Chapter

Immunogenicity Study of Biosimilar Candidates

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Abstract

Therapeutic proteins can induce undesirable immune reactions in the patient and constitute a major concern as they may compromise therapy safety and efficacy. During the comparability study between a biosimilar product and the innovator, several attributes are considered. Among them, the comparative immunogenicity analysis in preclinical and clinical stages has a major relevance. In this chapter, we will describe the most used experimental platforms for biotherapeutic immunogenicity characterization. Special emphasis will be placed on *in vitro* assays for the detection of contaminants that modulate innate immune responses, as well as tools for the identification of biologic-derived T-cell epitopes. Likewise, we will also review the current trials used for the detection of host cell proteins (HCPs) and their potential impact on protein immunogenicity. Finally, we will analyze the admissibility criteria established by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA), when comparing the immunogenicity of reference products and biosimilar candidates.

Keywords: immunogenicity, contaminants, T-cell epitopes, host cell proteins, *in vitro* assays, *in vivo* assays, admissibility criteria

1. Introduction

Therapeutic proteins or biotherapeutics represent a solution for the treatment of numerous human diseases [1]. Due to the complexity of these drugs and their manufacturing process, exhaustive quality control is required. Biosimilar products do not escape these control mechanisms and must comply with the eligibility criteria established by international regulatory agencies. Regarding the immunogenicity analysis of these products, the quality standards required are those achieved by the innovator product.

Protein immunogenicity represents a major concern for biologic's manufacturers due to its impact on therapy efficacy and safety. The immunogenicity of therapeutic proteins, including biosimilars, depends on several factors, including therapy-related, patient-related, and product-related issues. Here, we will focus mainly on those factors related to the biopharmaceutical product.

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In humans, the immune system exhibits different checkpoints to provide cellular and molecular components that recognize and ultimately eliminate rapidly and accurately disease-causing pathogens, while avoiding damage to healthy tissue itself. This training of the immune system is widely known as immune tolerance. After the biologic is given to the patient, the therapeutic protein is screened by complex immune system surveillance processes.

For this reason, an important aspect to consider when evaluating product immunogenicity is to detect the presence of contaminants derived from the manufacturing process that can trigger innate immune responses in the patient. These entities, previously described as innate immune response modulating impurities (IIRMIs) can enhance protein immunogenicity even when present at trace levels and include microbial structures and host cell proteins (HCP) [2]. Indeed, the International Council for Harmonization (ICH) considers these impurities as critical quality attributes (CQA), that is, a product characteristic that should be within an appropriate range to ensure product quality [3].

In this chapter, we will address the state-of-the-art in current *in vitro* assays for the detection of IIRMIs. In this regard, we will establish the advantages and limitations of primary cell cultures and established cell lines designed for the specific detection of immunogenic entities.

Host cell-derived proteins (HCPs) that co-purify with the product can lead to a breakdown of immune tolerance. This unwanted immune response is triggered against the contaminating protein and, in some cases, also against the therapeutic protein [4]. While some HCPs can modulate the activity of human innate immune cells, indirectly they can also have an impact on a patient's adaptive immune responses. Once these entities bind to their receptor, they can increase the endocytic and phagocytic capacity of professional antigen-presenting cells (APCs), such as dendritic cells and macrophages, and also modulates antigen processing and peptide presentation. Consequently, these APCs can activate specific T cells, which in turn can activate B lymphocytes that differentiate into neutralizing antibodies (Nab)-producing plasma cells. In conclusion, some HCPs can act as adjuvant and modulate an undesired immune response in the patient, characterized by the generation of a pro-inflammatory scenario and subsequently by the development of antibodies against the drug and/or against the HCP. Therefore, HCPs monitoring constitutes one of the most important analytical requirements in the production of biotherapeutics.

Biotherapeutic-induced immune tolerance breakdown may also involve the adaptive immune system. As is the case with numerous proteins, biologics may contain immunogenic B- and T-cell epitopes [5–7]. As mentioned above, the presence of B-cell epitopes is closely related to the molecule's capacity to induce the production of antidrug antibodies. These antibodies can either bind to the biologic without producing a direct impact on its function or bind to the protein regions responsible for its biological effect and cause a partial or total neutralization. For this reason, Ab-mediated immune response studies in individuals treated with the biopharmaceutical have become the focus of attention during clinical trials and as a pharmacovigilance strategy [8–12].

It is widely accepted that an immune response involving the formation of high-affinity antibodies requires the collaboration of specific T cells that recognize biologic-derived epitopes [13–16]. For this reason, we will also review the most relevant *in vitro* strategies for the characterization of T-cell-mediated immune responses induced by therapeutic proteins. Specifically, we will describe the immune cell-based bioassays used to elucidate biotherapeutic-induced T-cell response profiles.

2. Detection of impurities that activate innate immune responses

Several product-specific factors can affect the immunogenic risk of biotherapeutics: product origin, primary molecular structure, posttranslational modifications, product aggregates, glycosylation/pegylation, impurities, formulation, and container closure considerations, among others [17]. In this section, we will focus on the presence of impurities in biotherapeutics and the currently available tools for their detection, and the impact prediction on product immunogenicity.

