The Interferons: 50 Years after Their Discovery, There Is Much More to Learn

Sidney Pestka
From the Department of Molecular Genetics, Microbiology, and Immunology, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

The interferons (IFNs) and their receptors represent a subset of the class 2 α-helical cytokines that have been in chordates for millions of years. This brief review focuses on the discovery and purification of interferons, cloning of human IFN-α and IFN-β, interferon receptors, activities and therapeutic uses of interferons, and the side effects of interferons.

**Discovery and Purification of Interferons**

In studying the phenomenon of viral interference, Isaacs and Lindenmann (3) (see supplemental Fig. 1) discovered interferon in 1957. The first experiments were performed in chick choirdoallantoic membranes in a nutrient fluid where the addition of influenza virus stimulated the production of interferon, but in a short time interferons were found to be produced in many animals, tissues, and cells. Later on, infection of human white blood cells with viruses induced the expression of human interferons in the medium from which interferons were partially purified for use in clinical trials (4). This interferon preparation used in experiments as well as in initial human clinical trials was essentially a crude protein fraction less than 1% of which consisted of interferon. Thus, it was not clear which activities of these preparations were due inherently to the interferon present or to the contaminating proteins. However, even after a great deal of effort by many investigators to purify the interferons produced from various tissues and cells, purification of interferons to homogeneity was not achieved until about two decades after their discovery.

It was not until 1978 that an interferon was purified to homogeneity so that it could be analyzed and characterized (5–9). The introduction of reverse-phase and normal-phase high performance liquid chromatography for the purification of proteins (5, 7, 9) led to the first successful purification of IFN-α and IFN-β so that sufficient amounts were available for chemical, biological, and immunological studies.

Production of Leukocyte Interferons—Interferon was produced by incubating human leukocytes from donors with Newcastle disease virus or Sendai virus for 6–24 h (4, 7, 8). Milk casein was substituted for human or bovine serum in the culture medium. The use of casein, a single protein, instead of serum, which contains many different and uncharacterized proteins, simplified the initial concentration and purification steps. Although leukocyte interferon consisted predominantly of IFN-α, small amounts of IFN-β and IFN-ω were also present. To carry out the purification, it was necessary to assess the quantity and recovery of interferon at each step by a cytopathic effect (CPE) inhibition assay, an assay that took 3 days and would enable measurement of interferon titers only once or at most twice a week. To speed up the procedure, a cytopathic effect inhibition assay was developed that could be done in 12–16 h, which accelerated purification immensely (10).

**High Performance Liquid Chromatography (HPLC) for Protein Purification**—The initial steps for purification of IFN included selective precipitations and gel filtration followed by HPLC. The HPLC steps were reverse-phase chromatography at pH 7.5 on LiChrosorb RP-8, normal partition chromatography on LiChrosorb Diol, and reverse-phase chromatography at pH 4.0 on LiChrosorb RP-8 (6–8). Prior to purification by HPLC, interferon in media was concentrated and then was passed through a Sephadex G-100 fine gel filtration column to isolate protein in the M, 20,000 range (6–8). Because standard methods for protein purification were not significantly successful in purification of the human interferons, we developed HPLC for the purification of proteins. Unfriedlin, Stein, and co-workers (11–13) had developed sensitive fluorescent techniques for detection of amino acids and peptides that we used to detect the interferons. After gel filtration, the major interferon fraction was applied directly to a LiChrosorb RP-8 column, and the interferon was eluted with an n-propanol gradient (6–8). As subsequently demonstrated with fibroblast interferon (5), a large number of different columns and solvent systems could be used to effect resolution of proteins. By applying normal-phase chromatography with a diol silica column between the two reverse-phase columns, it was possible to use just three sequen-
MINIREVIEW: Interferons: 50 Years after Their Discovery

