

Expert Opinion

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Clinical immunotoxicity of therapeutic proteins

Jacques Descotes[†] & Aurore Gouraud

[†]Centre Antipoison – Centre de Pharmacovigilance, 162 Avenue Lacassagne, 69424 Lyon cedex 03, France

Therapeutic proteins are proteins engineered in the laboratory for pharmaceutical use. Although they tend to offer a better safety margin than most synthetic small molecules because they are processed by the same pathways as natural proteins in the human body, or mimic or replace natural proteins that are missing or defective, they can induce a variety of adverse effects in human patients among which immunotoxic effects deserve particular attention. Infectious complications and virus-induced neoplasias are the main consequences of intended or unexpected immunosuppression. They have been primarily described with anti-TNF- α drugs and anticancer monoclonal antibodies (mAbs) whose immunosuppressive effects can be largely anticipated from their mechanism of action. Cytokine release-associated adverse reactions ranging from mild-to-moderate flu-like reactions to severe cytokine release syndromes, or exceptionally cytokine storm, are now an issue of chief concern. They have been observed with most of the therapeutic proteins in current use. Acute infusion reactions whose clinical features are somewhat similar have been variably described with many mAbs. Immunostimulatory properties are suspected to account for the occurrence of more frequent autoimmune diseases as seen in patients treated with recombinant cytokines such as the IFNs- α and rIL-2. The risk of more frequent hypersensitivity reactions to unrelated allergens is also suspected to be a potential consequence of immunostimulation but compelling evidence is limited to radiocontrast media-induced hypersensitivity reactions in IL-2 treated cancer patients. In addition, recombinant cytokines can inhibit cytochrome P450 dependent biotransformation pathways with resulting alterations in the pharmacokinetics of the combined drugs at therapeutic dose. Because of their structure and origin, therapeutic proteins are intrinsically immunogenic. Despite extensive laboratory and clinical studies that were instrumental in delineating general concepts about key factors involved in immunogenicity, it is impossible nowadays to anticipate to what extent a novel therapeutic protein will be immunogenic in human patients. Specific antibodies are frequently detected in the sera of treated patients. They are often inconsequential but can also be neutralizing and result in decreased efficacy. Anaphylactic reactions induced by human therapeutic proteins have been rarely reported and true serum sickness extremely rarely. Because the immunotoxic effects of therapeutic proteins are frequent, sometimes severe and even potentially life-threatening, there is an urgent need to improve our capacity to anticipate the immunotoxic risks of novel therapeutic proteins and this could be achieved by the development of clinical immunotoxicology.

Keywords: anti-TNF- α , drugs, autoimmunity, clinical immunotoxicology, cytokines, hypersensitivity, immunogenicity, immunostimulation, immunosuppression, monoclonal antibodies, therapeutic proteins

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1. Introduction

Therapeutic proteins can be defined as proteins engineered in the laboratory for pharmaceutical use. So far, (> 150, therapeutic proteins have been approved by regulatory agencies throughout the world, and many more are under preclinical or clinical development. Their intended indications are extremely varied including the replacement of a natural protein that is deficient or abnormal as in a variety of endocrine and metabolic disorders (e.g., recombinant insulin or growth hormone); the augmentation of an existing biological process, such as hematopoiesis (e.g., recombinant erythropoietins and hematopoietic growth factors); the interference with immunoregulation (e.g., recombinant cytokines) or selected cellular functions, most notably by means of binding to a well-defined target (e.g., monoclonal antibodies); the implementation of a novel function or activity (e.g., recombinant lepirudin or hyaluronidase); and the delivery of other therapeutic or diagnostic molecules (e.g., ¹³¹I-tositumomab or arcitumomab) [1]. Therapeutic proteins can also be used as prophylactic or 'therapeutic' vaccines.

One attractive advantage of therapeutic proteins over most conventional small synthetic molecules is that they can be expected to exert far more specific effects on a selected target of interest. In addition, they tend to offer a better safety margin either because they are processed by the same pathways as natural proteins in the human body or because they mimic or replace natural proteins that are either missing or defective. Nevertheless, therapeutic proteins have been shown to induce adverse effects in human patients. Among these, immunotoxic effects deserve particular attention. Indeed, because of their origin (foreign, non-self molecules), size (large molecules), mechanism(s) of action – the immune system is the intended (or unintended) target of many therapeutic proteins – and the characteristics of the treated patients (underlying immunopathological conditions), several adverse effects in relation to immunosuppression, immunostimulation, hypersensitivity and autoimmunity have been described, or can be expected to occur.

Relatively limited information on the immunotoxic potential of therapeutic proteins is gained from preclinical (nonclinical) safety studies using procedures available at present. Indeed, it is widely recognized that the nonclinical safety evaluation of therapeutic proteins is fraught with many difficulties and challenges [2-5]. To achieve minimal immunogenicity and thus avoid related adverse effects in treated patients, increasingly humanized or even 'human-like' proteins are being developed. Quite expectedly, these proteins in turn prove to be markedly immunogenic in laboratory animals. Although immune-mediated adverse effects such as anaphylaxis or immune-complex-mediated hypersensitivity that are sometimes observed in nonclinical studies should not be considered of any relevance to man, therapeutic proteins when markedly immunogenic in laboratory animals, including nonhuman primates, can lead

to the production of high levels of neutralizing antibodies, which may result in the continuing studies being aborted and thus reduce their predictability, especially as regards possible long-term adverse effects. Another related issue is animal species relevance. Therapeutic proteins are increasingly directed towards exquisite targets that tend to exist only in man and to some extent in nonhuman primates but not in other conventional mammal species. Therefore, the safety evaluation of many therapeutic proteins has to be performed in only one animal species, that is, the Cynomolgus or Rhesus monkey whose relevance is actually far less optimal than too often claimed. In contrast to small synthetic molecules, the 'traditional' use of both a rodent and non-rodent species for the nonclinical safety evaluation of therapeutic proteins is often impossible unless a surrogate is produced for toxicity assessment, which is a lengthy and costly alternative so far seldom used [6]. Thus, the immunotoxicity directly related to the immunopharmacological properties of quite a few therapeutic proteins is often beyond the reach of current nonclinical studies. Finally, few if any relevant and validated predictive animal models or assays are available to predict other critical immunotoxic adverse effects such as hypersensitivity and autoimmunity, a situation that applies to therapeutic proteins as well as to small molecules [7].

