of this study determined that synthesized human IFN γ mimetic lipopeptide (95-133L) is able to induce an antiviral state in cell culture and C57BL/6 mice. The data presented here demonstrate that intracellular IFN γ and IFN γ mimetics with an NLS possess the full IFN γ activity as per the chaperone functions suggested above with extracellular IFN γ.

CHAPTER 2
MATERIALS AND METHODS

Cell Culture And Recombinant Adenoviruses

Propagation of recombinant adenoviruses was accomplished in the human embryonic kidney cell line 293 (ATCC, Gaithersburg, MD) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Mouse L929 cells and human WISH cells were grown in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum. All cell lines were grown in a 37^0 humidified chamber with 5% CO_2.

AdEasy adenoviral vector system from Stratagene (La Jolla, CA) was used. Construction and propagation of adenoviral vectors was carried out according to the manufacturer's protocol. A plasmid containing human IFN γ (ATCC) was used to carry out PCR using the following primers:
CGGTCGACGAACGATGAAATATACAAGTTATATC (forward) and
GCAAGCTTCATTACTGGGATGCTCTTCGAC (reverse). To obtain the nonsecreted IFN γ sequence, a forward primer was used that contained the initiating methionine and the remainder of the coding sequence from the first amino acid in mature polypeptide, along with the following sequence,
CGGTCGACGAACGATGTGTTACTGCCAGGACCCATA. Reverse primer was same as above for secreted IFN γ. NLS-modified IFN γ sequence was obtained by using a reverse primer in which the coding sequence was changed to replace lysine and arginine with alanines and the same forward primer as for the nonsecreted IFN γ. To obtain the

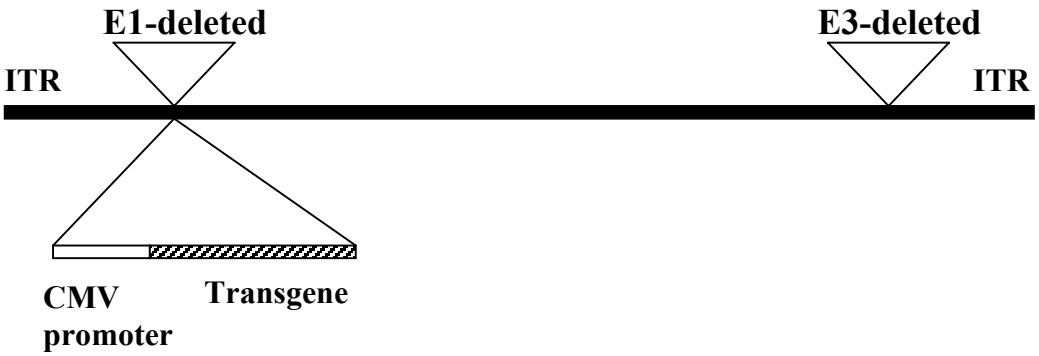
human IFN γ mimetic peptide IFN γ (95-134) the following primers were used: CGGTCGACGAACGATGCTGACTAATTATTCGGTAAC (forward) and GCAAGCTTCATTACATCTGACTCCTTTTTC (reverse). To obtain the murine IFN γ mimetic peptide IFN γ (95-133) the following primers were used: CGGTCGACGAACGATGGCCCAAGTTTGAGGTCAACAA (forward) and GCAAGCTTCATCAGCAGCGACTCCTTTTCC (reverse). The sequences produced for the two mimetic peptides are seen in Table 3. PCR products were digested with Sal I (5'end) and Hind III (3' end) and the resulting fragments were cloned in the multiple cloning site in the plasmid, pShuttleCMV. For the control plasmid, pShuttle MCS, which does not have a transgene, was used. Linearized plasmids as above were cotransformed with pAdeasy plasmid in BJ5183 to obtain recombinant adenovirus sequence. Recombinant plasmids were used to infect human embryonic kidney 293 cells to obtain viruses. Purification of viruses was carried out by using two CsCl gradients. Restriction enzyme digestion and DNA sequencing across the coding sequence characterized these viruses. Cells that were about 50% confluent were infected with different recombinant adenoviruses at a multiplicity of infection (m.o.i) of 10 for one hour, followed by growth in EMEM medium for periods indicated. Genomic maps for the adenoviral vectors can be seen in Figure 2.

Table 3. Sequences of murine and human IFN γ mimetic peptides and mimetic lipopeptides used in this study.

Peptide	Sequence[a]
huIFN γ mimetic peptide (95-134)	LTNYSVTDLNVQRKAIHELIQVMAELSPAAKTGKRKRSQM
muIFN γ mimetic peptide (95-133)	AKFEVNNPQVQRQAFNELIRVVHQLLPESSLRKRKRSRC
muIFN γ mimetic lipopeptide (95-133L)	LIPO-KFEVNNPQVQRQAFNELIRVVHQLLPESSLRKRKRSR
muIFNGR1 control lipopeptide (253-287L)	LIPO-CFYTKKINSFKRKSIMLPKSLLSVVKSATLETKPESKYVS

[a]NLS sequences are highlighted in bold type.

Recombinant Adenovirus Vectors



Empty Vector	**rAde**
IFNγ	**rAdIFNγ**
nsIFNγ	**rAdnsIFNγ**
nsIFNγmut	**rAdnsIFNγmut**
hIFNγpep(95-134)	**rAdhIFNγpep(95-134)**
mIFNγpep(95-133)	**rAdmIFNγpep(95-133)**

Figure 2. Genomic map of adenovirus vectors**.** A replication-deficient form of adenovirus deleted in early regions 1 and 3 was used. CMV-promoter driven genes were placed in E1 region. Different constructs made and their names are shown.

Western Blot Analysis and Immunoprecipitation

Cells were washed with phosphate buffered saline (PBS) and harvested in lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.1% NP-40, 50 mM NaF, 5 mM EDTA and protease inhibitor cocktail (Roche Biochemicals, Indianapolis, IN). Protein concentration was measured using BCA kit from Pierce (Rockford, IL). Protein (10 μg

each) was electrophoresed on an acrylamide gel, transferred to nylon membrane and probed with the antibodies indicated. Horseradish peroxidase conjugated secondary antibodies were used and detection was carried out by chemiluminescence (Pierce). Immunoprecipitation was carried out by incubating specific antibodies with cell extracts followed by incubation with IgG-Sepharose (Sigma Chemicals, St. Louis, MO), followed by centrifugation and washings. Phospho-STAT1α antibody was from Cell Signaling (Beverly, MA), and polyclonal antibody to STAT1α was from R&D chemicals (Minneapolis, MN). Antibodies to NPI-1 and IFNGR1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody to IFN γ used to probe the immunoprecipitate was obtained from PBL Biomedical (New Brunswick, NJ). ELISA kit for IFN γ was obtained from Biosource International (Camarillo, CA).

Intracellular IFN γ Antiviral Assay

Antiviral assays were performed by using a cytopathic effect (CPE) reduction assay utilizing VSV (Familetti et al., 1981). Mouse fibroblast L929 (4×10^3) were plated in a microtiter dish and allowed to grow overnight at 37^0 C. These cells were then infected with different adenoviruses and incubated for various times followed by growth in EMEM medium for 24 hours at 37^0 C. VSV at 100 pfu/ml was then added to these cells and incubated for 24 hours at 37^0 C. Cells were stained with crystal violet. The dye retained was extracted in methylcellusolve and absorption at 550 nM was measured.

Expression of MHC Class I

Human WISH cells were transfected with different recombinant adenoviruses for one hour, followed by growth in EMEM medium for 48 hours at 37^0 C. Cells were then washed and incubated with a monoclonal antibody to human MHC class I molecules

conjugated with R-phycoerythrin (R-PE). Mouse IgG2a conjugated with R-PE was used as a control. Both of these R-PE conjugated antibodies were from Ancell (Bayport, MN). Cells were analyzed for immunofluorescence (FL-2) in a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Data were collected in list-mode format and analyzed using CellQuest software (Becton Dickson Immunocytometry Systems).

