Understanding drug resistance to biologic therapy

Development of resistance to biologic therapies with reference to IFN-β

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Abstract

All biotherapeutics have the potential to generate anti-drug antibodies (ADAs) in patients. The main factors leading to an immune response are thought to be product, treatment and patient related. In this review, reasons for the formation of ADAs, and particularly neutralizing antibodies (NAbs), are considered, with a focus on IFN-β as a well-studied example. The time course for the production of NAbs, the measurement of NAbs, the defining of IFN-β responders and non-responders, the implications for disease progression in patients, and future methods for avoiding the production of ADAs and of tolerizing patients are considered.

Key words: biotherapeutic, immunogenicity, interferon-β, neutralizing antibodies, anti-drug antibodies, multiple sclerosis, tolerance.

Introduction

From the earliest use of biological therapies, such as the animal insulins introduced in 1922 [1], the development of resistance through the formation of anti-drug antibodies (ADAs) has been a cause for concern. Such immune responses lead to loss of activity of the drugs and are associated with injection site and allergic/anaphylactoid reactions [2]. There are now many licensed biological therapies and a large number in development. All of these therapies have the potential to induce ADAs. However, the reasons for their production are complex and not fully understood. This situation is made more difficult by the lack of suitable animal models, due to their normal immune response to human proteins. Once a biological therapy is in clinical use, assessment of ADAs is difficult due to the lack of suitable assays, standardization or agreement of a positive threshold. In this review, we discuss the development of antibodies against biological therapies using IFN-β, the main therapy for patients with relapsing-remitting multiple sclerosis (RR-MS), as an example. Many of the factors discussed here are applicable to other biological therapies, for example the anti-TNF-α drugs, widely used for the treatment of RA and Crohn’s disease, natalizumab, an anti-α4-integrin drug used in MS and Crohn’s disease, and alemtuzumab, an anti-CD52 drug used in oncology and MS. This review will include possible reasons for the formation of ADAs, the time course for the production of ADAs and neutralizing antibodies (NAbs), the measurement of ADAs and NAbs, the defining of IFN-β responders and non-responders, implications for patients and future methods to avoid the production of ADAs or to induce tolerance.

Factors important for the production of ADAs

The main factors [3] proposed for the production of ADAs in patients are product, treatment and patient related. These are summarized in Table 1 and are described in more detail elsewhere [3]. Great efforts are made by the pharmaceutical industry to reduce immunogenicity through the use of computer-based modelling to identify...
aggregation-prone regions and T-cell and B-cell epitopes and improved methods of manufacturing and delivery.

IFN-β is an ideal model for assessing the factors affecting the immunogenicity of biotherapies as two forms are produced; many companies are involved in their manufacture, large numbers of patients are treated and there are established assays and definitions of positivity [11, 12]. Patents on the earliest IFN-β products have expired and there is now a proliferation of companies producing biosimilars of varying quality [13]. Some of these new products are likely to be better than the originals due to improved methods of production while some will be more immunogenic due to poor regulation and practice. Formation of an immune response against IFN-β, in fact an alloimmune reaction, involves the breaking of immune tolerance. As IFN-β ADAs do not cause adverse effects and high doses of IFN-β can be used, it is also a good model system for investigating tolerance-induction strategies and, as such, it has wider implications for the development of potential treatments for autoimmune disorders.