TABLE 1
Human interferons and interferon-like proteins

| Ligand types | Names    | Receptor chain 1 | Receptor chain 2 |
|--------------|----------|------------------|------------------|
| Type I IFN   | IFN-α    | IFN-αR1          | IFN-αR2          |
|              | IFN-β    | (Also IFN-αRα)   | (Also IFN-αRβ)   |
|              | IFN-κ    | IFNAR1           | IFNAR2           |
|              | IFN-ω    |                  |                  |
| Type II IFN  | IFN-γ    | IFN-γR1          | IFN-γR2          |
|              | (Also IFN-γRα) | IFNGR1       | (Also IFN-γRβ, IFNGR2) |
| IFN-like proteins | IL-28A | IL-28R1         | IL-10R2         |
|               | IL-28B |                  |                  |
|               | IL-29  |                  |                  |

tial HPLC steps to purify human leukocyte interferon (IFN-α) to homogeneity. Sufficient amounts were purified in high yield for initial chemical characterization of the protein and for determination of amino acid composition (7). High recoveries of interferon activity were obtained in each chromatographic step, a requirement when small amounts of initial starting material are present. The overall purification was about 80,000-fold, and the specific activity of purified interferon was 2–4 × 10^8 units/mg (7). The first interferon prepared by this procedure yielded a single band of Mr 17,500 on polyacrylamide gel electrophoresis, and the antiviral activity was associated with the single protein band (6).

Although the initial experiments were performed with leukocytes from normal donors (6, 7), it was found that leukocytes from patients with chronic myelogenous leukemia who were undergoing leukapheresis to lower their peripheral white blood cell counts were a rich source of interferon, similar to the leukocytes from normal donors (14).

Multiple Species of Leukocyte Interferons—During the purification of leukocyte interferon, it became evident that multiple species existed. Peptide mapping and sequencing supported this conclusion. As additional leukocyte interferon species were isolated from cultured myeloblasts (15, 16) and other sources (17–19), the concept that IFN-α are a family of interferons was established. A detailed summary of all of these purification procedures are available in a Scientific American article with graphic presentations (20).

Glycosylation of IFN-α Subtypes—Although five purified species of leukocyte interferon that were initially isolated by HPLC, as described above (8), and interferon produced by Namalwa cells (17) were found to contain no detectable carbohydrate, a more extensive analysis of the IFN-α subtypes derived from patients with chronic myelogenous leukemia and from normal donors demonstrated that some subtypes exhibited O-linked glycosylation (21, 22). Considering that the recombinant human IFN-α species produced in Escherichia coli do not contain carbohydrate, it was useful to discover that most of the human IFN-α species were devoid of carbohydrate.

Cloning Recombinant Human IFN-α and IFN-β

Because development of recombinant DNA technology offered an opportunity to produce large amounts of Hu-IFNs economically compared with production of interferons in leukocytes or other cells by virus infection, many investigators set out to clone the interferons in E. coli. Several groups isolated recombinant Hu-IFN-α subtypes (23–25) and IFN-β recombinants (23, 26–29), obtaining the clones by somewhat different but analogous approaches. Since the interferon proteins had not been identified, isolating Hu-IFN DNA sequences was a formidable task. In fact, identifying a cDNA had never been accomplished previously for a protein with an unknown structure that had not been purified. In order to reconstruct DNA recombinants that would express natural IFN, it would be useful to know the partial amino acid sequence of the proteins, particularly at the NH₂- and COOH-terminal ends. Without this information, synthesis of natural Hu-IFN in bacterial cells was challenging. A summary of our cloning and expression of Hu-IFN-α2a (Hu-IFN-αA) is illustrated here.