Despite the rather limited preclinical information thus provided today, the immunotoxic potential of therapeutic proteins is being more clearly defined largely because of the clinical experience gained with their expanding use in more and more patients. This review is intended to describe the current knowledge on the clinical immunotoxicity of therapeutic proteins from the perspective of the four fundamental categories of immunotoxic effects, namely, immunosuppression, immunostimulation, hypersensitivity and autoimmunity [8,9].

2. Clinical immunotoxicity of therapeutic proteins in relation to immunosuppression

Historically, immunotoxicologists focused most of their attention on immunosuppression [10]. This resulted in the design and validation of quite a few animal models and assays enabling the reasonable prediction of the unexpected or unintended immunosuppressive effects of candidate drugs, which is reflected in the harmonized ICH S8 guideline released in September 2005 [11]. It is, however, noteworthy that the importance given to immunosuppression was initially fueled by the description of infectious complications shortly after the introduction of potent immunosuppressive drugs in the clinic [12].

2.1 Clinical immunotoxic effects as related to immunosuppression

Immunosuppression results in more frequent, often more severe and relapsing infections as well as atypical or

opportunistic infections that are caused by uncommon pathogens and/or develop in unusual sites compared to fully immunocompetent human beings [13]. Because of these characteristics, opportunistic infections should be considered as significant warnings ('flags') to suspect the immunotoxic (immunosuppression-related) potential of a drug treatment when other overt drug-unrelated causes are ruled out. It is, however, important to keep in mind that unremarkable but more frequent infections can also develop and will remain overlooked if no carefully dedicated clinical studies or follow-up are undertaken. In addition, immunosuppression can be associated with the development of more frequent virus-associated neoplasias including skin and lips cancers, lymphoproliferative disorders in particular B-lymphomas and Kaposi's sarcomas.

Adverse events related to immunosuppression have been reported to be associated with several therapeutic proteins. Also, in most instances, such adverse effects were described with those therapeutic proteins whose negative influence on the immune responsiveness of treated patients could be anticipated either from their mechanism of action or from the results of preclinical safety studies.

2.2 Infectious complications of anti-TNF- α drugs

The anti-TNF- α drugs represent the most illustrative example of therapeutic proteins associated with a variety of adverse clinical events related to immunosuppression among which infections are the most frequent. TNF- α is primarily produced by macrophages and T cells. Initially membrane-bound, it undergoes cleavage by a specific converting enzyme generating a soluble form of TNF- α . Both the transmembrane and the soluble forms function through binding to one of two receptors expressed on the surface of most mammalian cell types, which accounts for a broad spectrum of biological effects. As TNF- α levels are elevated systemically or at inflammation sites in chronic inflammatory diseases, such as rheumatoid arthritis or Crohn's disease, anti-TNF- α drugs were developed in an attempt to reduce tissue damage. The first anti-TNF- α drug to be approved was infliximab, a chimeric IgG₁ mAb, soon to be followed by etanercept, a recombinant, dimeric, soluble TNF- α receptor protein, then adalimumab, a human IgG₁ mAb and, more recently, certolizumab pegol, a PEGylated anti-TNF- α Fab' antibody fragment.

In keeping with the crucial physiological role of TNF- α in the body's defense against bacterial and viral infections, anti-TNF- α drug therapy has been shown to result in potentially severe infections [14-17]. The first main report of infections associated with anti-TNF- α drugs consisted of the review of 70 cases of tuberculosis in infliximab-treated patients recorded by the US FDA Adverse Event Reporting System [18]. Since then, reports of tuberculosis associated with every anti-TNF- α drug have been published [19], although the risk is considered to be lower in patients treated with etanercept than either infliximab or adalimumab [17].

Wide variations in the rates of tuberculosis among different populations have been observed, and this might be owing to the effect of ethnicity, country of origin or incidence of latent tuberculosis. Indeed, reactivation of latent tuberculosis is considered to be a key event. The recent application of recommendations from varying institutions to screen patients for latent tuberculosis before initiation of treatment including a tuberculin skin test, chest radiograph and/or whole blood interferon gamma assay led to a decreased incidence of reactivation [19]. Interestingly, neutralization of TNF- α by a specific anti-TNF- α mAb in mice with latent tuberculosis was shown to result in reactivation and outbreak of the disease, even before infliximab was approved [20]. This early finding was subsequently confirmed [21,22], which suggests that a more thorough assessment of the biological function(s) of the target(s) is likely to prove instrumental to achieve a better safety evaluation of novel therapeutic proteins before clinical trials.

Even though tuberculosis is a chief safety concern, anti-TNF- α drugs have been also reported to be associated with a variety of other infectious complications including bacterial and fungal opportunistic infections such as histoplasmosis, listeriosis, aspergillosis, candidiasis, *Pneumocystis carinii* pneumonia and coccidioidomycosis [23-25]. The risk of lower respiratory tract, skin, urinary tract and bone and joint infections in patients treated with anti-TNF- α drugs is suspected to be increased but this is not firmly substantiated [17].