Immunofluorescence Analysis

Human WISH cells (3×10^5) were grown overnight on tissue culture-treated slides (Falcon, Becton Dickinson, Franklin Lakes, NJ) before infecting with andenovirus vector for one hour. This was followed by growth in EMEM medium for 8 hours at 37^0 C. Cells were then fixed in methanol (-20^0 C) and dried. Cells were permeabilized using 0.5% Triton X-100 in 10 mM Tris-HCl, pH 8.0, 0.9% NaCl (TBS) for 10 min. Slides were washed with TBS and nonspecific sites were blocked with 5% nonfat milk in TBS. Slides were then incubated at room temperature for one hour in the same blocking buffer containing rabbit polyclonal antisera against IFNGR1 (Santa Cruz Biotechnology, Santa Cruz, CA) and goat polyclonal antisera to human STAT1α (R&D Systems). Cells were washed four times with TBS containing 0.1% Triton. This was followed by incubation at room temperature with secondary antibodies, which were Cy-2 conjugated donkey anti-rabbit (Jackson Immunochemicals) and Alex Fluor 594 conjugated donkey antisera (Molecular Probes, Eugene, OR) for one hour. After four washings in TBS with 0,1% Triton, slides were mounted in Prolong antifade solution (Molecular Probes), covered with a cover slip and sealed with nail varnish. To view the nuclear translocation of IFN γ, slides were incubated with a monoclonal antibody to human IFN γ (BD

Pharmingen, San Diego, CA) as the primary antibody and Alex Fluor 488 conjugated anti-mouse antibody (Molecular Probes) as the secondary antibody. Images were recorded on epifluorescence microscope attached to a Macintosh computer running IP Lab software and deconvolution software (Scanylatics Corp). Images were recorded and a portion of out-of-focus haze from each image removed using the MicroTome software (Vaytek) to improve clarity. Quantitation of these images was done by measuring mean pixel intensity in cytoplasmic (Fc) and nuclear (Fn) regions in each cell using IP Lab software (Scanylatics Corp). The ratio Fn/Fc was determined for each cell and the average of the ratio Fn/Fc across at least seven different fields was measured and is presented as Fn/Fc for a given treatment.

Synthetic Peptides

Murine IFN γ mimetic peptide (95-133), and the murine IFNGR cytoplasmic receptor control peptide (253-287) were synthesized with a PerSeptive Biosystems 9050 automated peptide synthesizer using fluorenyl-methyloxy carbonyl (Fmoc) chemistry (Chang and Meienhofer, 1978). A lipid moiety (palmitoyl-lysine residue) was incorporated into the amino terminus of each of the two peptides. Trifluoroacetic acid/phenol/water/triisopropylsilane at a ratio of 88:5:5:2 was used to cleave the peptides from the resins. The cleaved peptides were then extracted in ether and ethyl acetate, dissolved in water, and lyophilized. The crude peptides were analyzed by reverse-phase HPLC showing one major peak in each profile. Sequence analysis of these peptides showed that the amino acid composition corresponded closely to theoretical values. The sequences of the peptides synthesized are shown in Table 3. Wild-type murine IFN γ for

use in IFN γ mimetic lipopeptide experiments was purchased from Pepro Tech Inc. (Rocky Hill, NJ).

Toxicity Assay and Antiviral Assay of Mimetic Lipopeptide

The toxic effects of murine IFN γ, murine IFN γ mimetic lipopeptide (95-133L), and the murine IFNGR control lipopeptide were measured on mouse fibroblast L929 cells. Cells were plated at 6 x 10^4 cells/well in a microtiter dish and allowed to grow overnight at 37^0 C. EMEM medium, IFN γ, IFN γ lipopeptide (95-133L), and IFNGR control lipopeptide were incubated with cells at various dilutions for 24 hours at 37^0 C. Cells were then stained with crystal violet. The dish was scanned on an Astra 2100U scanner and mean pixel intensities were determined using ImageJ 1.29X software (NIH, Bethesda, MD).

Concentration endpoints of murine IFN γ and murine mimetic lipopeptides that resulted in 50% protection from EMC virus were obtained using an antiviral assay. Mouse fibroblast L929 (6 x 10^4) cells were plated in a microtiter dish and allowed to grow overnight at 37^0 C. Cells were pretreated with EMEM medium, IFN γ, IFN γ mimetic lipopeptide (95-133L), and IFNGR control lipopeptide (253-287L) for 24 hours at 37^0 C. EMC virus at 100 pfu/ml was then added to these cells and incubated for 1 hour at 37^0 C. Cells were washed and incubated with EMEM media for 24 hours, and then stained with crystal violet.

Viral Yield Assay

Mouse fibroblast L929 cells, seeded to confluency in 25 cm^2 flasks, were pretreated with murine IFN γ, murine IFN γ mimetic lipopeptide (95-133L), and the murine IFNGR control lipopeptide for 24 hours at 37^0 C. EMC virus at 100 pfu/ml (2.1 x 10^{-4} m.o.i.)

was then added to these cells and incubated for 1 hour. Cells were washed and incubated with EMEM media for 24 hours at 37^0 C. Virus produced was harvested and titered on murine L929 cells plated in a 24 well plate at 3 x 10^5 cells/well. Plates were stained with crystal violet after 24 hours and plaques were counted. Plaques for IFN γ, IFN γ mimetic lipopeptide (95-133L), and IFNGR control lipopeptide (253-287L) were counted at the 1:200,000 dilution. Media only plaques were counted at the 1:2,000,000 dilution. The original sample concentrations are reported as pfu/ml.

Animal Studies of EMC Infection

Mouse fibroblast L929 cells, seeded to confluency in 25 cm^2 flasks, were pretreated with EMEM medium, murine IFN γ, or murine IFN γ mimetic lipopeptide (95-133L) for 24 hours. Cells were then incubated with 100 pfu/ml of EMC virus for one hour. The cells were then washed and resuspended in EMEM media for 24 hours. Supernatants were collected for intraperitoneal (IP) injection of female C57BL/6 mice (Jackson Laboratories) on Day 0. The three sets of supernatants were diluted 1:200,000 with media prior to IP injection (200μl) of mice (4 per group). Mice were housed according to IACUC standards in a BSL-2 room, and their condition was checked every 24 hours. Mortality from EMC infection was recorded and the percent of mice surviving for each group is reported over a 14-day period.

CHAPTER 3
RESULTS

Expression Vectors

This study utilizes the pAdEasy adenoviral vector system for the expression of different polypeptides. As seen in Figure 2, pAdEasy is a derivative of ad5 constructed with deletions in early regions I and III. The deletion of region I ensures these vectors are replication-deficient. In the place of early region I, a cassette of CMV promoter driven transgene was inserted. rAde represents the vector without the transgene. As follows, CMV promoter driven nonsecreted IFN γ and NLS-modified nonsecreted IFN γ were termed rAdnsIFNγ and radnsIFNγmut, respectively. Restriction enzyme digestion and subsequent DNA sequencing across the transgene helped characterize these vectors.

WISH cells were transduced with the empty control vector or IFN γ expressing vectors to examine the synthesis of IFN γ. Two days post-transfection, proteins from cell extracts and supernatants were electrophoresed and probed with an antibody to IFN γ (Figure 3A). Cells treated with nonsecreted IFN γ and NLS-modified nonsecreted IFN γ expressing vectors showed the presence of IFN γ in the cell extracts, while IFN γ was undetected in the supernatants. Cells transduced with the empty control vector failed to express IFN γ, suggesting that the infection with recombinant adenovirus in itself does not induce endogenous IFN γ expression.

To examine if murine L929 cells were infectable with the recombinant adenovirus vectors, cellular extracts and supernatants from cultures of cells previously infected with

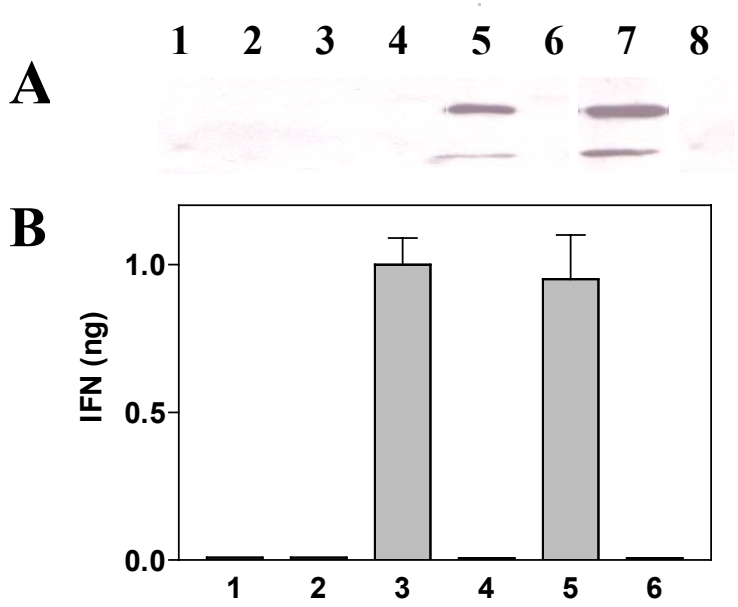


Figure 3. Synthesis and intracellular retention of IFN γ. (A) WISH cells, untreated (lanes 1 and 2), or those transduced for two days with an empty vector control (lanes 3 and 4), a vector expressing nonsecreted IFN γ (lanes 5 and 6) or a vector expressing nonsecreted IFN γ mutated in the NLS (lanes 7 and 8) were used. Proteins from cell extracts (odd numbered lanes) or supernatants (even numbered lanes) were separated by SDS-PAGE and probed with an antibody to IFN γ. Detection was carried out by using chemiluminescence. (B) Quantitation of IFN γ produced in L929 cells by ELISA. Cell extracts (odd numbers) and supernatants (even numbers) from L929 cells, transduced for two days with the empty adenoviral vector (column 1 and 2) or vector expressing non-secreted IFN γ (column 3 and 4), or vector expressing non-secreted IFN γ mutated in the NLS (column 5 and 6) were assayed for IFN γ by ELISA.

rAde, rAdnsIFNγ, and rAdnsIFNγmut were assayed for IFN γ by ELISA (Figure 3B). Cells treated with rAdnsIFNγ and rAdnsIFNγmut showed the synthesis of IFN γ in their cell extracts, while the supernatants from the same cells did not produce detectable IFN γ. There was no detectable IFN γ in the cell extracts or the supernatants of L929 cells infected with the empty vector control. Additionally, purified virus preparations were found to be free of IFN γ. Therefore, the effects reported below were not from free contaminating IFN γ acting extracellularly. Thus, IFN γ synthesized by rAdnsIFNγ or rAdnsIFNγmut vectors was expressed intracellularly and not secreted.