### IFN-β biotherapies

IFN-β, the first disease-modifying therapy licensed for RR-MS, is produced in two forms: IFN-β-1a and IFN-β-1b. Their production methods are very different. IFN-β-1a is produced as a recombinant protein in Chinese hamster ovary cells and has an identical amino acid sequence to endogenous human IFN-β and the glycosylation pattern [14] is similar to other mammalian proteins. IFN-β-1b is produced in *Escherichia coli*, it is Unglycosylated, the cysteine at position 17 has been substituted for a serine and the methionine at position 1 has been removed. The differences in the design of the IFN-β-1a and 1b have important consequences for their relative immunogenicities and activities, with IFN-β-1b being more immunogenic and about 10 times less active [15] than 1a preparations. The greater immunogenicity (possibly due to aggregate formation [16]) and lower activity of IFN-β-1b have been shown to be related to the lack of glycosylation and not to the sequence changes. The sequence changes were necessary to increase stability during storage [17]. IFN-β-1a is glycosylated on the asparagine at position 80, and this helps to stabilize the structure and prevent exposure of a relatively hydrophobic region of the molecule. Deglycosylation of IFN-β-1a leads to the production of aggregates (a mixture of disulphide-linked complexes) and loss of activity [15]. Aggregates have been strongly associated with the induction of immune responses, and regions prone to aggregation may also contain T-cell and B-cell immune epitopes [18]. At present, the size of aggregates and quantity required to produce an immune response are a matter of debate [10]. An understanding of some of the factors that lead to increased immunogenicity

### Table 1  Major factors determining the immunogenicity of biological therapies

<table>
<thead>
<tr>
<th>Product related</th>
<th>Treatment related</th>
<th>Patient related</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular design: important factors are the presence of non-human sequences, antigenic regions and parts that promote aggregation</td>
<td>Mode of delivery: generally s.c. delivery is most likely to produce an immune response, whereas i.m. and i.v. are less likely</td>
<td>Type and state of disease including concurrent illnesses</td>
</tr>
<tr>
<td>Presence and type of post-translational modification: depending on the type of modification the molecule will either be more of less immunogenic</td>
<td>Dosage: generally the higher the dose the greater the likelihood of developing an immune response; however, tolerance induction may be more likely</td>
<td>Genetic background: HLA alleles</td>
</tr>
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<td>Manufacturing: the presence of damaged proteins, contaminants and impurities may be antigenic or act as adjuvants</td>
<td>Frequency of dosing: greater frequency and episodic therapy [2] are associated with an increased risk of an immune response</td>
<td></td>
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<tr>
<td>Presence and size of aggregates: aggregates are particularly immunogenic [10]</td>
<td>Treatment length: longer treatment periods increase the risk of an immune response</td>
<td>Additional treatments: pre-medication with hydrocortisone [7] and concomitant use of immunosuppressives, such as MTX [8] and AZA [9] reduce antibody formation</td>
</tr>
<tr>
<td>Formulation: formulation will determine the stability of the protein during storage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Packaging: the type is important to ensure stability, prevent adsorption and to avoid contamination through leaching</td>
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Table adapted from Singh [3].
Use of IFN-β in MS

IFN-β was originally developed as a therapeutic agent because of its anti-viral activity [30]. Although this may partially explain the effectiveness in MS, the exact mechanism of its action is complex and not understood. Recent genome-wide blood RNA expression studies [31-34] have been performed to elucidate the key elements involved and to identify markers of treatment efficacy. In a 2-year longitudinal study [32] in MS patients treated with IFN-β, 269 differentially expressed genes were identified and, of these, 19 genes were consistently modulated over the study period. Modulated genes were mainly involved in immune, inflammatory and defence responses. Important immunomodulatory activities of IFN-β include altering the Th-1/Th-2 balance [35], antagonizing pro-inflammatory cytokines (IFN-γ, IL-12 and TNF-α), down-regulating MHC class II expression affecting antigen presentation [36], anti-proliferative effects on T-cell expansion, differentiation and increased T-cell apoptosis [37, 38]. There is also evidence that Type 1 IFNs inhibit transmigration of immune cells across the blood-brain barrier [39].

In 1993 the first trial of IFN-β in MS used IFN-β-1b; since then, separate and comparative trials have been conducted for each new product. On average all IFN-β products reduce the annualized relapse rate by approximately one-third [40, 41]. MRI indices show a 50-70% reduction in disease activity using conventional markers. Evidence of effect on disability measures has been variable [41], as these studies were not powered to show it. Early use of IFN-β in subjects with a clinically isolated syndrome has shown delayed time to first relapse and conversion to clinically definite MS, and has reduced the progression of disability [42-45]. There is some evidence [46] that individual patients, in the absence of ADAs, may respond better than others to IFN-β. In the future, markers of efficacy may be used to identify suboptimal responders.