2.3 Infectious complications of other therapeutic proteins

Infectious complications have been reported in human patients treated with other therapeutic proteins. Several mAbs are being used or evaluated as new approaches for cancer therapy. Overall, the incidence of associated infectious complications is in agreement with their mechanism of action [26]. Rituximab, a chimeric anti-CD20 mAb, when used as a monotherapy in B lymphomas or autoimmune diseases, was found to be associated with bacterial or viral infections in $\leq 30\%$ of treated patients but severe or opportunistic infections were rather infrequent, which is in agreement with conserved T cell functions in rituximab-treated patients [27]. Infectious complications were noted in 30 – 97% of patients with chronic lymphocytic leukemia as a consequence of treatment with alemtuzumab, a humanized IgG1 mAb that targets the CD52 antigen expressed by most human mononuclear cell subsets, thus resulting in profound cellular immune dysfunction [28]. Severe and sometimes lethal infections including disseminated viral infections, systemic *Candida* infections, tuberculosis reactivation and invasive fungal infections were frequently observed. In sharp contrast but not unexpectedly, there was only a small trend towards increased infections in breast cancer patients treated with trastuzumab, a humanized mAb targeting the human epidermal growth factor receptor-2 [29]. Similarly, only rare

and unremarkable infectious complications were observed in patients treated with either palivizumab, a humanized mAb directed against the human respiratory syncytial virus (HRSV) fusion glycoprotein, or omalizumab, a humanized anti-IgE mAb.

Beside mAbs, infectious complications were initially suspected to develop in patients treated with either recombinant IL-2 or the interferons- α but these findings were not confirmed and this is also in general agreement with the known mechanism of action of either compound [29]. Similarly no more frequent or abnormal infections have seemingly been reported in relation to the therapeutic use of recombinant hematopoietic growth factors such as the granulocyte-macrophage colony-stimulating factor (GM-CSF) or granulocyte colony-stimulating factor (G-CSF).

However, the parallel between the risk of infectious complications and the known mechanism of action of therapeutic proteins may not be that straightforward as illustrated by the occurrence of progressive multifocal leukoencephalopathy (PML) in several patients treated with natalizumab [30]. Natalizumab is a humanized mAb that targets $\alpha 4$ integrin and thus prevents the entry of inflammatory cells into tissues, which is the basis for its therapeutic application in autoimmune diseases including multiple sclerosis and Crohn's disease. On the 28 February 2005, natalizumab was withdrawn from the market following the observation of three patients treated with PML. Progressive multifocal leukoencephalopathy is a rare disease typically associated with immunosuppression in particular when T cell functions are impaired. The JC virus, a ubiquitous virus affecting $\leq 80 - 95\%$ of some populations, is considered to be the causative agent. Although recent findings suggest that natalizumab can alter the immune responsiveness in the CNS of treated patients [31], it remains unexplained why it seems to uniquely predispose to the development of PML. In any case, neither the mechanism of action nor the results of preclinical safety studies were helpful to anticipate this adverse effect. Even though it could also have been problematic to predict this risk with a small molecule than it proved to be with natalizumab, it is unsure that any small molecule could ever bind so exquisitely to the specific target of natalizumab, and this highlights current difficulties in the safety evaluation of novel therapeutic proteins. The return of natalizumab on the market was approved under very strict conditions.

2.4 Virus-induced neoplasia and therapeutic proteins

Virus-induced neoplasias are well-recognized complications of immunosuppressive agents [32]. Overall, virus-induced neoplasias have been much less frequently observed than infectious complications in patients treated with therapeutic proteins. Several factors may explain this finding. Virus-induced neoplasias typically develop in immunocompromised patients and most therapeutic proteins exert only mild-to-moderate immunosuppressive effects, if any. So far, most of the

therapeutic proteins with the notable exception of the anti-TNF- α drugs and the interferons α and β or those used for replacement therapy are administered on a relatively short period of time and daily administrations are rarely recommended.

Again, anti-TNF- α drugs have been the focus of most clinical studies because of their immunosuppressive effects, their long-term therapeutic indications and the nature of the treated diseases. Rare case reports of lymphoma or skin cancer in patients treated with anti-TNF- α drugs have been published, and the results of most meta-analyses evidenced a lack of convincing association [33-35], even though conflicting results have been reported [36]. Differences in the methodology and studied populations as well as the increased background incidence of lymphomas in rheumatoid patients are obvious confounding factors. Anyway, so far, the risk of virus-associated neoplasia in patients treated with anti-TNF- α drugs does not seem to be an issue of concern. Although solid tumors are generally not considered to be a direct consequence of even long-term immunosuppression owing to drug treatments [32], the possible role of anti-TNF- α drugs in human carcinogenesis is a matter of recent and unsettled debate [36].

3. Clinical immunotoxicity of therapeutic proteins in relation to immunostimulation

For the sake of simplicity, the term 'immunostimulation' will be used later to address all the clinical immunotoxic effects resulting either from an enhanced immune responsiveness (as opposed to immunosuppression) or from a direct (non-antigen-specific) activation of some components of the immune system (immuno-activation). It is outside the scope of this review to feed the long-lasting debate whether it is deemed impossible or not to enhance the immune responsiveness of a previously immunocompetent living being by pharmacological agents, or whether immuno-activation is not more than an inflammatory response.

In contrast to immunosuppression, the adverse effects related to immunostimulation have long been ignored, if not overlooked [37], in part because of the lack of potent immunostimulatory agents for many years. The introduction of recombinant cytokines in the clinic totally changed the scene, so that nowadays the clinical immunotoxic effects of immunostimulation are rather well established [13]. They include cytokine release-associated adverse reactions, more frequent autoimmune diseases and hypersensitivity reactions towards unrelated allergens and the inhibition of some drug metabolism pathways.