Biological Activity of Nonsecreted IFN γ is Dependent on the Presence of NLS

Next, we assessed if intracellularly expressed IFN γ possessed biological activity. Mouse L cells, untreated, or transduced with an empty vector control, nonsecreted IFN γ, or NLS-modified nonsecreted IFN γ expressing vector were allowed to grow for one day after which they were challenged with VSV. Twenty-four hours later, these cells were compared for relative resistance to VSV induced cytopathic effect (Figure 4). Intracellular expression of IFN γ, produced a three-fold increase in cell survival, compared to untreated cells or cells treated with empty vector. Cell survival was reduced to nearly the same as with the empty vector control with expression of NLS-mutated IFN γ. Intracellularly expressed IFN γ thus induced antiviral activity in cells, and this activity was dependent on the presence of the NLS in its C terminus. Given that mouse L cells do not recognize human IFN γ via the extracellular domain of the receptor, the data further support an intracellular effect of human IFN γ.

Previous studies have established that extracellularly added IFN γ induces MHC class I molecule expression on the cell surface (Szente et al., 1994). We examined the

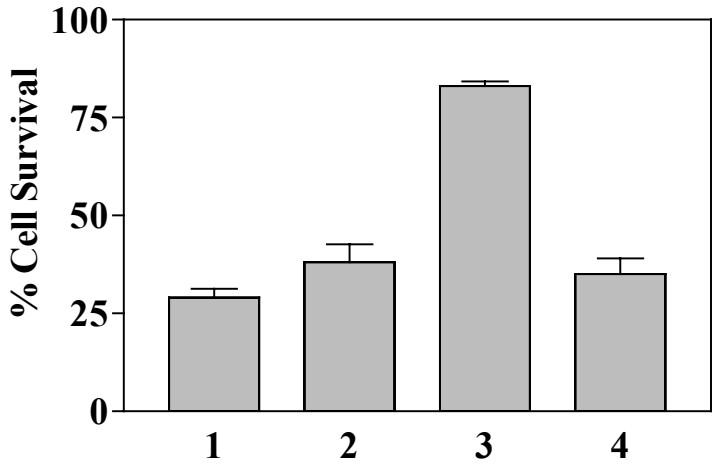


Figure 4. Resistance to viral infection by intracellular IFN γ is dependent on the presence of the NLS. Mouse fibroblast L929 cells, untreated (column 1), or those transduced for one hour with and empty vector control (column 2), a vector expressing nonsecreted IFN γ (column 3), or a vector expressing nonsecreted IFN γ with a mutation in the NLS (column 4) were allowed to grow for 24 hours, followed by infection with VSV for 24 hours. Cells were then stained with crystal violet, extracted with methylcellusolve and the absorbance was measured at 600 nM. Absorbance after different treatments is expressed as percent of cells that were not exposed to any virus, which was taken as 100%. Results represent the mean +/- SD of three independent determinations.

effects of nonsecreted IFN γ on MHC class I induction by transducing WISH cells with an empty vector control, nonsecreted IFN γ or NLS-modified nonsecreted IFN γ expression vector. Two days later, these cells were stained with R-phycoerythrin (R-PE) conjugated monoclonal antibody to human MHC class I molecules and underwent flow cytometry analysis. Murine IgG2a antibodies conjugated with R-PE were utilized as an isotype control. Relative mean fluorescence profiles are presented in Figure 5. Cells transduced with the empty vector control, nonsecreted IFN γ or NLS-mutated IFN γ expression vectors showed 417 +/- 9, 778 +/- 10, and 362 +/- 29 units of mean fluorescence, respectively. Thus, an approximate two-fold increase in expression of MHC I molecules was seen with the intracellular expression of IFN γ. This induction was abolished with the removal of the NLS. Intracellularly expressed IFN γ therefore induces antiviral activity and upregulation of MHC class I molecules only with an intact NLS in its C-terminus.

Activation of STAT1α and its Association with IFN γ, IFNGRI, and NPI-1

To determine if the biological activity exhibited by nonsecreted IFN γ involved the activation of STAT1α, whole cells extracts from WISH cells transduced with an empty vector control, nonsecreted IFN γ or the NLS-mutated nonsecreted IFN γ expression vector were analyzed (Figure 6). Probing with Tyr^{701} phospho-STAT1- specific antibody traced the phosphorylation of STAT1α in response to intracellular expression of both wild-type IFN γ and its NLS mutant. Re-probing this filter to observe STAT1α showed similar amounts of STAT1α in all cell extracts. Further, intracellular expression of NLS-mutated IFN γ induced STAT1α tyrosine phosphorylation comparable to the wild-type IFN γ.

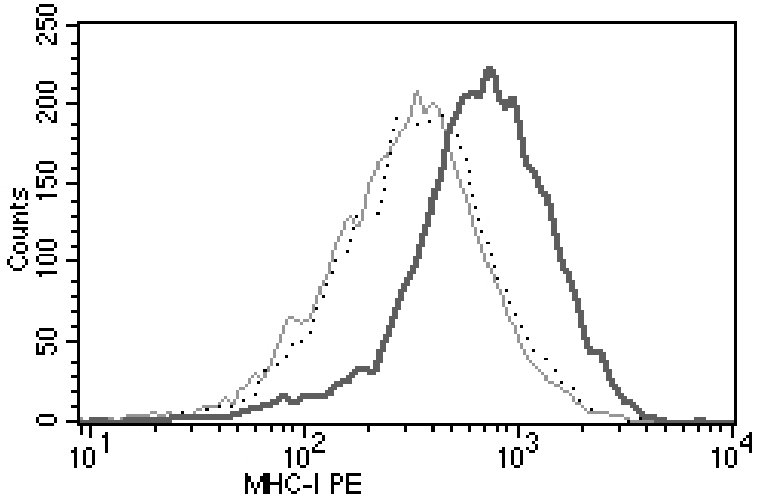


Figure 5. Induction of MHC class I by intracellular expression of IFN γ is abolished by removal of the NLS. WISH cells were transduced for one hour with an empty vector control (dotted line), a vector expressing nonsecreted IFN γ (solid line), or a vector expressing nonsecreted IFN γ mutated in the NLS (thin line). Cells were then allowed to grow for 48 hours in regular medium followed by staining with R-PE conjugated monoclonal antibody to human MHC class I and analyzed by flow cytometry. R-PE conjugated murine IgG2a was used as a control. A similar profile was noted in three independent experiments.

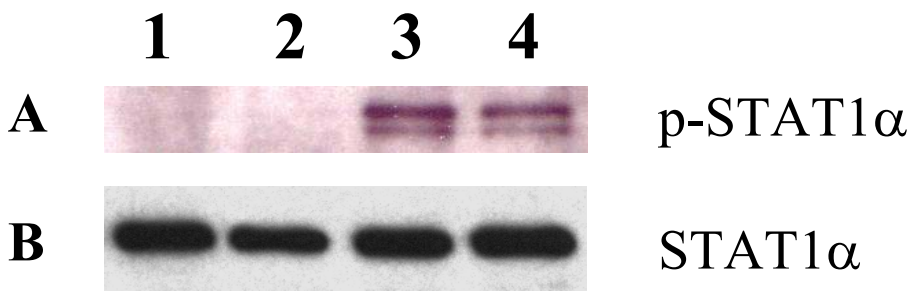


Figure 6. Phosphorylation of STAT1α by intracellular IFN γ is independent of the IFN γ C-terminal NLS. WISH cells, untreated (lane 1), or with an empty vector control (lane 2), a vector expressing nonsecreted IFN γ (lane 3), or a vector expressing nonsecreted IFN γ with a mutated NLS (lane 4) were allowed to grow for 8 hours. Equal amounts of proteins from whole cell extracts were electrophoresed and probed with an antibody to phospho-STAT1α (upper row). Filter was stripped and re-probed with an antibody to STAT1α (lower panel). Detection was by chemiluminescence.