To isolate recombinants containing the human DNA corresponding to IFN-α, we developed a number of procedures. First, it was necessary to isolate and measure the IFN mRNA. This had been accomplished several years earlier when IFN mRNA was translated in cell-free extracts (30, 31) and in frog oocytes (32–34). The next step was to prepare sufficient mRNA from cells synthesizing IFN, and this was accomplished with both fibroblasts and leukocytes (35, 36). A library of complementary DNA (cDNA) was prepared from a template of partially purified mRNA isolated from human leukocytes synthesizing IFN. The next step was to find in this vast library of recombinant plasmids those that contained DNA encoding IFN. We devised an indirect two-stage procedure to identify clones containing interferon sequences. In the first stage, we screened all of the bacterial colonies to find those with cDNA made from the RNA of induced cells; among these there might have been some carrying IFN cDNA. We, therefore, screened all of the recombinants for their ability to bind to mRNA from cells synthesizing IFN (induced cells) but not to mRNA from uninduced cells (those not producing IFN). To do this, individual transformed colonies were screened by colony hybridization for the presence of specifically induced sequences with 32P-labeled IFN mRNA (mRNA from induced cells) as probe. In the presence of excess mRNA from uninduced cells, recombinants that were representative of mRNA sequences existing only in induced cells should be evident on hybridization. This screening procedure allowed us to discard about 90% of the colonies; because their plasmids carried no induced cDNA, these could not encode IFN (23, 37).

In the second stage, we identified those recombinants containing the IFN DNA sequences among the remaining 10%. To accomplish this, we pooled the recombinant plasmids in groups of 10 and examined them for the presence of IFN-specific sequences by an assay that depends upon hybridization of IFN mRNA to plasmid DNA (23, 38). Plasmid DNA from 10 recombinants was isolated and covalently bound to diazobenzoxymethyl paper. The mRNA from induced cells was hybridized to each filter. Unhybridized mRNA was removed by washing. After the specifically hybridized mRNA was eluted, both fractions were translated in Xenopus laevis oocytes. Once a positive group had been found (one in which the specifically hybridized mRNA yielded IFN after microinjection into frog oocytes), it
were able to obtain the first crystal of a cytokine, IFN-α, and were able to express the IFN-α protein in bacteria (20). With a substantial quantity of IFN-α produced, we first purified the recombinant IFN-α cDNA sequence, which could be used for expression of the IFN-α protein. The cloned Hu-IFN-α cDNA sequence, which could be used for expression of the IFN-α protein, was determined and found to correspond to what was then known of the amino acid sequence of purified Hu-IFN-α (39, 40). The cDNA insert in plasmid p104 contained the sequence corresponding to more than 80% of the amino acids in IFN-α2a, but not for those at its amino-terminal end. It was therefore used as a probe for finding a full-length copy of the IFN cDNA sequence, which could be used for expression of the mature Hu-IFN-α2a in E. coli (25). In addition, p104 DNA was used to isolate DNA sequences corresponding to other IFN-α subtypes by hybridization.

Examination of the coding regions of the IFN-α genes that have been isolated in our laboratory and others has shown that these correspond to a family of homologous proteins (7, 41) that are closely related to each other (Table 2). Thus, the previously discovered heterogeneity in Hu-IFN-α was the result of distinct genes representing various expressed Hu-IFN-α sequences. The cloned Hu-IFN-α2a, the first one we isolated, corresponds to one of the natural Hu-IFN-α that we purified by HPLC. By procedures similar to those described for plasmid p104, plasmid p101 was shown to contain the sequence for one of the natural Hu-IFN-α2a (also called IFNAR1) and IFN-α2R (IFNAR2), which is the major ligand binding chain (Table 1; supplemental Fig. 3). The various Type I interferons interact with the receptor differently, thereby accounting at least in part for their differential activities. The conservation of the multitude of Type I interferons, but not their sequences, throughout evolution of the mammals is consistent with the unique functional roles for each of the Type I interferons (57). Understanding these events is further complicated by the multiplicity of IFN-α (2, 58), which exhibit different activities although they interact with the same receptor. This is an area that has begun to be explored to understand the subtle details of this family of proteins and their mechanisms of action (59). Although IFN-αR1 and IFN-αR2 are the main receptors for Type I interferons, human complement receptor type 2 (CR2/CD21) serves as a receptor for IFN-α in B-cells (60); elucidating the functions of this interaction will likely be highly informative, particularly in relation to immune modulation by interferons. All of the Type I interferons activate Stat1, Stat2, Tyk2, and Jak1 and induce genes that have the IFN-α-stimulated response element (ISRE) in the promoter (52, 61).