The IFN-βs are well tolerated and side effects include flu-like symptoms, injection site reactions, myalgia, abnormal liver function tests, anaemia, leucopenia and thrombocytopenia. Various strategies have been proposed to manage these side effects [47].

NAbs to IFN-β

A significant problem with IFN-β treatment is the production of drug-specific antibodies (ADAs). Antibodies produced in patients to IFN-β are described as binding antibodies (BAbs) and a subset of these are NAbs. IFN-β induces its biological actions by binding to the Type 1 IFN receptor complex on the surface of cells, which leads to the activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and other pathways [48, 49]. BAbs bind to IFN-β, but do not necessarily inhibit (they may have other effects, such as on pharmacokinetics) the binding to the receptor complex, whereas NAbs do prevent binding. BAbs occur earlier and may be detected within the first month of treatment. Their rate of appearance depends on the product used, with the majority of patients developing BAbs within 3 months of commencing IFN-β-1b treatment; in contrast, only 10% of those treated with IFN-β-1a developed BAbs in the same period. By 12 months, 58% of those treated with i.m. IFN-β-1a and 89% of those treated with s.c. IFN-β-1a had developed BAbs [50]. Up to 45% (mean ~25%) of patients develop NAbs to IFN-β products. NAbs may be detected as early as 3 months after commencement of therapy, but usually appear between 6 and 18 months [50].

The frequency of BAbs and NAbs differs between the two IFN-β-1a products; s.c. IFN-β-1a is more immunogenic than i.m. IFN-β-1a and antibodies are more frequently induced by IFN-β-1b than 1a [51-54]. The relationship between the development of NAbs and their
clinical significance has been an area of controversy. A number of studies have shown that NAbs are a significant factor contributing to treatment failure, and the reduction in relapse rate in subjects who remain NAb negative may be as high as 50% [55]. Patients who become antibody positive have higher relapse rates, more lesion activity on MRI and greater rates of disease progression [56, 57]. Supporting evidence for the failure of treatment in the presence of NAbs comes from the identification and measurement of biomarkers of IFN-β-induced activity.

NAbs and biomarkers

As described above, IFN-β affects the expression of many genes and their products. Some have been identified as being relatively specific to Type 1 IFNs. Myxovirus resistance protein (MxA) [58, 59], viperin and IFN-induced protein with tetratricopeptide repeats 1 (IFIT-1) [60] are good examples of this. Many, such as oligoadenylate synthetase [61], neopterin [53, 62, 63], β-2 microglobulin [57, 62, 63] and TNF apoptosis-inducing ligand (TRAIL) [64, 65] can also be induced by other factors. Through the use of biomarkers it has been shown that the presence of NAbs reduces the bioactivity of IFN-β. The significant rise in MxA mRNA and MxA protein that occurs in response to IFN-β administration is lost in the presence of NAbs [52, 66-68]. A study in 718 IFN-β-treated patients, using the biomarkers MxA, viperin and IFIT-1 [60], showed the reduction in bioactivity was NAb titre dependent. Complete suppression of responses was found in those with titres >100 ten-fold reduction units (TRUs). A second study [69] gave a cut-off >600 TRU as the point above which there was no in vivo response. This reduction in activity has been further confirmed using a gene expression study where it was found that 1077 genes were regulated by IFN-β, while none were regulated in NAb-positive patients [34].

Measurement of ADAs in patients receiving IFN-β

Assays for the measurement of BAbs and NAbs are discussed below and have been reviewed in detail elsewhere [70].

