Origins of..

Cytokine research: the interferon paradigm

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The term cytokine was introduced in the early 1970s by Cohen *et al* to extend the lymphokine concept.¹ Dumonde and coworkers in the late 1960s had introduced the term lymphokine to denote physiologically active substances (factors) produced by lymphocytes in response to antigenic stimulus (excluding antibodies).² Cytokines are cell-active factors produced by a range of cell types in response to a range of stimuli (not only antigens) and indeed may be constitutively produced. Their essential attribute is that they interact with cells and alter their behaviour in some way. Evidently, with such a definition, cytokines include all the polypeptide hormones such as insulin, and growth factors such as epidermal growth factor. However, the classic endocrine hormones are by convention excluded. To exclude mediators such as arachidonic acid derivatives only proteins are defined as cytokines. The term interleukin was introduced in 1979 to denote polypeptide factors produced by leucocytes and active on leucocytes.³ The numerical interleukin nomenclature was considered superior to trivial names dependent on the bioassay used to detect the factor, and a factor is only admitted as an interleukin when a full biochemical characterisation (gene cloning) is available. Many interleukins, which are a subset of the cytokines, are produced by cells other than leucocytes and are certainly active on non-leucocytes, therefore, the term is a misnomer, and many factors that if discovered now would be regarded as interleukins retain their older names, such as the interferons (IFN).

The IFNs were the first cytokines to be discovered and characterised. The origins of IFN research in a very real sense are the origins of cytokine research, and in this 40th anniversary year of the discovery of IFNs it seems appropriate to review how the field of IFN research has developed. Table 1 summarises the key events of the IFN story. The biology of the IFNs is a paradigm for the biology of cytokines in general: likewise, the style and flavour of IFN research—particularly the involvement of major commercial interest, and its highly competitive, sometimes frenetic, nature—is characteristic of cytokine research as a whole.

Discovery of “the interferon” and early research into the nature of the IFNs

The discovery of IFNs grew out of the study of the phenomenon of viral interference—that

replication of one virus may prevent the replication of another in the same cells. This phenomenon had been reported as early as 1804 by Jenner who had observed that vaccination was not successful in individuals suffering “herpetic affections”. However, the key experiment was that of Isaacs and Lindenmann published in 1957⁴: they found that the supernatant of cells treated with heat killed virus was able to prevent infection by live virus of other cells. They showed that the factor was probably protein, probably not viral in origin, not an antibody, and that its effects were not virus specific. Furthermore, the factor acted on target cells (not the virus) and some time was required to render the cells resistant to infection—to induce the “antiviral state”. The factor was named “the interferon”. That IFN had clinical potential that might be immense was recognised almost immediately. Mindful of the penicillin fiasco, the British authorities set up a committee to coordinate and exploit IFN research: the Scientific Committee on Interferon. This committee included representatives from the pharmaceutical industry and so one of the sociological features we see in cytokine research, big industrial money ploughed in with the expectation of huge profits through development of wonder drugs, was present in cytokine research almost from the very beginning.

It was quickly found that IFN could be produced by a range of cell types in response to many viruses and some non-viral inducers, of which the most efficient were double stranded polynucleotides. A little later it was found that IFN activity was present in the supernatants of cultured leucocytes treated with mitogens,⁵ but in relatively small amounts; therefore linking IFN with the immune system. Somewhat later it was shown that IFN was produced by T lymphocytes in a strictly antigen specific fashion,⁶ conforming with Dumonde’s definition of lymphokine.

By the mid-1970s it was clear that there was not one IFN but several. These differed in physicochemical properties, cellular origins, and biological properties, but the operational distinction was in their antigenic properties. Thus were defined IFN α (leucocyte) and IFN β (fibroblast), both regarded as type I IFNs because they were induced by viruses, in distinction to IFN γ (immune) that was produced by T lymphocytes on mitogenic stimulation, type II IFN.⁷

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Table 1 Chronology of interferon research

Date	Event
1957	Discovery of type I IFNs
1964	Discovery of type II IFNs
Mid-1960s	First trials of human IFNs in treatment of viral infection
Early 1970s	First trials of human IFNs in malignant disease
Mid-1970s	Purification of type I IFNs
1979 to early 1980s	Cloning and expression of IFN genes
1982	Demonstration that IFN γ is a T cell lymphokine
1980s	Demonstration of IFNs wide biological effects in immune system
1980s	Extensive clinical trials show that IFNs have little therapeutic efficacy
Early 1990s	Definition of IFN signal transduction mechanisms
Early 1990s	Gene knockout experiments confirm importance of IFNs in resistance to infectious diseases
1997	Worldwide sales of IFNs exceed US\$2 billion dollars annually?