3.1 Cytokine release-associated adverse reactions

Although these reactions have been and still are a matter of acute concern for industry and regulatory bodies following the report of a 'cytokine storm' that developed on 13 March 2006 in six human healthy volunteers

administered TGN1412, an anti-CD28 superagonistic mAb during a Phase I clinical trial [38], most if not all of our current understanding of these reactions has been available for some time [39]. Indeed, flu-like reactions have long been known to occur following the administration of immunostimulatory agents, including microbial extracts, synthetic immunomodulators and vaccines, and were then suggested to involve direct macrophage activation.

Flu-like reactions typically consist of mild-to-moderate fever (38 – 39.5°C) associated with chills, fatigue, myalgias, headache or nausea of variable severity. In some instances, however, the severity of the reaction is more pronounced. Hyperpyrexia may rise well above 40°C and be associated with various cardiovascular and neurological disturbances including blood pressure drop or even cardiovascular collapse potentially leading to cardiac ischemia, tremor, rigor, confusion, obtundation or seizures. The term 'cytokine release syndrome' is usually preferred to describe such severe reactions. Finally, the term cytokine storm refers to a multi-organ dysfunction syndrome resulting from an inappropriate generalized inflammatory response to a variety of acute insults [40]. The clinical features are similar but usually more severe than in cytokine release syndromes, and they also include lung and renal failure. The lung is indeed a key target, so that cytokine storm is often associated with SARS (severe acute respiratory syndrome).

In general, clinical signs and symptoms develop within 2 – 4 h after the administration, including a first administration [39]. In flu-like reactions, they normally abate spontaneously. Symptomatic or supportive measures may be required depending on the severity of the reaction as no specific treatment is available. The administration of a minor antipyretic/anti-inflammatory drug such as paracetamol (acetaminophen) or ibuprofen is usually effective to relieve the patient's complaints related to flu-like reactions. It can also serve as an effective preventive measure. The pretreatment administration of a corticosteroid such as prednisone has not been found to prevent or alleviate severe cytokine release-associated symptoms noticeably and does not seem to be more effective than either paracetamol or ibuprofen. Another preventive measure is dose reduction, which may, however, result in decreased therapeutic efficacy. In several patients, treatment discontinuation cannot be avoided. It is noteworthy that the severity of reactions may decrease with repeated administrations in some patients.

Also, whatever the severity of the reaction or the term used to describe it, the release of pro-inflammatory cytokines including primarily IL-1 β , TNF- α , IFN- β , IFN- γ , IL-6 and IL-8 is considered to be the main underlying mechanism [39]. Cytokine release is the consequence of a direct activation of various immunocompetent cells including macrophages, monocytes, lymphocytes and NK cells. Depending on the intrinsic properties of the compound, activation may affect one or several cell populations with variable intensity [41]. In addition to their peripheral

pleiotropic effects affecting many organs and functions, the released cytokines can pass or bypass the blood-brain barrier to enter the circumventricular organs in the brain where they induce the production of prostaglandins such as PGE₂ by neurons, microglia and astrocytes. The signal for the production of fever is then relayed to cells in the hypothalamus and brain stem that coordinate the febrile response [39]. Because of the short latency in fever induction, the role of the anaphylatoxins C4a, C3a and C5a generated during activation of the complement cascade has been suggested to be involved as well. Activation of the complement cascade has been evidenced in acute infusion reactions caused by various mAbs such as rituximab [42].

Since 1989 when the first case reports involving muromonab (OKT3), a murine anti-CD3 Mab, were published [43], cytokine release-associated adverse reactions have been reported following the administration of many therapeutic proteins. Nearly all patients develop a reaction within 2 – 4 h following recombinant IFN- α administration [44]. The severity is clearly dose-dependent with severe symptoms in ≤ 40 – 60% of patients when a high dose is used [39]. No differences were seemingly noted depending on the type of IFN- α . A flu-like reaction is also very frequently observed in patients treated with recombinant IFN- β but the incidence or severity does not seem to be dose-dependent. Fever and flu-like symptoms were universal in the few patients treated with recombinant IL-1 α and IL-1 β . They are extremely frequent in rhIL-2 treated patients whatever the dose, route or schedule of administration but are usually mild, except when high doses are used. Similar reactions have also been noted with rhIL-3 and rhIL-4 during clinical trials. They were more severe and clearly dose-limiting in patients treated with either recombinant IL-6 or TNF- α . Flu-like symptoms have also been observed following the administration of hematopoietic growth factors, such as GM-CSF or G-CSF [39].

The administration of mAbs can result in acute adverse reactions that are alternatively depicted as acute infusion reactions or cytokine release syndromes. In ~ 50% of patients initially treated with OKT3, the first doses caused hyperpyrexia, chills, tremor, nausea, vomiting and diarrhea, joint pains and hypotension [43]. The symptoms were associated with a sharp increase in TNF- α and IFN- γ concentrations suggesting the involvement of cytokine release. Reduction in the dose and speed of administration, pretreatment with acetaminophen or indometacin decreased the incidence and severity of these complications [44]. Acute infusion reactions have also been reported with infliximab, rituximab, cetuximab, alemtuzumab, trastuzumab, panitumumab and quite a few other mAbs [45]. Rituximab and trastuzumab produce the highest incidence of infusion reactions. Severe but usually reversible reactions were reported in ~ 10% of patients. Of the fatal reactions, ~ 80% were associated with the first infusion of rituximab. In contrast, the first infusion of trastuzumab produced mostly

mild-to-moderate reactions in ~ 40% of patients, and only infrequently during subsequent infusions. Reactions to the first infusion of cetuximab were also usually mild-to-moderate and affected 12 – 19% of patients. Severe reactions were seen in 3% of patients and were fatal in 1 of 1,000. Finally, infusion reactions occurred in 4% of patients given a first dose of panitumumab and were severe in 1%.