Measurement of BAbs

BAbS are relatively easily measured and a large number of methods, including western blotting, RIA, affinity chromatography and ELISA, have been developed. ELISA methods are most commonly used, and in particular capture ELISA, which has been found to correlate better with NAb titres [71]. In this method an anti-IFN-β monoclonal or polyclonal antibody is used to bind IFN-β to a microtitre plate. Serum is incubated with the captured IFN-β and the bound human antibodies detected by adding a labelled anti-human antibody.

Measurement of NAbs

Measurement of NAbs exploits the biological actions of IFN-β in cellular systems. As discussed above, the binding of IFN-β to its receptor in vivo leads to the altered expression of genes that have anti-viral, anti-proliferative and immunological properties. In the presence of NAbs, expression of these genes is lost [34]. A number of in vitro NAbs assays, described below, have been developed that make use of the anti-viral properties of IFN-β or the proteins or mRNAs induced by IFN-β. Also described is the in vivo induction of MxA in patients and measurement of the expressed mRNA.

The cytopathic effect assay

The cytopathic effect (CPE) assay is considered the gold standard method for measuring NAbs [72]. Virus susceptible cells are incubated with IFN-β and patient serum for 12-24 h and virus added. After a further 24 h, viable cells (protected by IFN-β-stimulated anti-viral factors) are quantified. The NAb titre is calculated using the Kawade method [73, 74]. Large efforts have been made by the World Health Organization (WHO) to standardize the assay; however, it has not been totally successful. The assay is prone to variation, very time-consuming and not specific (other factors within the serum may also have anti-viral properties).

Assays based on the quantification of MxA

One of the major proteins induced by IFN-β is MxA. The CPE assay has been modified by measuring the amount of MxA protein or MxA mRNA produced following stimulation with IFN-β, in the presence of patient serum, rather than cell viability following treatment with a virus. Stimulation with IFN-β leads to a dose-dependent increase in MxA protein and MxA mRNA. Quantification of the MxA protein has been performed by ELISA, chemiluminescence and by FACS. Measuring MxA production has the advantage of being faster; however, assay variability remains quite high. Extraction of MxA mRNA followed by reverse transcription and quantification by real-time PCR, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene, has been performed to measure the amount of MxA mRNA produced [75]. The assay is faster than the CPE assay and is much more reliable and reproducible than both the CPE assay and MxA protein assay, but the relatively high cost may limit its adoption as a routine assay in clinical laboratories.

Assays based on the in vivo induction of MxA

Treatment of patients with IFN-β, as described above, leads to the production of the biomarker MxA and, in the presence of NAbs, this response is lost [34, 52, 76]. Maximal MxA mRNA concentrations are achieved about 12 h after the dose of IFN-β is given. To assess IFN-β activity, blood samples are collected 12 h post-IFN-β injection [77] into tubes designed to preserve mRNA. The mRNA is extracted and reverse transcription used to produce cDNA. This is quantified by real-time PCR [75], and MxA expression values normalized by reference to GAPDH. Results are reliable and reproducible; however, costs are relatively high.
An assay utilizing a cell line containing a luciferase reporter gene

A luciferase reporter gene assay is used within our laboratory to measure NAbs to IFN-β. The assay was developed by R.A.F. and G.G. [78, 79] and uses a genetically engineered human fibrosarcoma HT 1080 cell line (clone HL116). The HT1080 cell line was stably transfected with the Firefly luciferase gene under transcriptional control of the IFN-stimulated response element [80]. Binding of IFN-β to its receptor leads to activation of the reporter, via the JAK/STAT intracellular signalling mechanism, and luciferase is expressed. Addition of the luciferase substrate Luciferin to cell lysates produces chemiluminescence. In the assay, patient serum is pre-incubated with IFN-β for 1 h, added to HL116 cells in a 96-well plate and further incubated for 6 h. Luciferin substrate is added and the plate read in a luminescence plate reader. In the presence of NAbs, luciferase production is inhibited, and the titre quantified using the Kawade method [73, 74]. The assay is reliable, reproducible and relatively simple and can be completed in a day.