We have previously established that cells stimulated with extracellular IFN γ resulted in the formation of a complex of IFN γ/IFNGR1/STAT1α in the cytoplasm, and that the nuclear importin α homolog, NPI-1, bound to the complex via the NLS in the C-terminus of IFN γ (Larkin et al., 2000; Subramaniam et al., 2000). In addition, we have provided evidence that the NLS of IFN γ is responsible for the nuclear transport of STAT1α (Subramaniam et al., 2000), which otherwise lacks an intrinsic NLS as demonstrable via the standard digitonin-based nuclear import assay (Subramaniam and Johnson, manuscript in preparation). The binding of IFN γ to the cytoplasmic domain of IFNGR1 as well as to NPI-1 is critical to the chaperoning of STAT1α to the nucleus. This feature of IFN γ was examined by obtaining the extracts of cells expressing IFN γ intracellularly 8 hours after transduction with the empty vector or the vectors expressing wild-type IFN γ and NLS-mutated IFN γ. Immunoprecipitated proteins were then electrophoresed and identified individually with antibodies specific for IFNGR1, IFN γ, phosphorylated STAT1α (p-STAT1α) and NPI-1 signaling elements (Figure 7). IFNGR1, IFN γ, and p-STAT1α were found in the anti-NPI-1 immunoprecipitate of the cells expressing wild-type IFN γ intracellularly (Figure 7, lane 3), while anti-NPI-1 precipitate from untreated cells (Figure 7, lane 1) or cells transduced with NLS-mutated IFN γ vector (Figure 7, lane 2) were negative for IFNGR1, IFN γ, and p-STAT1α. Similar concentrations of NPI-1 were present in the immunoprecipitates from each of the cell extracts. Therefore, intracellular wild-type IFN γ formed a complex of

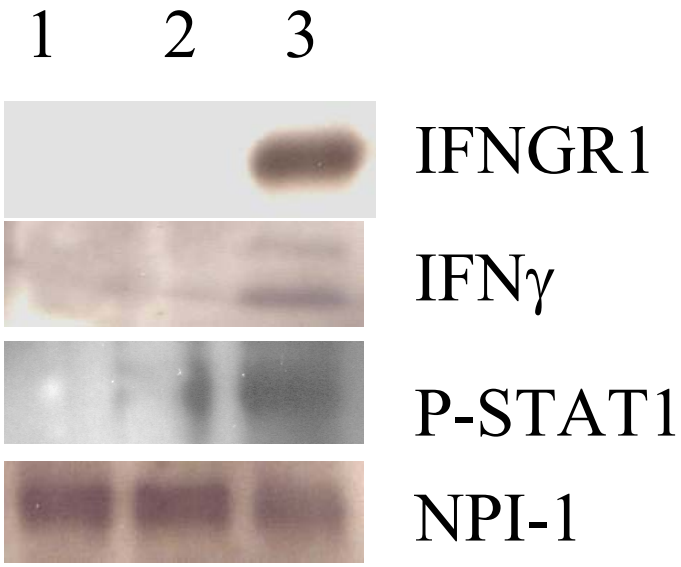


Figure 7. Association of activated STAT1, IFN γ and IFNGR1 with nuclear importer, NPI-1. Cell extracts from WISH cells transduced for 8 hours with an empty vector (lane 1), NLS-mutated IFN γ expression vector (lane 2), or a nonsecreted IFN γ expression vector (lane 3) were used for immunoprecipitation with an antibody to NPI-1. Equal amounts of immunoprecipitates were electrophoresed and probed individually with antibodies to IFNGR1 (first row), IFN γ (second row), phospho-STAT1 (third row), or NPI-1 (fourth row).

IFN γ/IFNGR1/STAT1α/NPI-1, but this complex was absent in cells expressing the IFN γ NLS-mutant. Taking into account, that the NLS mutant IFN γ was determined to activate STAT1α phosphorylation similar to wild-type IFN γ (Figure 6), these data would suggest that the IFN γ NLS is required for the binding of phosphorylated STAT1α to NPI-1 but not for STAT1α activation.

Nuclear Translocation of STAT1α, IFNGR1, and IFN γ

Further support of nuclear translocation of STAT1α and IFNGR1 in cells expressing intracellular IFN γ was determined by immunofluorescence analysis and found to be consistent with intracellular activation of STAT1α and the association of IFNGR1 with nuclear import machinery. WISH cells underwent simultaneous staining with antibodies to STAT1α and IFNGR1 in order to track the nuclear translocation of the signaling elements. Figure 8 exhibits the translocation of these molecules into the nucleus with the intracellular expression of IFN γ, while the NLS-modified IFN γ or the empty vector failed to induce similar translocation of STAT1α or IFNGR1. As was found with previous studies (Larkin et al., 2000), cells that were simultaneously stained with IFNGR2 and STAT1α showed translocation of only STAT1α, while IFNGR2 was not translocated with the expression of intracellular IFN γ. Similarly, neither of these was translocated with the NLS-modified IFN γ or the control vector (data not shown). Quantitation of the ratio of fluorescence in nuclei (Fn) to the fluorescence in cytoplasmic (Fc) fractions for STAT1α and IFNGR1 was obtained from at least seven different fields among these fluorescence images (Figure 9). The results show that cells expressing nonsecreted IFN γ induced translocation of both STAT1α and IFNGR1 into the nucleus,

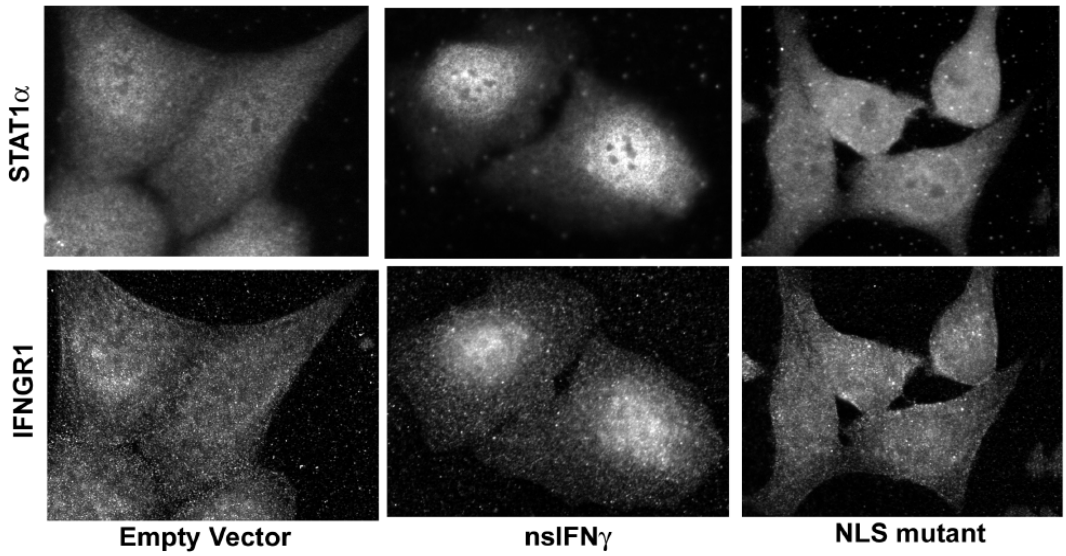


Figure 8. Nuclear translocation of STAT1α and IFNGR1 by intracellular IFN γ requires the NLS of IFN γ. WISH cells were transfected for 8 hours with an empty vector (column 1), a vector expressing nonsecreted IFN γ (column 2), or a vector expressing NLS-mutated IFN γ (column 3) and stained simultaneously with antibodies to STAT1α and IFNGR1. Secondary antibodies to STAT1α conjugated to Alexa 594 (top row) or to IFNGR1 conjugated to Cy-2 (bottom row) were used and analyzed by fluorescence microscopy.