IFN-γ uses receptor chains IFN-γR1, which is the major ligand binding chain, and IFN-γR2 (Table 1; supplemental Fig. 4) and activates Jak1, Jak2, and then Stat1, which in turn induces genes containing the γ-activation sequence in the promoter (62). With the use of fluorescence resonance energy transfer, we demonstrated that the IFN-γR1 and IFN-γR2 receptor chains are preassembled into a four-chain complex and that the intracellular domains move apart on binding the ligand IFN-γ (supplemental Fig. 4), in contrast to common

---

**Table 2**

| Genes     | Proteins |
|-----------|----------|
| IFNA1     | IFN-α, IFN-γ |
| IFNA2     | IFN-α, IFN-γ |
| IFNA3     | IFN-α, IFN-γ |
| IFNA4     | IFN-α, IFN-γ |
| IFNA5     | IFN-α, IFN-γ |
| IFNA6     | IFN-α, IFN-γ |
| IFNA7     | IFN-α, IFN-γ |
| IFNA8     | IFN-α, IFN-γ |
| IFNA9     | IFN-α, IFN-γ |
| IFNA10    | IFN-α, IFN-γ |
| IFNA11    | IFN-α, IFN-γ |
| IFNA12    | IFN-α, IFN-γ |
| IFNA13    | IFN-α, IFN-γ |
| IFNA14    | IFN-α, IFN-γ |
| IFNA15    | IFN-α, IFN-γ |
| IFNA16    | IFN-α, IFN-γ |
| IFNA17    | IFN-α, IFN-γ |
| IFNA18    | IFN-α, IFN-γ |
| IFNA19    | IFN-α, IFN-γ |
| IFNA20    | IFN-α, IFN-γ |
| IFNA21    | IFN-α, IFN-γ |
| IFNA22    | IFN-α, IFN-γ |

---

**Recombinant Human IFN-α genes and Proteins**

A summary of the IFN-α genes and proteins is listed in Table 2. There are 14 human genes that comprise the IFN-α family. Minor variants that exhibit one- or two-amino acid differences account for the multiple alleles (1, 20, 41, 50, 51). Excluding the pseudogene IFNAP22, there are 13 genes. One of them, IFNA10, is also a pseudogene in one allelic form. There are 13 proteins expressed from these genes. The protein produced from gene IFNA13 is identical to that produced from IFNA1. Thus, there are 12 distinct IFN-α (and allelic forms) produced from these 14 genes (Table 2), but only one of them (IFN-α2) was predominantly used as a therapeutic. Hopefully, the other 11 and new IFN-α will enter the therapeutic arsenal.
MINIREVIEW: Interferons: 50 Years after Their Discovery

beliefs indicating that binding of ligands assemble the receptor chains (57). Similar experiments have not yet been completed for the IFN-αR1 and IFN-αR2 receptor chains.

In addition to the JAK-STAT pathway (see supplemental Table 1), the Type I and Type II interferons utilize other pathways, independently of JAKs and/or STATs, to initiate signal transduction by the interferons. For example, IFN-α and IFN-β can modulate proliferative responses in phagocytes from Stat1-deficient mice, and Stat1 is not necessary for some functions of IFN-γ (63).