Likewise, it was becoming clear that the IFNs had multiple effects, accepted now as a characteristic property of cytokines. One of the earliest “non-antiviral” effects to be noted was that IFN inhibited cell growth. This observation was germinal for the subsequent enthusiasm for IFN as an anticancer drug. Similarly, effects on histocompatibility antigen expression and natural killer cell activity were found, again linking IFNs with the immune response, in a reciprocal manner. But these non-antiviral effects of IFN (misinterpretation) were usually dismissed as resulting from non-IFN factors in the preparations that were notoriously crude. Hence the demonstration that IFNs did indeed possess multiple activities required purification to homogeneity. As purifications were attempted, it became clear that IFNs were enormously potent—preparations with specific activities in the order of 10^6 antiviral units per milligram of protein were still impure. This potency again is characteristic of many other cytokines. However, by the early 1970s, homogenous preparations of IFNs were available, largely because of the efforts of Gresser and associates, and it was possible to demonstrate that the multiple effects of IFNs indeed resulted from a single molecular species⁸ (or as it subsequently became apparent, to a mixture of very closely related molecules).

A further important consequence of the availability of pure IFNs was the ability to prepare monospecific antibodies, again initially developed by Gresser in association with De Maeyer. These allow not only the unequivocal definition of IFN types but more importantly allow the demonstration by neutralisation *in vivo* that IFNs do play an important role in resistance to disease. Of course the application of monoclonal antibody technology to IFN, and to cytokine research in general, has obviated the need to purify the factor for the preparation of antibodies, and indeed may be applied to the purification and assay of the factor. The preparation and use of monoclonal antibodies to cytokines was pioneered in IFN research—for example, through the work of Morser and colleagues who prepared a monoclonal antibody to IFN α ⁹ much used in purification and immunoassay.

Genes, gene cloning, and heterologous expression

IFN research and genetic engineering were, during the 1970s, powerfully symbiotic. Early

clinical trials had foundered through lack of sufficient quantities of the drug (or so it was thought at the time). The demonstration that human genes may be cloned and expressed in heterologous systems (bacteria) suggested that IFN could be produced by this route, and the availability of generous funding from pharmaceutical companies resulted in a tremendous flurry of activity so that by 1980 both IFN α and β cDNAs had been cloned^{10–12} and IFN γ cDNA a little later.¹³ The expression of these genes in *Escherichia coli* and other heterologous systems rapidly followed, but this was not without its difficulties as yields were initially low. When more powerful expression vectors were used, the product precipitated. This was not a problem with IFN α and β , as these can be easily renatured after solubilisation, but was troublesome with IFN γ . By the mid-1980s unlimited amounts of recombinant IFNs were available for clinical use, as was natural IFN α being produced by large scale lymphoblastoid cell culture by Wellcome. This is a pattern that has been replicated for many of the other cytokines, and the development of the necessary gene cloning and expression technology was a breakthrough.

Subsequent cloning of the IFN genes (rather than to cDNA) clarified the genetic organisation of the IFN system, and it is now clear that there are multiple similar genes for IFN α (divided into two subgroups, α and ω), a single gene for IFN β related to the IFN α gene, and a single gene for IFN γ that is completely unrelated to the other IFNs.¹⁴ This makes sense of the different IFN antigenic types but leads to a very confusing and potentially misleading nomenclature as pointed out by Finter.¹⁵

Biological roles of IFNs

IFN α , ω , and β are biologically very similar but IFN γ has an overlapping but distinct set of properties (table 2). This phenomenon is also characteristic of other cytokines—unrelated molecules may share properties, and there may be multiple closely related molecules that are produced by different cell types under different conditions but with very similar biological activities. All IFNs by definition exert antiviral effects, but the type I IFNs are considerably more potent than IFN γ . IFNs diverge in other important ways, perhaps most significant is that only IFN γ activates expression of class II histocompatibility antigens^{16–18} (all IFNs activate class I expression) together with other components of the antigen presenting machinery of cells,^{19–20} activates macrophages,²¹ and inhibits the proliferation of the Th2 subset of helper T cells²² (and so potentially controls the balance between cellular and humoral immune responses). Such findings imply that the biological roles of the different IFNs are distinct, and that apart from the common antiviral effect they are distinct cytokines with type I IFNs being important in resistance to viral infections but IFN γ with a wider role in immune responses.