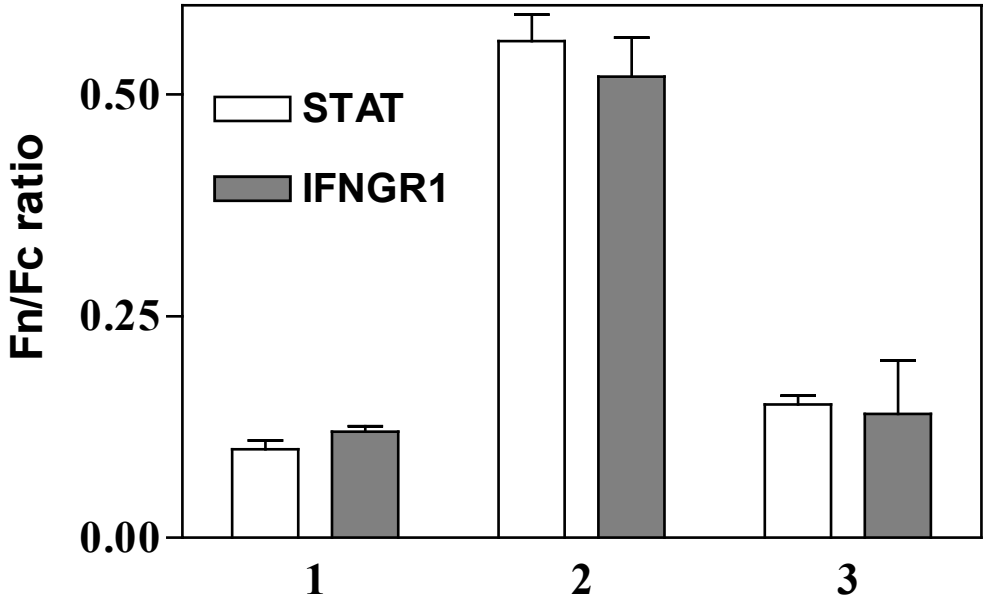


Figure 9. Nuclear translocation of STAT1α and IFNGR1 is abolished with the removal of NLS in IFN γ. Images of cells from Figure 8 transduced with an empty vector control (left lanes, 1), or a vector expressing nonsecreted IFN γ (middle lanes, 2), or a vector expressing NLS-mutated IFN γ (right lanes, 3) were viewed in seven different fields to obtain a mean ratio +/- SD of nuclear pixel intensity (Fn) to cytoplasmic pixel intensity (Fc). STAT1α and IFNGR1 Fn/Fc for nsIFN γ versus mutant were both significant at $P<0.002$ by t-test. Calculations for fluorescence in the nucleus (N) versus cytoplasm (C) were also done by using N/N+C. Four independent measurements showed a $P<0.03$ by t-test for the nuclear translocation of STAT1α and IFNGR1 for the wild-type IFN γ versus the NLS-mutated IFN γ.

while cells treated with NLS-modified IFN γ or the empty vector did not show nuclear translocation.

Immunofluorescence analysis was also used to visualize the effects removal of IFN γ's NLS would have on its translocation into the nucleus (Figure 10A). Indeed, the intracellular expression of NLS-mutated IFN γ resulted in its lack of nuclear translocation, while the expression of intracellular IFN γ showed clear nuclear translocation. There was a complete lack of IFN γ signal with the empty vector control (Figure 10A). Mean fluorescence intensities for the nonsecreted and NLS-mutated forms of IFN γ were compared by measuring mean pixel intensity across several lines drawn through the cells as shown in Figure 10B. The results show approximately 40% more fluorescence in nuclei treated with nonsecreted IFN γ compared with the NLS-mutated IFN γ. Quantitation of the images (Fn/Fc ratios) is shown in Figure 11. Association of IFN γ together with IFNGR1 and STAT1α points to a role for this ligand and one of its receptor subunits in chaperoning STAT1α into the nucleus.

Biological Activity of Human and Murine IFN γ Mimetic Peptides

We first examined the antiviral activity of the mimetic peptides. WISH cells, untreated, or transduced with an empty vector control, human IFN γ mimetic peptide (95-134), or murine IFN γ mimetic peptide (95-133) expressing vector were allowed to grow for one day followed by a challenge with VSV. Twenty-four hours later, the cells were compared for relative resistance to VSV induced cytopathic effect (Figure 12). Both sets of cells treated with human and murine mimetic peptides showed a 4-fold increase in cell survival compared to the untreated or cells treated with the empty vector control.

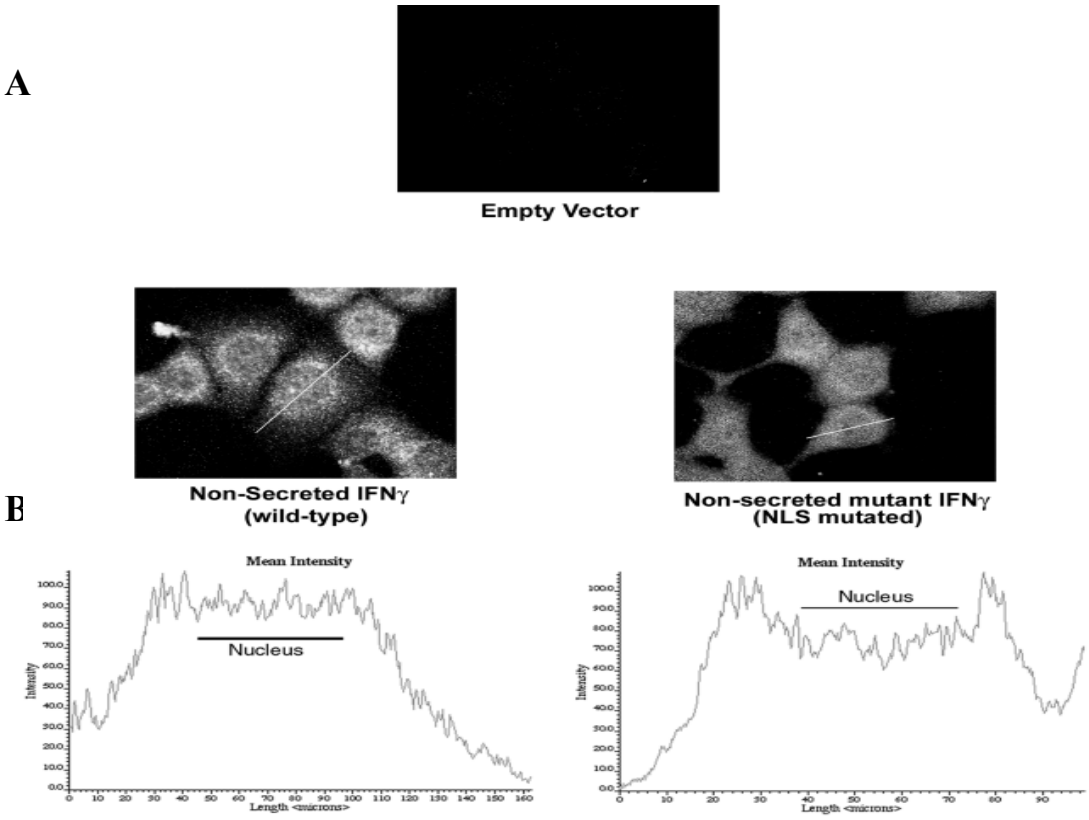


Figure 10. Nuclear translocation of IFN γ requires the presence of the C-terminal NLS. (A) WISH cells were transduced for 8 hours with an empty vector (top), a vector expressing nonsecreted IFN γ (middle left), or a vector expressing NLS-mutated IFN γ (middle right) were probed with a monoclonal antibody to IFN γ and stained with Alexa Fluor 488 conjugated secondary antibody and analyzed by confocal microscopy. (B) Mean fluorescence intensity comparisons for nonsecreted IFN γ and NLS-mutated IFN γ. Measurements of fluorescence intensities were done with IP Lab using the MP-line measure tool. Mean pixel intensity was measured across a line drawn through the cells in the plane of the images shown. A representative measurement line is shown in white. The resultant graphs were generated by the software averaging the intensities of a total of 100 pixels on either side of the line at each point along the line. Triplicate determinations showed a significance of $P<0.25$ (t-test) for nuclear presence of IFN γ versus NLS mutant.

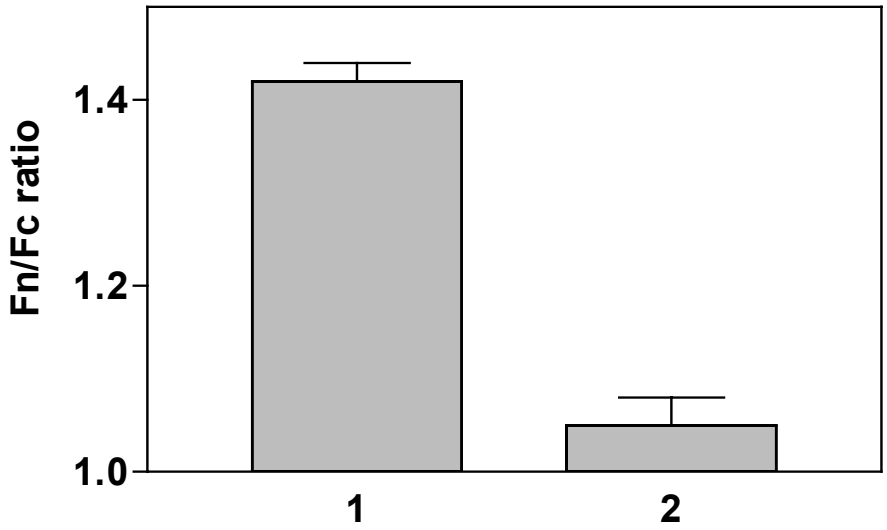


Figure 11. Nuclear translocation of IFN γ is abolished with the removal of the C-terminal NLS. Images of cells from Figure 10 transduced with nonsecreted IFN γ or NLS-mutated nonsecreted IFN γ were used to determine Fn/Fc values, which are shown in columns 1 and 2, respectively. Seven fields were examined and the results were averaged +/- SD. The significance was $P<0.02$ by the t-test. Calculations for fluorescence in the nucleus (N) versus cytoplasm (C) were also done by using N/N+C. Four independent measurements showed a $P<0.025$ for the nuclear translocation of IFN γ for the wild-type IFN γ versus the NLS-mutated IFN γ.

