Protein Potential

Ronald G Jubin of PBL discusses the techniques currently employed to study interferon levels in order to help further comprehend what is a potentially very useful cytokine

Interferon alpha (IFNα) was one of the first FDA-approved biotherapeutic treatments. Since its approval, it has proved to be a powerful cytokine with potent therapeutic activity but, unfortunately, strong side effects. The overall use of IFNα increased dramatically when it was approved as the treatment of choice for hepatitis C. However, nearly half of the individuals infected with genotype 1 of the virus still fail to respond to therapy (1). Consequently, several pharmaceutical companies are now trying to turn on the body's own interferon alpha family of proteins using immunomodulatory molecules in the hope that this will elicit a more complete antiviral response. Both therapeutic approaches are not without risk, due to the side effects associated with IFNα. Additionally, there is a growing amount of published scientific articles suggesting that IFNα may be involved in certain autoimmune diseases, including systemic lupus erythematosus (SLE) (2).

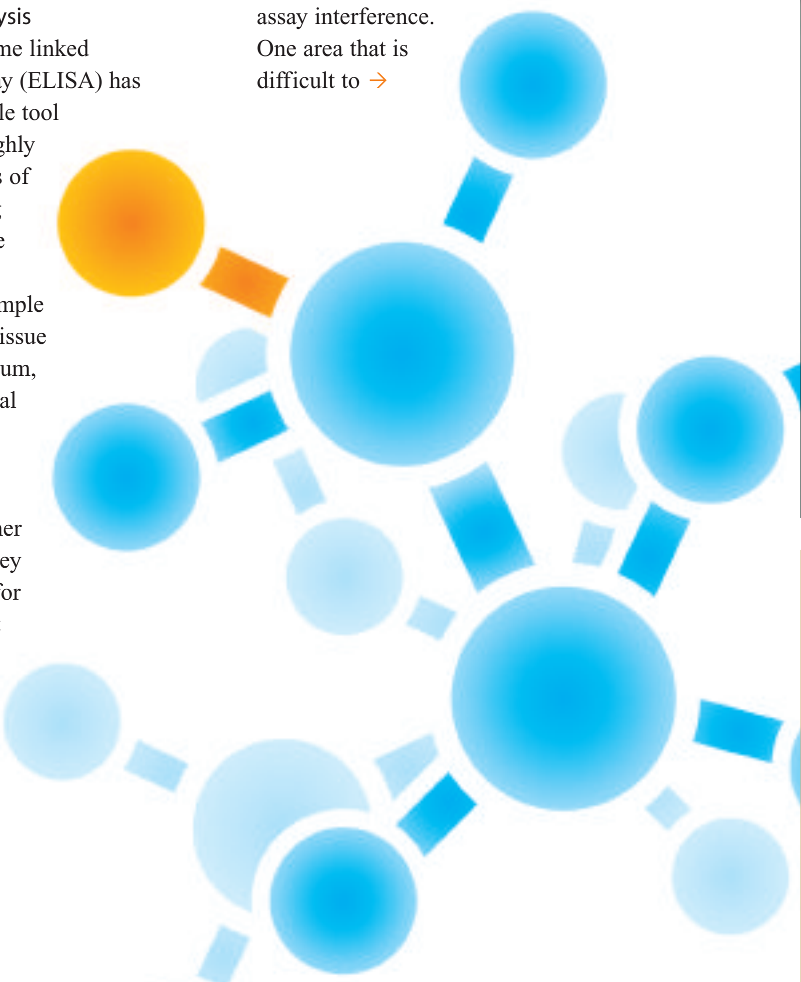
Combined, these observations clearly suggest an increased need to monitor IFNα levels in both normal and diseased individuals along with patients undergoing therapy. How much IFNα produced by the body in response to new immunomodulatory therapies will be functionally equivalent to the current IFNα exogenous treatment regimen? How much 'basal' IFNα is beneficial to prevent or limit viral infection and how much is too much, thereby predisposing an individual to autoimmune disorders? Although IFNα has been studied for over 50 years, we are only now beginning to understand that interferon can have vastly dichotomous activities. This article will highlight the primary methods currently employed to study interferon levels in research and in clinical settings of this very useful, yet only moderately understood cytokine.

MEASURING INTERFERON ALPHA LEVELS

IFNα protein levels can be determined by direct methods, including directly determining mass levels, and indirectly by many unique biological activity assays. There have been many assays developed over the years for the detection of IFNα. While many were important for increasing our understanding of IFNα expression and activity, many were very laborious and low-throughput. The assays highlighted here are those of a medium- to high-throughput in nature that could be adapted to large scale drug screening and/or clinical sample analysis. Both purified IFNα preparations as well as IFNα from stimulated cells can be analysed by the methods described below.