Activities and Therapeutic Uses of Interferons

IFN-αs and IFN-β exhibit a wide breadth of biological activities: antiviral and antiproliferative stimulation of cytotoxic activity (64) of a variety of cells of the immune system (T-cells, natural killer cells, monocytes, macrophages, dendritic cells); increasing the expression of tumor-associated surface antigens and other surface molecules such as major histocompatibility complex (MHC) class I antigens (65); induction and/or activation of proapoptotic genes and proteins (e.g. TRAIL, caspases, Bak, and Bax); repression of antiapoptotic genes (e.g. Bcl-2, IAP (inhibitor of apoptosis protein)); modulation of differentiation; and antiangiogenic activity. All of these actions make interferon a most promising agent for the treatment of various diseases (supplemental Fig. 5).

The U. S. Food and Drug Administration approved human IFN-α2a and IFN-α2b (allelic versions of IFN-α2) in 1986 for the treatment of hairy cell leukemia. About 10 years later, IFN-β1a and IFN-β1b were approved for the treatment of relapsing-remitting multiple sclerosis. Clinical trials for IFN-α, before and after approval, focused on cancers and viral diseases. To date, IFN-αs are approved for the treatment of hairy cell leukemia, malignant melanoma, follicular lymphoma, condylomata acuminata (genital warts), AIDS-related Kaposi sarcoma, and chronic hepatitis B and C; IFN-βs for multiple sclerosis; and IFN-γ for chronic granulomatous disease and malignant osteoporosis. In addition, off-label use of IFN-α is prevalent in many cancers, especially in bladder and renal cancers, often as an adjuvant in conjunction with other therapeutics.

Side Effects of Interferons

The side effects of most therapeutics limit dosage, and the interferons are no exceptions. A common adverse reaction to the interferons (IFN-α, IFN-β, and IFN-γ) is lymphopenia and occasional mild injection site reactions. The lymphopenia seems to be a direct inhibitory effect on the production, recirculation, and/or proliferation of the lymphocytes and is reversed once interferon treatment ceases. Although the mechanism of lymphopenia has been reported, the mechanisms of most of the other side effects remain obscure (Fig. 1). A summary of the percentage of patients exhibiting other side effects when treated with IFN-α is presented in Fig. 1 and is generally also applicable to IFN-β and IFN-γ. Common flu-like symptoms (headache, malaise, fever, chills, fatigue, myalgia, back and joint pain) are experienced by most patients. Although these symptoms usually disappear within hours after administration of interferon, these side effects significantly limit the dosage of interferon that can be administered systemically. While these symptoms have been recognized virtually since the discovery of interferons, no mechanism has been identified. High doses of interferon have been shown to have substantial efficacy, but unfortunately patients often cannot tolerate the high doses necessary to control the cancers or viral diseases. Thus, despite the enormous potential of the interferons in preventing and/or treating diseases, the debilitating side effects often prevent the administration of high doses necessary to prevent or cure the disease. The development of methods to minimize or eliminate these side effects will have a great effect in enhancing the efficacy of the interferons. Having the appropriate technology to deliver the interferons locally to tumors and virally infected tissues would be a major contribution toward overcoming the problem of systemic side effects. Once this is achieved, it is highly likely that interferons will play additional vital roles in the next generation of novel antitumor and antiviral therapies. Furthermore, although IFN-α and IFN-β are not usually used to prevent diseases, it is likely that these interferons will play major roles in the prevention of some cancers and viral diseases in the not too distant future.

Acknowledgments—For the work done in my laboratory that is discussed in this review, I thank my many colleagues who have carried out the studies over the past several decades. I thank Ellen Feibel for her diligent assistance in the preparation of this manuscript.

REFERENCES
1. Krause, C. D., and Pestka, S. (2005) Pharmacol. Ther. 106, 299–346
2. Pestka, S., Krause, C. D., and Walter, M. R. (2004) Immunol. Rev. 202, 8–32
3. Isaacs, A., and Lindenmann, J. (1957) Proc. R. Soc. Lond. Ser. B 147, 258–267
4. Cantell, K., Hirvonen, S., Kauppinen, H. L., and Myllyla, G. (1981) Methods Enzymol. 78, 29–38