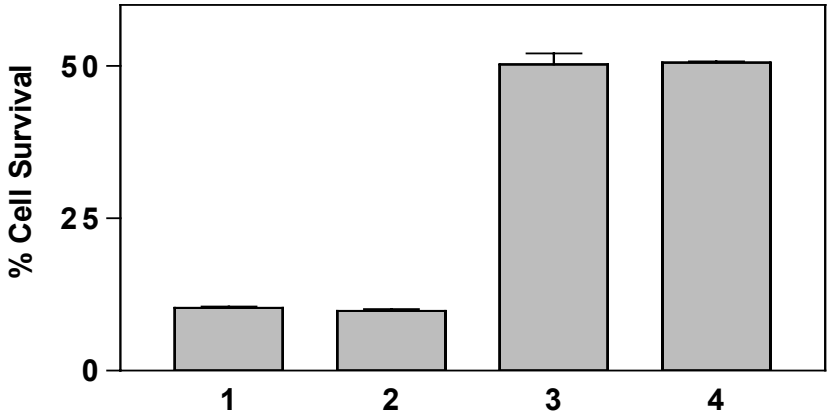


Figure 12. IFN γ mimetic peptides offer protection against Vesicular Stomatitis Virus (VSV). Human IFN γ (95-134) or murine IFN γ (95-133) mimetic peptides were expressed by using adenovirus expression system. WISH cells, either untreated (lane 1), or those infected for 1 hour with an empty vector control (lane 2), a vector expressing human IFN γ mimetic peptide (lane 3), or a vector expressing murine IFN γ mimetic peptide (lane 4) were allowed to grow for 24 hours and subsequently challenged with VSV. After 24 hours, cells were stained crystal violet, the dye was extracted with methylcellusolve and the absorbance was measured at 600 nM. Absorbance after different treatments is expressed as percent of cells that were not exposed to any virus +/- SD, which was taken as 100%. Results represent the mean of three independent determinations.

Therefore, these mimetic peptides induce antiviral activity much like the wild type IFN γ. Given that human WISH cells do not recognize murine IFN γ via the extracellular domain of the receptor, the data further support an intracellular effect of the murine IFN γ mimetic peptide.

As stated earlier, both extracellularly added IFN γ and nonsecreted IFN γ induce MHC class I molecule expression at the cellular surface. To examine if human IFN γ mimetic peptide (95-134) or murine IFN γ mimetic peptide (95-133) also induce MHC class I expression, WISH cells were transduced with an empty vector control, or human IFN γ mimetic peptide (95-134), or murine IFN γ mimetic peptide (95-133) expression vectors. After two days, the cells were stained with R-PE conjugated monoclonal antibody to human MHC class I molecules and underwent flow cytometry analysis. Murine IgG2a antibodies conjugated with R-PE were utilized as an isotope control. Relative mean fluorescence profiles are presented in Figure 13. Cells transduced with empty vector control, human IFN γ mimetic peptide (95-134), or murine IFN γ mimetic peptide (95-133) expression vectors showed 142 +/- 3.5, 306 +/- 11, and 313 +/- 16 units of mean fluorescence, respectively. Therefore, expression of both human and murine IFN γ mimetic peptides induce more than 2-fold expression of MHC class I molecules. Thus, human and murine IFN γ mimetic peptides induce both antiviral activity and induction of MHC class I molecules.

Antiviral Activity of Murine IFN γ Mimetic Lipopeptide (95-133L) *in vitro* and *in vivo*

Three independent experiments were used to characterize the antiviral capacity of a new murine IFN γ mimetic lipopeptide (95-133L). The penetration of peptides into cells is enhanced by the addition of a lipophilic group in the synthesis of the mimetic peptides.

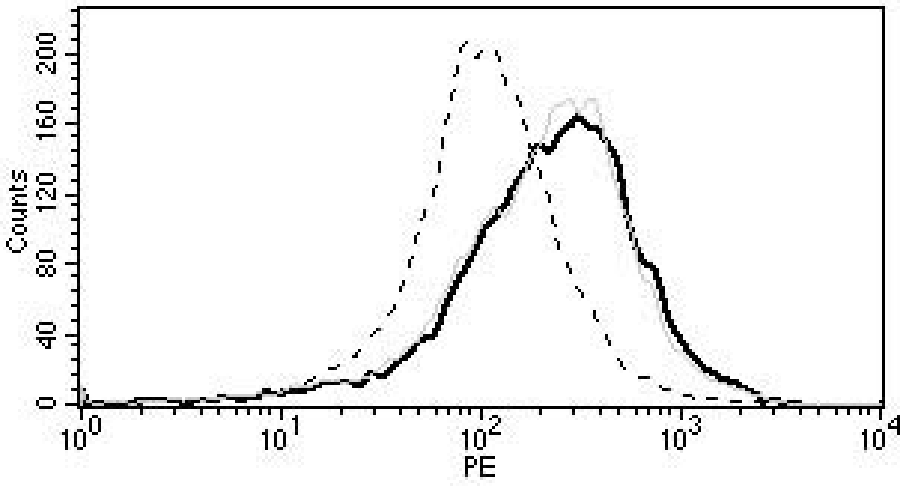
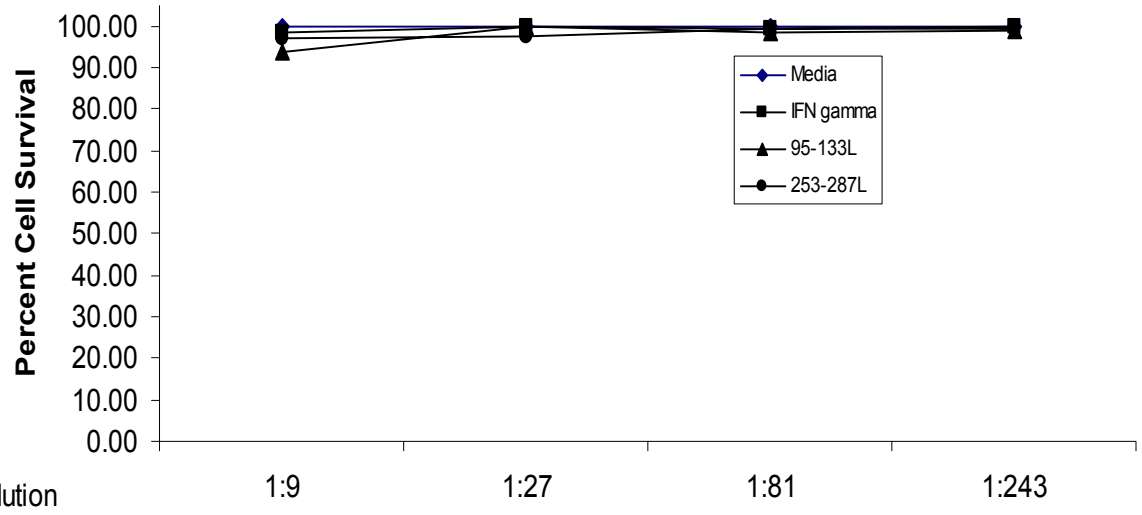


Figure 13. Induction of MHC class I by human IFN γ (95-134) or murine IFN γ (95-133) mimetic peptides. WISH cells were transduced for one hour with an empty vector control (dotted line), a vector expressing human IFN γ mimetic peptide (95-134) (grey thin line), or a vector expressing murine IFN γ mimetic peptide (95-133) (thick black line). Cells were then allowed to grow for 48 hours in regular medium followed by staining with R-PE conjugated monoclonal antibody to human MHC class I and analyzed by flow cytometry. R-PE conjugated murine IgG2a was used as a control. A similar profile was noted in three independent experiments.

An initial toxicity study was performed on the synthesized lipidated peptides to ensure the cytopathic effects reported below were due to virus alone. Mouse L929 cells were treated with media or with a serial dilution of IFN γ, IFN γ mimetic lipopeptide (95-133L), or IFNGR control lipopeptide for 24 hours. Results showed that IFN γ is not toxic to cells at concentrations less than 4700 U/ml, while both IFN γ mimetic lipopeptide (95-133L) and IFNGR control lipopeptide (253-287L) tend to be nontoxic at concentrations less than185 μg/ml (Figure 14). Thus, subsequent experiments utilized peptides at appropriate concentrations below the toxic level, and reported results are due to the cytopathic effects of EMC virus alone.