Direct Protein Analysis

The sandwich enzyme linked immunosorbent assay (ELISA) has become an invaluable tool for the rapid and highly quantitative analysis of cytokines, including IFNα. Most of these commercial assays work in complex sample matrices including tissue culture medium, serum, plasma, cerebrospinal fluid and urine. Advantages of this approach are that these assays are rather simple, rapid and they are highly specific for IFNα (meaning that other cytokines including IFNβ, IFNω and TNFα, are not recognised). Limitations are the possibility of false positive results due to the presence of heterophilic binding agents, and false negatives due to the interaction of the IFNα with soluble receptors, antibodies or other factors that would inhibit IFNα capture and detection. However, these can usually be overcome by incorporating various agents into kit buffers to mitigate assay interference. One area that is difficult to →



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[T] +1-732-777-9123 [F] +1-732-777-9141 [E] info@interferonsource.com [W] www.interferonsource.com control is individual IFNα subtype detection capacity. Many IFNα ELISA kits are developed to detect IFNα2. One must make sure when using an IFNα ELISA kit that it has been developed to detect most if not all of the IFNα subtypes. This way, accurate global expression data can pertain to the IFNα family of proteins, and just not IFNα2. Newer versions in addition to the standard colorimetric ELISA are available that can offer increased sensitivity and dynamic range, but usually at a premium price, and they often require costly plate reading equipment. The requirements for these more expensive assay systems need to be carefully weighed against the detection levels that are anticipated and those level ranges needed to make proper assessments.

An alternative to solid-phase ELISA are the solution-based, bead-based technology systems. Instead of the capture antibody being passively adsorbed on a microplate, it is attached to a microbead. The assay sample is mixed with the anti-IFNα beads and the detection antibody in solution. The sample is then passed through a reader that detects and quantifies the level of bound IFN. Similar to the more complex ELISA systems, these reagents are more costly and require dedicated reader systems. A distinct advantage of these systems over standard ELISA is the potential to detect multiple cytokines simultaneously within a single sample. Another method which is limited to cells in culture involves the detection of cells expressing IFNα by flow cytometry methods. This is typically done with peripheral blood mononuclear cells from *in vivo* or *ex vivo* treatments. This is not as sensitive as ELISA and does require a flow cytometer and a skilled operator. However, these assays are useful when performing studies to look for distinct IFNα expressing cell populations in mixtures by multi-sorting peripheral blood mononuclear cells (PBMCs).

To summarise, the single analyte colorimetric anti-IFNα continues to be the simplest and most affordable means by which to monitor IFNα protein levels. The more expensive and laborious procedures are valid alternatives if the proposals warrant the sensitivity or multiplex capabilities of these approaches.

Biological Activity Assays

The measurement of IFNα biological activity by most methods is by nature prone to variation. Early studies conducted during the clinical development of IFNα2 showed that significant variation was common between different laboratories and operators (3,4). Different cell line origins, passage numbers, media constituents and virus preparations were all sources of possible assay variation. Consequently, reference standards were prepared to be analysed in parallel with test samples to calculate activity levels. These reference standards are available from several sources and are the utmost critical reagent for any assay method chosen. Since these standards are in finite levels, they are only used to calibrate internal lab standards that are run in all assays each and every time and, preferably, on each assay plate. In fact, many reagent and pharmaceutical preparations of IFNα are sold based on units per millilitre determined using reference standards.

The cytopathic protective effects (CPE) assay continues to be the most widely-used assay to determine IFNα biological activity. Briefly, IFNα responsive cells are treated with serial dilutions of test samples and known dilutions of lab standards. Following a brief period, cells are challenged with a single concentration of cytopathic virus. A second incubation step is carried out and completed when cells treated with only the virus show complete killing and lysis. IFNα activity values of the experimental samples are determined by comparing dilution endpoints to those of the lab standards. The principle advantages of this approach are low reagent costs and high sensitivity. Disadvantages include cross-reactivity with other type I cytokines (and potentially other cytokines depending on the cell line and virus used), extensive training required to perform assays on a reproducible level, multiple addition steps and overall assay times (usually 24 to 72 hours). Moreover, biological activity can vary greatly in a cell and virus type manner. Therefore, if these experiments are going to be used as screening assays, one must have sufficient stocks of cells and virus to maintain assay continuity over time.

Another clinical application of IFNα proteins is as an anti-cancer agent. Consequently, IFNα is also screened for the ability to inhibit the replication of cancer cells in culture and therefore a desired screening assay can be to measure the antiproliferation capacity of IFNα. These assays are similar to antiviral assays in that they are carried out by treating cells with experimental sample and lab standard dilutions in parallel and activity is determined by comparison of experimental and lab standard endpoints. The advantages of these assays are that they require fewer reagent addition steps than antiviral assays and have good sensitivity. Disadvantages include an extended three- to seven-day overall assay time and, similar to antiviral assays, the potential for cross-reactivity with other cytokines. Nevertheless, these assays are the standard methods for monitoring the antiproliferative effects of interferon alpha.