Available data on the functioning of IFNs in *in vivo* animal models of infectious disease support this contention. The administration to

Table 2 Major biological effects of interferons

Effect	IFN $\alpha\beta$	IFN γ
In vivo resistance to viruses	+	+/-
In vivo resistance to parasites	-	+
Antiviral effect	+	+
Activation of class I histocompatibility antigens	+	+
Activation of class II histocompatibility antigens, TAP, proteasomes	-	+
Macrophage activation	-	+
Inhibition of type 2 T cells	-	+
Inhibition of IgE synthesis	-	+
Activation of natural killer cells	+	+
Inhibition of cell proliferation	+	+

TAP, transporter important in antigen presentation.

virus infected animals of antibodies to type I IFNs exacerbates disease irrespective of the virus. On the other hand, the limited data available from experiments using anti-IFN γ implies that this is not important in viral diseases. More recently gene knockout mice nullizygous for type I IFN receptor (it is impossible to knock out all the multiple type I genes) have been shown to be generally more sensitive to viral infection.²³ Gene knockout mice lacking the IFN γ system (either receptor or IFN itself) do not have greatly exaggerated sensitivity to virus infection (with the exception of vaccinia)^{23, 24} but they are more sensitive to bacteria (*Listeria monocytogenes*, *Mycobacteria tuberculosis*^{18, 25}) and parasite (*Leishmania major*²⁶) infections.

It seems reasonable to attribute the increased sensitivity to viruses of IFN $\alpha\beta$ knockout mice to the failure of the direct antiviral mechanism in these animals. The sensitivity of IFN γ knockout mice to bacterial and parasitic infections is best interpreted as resulting from the failure of activation by IFN γ of macrophages and granulocytes for the "oxidative burst", the production of oxygen radicals and nitric oxide. Clearly, IFN γ is involved in classic inflammatory mechanisms: inappropriate or uncontrolled production of IFN γ (together with other pro-inflammatory cytokines) is widely accepted as one of the principal mechanisms of tissue injury, and may be of key importance in the development of autoimmune diseases of many sorts.

Interaction of IFNs with cells: receptors and signal transduction

Perhaps the most exciting area of cytokine research today is the elucidation of the intracellular pathways by which they exert their effects. Again, the IFNs provide an excellent model for other cytokines. By definition, cytokines act on cells. As they are proteins, they are unable to diffuse into the cell (unlike—for example, steroid hormones) and so must achieve their effects through interaction with cell surface receptors. By the early 1980s it was shown that IFN α and β shared a common receptor but that IFN γ had a different receptor (beginning to provide an explanation for their differing biological activities). From the early days of IFN research, it was known that the development of the antiviral state in cells was dependent on gene expression as it took time and was prevented by the inhibition of

transcription. Therefore, IFNs, like all cytokines, act by binding to their receptors and transmitting a signal to the cell nucleus that results in the activation of new gene expression: transcription of new mRNA and its translation. There are therefore new mRNAs and proteins present in IFN treated cells (overlapping but not identical sets are induced by type I and type II IFNs). By the late 1970s, in large part resulting from the work of McMahon and Kerr, the induced proteins involved in the antiviral state were identified and their biochemistry understood.²⁷ Many IFN induced genes were identified during the 1980s through molecular genetic techniques. At the same time it was becoming clear that transcription of some genes is directly induced by IFNs without requirement for protein synthesis (called "immediate early" genes by analogy with certain viral genes) while others required protein synthesis (the production of IFN response factors (IRFs)^{28, 29}) to turn on a second wave of gene induction.

Molecular genetic techniques have clarified the nature of the interaction of IFNs with their receptors and how this changes the cell's behaviour (fig 1). The cloning of the receptor for IFN α and β was initially uninformative. It was unrelated to growth factor receptors and G-protein linked receptors and had no obvious enzyme activity (such as tyrosine kinase). When transfected into heterologous cells (IFNs are to a large extent species specific, therefore, mouse IFN will not bind to or act on human cells), although these now bound IFN, no signal for induction of the antiviral effect was transmitted.³⁰ The explanation is simple, the ligand binding component of the receptor had been cloned, but this requires specific interaction with other components for efficient signal transduction. The IFN receptors are in fact prototypes for a new family of multicomponent cytokine receptors, which function in a manner distinct from the other major receptors, growth factor and G-protein linked receptors.

The IFN γ receptor has been studied in considerable detail. It consists of an IFN binding component (IFN γ R α , also known as IFN γ R1),³¹ a 472 amino acid transmembrane protein with roughly equal extra and intracytoplasmic domains (about 220 amino acids each), and a 310 amino acid transmembrane accessory factor³² (IFN γ R β , also known as IFN γ R2 or AF-1) with an intracellular domain of 66 amino acids. The chain is associated, independently of ligand binding, with a tyrosine kinase (phosphorylates tyrosine residues in proteins) of the JAK family (Janus kinase—so called because they have a second kinase-like domain, and are thus two-faced like the Roman god³³) JAK1,³⁴ and the chain with JAK2.^{35, 36}