Murine IFN γ lipopeptide (95-133L) contains the putative NLS shown to be key in establishing an antiviral state in infected cells (Subramaniam et al., 2001). Recently, it was shown that a lipid-modified peptide derived from murine IFN γ was able to gain access to cytoplasmic targets in nonphagocytic cells that were otherwise nonaccessible by its unmodified IFN γ peptide counterpart. In addition, the murine IFN γ lipid-modified peptide was reported to intracellularly induce biologically activity upon entering both human and murine cells (Thiam et al., 1999; Thiam et al., 1998). We examined the effect of adding a lipid moiety to the N-terminal end of the murine IFN γ mimetic (95-133) on its antiviral capabilities. Murine L929 cells were treated with media, or with 2.5 x 10^3 U/ml of IFN γ, 20 μM of IFN γ lipopeptide (95-133L), or 20 μM of IFNGR control lipopeptide (253-287L) for one day followed by treatment with EMC at 100 pfu/ml for one hour. Cells were reincubated in media for 24 hours after which, the virus was titered in a standard plaque assay and treatments were compared for EMC virus yield and fold



IFN γ (U/ml)	4700	1600	530	530
95-133L (μg/ml)	555 (110 μM)	185 (37 μM)	62 (12 μM)	21 (4 μM)
253-287L (μg/ml)	555 (124 μM)	185 (41 μM)	62 (13.7 μM)	21 (4.6 μM)

Figure14. Toxicity study of IFN γ, mimetic lipopeptide IFN γ (95-133L), and IFNGR control peptide (253-287L) on murine fibroblast L929 cells. Cells were plated at 6 x 10^4 cells/well in a microtiter dish and incubated overnight. Media, IFN γ, IFN γ mimetic lipopeptide (95-133L), and IFNGR control lipopeptide (253-287L) were incubated with cells at various dilutions for 24 hours. Cells were then stained with crystal violet and the plate was scanned on a Astra 2100U scanner. Mean pixel intensities were determined using ImageJ 1.29 software (NIH). Values were divided by media control taken as 100% cell survival, and reported as the average percent cell survival of duplicate treatments .

reduction (Table 4). Wild-type IFN γ showed 37.3 fold reduction compared to cells treated with media only, while IFN γ lipopeptide (95-133L) showed a 24.1 fold reduction in virus yield compared to the media treated cells. The cells treated with control lipopeptide (253-287L) only showed a 0.7 fold reduction in virus yield compared to those cells treated with media alone. These findings suggest that the IFN γ mimetic lipopeptide (95-133L) maintained its antiviral activity with the addition of the lipid group and that this antiviral activity was not due to the lipidation of the sequence itself.

A similar experiment was conducted to determine the concentration of the IFN γ mimetic lipopeptide (95-133L) that offered 50% protection from infection by EMC virus. Again, mouse L929 cells were treated with a serial dilution of 2.5 x 10^3 U/ml of IFN γ, 1.6 mg/ml of IFN γ mimetic lipopeptide (95-133L), or 1.6 mg/ml of IFNGR control lipopeptide (253-287L) for one day followed by challenge with 100 pfu/ml of EMC virus. The cells underwent washing and 24 hours of incubation before results were calculated. As shown in Table 5, wild-type IFN γ treated cells showed 50% protection at 200 U/ml. Cells treated with the IFN γ lipopeptide (95-133L) showed 50% protection at only 6 μg/ml while the control peptide showed 50% protection at 177 μg/ml. These results show a 30-fold difference in the concentrations that provide 50% protection for these two lipopeptides.

The final experiment examines the protective effect the IFN γ mimetic lipopeptide would have against EMC virus induced lethality in C57BL/6 mice. Previous studies have shown that mice injected with 100 pfu/ml of EMC resulted in 100% mortality in 14 days (Shalaby et al., 1985). Murine L929 cells were treated with media, or with 2.5 x 10^3 U/ml of IFN γ, or 20 μM of IFN γ lipopeptide (95-133L), or 20 μM or IFNGR control

Table 4. IFN γ and mimetic lipopeptide reduction of EMC yield[a]

IFN γ or Peptide concentration		Virus Yield (pfu/ml)	Fold Reduction
IFN γ	2.5×10^3 U/ml	1.1×10^7	37.3
IFN γ Lipopeptide (95-133L)	20 μM	1.7×10^7	24.1
IFNGR Lipopeptide (253-287)	20 μM	6.3×10^8	0.7
Media only		4.1×10^8	-----

[a]Mouse fibroblast L929 cells seeded to confluency in a $25cm^2$ flask were pretreated with 2.5×10^3 U/ml of IFN γ , 20 μM of IFN γ mimetic lipopeptide (95-133L), and 20 μM of IFNGR control lipopeptide (253-287L) for 24 hours after which the cells were challenged with EMC virus at 100 pfu/ml (2.1×10^{-4} m.o.i.) for 1 hour. The cells were washed and incubated with EMEM media and incubated for 24 hours. Virus produced was harvested and titered in a standard viral plaque assay. Cells were stained with crystal violet. Plaques for IFN γ, IFN γ mimetic lipopeptide (95-134L), and IFNGR control lipopeptide (253-287L) were counted at 1:200,000 dilution. Media only plaques were counted at 1: 2,000,000 dilution. The original sample concentrations are reported as pfu/ml.

Table 5. Concentration of IFN γ, IFN γ mimetic lipopeptide (95-133L), and IFNGR control lipopeptide (253-287L) that resulted in 50% protection of mouse L cells against 100 pfu/ml of EMC[a]

IFN γ or peptide	50% endpoint
IFN γ	200 U/ml
IFN γ lipopeptide (95-133L)	6 μg/ml
IFNGR lipopeptide (253-287L)	177 μg/ml

[a]Mouse fibroblast L929 cells were plated at $6x10^4$ cells/well in a microtiter dish and pretreated with $2.5x10^4$ U/ml of IFN γ, 1.6 mg/ml of IFN γ mimetic lipopeptide (95-133L), and 1.6 mg/ml of IFNGR control lipopeptide (253-287L) for 24 hours. Cells were then challenged with encephalomyocarditis virus (EMC) virus at 100 pfu/ml for 1 hour. Cells were washed, incubated with EMEM media for 24 hours, and stained with crystal violet. The dilution at which 50% of the cells were stained was taken as the 50% endpoint.

lipopeptide (253-287L) for one day followed by challenge with EMC at 100 pfu/ml for one hour. Cells were washed and reincubated in media for 24 hours. All supernatants harvested from the treated cells were diluted 1:200,000 prior to their intraperitoneal injection of mice on day 0. The percent of mice surviving out of four for each group was determined on a daily basis. As shown in Table 6, after seven days, the mice administered the media supernatant showed only 25% survival, while those mice administered the supernatants of the wild-type IFN γ and the IFN γ mimetic lipopeptide (95-133) showed 75% and 100% survival, respectively. Furthermore, after two weeks only the mimetic group exhibited a survival rate of 75% or higher. Thus, treatment with IFN γ mimetic lipopeptide (95-133L) prolongs the survival of C57BL/6 mice under EMC viral challenge. Therefore, the results seen from these three trials offer evidence that IFN γ mimetic lipopeptide (95-133L) may provide a similar antiviral activity to that of wild-type IFN γ *in vitro* and *in vivo*.

Table 6. Treatment with IFN γ mimetic lipopeptide (95-133L) prolongs the survival of mice with EMC infection

	Days after EMC infection (% surviving)											
Treatment[a]	0	4	5	6	7	8	9	10	11	12	13	14
Media n=4[b]	100	100	50	50	25	25	25	25	25	25	25	25
IFN γ n=4[b]	100	100	100	100	75	50	25	0	0	0	0	0
(95-133L) n=4[b]	100	100	100	100	100	100	100	75	75	75	75	75

[a]Mouse fibroblast L-929 cells were treated with media, 2.5 x 10^3 U/mL of IFN γ, or 20 μM of IFN γ, or IFN γ mimetic lipopeptide (95-133L) for 24 hours after which cells were incubated with 100 pfu/mL of encephalomyocarditis virus (EMC) for one 1 hour. Cells were then washed three times and resuspended in EMEM media for 24 hours and supernatants collected. All supernatants were diluted with media (1:200,000) prior to intraperitoneal injection of C57BL/6 mice on Day 0. Mortality from EMC infection was recorded and the percent of mice surviving for each treatment group is reported above. The findings were found to be significant at $P < .001$ for the lipopeptide compared to the media control group and $P < .02$ for the IFN γ peptide compared to the media control group by chi-squared test.