Another cell-based assay option that indirectly measures IFNα levels involves monitoring specific gene expression induced by IFNα in a dose-dependent manner. Preferably, cell lines containing a stably integrated gene construct that contain a promoter region from an interferon stimulated gene (ISG) controlling translation of a reporter gene are employed. Addition of serial dilutions of experimental and lab standards are conducted in parallel, and unknown values are determined by backfitting values against the lab standard curve (similar to ELISA data analysis). The primary advantages of these types of assays are comparable sensitivity to antiviral assays without the multiple reagent addition steps and extended incubations times. These assays are simple and rapid when compared to other cell-based assays, while still maintaining the sensitivity. Another advantage is that one can employ the use of cryopreserved cells to limit the need for continuous cell culture, which also limits assay variability. Disadvantages are similar to all other bioassays in that interfering substances can artificially dampen or enhance the reporter signal. Nevertheless, these newer assays represent a quicker, simpler and more reproducible means to assess IFNα biological activity. Consequently their use is likely to increase greatly in the future to keep up with the increased demands for IFNα testing. Lastly, flow cytometry can also be employed for indirect IFNα gene upregulation. Class I major histocompatibility complex is up-regulated by IFNα. While more labour intensive and less quantitative, it does allow for differentiating the cell-types that respond to IFNα by sorting populations for other cell markers. Therefore this would be a highly useful research tool to determine if IFNα expression levels could be traced to specific cell types.

In summation, biological assays – although generally more labour intensive than direct protein assays – are critical for the proper evaluation of IFNα biological activity. One cannot assume biological activity based on protein levels. As such, if activity levels are required for your studies, a bioassay must be carried out. Lastly, the appropriate cell line and additional reagents described for the assays aforementioned need to be carefully evaluated for sensitivity, specificity and reproducibility in relation to the origin of your samples and the assay sensitivities required.

Other Options

There are other assays that can be used as well. For example, signal transducer and activator of transcription proteins 1 and 2 (STAT1 and STAT2) are phosphorylated in response to IFNα treatment and form the core of the transcription unit (along with interferon regulatory factor 9; IRF9) that upregulates ISG expression. These assays can be performed either by ELISA with cell lysates or by high content screening assays. The primary advantages of analysing STAT1 or STAT2 is that phosphorylation occurs in minutes and assays can be performed and analysed within a single day. Drawbacks include the need for cell lysis in the ELISA, and the cost of high content screening equipment. However, the rapidity of these assays make them a plausible choice, especially for screening large compound libraries. In addition, qRT-PCR based detection is possible, but not established for detecting all the IFNα subtypes. If developed, this could be a useful tool for the early and rapid IFNα production in stimulated cells.

CONCLUSION

Are there any perfect IFNα assays? Clearly, there is no single, absolute assay for measuring IFNα. This is due in part to their nature in that they are a family of proteins and also that they are usually expressed with several other pro-inflammatory cytokines that may also produce overlapping biological effects. Perhaps the best approach is to screen both protein mass and biological activity in parallel. Following a series of initial spike recovery studies, it should be possible to have a strong correlation between the two assays. This way, each assay will indirectly serve as an assay control for the other. One additional cautionary note for IFNα proteins is how to collect, store and handle samples. In short, the less manipulation the better. Rapid collection, aliquoting (if multiple testing or repeat testing is anticipated), flash freezing and gentle thawing in a cold water bath are recommended to maintain high biological activity. It is also important not to aggressively vortex the samples, as some IFNs have been shown to lose activity when agitated.

Today, the advancement of immune response modulating drugs into clinical development is demanding better assays to monitor cytokines like IFNα. In one instance, toll-like receptor agonists are being developed to specifically enhance IFNα expression, while limiting the expression of TNFα (5). Likewise, newer cancer therapies are being developed for which IFNα is a critical component. Separately, there is a growing body of evidence that IFNα may be a causative agent either predisposing or advancing autoimmunity diseases like SLE, and that monitoring IFNα levels may be important for controlling disease progression. A recent search of the NIH clinical trials site using 'interferon alpha' as the query returned over 560 hits including recently completed, recruiting and ongoing clinical trials (6). Monitoring this cytokine will remain an important goal for many areas of therapies for years to come.

Lastly, one higher order question remains: are there really 'good' and 'bad' IFNα subtype proteins? Would some IFNα subtypes be better therapies for antiviral and cancer treatments, producing more potent desired activities with fewer side effects? Conversely, could bad IFNα subtypes associated with autoimmune diseases be selectively blocked, preventing the disease effects but still allowing ample host response to pathogens? These questions cannot be adequately addressed in large-scale studies to date, because no reagents are commercially available to differentiate all of the individual IFNα proteins. Hopefully, current studies will help to better determine global levels of IFNα in a variety of settings so that this information can be used as a base for deconvolution of the individual IFNα subtypes when these assays are established.

About the author

Ronald G Jubin PhD is the current Research and Development Director at PBL InterferonSource. Previously, he was a Research Scientist with Schering-Plough from 1992 to 2004 in the antiviral therapy department, working on hepatitis C virus and interferon antiviral mechanisms. Whilst there, he published several papers that helped better define the mechanism of HCV translation. Ronald received his BA in Microbiology from Kean University, his MS degree in Microbiology from Seton Hall University and his PhD in Microbiology and Molecular Genetics from the University of Medicine and Dentistry of New Jersey, US. Email: rjubin@pblbio.com

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