Defining IFN-β responders and non-responders

Individuals treated with IFN-β have highly variable responses. The most essential aspect is the reduction in relapses and disability progression over time. The difficulty in monitoring response clinically lies in the fact that relapse rate naturally declines over time and disability progression, for the majority, is a process that develops slowly over many years (decades). Patients who continue to experience disease activity are non-responders or sub-optimal responders, but it is difficult to know if an individual is responding to therapy and to what degree. MRI is the most sensitive biomarker of subclinical MS disease activity that may continue in the absence of clinically apparent signs of progression. The numbers of new lesions predict future disability [81]; however, there is a lack of correlation between lesions and relapses at an individual level, suggesting a need for other biomarkers [46].

NAbs are a predictor of increased disease activity on MRI and clinically; however, they have not been universally incorporated into routine clinical practice. Measurement of biomarkers, such as MxA and NAbs [77], provides a discriminatory tool to identify biological responders and non-responders, and complements paraclinical and clinical measures. The algorithm in Fig. 1 is proposed by us for the routine monitoring of IFN-β efficacy in MS patients. Non-responders are initially identified by a low in-vivo MxA mRNA response to a dose of IFN-β and the presence of NAbs is confirmed using the luciferase cell-based reporter gene assay.

Incorporating response markers into clinical practice

There is much controversy with respect to the significance of NAbs in IFN-β-treated MS patients and how to manage them [82, 83]. Currently, separate European and North American guidelines govern the incorporation of NAbs testing into clinical practice. In 2007, the American Academy of Neurology (AAN) reported in their recommendations, with regard to the effect of NAbs on clinical and radiological outcomes [84], that there is probably a reduction in efficacy of treatment because of NAbs and there is likely to be greater antibody production in response to IFN-β-1b than to 1a, but were unable to make definite recommendations for changing therapy.

In contrast, the European Federation of Neurological Societies (EFNS) recommended that patients treated with IFN-β be tested for the presence of NAbs at 12 and 24 months of therapy (Level A recommendation). For those who remain NAb negative at 18–24 months further testing is not routinely required (Level B recommendation). Class I evidence shows the presence of NAbs significantly reduces the effect of IFN-β on relapse rate and active lesions and burden of disease seen with MRI. In patients who are NAb positive, measurements should be repeated at intervals of 3–6 months and therapeutic options should be re-evaluated (Level A recommendation). Therapy with IFN-β should be discontinued in patients with high titres of NAbs at repeated measurements at intervals of 3–6 months (Level A recommendation) [70]. Subsequently, new guidelines from a European consortium stated that in patients with sustained high positive NAbs, even if doing well clinically, a switch to a non-IFN-β treatment should be considered [12].

Future developments

As our understanding of why biotherapeutics generate an immune response in some patients increases, new less immunogenic products and methods for inducing tolerance will be developed. These two areas are discussed below with reference to IFN-β.

Development of new less immunogenic IFN-β products

Reformulation of Rebin IFN-β-1a, as described above, resulted in a product with an improved patient safety profile and low immunogenicity. This process has been performed with other biotherapeutics and is likely to occur more frequently. An alternative for reducing immunogenicity is PEGylation [85–87]. PEGylation involves the covalent bonding of the polymer polyethylene glycol (PEG) to proteins and was first approved by the U.S. Food and Drug Administration (FDA) in 1990 for PEGylated adenosine deaminase (Enzon Pharmaceuticals) for the treatment of severe combined immunodeficiency disease. Currently more than nine PEGylated biotherapies have been approved, and many more are in clinical trials, including enzymes, cytokines, antibodies and growth factors. PEGylation provides the molecule with a hydrophilic coating and increases size. The increased size helps to prolong circulatory lifetimes by reducing clearance through the kidneys. In addition, the greater steric hindrance helps to protect against enzymatic degradation, inhibits recognition by the immune system and reduces
Two PEGylated IFN-β products in clinical trials are Biogen Idec’s PEGylated IFN-β-1a (BIIB017) and Allozyne’s PEGylated IFN-β-1b (AZ01).