[b]Four mice per group

CHAPTER 4
DISCUSSION

The toxic side effects of interferon therapy have limited its potential in the clinical setting. Yet, continuing work in the field of interferons only strengthens the evidence of their efficacy in antitumor and antimicrobial host invasion. In order for those in the medical industry to exploit these properties researchers must develop more efficient and less toxic methods of delivering interferons. This study makes use of some of the latest attempts at fine-tuning interferon therapy.

In recent years, gene therapy vectors were applied successfully to interferon therapy. In particular, adenoviral vectors have become the most commonly used systems in gene therapy experimentation. Reports on adenoviral vector delivery of interferons reveal this combination's ability to yield high, localized concentrations devoid of pervasive distribution (Ahmed et al., 2001). The first phase of this study confirmed the biological activity of an intracellular form of IFN γ expressed by an adenoviral vector. Further, this biological activity was found to be dependent on the polybasic NLS sequence identified in previous works (Subramaniam et al., 1999). Intracellular IFN γ antiviral activity in mouse fibroblast cells and MHC class I upregulation in human WISH cells was lost when the wild type NLS sequence, ^{128}KTGKRKR^{134}, was mutated to ^{128}ATGAAAA^{134}. In addition, nuclear import of STAT1α was abolished in cells expressing the mutated version of the NLS. These findings suggest that intracellular IFN γ initiates the biological activity without interaction with the extracellular domain of the

IFN receptor. This phenomenon is consistent with previous reports of intracellular forms of cytokines. As with intracellular IFN γ, human IFN α2b and IFN α2α1 (Ahmed et al., 2001), IFN α consensus (Rutherford et al., 1996), murine IFN γ (Will et al., 1996), IL-6 (Roth et al., 1995), and IL-3 (Dunbar et al., 1989) were each found to be biologically active. Perhaps the nuclear import of STATs by these cytokines is originated by a receptor motif similar to that identified for IFN γ at the C-terminus of IFNGR1 (Szente et al., 1994; Szente et al., 1995). Moreover, the NLS or NLS-like sequences identified for these cytokines may also be involved in the chaperoning of their appropriate STATs to the nucleus (Subramaniam et al., 2001).

The IFN γ receptor complex is comprised of two subunit chains termed IFNGR1 and IFNGR2 (Bach et al., 1997). IFNGR1 plays a more comprehensive role in the signaling cascade initiated by IFN γ ligand binding. Various structure-function analyses revealed the extracellular ligand binding site as well as the intracellular binding sites for JAK1 and latent STAT1. Initial work with polypeptides from the C-terminus of murine and human IFN γ possessing a NLS demonstrated the ability of these peptides to bind to the intracellular domain of IFNGR1 (Szente et al., 1995; Szente et al., 1994). Subsequently, the polypeptides were found capable of inducing an antiviral state and upregulation of MHC class II molecules. Antibodies raised against the murine polypeptide encompassing the NLS were microinjected into cells and treated extracellularly with IFN γ. The cells ceased to exhibit STAT1α nuclear translocation (Subramaniam et al., 1999). However, when the NLS from the SV40 T antigen was used to replace the NLS in the C-terminus polypeptide of IFN γ the cells regained their biological activity (Subramaniam et al., 2001). This suggests that a requirement of

biological activity is the association of the NLS region of the IFN γ ligand with the IFNGR1 chain. Two additional studies that corroborate these findings state that IFNGR1 -/- cells do not respond to murine intracellular IFN γ (Will et al., 1996), or agonist peptide (Thiam et al., 1998). The preferential nuclear translocation of IFNGR1 is another aspect of the signaling paradigm that requires further examination. Interestingly, it was shown that IFNGR1 is transported to the nucleus upon treating cells with IFN γ, but that IFNGR2 is found to remain at the cellular surface (Larkin et al., 2000). The IFN γ receptor subunit is included in a list of many cytokine and growth factor receptor subunits exhibiting nuclear translocation (Subramaniam et al., 2001; Jans and Hassan, 1998). Work with the EGF receptor subunit revealed a transcription factor-like activity upon nuclear translocation (Lin et al., 2001). Perhaps, these types of receptors play a role in conferring signaling specificity by allowing their associated STATs to utilize promoters designated for that particular ligand and/or receptor.

Sekimoto et al. have shown that STAT1α achieves nuclear translocation by NPI-1 via the Ran/importin pathway. Taking into account that their mutational sequence analysis of STAT1 failed to reveal a clear NLS (Sekimoto et al., 1997), our lab explored the possibility of human IFN γ containing a NLS sequence by means of a digitonin-permeabilization assay (Subramaniam et al., 1999). Although the 17-kDa IFN γ ligand meets the NPC's restriction for nuclear import by way of passive diffusion a strong NLS was identified by the assay. Since various cytokines utilized the seven STATs to give rise to a myriad of biological responses, it is conceivable that the strong NLS of IFN γ serves a chaperoning function for STAT1α as well as a means of achieving signal specificity (Subramaniam et al., 2001). This study also determined that the

phosphorylation of STAT1 alone was insufficient for its nuclear translocation, as demonstrated with the angiotensin II receptor (Sayeski et al., 2001). However, activated STAT1α does undergo nuclear import in an NLS-dependent fashion with intracellular IFN γ. Both the tyrosine phosphorylation of STAT1α and the formation of the IFN γ/NPI-1/STAT1α nuclear targeted complex were found to be reliant on the NLS of intracellular IFN γ. In conclusion, these findings are beginning to define a broader role for IFN γ in the events of the IFN γ signaling pathway.

The second phase of this study determined that adenoviral vector delivery of mimetic human IFN γ (95-134) and murine IFN γ (95-133) resulted in upregulation of MHC class I molecules and establishment of an antiviral state similar to that of wild-type IFN γ. This suggests that the intracellular delivery of these mimetics stimulate IFN γ signaling events in a fashion similar to the pathway outlined for the intracellular human IFN γ during the first phase of this study. Again it was noted that the intracellular domain of the IFNGR1 receptor chain was species non-specific, as the murine IFN γ mimetic peptide (95-133) was able to induce an antiviral response in human WISH cells. Further characterization of these mimetics is currently underway.

Biologically active lipopeptides are another form of cytokine delivery being explored for interferon therapy. The characteristic stability and cytoplasmic accessibility achieved by these lipopeptides make them good candidates for interferon therapy. *In vitro* the lipopeptide was able to reproduce antiviral activities of wild-type IFN γ in L929 cells, as has been described for a similar lipophilic peptide with the lipid moiety added to the C-terminal end (Thiam et al., 1998). Our findings suggest that our murine IFN γ mimetic lipopeptide (95-133L) offers resistance to EMC infection *in vivo* and *in vitro*.

The surprising increased survival of C57BL/6 mice injected with the mimetic lipopeptide (75%) after 14 days compared to the decreased survival of mice injected with media (25%) and IFN γ (0%) treated cells may suggest that the mimetic maintained a longer half life than IFN γ. Others have suggested that similar IFN γ lipopeptides gained an increased efficacy due to the lipid moiety's indirect effect on the stabilization of the helical organization of the entire lipopeptide (Thiam et al., 1997). Structure-function studies of our IFN γ mimetic lipopeptide (95-133L) may offer greater insight into the antiviral activity noted.

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BIOGRAPHICAL SKETCH

Margi Anne Burkhart was the third child born to Dr. Robert Davis Burkhart Jr. and Suzanne B. Burkhart on March 8, 1978. Although she was a "surprise" her parents always felt like the third time was the charm. However, her older brother, Geoff, and older sister, Jody, were not too sure about the new addition to the family, but loved her anyway. Together the Burkhart family spent many weekends enjoying the sun and fun of Florida's waterways on their boat. Her parents were naturals at teaching their children about the land and the sea during these weekend getaways. Margi was very curious about her surroundings, and it was not uncommon to see her dissecting the day's catch from the waters off St. Augustine's coast or catching baby alligators in Silver Glen State Park. Thus, her interest in science began very early in her childhood and is still growing. When the Burkhart family was not on the boat they were at a soccer field. Geoff, Jody, and Margi were awesome soccer players with a knack for defense. Margi's soccer abilities landed her a starting spot on the varsity team as a freshman and a soccer scholarship to the school of her choice. Margi accepted a soccer scholarship and an academic/leadership scholarship from Elon College in North Carolina. At Elon Margi was part of a wonderful soccer program and made friends that will last her a lifetime. After four years Margi reluctantly left Elon for graduate studies at the University of Florida. She was very happy to be a Gator like her brother and sister before her. After being accepted into the research laboratory of Dr. Howard M. Johnson in the Department of Microbiology and Cell Science she was assigned to a project concerning intracellular

interferon gamma and placed under the supervision of Assistant Scientist Dr. Iqbal Ahmed. Margi felt privileged to learn her research techniques from such a gifted scientist as Dr. Ahmed. After receiving her Master of Science in August 2003 Margi will be applying to Physician's Assistant programs along the East coast where she hopes her strong background in microbiology and immunology may be applied to her future studies in pediatrics.