So far, the mechanism of acute infusion reactions following the injection of mAbs is not fully explained. Although cytokine release is considered to play a pivotal role, the somewhat different clinical features, for example, the lack of marked fever in most infusion reactions as compared to flu-like reactions, suggest the involvement of extra mechanisms, such as complement activation [42], as already mentioned. Although an antigen-specific (IgE-mediated) hypersensitivity reaction can be easily ruled out after the injection of a first dose owing to the lack of the required previous sensitization, a convincing medical diagnosis may be more difficult to attain after repeated administrations. Readministration at a slower infusion rate can be considered provided close monitoring of the patient and sufficient evidence supporting a direct (non-antigen-specific) cytokine release are available [45].

3.2 More frequent autoimmune diseases

Although treatments with immunostimulatory agents have long ago been suggested to be associated with more frequent autoimmune diseases, the evidence was considered to be too elusive to allow any firm conclusion [37]. The introduction of therapeutic proteins, in particularly recombinant cytokines, expanded and confirmed this hypothesis. That therapeutic cytokines can be associated with a variety of more frequent autoimmune diseases, particularly thyroiditis, is indeed largely demonstrated by the available clinical data. Although, or because the underlying mechanisms are not known, a careful monitoring of patients, especially those with a history of autoimmune disease or preexisting serological abnormalities, is recommended [46].

Early claims that IFN- α treatment of patients with various myeloproliferative disorders had a higher incidence of lupus-like symptoms were subsequently not confirmed but further studies demonstrated that systemic lupus erythematosus (SLE) is indeed a complication of IFN- α therapy in chronic hepatitis C, even though it remains fairly uncommon [29]. Although diabetes mellitus was found in only 10 of 11,241 patients treated for chronic hepatitis C, several isolated reports of acute onset or worsening of diabetes mellitus shortly after IFN- α treatment initiation with subsequent improvement or complete recovery after IFN- α withdrawal suggest diabetes mellitus was probably more than coincidental. Other autoimmune diseases reported to be associated with IFN- α treatment include autoimmune thrombocytopenias and hemolytic anemias, myasthenia and thyroiditis. Thyroid disorders are indeed well-recognized complications of IFN- α therapy ranging from asymptomatic

antithyroid auto-antibodies to clinically overt hypothyroidism, hyperthyroidism or acute biphasic thyroiditis [47]. In large prospective studies, the incidence of thyroid abnormalities was 5 – 8% in treated hepatitis C patients, 1 – 3% in patients with chronic hepatitis B and 12% in patients treated for cancer [29]. Because the underlying treated disease, that is, chronic hepatitis C in most instances nowadays, can be associated with immunopathological disorders, the exact role of the recombinant IFNs- α in the occurrence of autoimmune disorders remains ill-ascertained [48]. The IFNs- α play a critical role in the pathogenesis and perpetuation of autoimmune diseases, including SLE, autoimmune thyroid disease and type 1 diabetes but the mechanisms by which they contribute to immune dysfunction are not explained. In addition, genetic and environmental factors are thought to be critical to the development of autoimmune diseases.

In contrast to the IFNs- α , the contribution of IFN- β to the development of autoimmune diseases is seemingly limited [29]: auto-antibodies and autoimmune diseases, for example, thyroiditis, have rarely been observed in treated patients. Only limited information is available on the effects of IFN- γ : most patients treated for hepatitis B developed auto-antibodies without clinical signs of autoimmune disease, whereas conflicting results have been reported in patients with rheumatoid arthritis. rIL-2 treatment is associated with a high incidence of thyroid dysfunction associated with antithyroid auto-antibodies but very occasionally with other autoimmune diseases [29].

Other therapeutic proteins that have been reported to induce more frequent autoimmune diseases are the anti-TNF- α drugs [49]. A recent review of 53 published case reports concluded that in most instances the diagnosis of drug-induced lupus would be excluded if the American College of Rheumatology criteria were strictly applied. The mechanism by which anti-TNF- α drugs can induce auto-antibodies is not known.

3.3 More frequent hypersensitivity reactions towards unrelated allergens

Although it may seem logical to suspect that stimulating the immune system could result in enhancing normal (physiological) as well as abnormal (hypersensitivity) response, extremely few data have been provided to substantiate this suspicion. From at least a theoretical perspective, an enhanced recognition of the antigens or a nonspecific adjuvant-like effect can be suspected to be involved. Few and mostly unpublished data suggested that the therapeutic use of immunostimulatory agents may be associated with more frequent hypersensitivity reactions towards unrelated allergens, for example, asthma, hay fever, eczema or urticaria, that should be clearly differentiated from hypersensitivity reactions directed against the therapeutic agent [37].

Surprisingly, only one example provides evidence, but compelling evidence, that treatment with immunostimulatory agents can be associated with more frequent hypersensitivity

towards unrelated allergens. Indeed, hypersensitivity reactions to radiological contrast media were shown to be significantly more frequent in cancer patients treated with rIL-2 than in matched untreated patients in several retrospective or prospective studies [50-52].

3.4 Inhibition of some drug metabolism pathways

That immunostimulating agents can inhibit some cytochrome P450 dependent pathways of drug metabolism is largely overlooked despite the available data accumulated over the years [53,54]. The basis for this interaction is predominantly through cytokine mediated pathways. IL-1 α , IL-1 β , IL-2 β , IL-6, TNF- α , IFN- α , IFN- γ and TGF- β are known to downregulate the principal forms of cytochrome P450 (CYP450) at the level of gene transcription, which decreases the corresponding mRNA, protein and enzyme activity.