The α and β subunits are not constitutively associated, but on IFN γ binding they come together. This results in mutual phosphorylation of JAK1 and 2, augmenting their kinase activity, and of IFN γ R α by JAK2 at tyr⁴⁴⁰.³⁷ This generates a binding site for another component of the multimolecular transduction complex, STAT1, a protein of molecular

Figure 1 Interferon signal transduction. IRE, interferon response element; JAK, Janus kinase; Ra, Rb, subunits of interferon receptor; STAT1, signal transducer and activator of transcription 1; P, phosphate.

weight 91 kDa hence often referred to as p91.^{37, 38} This is one of a rapidly expanding group of proteins called signal transducers and activators of transcription (STAT) that appear to be of major importance in cytokine signalling and probably interact with other signalling pathways for other factors—for example, growth factors.³⁹ The binding of STAT1 with the IFN γ Ra results from the interaction between a domain in STAT1 (SH2) that has homology with the src oncogene and the region about tyr⁴⁴⁰ of the R chain (SH2 domains recognise specific domains containing phosphotyrosyl residues). STAT1 is phosphorylated on tyr⁷⁰¹, within the SH2 domain, this results in its homodimerisation and subsequent translocation to the nucleus. Here it binds to specific sequences in promoters of IFN γ responsive genes, the IFN γ receptor element (sometimes referred to as GAS, the γ IFN activated sequence), so turning on transcription.

Other cytokine receptors (including that for the type I IFNs) function in a broadly similar way, but interacting with different JAKs (of which there are four) and different STATs (of which there are seven). For example, the IFN α R complexes with the kinases TYK2 and JAK1 and STAT1 and 2. Therefore, through the formation of a series of activated STAT homodimers and heterodimers, interacting differently with target response elements in gene promoters, we see how there is an element of

specificity combined with redundancy and interaction in cytokine action.⁴⁰

Clinical and commercial exploitation—the stimulus for research

The dream was that IFN would be the antiviral equivalent of penicillin, broad spectrum (because it acts on cells rather than the virus) and safe (because it is a natural product). That the IFN system is crucial for resistance to virus disease is undisputed. At the time of the 20th anniversary of the Isaacs and Lindenmann paper⁴ it was still possible to state “We have increasingly firm grounds for believing [IFN] can be a valuable clinical agent . . .”.⁴¹ But, after a further 20 years it is now the case that, with the exception of the viral hepatitises, IFN has no significant use as an antiviral, and even with hepatitis C (its main use) its effect is not great.⁴² In retrospect, this is not surprising. In acute infection with a virus, symptoms develop when the damage is done, and then treatment is too late. Prophylactic treatment has been tried but IFNs are not without side effects that may be very unpleasant at effective doses (for example, inflammation of the nasal passages resembling the symptoms of a cold with intranasal doses that prevent infection with rhinoviruses). Perhaps the main reason that IFNs are so ineffective is that during a viral infection, endogenous IFN synthesis is well under way, or the virus is little inhibited by IFN (for example, herpes viruses). In which case, more does not mean better. Additionally, injection of IFN either intravenously or intramuscularly does not guarantee that it reaches its target cells.

The finding that IFNs have growth inhibitory properties led to trials of its efficacy in cancer. As with the antiviral effect, the anticancer effect proved largely illusory. Only with a few, usually very rare cancers, has IFN treatment been effective. One example is hairy cell leukaemia, of which there are perhaps one or two cases a year in a large haematology unit.

IFNs have found some clinical efficacy in a one or two other diseases. The activation by IFN γ of antimicrobial mechanisms in macrophages (and other leucocytes) is probably the basis for the efficacy of IFN γ in chronic granulomatous disease, a rare disease with an inherited defect in the oxidative enzymes of myeloid cells.⁴³ Likewise, IFN β has some effect in multiple sclerosis, it reduces the relapse rate but as yet it is not clear whether there is long term benefit.⁴⁴ The high cost of the treatment (about £33 000 per relapse avoided) puts into doubt whether it will be used widely.⁴⁵

Despite this rather negative clinical picture, IFN as a drug is much used, perhaps predominantly against hepatitis C (it is rumoured that one eighth of the Japanese pharmaceutical market is IFN α) with annual world sales in the billion dollar region.

What next?

There have been several occasions when it has appeared that IFN research was over. This has usually been just before some new flurry of important experimental results of general interest to all involved in cytokine research.

There are still many gaps.⁴⁶ One cannot predict the future, but one feels it to be relatively rosy from an IFN researcher's point of view: papers published annually with IFN in the title are increasing: 1773, 1896, 2108, 2203, and 2646 hits from the Science Citation Index for the years 1992–96, respectively. There is continued interest in the biology of IFNs, which has been germinal for cytokine research since the beginning, with by now a longer history than many of those studying it.

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