Tolerance induction to biotherapies

The concept of tolerance induction stems from the work needed to support solid organ transplantation. Tolerance to self-antigens shows that the immune system recognizes both self and non-self and/or the environment in which these antigens are presented.

As IFN-β is a naturally occurring molecule, the development of NAbs in MS patients involves breaking the pre-existing tolerance. A similar situation occurs in patients who inject insulin, human factor VIII (hFVIII; for haemophilia) or acid α-glucosidase (GAA; for Pompe disease).

Many strategies have been tried in animal models and human trials to decrease and stop the production of the antibodies and to re-instate tolerance. Most of these strategies are based on inducing tolerance by the overexpression of the protein concerned. In animal models of Pompe disease, gene therapy with a low-dose adeno-associated virus (AAV) vector, expressing human GAA lowered antibody levels [88] to GAA. Similarly, lentiviral vector-mediated (expressing human GAA) haematopoietic stem cell gene therapy induced tolerance to GAA [89]. Administration of GAA, with short-term low-dose MTX, avoided NAb development [90]. In animal models of haemophilia, AAV expressing hFVIII was used to produce high levels of hFVIII in neonatal mice and prevented NAb development [91]. A single dose of IgG1 anti-CD20 pretreatment stopped the increase in hFVIII NAb formation in the majority of mice subsequently treated for 3 months with daily high-dose hFVIII [92]. In haemophilic dogs, sustained endogenous expression of hFVIII with AAV vectors encoding canine FVIII was shown to eradicate NAbs [93]. In patients with haemophilia and NAb against hFVIII, administration of high-dose hFVIII led to immune tolerance induction (ITI) more rapidly than low-dose protocols [94, 95].
ITI protocols that include additional immunomodulatory therapy, such as rituximab or i.v. immunoglobulins or von Willebrand factor, have an improved effect [96]. Relevant to this observation, the combination of a transient deletion of T cells followed by i.v. myelin antigen administration inhibited relapses in EAE, but only prevented EAE progression when used at an early stage [97]. High-dose ITI protocols have been used in a small number of people with MS who developed NAbS to IFN-β or natalizumab [98, 99]. Recently an elegant strategy has been developed to reduce NAbS to alemtuzumab and involves the administration of a non-functional antibody that differs by a single point mutation [100].

These examples illustrate a number of effective strategies that could be applied in clinical practice to induce tolerance to biotherapeutics and to reduce NAb production.

Conclusion

Biotherapeutics offer great potential for the management of many currently untreatable conditions. One of the major risks to the patient and challenges to industry, the prescribing clinician and the regulators is the development of an ADA response to a biotherapeutic. There is a greater understanding of the important factors for inducing ADA responses. This is reflected in the design and production of new drugs, which are likely to be much less immunogenic. However, the routine monitoring of patients receiving biotherapeutics is less advanced, mainly because of the lack of suitable assays and guidelines for clinicians. The existing guidelines and assays for IFN-β and FVIII are well developed and could therefore be used as models for other classes of biotherapeutics. There is greater scope for the use of concomitant selective immunosuppression with biotherapeutics, which is currently used to reduce the development of NAbS against TNF-α inhibitors. HLA and single-nucleotide polymorphism genotyping also offers potential for the identification of patients particularly at risk of developing NAbS. The long-term outlook for biotherapeutics is very exciting, with many new agents of low immunogenicity in clinical trials, and with the potential that good methods will be developed for inducing tolerance if patients do develop NAbS.

**Rheumatology key messages**

- All biotherapeutics are potentially immunogenic.
- NAbS produced in patients to biotherapeutics lead to a loss of drug efficacy.
- Guidelines and assays are required for monitoring the formation of NAbS and patient treatment.

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**References**


42 Jacobs LD, Beck RW, Simon JH et al. Intramuscular interferon beta-1a therapy initiated during a first...


73 Grossberg SE, Kawade Y, Kohase M et al. The neutralization of interferons by antibody. I. Quantitative and