The administration of various therapeutic cytokines including rIL2, rIL-10, recombinant IFNs- α or β to human beings has been shown to depress the levels of several CYP450-mediated pathways [54]. Although no clear evidence of adverse effects is available, these changes were shown to alter significantly the pharmacokinetics of several drugs in treated patients.

4. Clinical immunotoxicity of therapeutic proteins in relation to immunogenicity

Immunogenicity is a principal safety concern as regards therapeutic proteins. Whether immunogenicity assessment should be uncoupled from immunotoxicity assessment is a matter of debate among regulators. The US FDA guidance on the immunotoxicology evaluation of new investigational drugs includes immunogenicity in immunotoxicology evaluation but separates immunogenicity from hypersensitivity [55]. In contrast, the EMEA recently released a guideline focused on the immunogenicity assessment of biotechnology-derived therapeutic proteins [56]. Immunotoxicity encompasses all types of adverse effects involving the immune system. Even though one single animal model or *in vitro* assay cannot be expected to predict all types of immune-mediated toxicities, maintaining immunogenicity assessment within the broader scope of immunotoxicity assessment is deemed to be necessary to ensure a thorough and multifaceted evaluation of the immunological safety of pharmaceuticals including therapeutic proteins.

It has long been recognized that molecules of sufficient size and foreign (non-self) origin can act as direct immunogens, that is to say they can induce the production of specific antibodies that subsequently react with the eliciting (macro)molecule, or more accurately only small parts of it (the so-called epitopes). All therapeutic proteins that are available or publicly known at present can be considered to be potential direct immunogens as their molecular size is large enough, and their structure is different from endogenous proteins, even though sometimes

only marginally. Indeed, despite current efforts to produce highly humanized or so-called human-like therapeutic proteins, particularly mAbs, immunogenicity is not totally eradicated [57]. The immunogenic potential of therapeutic proteins ranges from non-immunogenic, where no antibodies are generated, to strongly immunogenic, where most treated subjects develop specific antibodies. The clinical experience shows that most of the therapeutic proteins induce the production of specific antibodies in treated patients. The clinical relevance of the antibodies is a result of the quantity generated, the region of the therapeutic protein the antibody binds to and the mechanism of action of the therapeutic protein. Thus, clinical consequences range from a lack of overt consequences to life-threatening complications.

4.1 Factors affecting the immunogenicity of therapeutic proteins

Factors shown to affect the immunogenic potential of therapeutic proteins have been the focus of many laboratory and clinical studies. The results of these studies have been extensively reviewed elsewhere [58,59] and, therefore, will only be briefly overviewed here.

The production of antibodies to a protein antigen by naive B cells and the resulting initiation of an immune response require several coordinated events. The first step is the internalization of the antigen by antigen-presenting cells (APC), such as mature dendritic cells and macrophages that process the antigen into small peptides, which are subsequently presented to T CD4⁺ cells, the full activation of which requires further (second) signals provided by the APC. Once activated, the T CD4⁺ cells produce various cytokines. An interaction between B cell surface immunoglobulins (Ig) and the protein antigen is required for the activation of B cells. The B cells process the antigen-Ig complex and present T cell epitopes on its surface. On encountering the epitope, T CD4⁺ cells deliver cytokines, which activate B cells that differentiate into memory B cells and antibody-secreting plasma cells.

The basis for the development of antibody responses to therapeutic proteins remains a matter of some debate [60]. When there is no specific immune tolerance, autologous proteins normally induce the formation of specific antibodies along the pathways briefly described above. With therapeutic proteins that are homologous to natural human proteins, the formation of antibodies is thought to be the consequence of breaking B tolerance. Various mechanisms have been hypothesized to be involved but none has been fully explained. Thus, the degree of non-self and the presence of T cell epitopes in the case of nonhuman proteins are critical factors for the production of antibodies.

The structure of therapeutic proteins is a key factor affecting immunogenicity [61]. The more therapeutic proteins deviate from the native sequence of the endogenous protein, the more likely is the immune system to identify these as foreign and respond by producing antibodies. Marked

divergences between animal and human proteins account for the high degree of immunogenicity evidenced in human patients treated with animal proteins, such as porcine or bovine insulin. A single amino acid change can be sufficient to create a neo-epitope that will be recognized by the immune system but this critically depends on the position and type of amino acid. Therapeutic proteins looking more like self are less immunogenic than proteins that seem unique to the immune system, such as mutated or fusion proteins containing novel antigenic B and T cell epitopes.

Glycosylation is a feature of some therapeutic proteins. Depending on the glycosylation characteristics, the masking or unmasking of immunogenic sites may ensue with variable consequences as regards immunogenicity [61]. As glycosylation is species- and cell-specific, recombinant human proteins are not exactly the same as the natural endogenous protein. The chemical conjugation of proteins with polyethylene glycol (PEGylation) is generally considered to decrease immunogenicity [62]. Proteins denatured during formulation can be more immunogenic as they may present new B cell or T cell epitopes that are not present in the parent molecule. Proteins can be fragile and must be protected from temperature extremes, vigorous shaking or excessive freeze-thaw cycles to avoid denaturation or degradation. When therapeutic proteins become unstable, one key event that can occur is aggregation [63]. An immune response is more likely as aggregated materials are more prone to phagocytosis and subsequent antigen processing that leads to an immune response.

Therapeutic proteins are formulated by adding reagents, such as human serum albumin, to help stabilize the protein and inhibit aggregation. The formulation of a therapeutic protein is specific for that protein. Certain contaminants and process-related impurities can lead to increased immunogenicity of the protein or they induce a specific immune response directed against themselves.

The route of administration can also influence the rate of immunogenicity [64]. A given protein when administered subcutaneously is more likely to generate an immune response than intramuscularly, and then than intravenously. However, there is no route of administration that can absolutely protect against raising an immune response, and there are no systematic studies comparing parenteral and non-parenteral routes.

The immune system responds best to a foreign protein when it is administered over a long period of time and when it is allowed to clear before subsequent administration. By contrast, if circulating levels of the therapeutic protein are maintained, the immune system is more likely to become tolerant. As it takes some time for the immune system to mount a robust response, those therapeutic proteins that are not administered chronically are less likely to induce a strong immune response. The biological properties of therapeutic proteins can also influence the immune response. Thus, immunostimulating proteins are considered to be

more likely to induce the formation of antibodies than immunosuppressive proteins. mAbs targeted to cell-bound epitopes are more likely to induce an immune response than those with a target in solution.

Finally, the characteristics of the treated patients including age, gender and genetic predisposition are suspected, if not likely to influence the immune response towards therapeutic proteins. Because many immune responses outside of the normal range are genetically based, specific genetic associations in patient populations that demonstrate adverse reactions to therapeutic proteins can be suspected to be involved. However, so far none has seemingly been evidenced.

4.2 Clinical consequences of antibodies to therapeutic proteins in treated patients

Treatment of human patients with therapeutic proteins is commonly associated with the development of specific antibodies. As already mentioned, the incidence of patients developing specific antibodies, the quantity generated (as reflected by serum levels) and the clinical relevance of these antibodies are extremely variable and largely impossible to anticipate either from the chemical structure of the protein or from the results of preclinical safety studies. Therefore, the search for specific antibodies in the sera of human subjects enrolled in clinical trials is a critical step in the evaluation of the immunogenicity of any novel therapeutic protein [65].

In some patients, specific antibodies are inconsequential with no overt clinical consequence.

Neutralizing antibodies are also frequently detected using bioassays or cell-based assays [66]. Neutralizing antibodies can block the biological activity of the therapeutic protein by binding directly to epitope(s) within the active site, or by blocking the active site by steric hindrance owing to binding to epitope(s) in close proximity to the active site. The presence of neutralizing antibodies may not result in a clinical effect but a decrease in efficacy may be observed requiring administration of higher doses of the therapeutic protein. In certain cases, non-neutralizing antibodies *in vitro* may also reduce the clinical response. In most instances, neutralizing antibodies despite the reduction or abrogation of efficacy have no other apparent adverse effects as shown with IFN- β in multiple sclerosis patients [67].

However, the antibodies can not only neutralize the administered protein but also cross-react with and neutralize the biological activity of the homologous, endogenous protein. Thus, severe thrombocytopenia was observed in healthy volunteers after the administration of PEGylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF) that induced antibodies cross-reacting with endogenous thrombopoietin [68]. The most illustrative example, however, is the recent occurrence of pure red cell aplasia (PRCA) resulting from neutralizing antibodies following the administration of recombinant human erythropoietin (rHu-EPO) [69]. Pure red cell aplasia

is a very rare disease defined by the lack of medullary erythroid progenitors, or by an arrest of erythroblasts' maturation. Epoetin- α was the first rHu-EPO to be marketed in 1988, followed by epoetin- β and then darbopoietin. From 1998, the manufacturing process of epoetin- α marketed in Europe and Canada excluded human serum albumin to comply with a new European recommendation and was replaced with polysorbate 80 and glycine. Since then, more than 200 cases of epoetin antibody-mediated PRCA have been notified worldwide with a sharp increase in incidence from 1998 to 2002. Most of the patients were treated with epoetin- α , primarily via the s.c. route. In April 2003, the prefilled uncoated stopper syringes were switched to FluroTec-coated stopper syringes. In July 2003, all uncoated stopper prefilled syringes were recalled. Since then, the reported occurrence of rHu-EPO-associated PRCA has dramatically decreased to (< 1 case per 10,000 patient-years).

Pure red cell aplasia occurs suddenly after several months of treatment with rHu-EPO (median: 7 – 9 months for the s.c. route; 18 months for the i.v. route). Clinically, the patient experiences a lack of efficacy of rHu-EPO resulting in the reoccurrence of severe normochromic, normocytic, aregenerative anemia. Circulating endogenous erythropoietin is undetectable, whereas anti-rHu-EPO antibodies are detected in the sera of patients. After rHu-EPO discontinuation, antibody levels slowly decrease but even at a very low concentration they retain a neutralizing activity. The treatment of rHu-EPO-associated PRCA consists in the administration of immunosuppressive therapy after discontinuation of the suspected rHu-EPO. The time to recovery defined as undetectable anti-EPO antibodies is several months [70]. Several factors have been considered to explain this surge in rHu-EPO-associated PRCA. The prefilled syringes contained silicone oil with an uncoated rubber stopper, and an excess of polysorbate 80 after 1998. Polysorbate 80 may leach through the rubber plunger and thus the high concentration of polysorbate 80 would promote micelle formation and increase epoetin- α immunogenicity, which is further enhanced via the s.c. route.

Surprisingly, the presence of specific antibodies to human therapeutic proteins is seldom associated with 'classical' immuno-allergic hypersensitivity reactions. Anaphylactic shock has seemingly not been reported in patients treated with IFN- α and exceptionally in patients treated with IFN- β [71]. Mild-to-moderate immediate hypersensitivity reactions have very occasionally been reported with recombinant cytokines including IFN- α IFN- β and rhIL-2 [29]. Similarly, anaphylaxis is a possible but rare adverse effect of recombinant Factor VIII [72] or human insulin [73]. Rare anaphylactic reactions associated with various therapeutic mAbs including cetuximab [74], infliximab [75,76], basiliximab [77] or abciximab [78] have been reported. In sharp contrast to quite a few misleading case reports in the literature, it is

essential to differentiate acute infusion reactions that can occur during the first infusion from anaphylactic shock than can only occur following a readministration of the same mAb.

Because of the presence of specific antibodies to therapeutic proteins in their sera, treated patients are considered to be at risk of developing serum sickness (type III hypersensitivity). In fact, extremely rare case reports of serum sickness associated with therapeutic proteins have been published. A small percentage of infliximab- and rituximab-induced infusion reactions are delayed and thus suspected to involve a type III mechanism. One case of serum sickness has been reported following the administration of infliximab [79].

5. Clinical immunotoxicity of therapeutic proteins and drug-induced autoimmune reactions

The need to carefully differentiate more frequent autoimmune diseases that may be caused by immunostimulating or immunomodulating agents from drug-induced autoimmune reactions should be highlighted. Indeed, any type of autoimmune disease clinically and serologically identical to an idiopathic disease is suspected to develop more frequently as a consequence of the facilitating, even though ill-understood role of immunostimulation. In contrast, systemic drug-induced autoimmune reactions typically bear key clinical and serological differences with their idiopathic counterpart, for example, drug-induced lupus versus SLE. In addition, as far as autoimmune reactions are concerned, one given drug is typically associated with only one characteristic type of autoimmune reaction; in contrast, immunostimulatory drugs including several recombinant cytokines, such as the IFN- α are associated with a wide array of more frequent spontaneous autoimmune diseases [13].

Overall, drug-induced autoimmune reactions are rarely observed. So far, no autoimmune reaction as defined earlier has been reported in association with the use of any therapeutic protein.

6. Conclusion

As reviewed earlier, therapeutic proteins can induce a large variety of immune-mediated (immunotoxic) adverse effects that are potentially severe, and sometimes life-threatening in human patients. Thanks to the clinical experience accumulated over the recent years, our knowledge of the incidence, clinical presentation and management of these adverse events has significantly improved.

Although developers and regulators have been assessing the clinical toxicology of the immune system for the past 25 years, at least to some extent, clinical immunotoxicology is the missing link in the immunological safety evaluation of pharmaceuticals [80]. This is particularly obvious for therapeutic proteins because of the many limitations and

pitfalls of current procedures applied to the prediction of their immunotoxic potential during nonclinical studies. The transition from animal to human studies and the expectations of clinicians to improve the immunological safety evaluation of drug candidates have been the matter of a recent workshop [81]. The release of guidance documents by regulatory authorities on issues specifically related to clinical immunotoxicity would certainly help trigger initiatives and efforts in this somewhat neglected area.

There is, indeed, an urgent need to design, standardize and validate relevant biomarkers of immunotoxicity to be used in clinical trials. Ideally, these biomarkers should also be applicable to animal studies as well to ensure the needed transition from animal to man. The use of new animal models, such as transgenics, or refined existing models, such as nonhuman primates, to conduct dedicated safety immunopharmacology studies should be promoted to improve our present limited prediction of the immunological safety of novel therapeutic proteins in humans.

In any case, a thorough understanding of the mechanism of action is essential to design dedicated trials to address suspected safety issues and also to implement more adequate preventive or corrective measures. Finally, the value of risk management plans to ascertain the suspected potential of therapeutic proteins to induce immune-mediated adverse effects should be emphasized.

7. Expert opinion

Despite several safety advantages compared to small synthetic molecules, therapeutic proteins can induce a variety of adverse effects among which immunotoxic effects are globally rather frequent and potentially severe.

Some adverse effects of therapeutic proteins are linked to their intended or unintended mechanism of action on specific target(s) of the immune system resulting in either immunosuppression or immunostimulation. A thorough understanding of the mechanism of action is, therefore, critical for the safety evaluation of therapeutic proteins

to enhance the prediction of potential immunotoxic effects. Up to now, far more attention has been focused on the nonclinical detection of immunosuppression-related than immunostimulation-related adverse effects and this gap needs to be filled.

Other clinically important adverse effects of therapeutic proteins involving the immune system include immunogenicity that can lead to decreased efficacy or hypersensitivity reactions and auto-immunity. Current nonclinical procedures are inadequate and efforts should be made to improve their predictability.

Because of their inherent limitations, of which some are related to the very nature of the studied therapeutic proteins, nonclinical safety studies cannot be expected to assure the required level of human safety. Nevertheless, progress can probably be made with the development of novel animal models such as transgenics or dedicated safety immunopharmacology studies.

There is an urgent need to conduct clinical studies focused on the evaluation of the immunological safety of therapeutic proteins. To do so, standardized and validated assays and biomarkers to assess immunotoxicity are absolutely necessary. Ideally, such assays and biomarkers should also be used in nonclinical studies. In addition, more attention should be paid to known but often ill-investigated potential adverse effects, such as infectious complications. Globally, the value of risk management plans to assess the immunological safety of therapeutic proteins should be emphasized.

Clinical immunotoxicology is the missing link of immunotoxicity evaluation. To achieve the needed transition from animal to man and ensure relevant clinical assessment, collaborative efforts should be focused on this aspect of immunotoxicology. Specific clinical immunotoxicology guidance would be very helpful in that respect.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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Affiliation

Jacques Descotes[†] MD PharmD PhD & Aurore Gouraud PharmD
[†]Author for correspondence
 Centre Antipoison – Centre de Pharmacovigilance,
 162 Avenue Lacassagne,
 69424 Lyon cedex 03, France
 Tel: +33 0 4 72 11 94 10;
 Fax: +33 0 4 72 11 69 85;
 E-mail: jacques-georges.descotes@chu-lyon.fr