Impurities can come from the host cell or the different manufacturing process steps and can be recognized by the patient's innate immune cells, even when present at trace levels. These contaminants are widely known as innate immune response modulating impurities (IIRMIs) and include DNA, dsRNA, lipid and carbohydrate complexes, organic and inorganic components, and host cell proteins (HCPs) [2].

At present, downstream purification processes are very efficient and allow the reaching of products with high purity levels. Despite this, some contaminants can remain along with the biological product at levels not detected by the routinely used test. Moreover, *in vivo* studies in nonhuman primates showed ADA formation, when minute amounts of impurities were coadministered with a therapeutic protein [18, 19]. In addition, it was also reported that trace levels of IIRMIs could act as adjuvant and induce *in vitro* polyclonal B cell activation on murine splenocytes and consequently increase *in vivo* antibody responses [2]. Altogether, this demonstrates that very low levels of these contaminants may foster product immunogenic risk and highlights the need for tools to detect and identify IIRMIs in therapeutic products.

Currently, the analysis of IIRMIs in biologics is limited to the detection of endotoxins through the Limulus amebocyte lysate (LAL) test, PCR assays to detect host cell DNA, and ELISA-based tests for host cell proteins [20–22]. For this reason, some experimental approaches are emerging to expand the repertoire of detection of IIRMIs potentially present in biological products. Among them, the use of peripheral blood mononuclear cells (PBMCs), human and mouse monocyte/macrophage cell lines, and cell lines expressing innate immune receptors was recently proposed [23].

PBMC samples are constituted of human immune cells endowed with diverse pattern recognition receptors (PRR) capable of detecting a repertoire of IRMIIs. The results obtained by using these primary cell cultures usually mimic the *in vivo* innate immune responses observed in the clinic. The assay is based on the incubation of PBMC samples with different amounts of the tested product. Thus, whether IIRMIs are present, an increase in the expression of proinflammatory genes is observed. This experimental platform is sensitive and has a low detection limit of impurities and, as mentioned above, allows replication of the potential *in vivo* immunogenicity risk. However, some disadvantages may limit its use, such as low reproducibility, which makes it difficult to validate, limited sample availability, and safety issues associated with the handling of these cell cultures [24, 25].

Another interesting strategy involves the utilization of monocyte and macrophage cell lines. These cell lines also bear multiple innate immune receptors but without having the disadvantages associated with the use of primary cell cultures. Currently, the most widely used cell lines to detect IIRMIs in biologics are RAW 264.7 (murine macrophage); human monocytic cell line macrophage-like-MonoMac6 (MM6); and THP-1, a human monocyte cell line-derived from a patient with acute monocytic leukemia [23, 26–28]. A comprehensive study revealed that the combined use of these monocyte/macrophage cell lines allowed the recognition of a wide repertoire of IIRMIs in biotherapeutic proteins, with a detection limit similar to that achieved with PBMC samples [23].

In addition, cell engineering has also contributed to the development of strategies to specifically detect IIRMIs in biopharmaceuticals. Thus, numerous HEK293 cell lines stably expressing one or multiple PRRs and an associated reporter protein are now available [23, 29–31]. In these assays, once the cell membrane receptor is engaged by its specific ligand, these cell lines initiate a signaling cascade that culminates in the transcription activation of a reporter gene, which generates a rapidly and easily quantifiable signal.

Therefore, to carry out the adjuvant activity analysis of IIRMIs present in the biologic, it is recommended to start the study using primary cell cultures (PBMC) in combination with human and murine immune cell lines. Then, if these experimental platforms reveal the presence of IIRMIs in the product, the study can be further complemented by identifying the nature of the ligand detected through the use of HEK293 reporter cell lines.

Some reports showed the successful use of these experimental platforms for the detection and analysis of IIRMIs and their impact on biologic's safety. For instance, Haile and collaborators revealed that two IFN-β products that proved to be immunogenic in the clinic included IIRMIs in their formulation [29]. Then, the authors further explored these findings in depth and found that the increased immunogenicity was due to the presence of TLR2 and TLR4 ligands. Interestingly, none of these products evidenced the presence of endotoxins when evaluated by the LAL method. This highlights the limitations of this method for detecting low levels of this contaminant and highlights the advantages inherent in the use of cell-based assays for the detection of IIRMIs in biotherapeutics.

Additionally, a current report revealed that some excipients could mask the presence of IIRMIs. Hence, product formulation is another important issue to be considered when developing cell-based assays for IIRMIs assessment [32].

Finally, considering that comparability exercises between a reference product and the biosimilar candidate are multifactorial, these experimental platforms appear as a highly attractive alternative for measuring the immunogenicity risk of these products in the preclinical stages of development.

3. Immune responses elicited by biologic-derived T-cell epitopes

Two mechanisms culminate in the production of antidrug antibodies: a T-cell independent (Ti) and a T-cell dependent (Td) pathway. The formation of high-affinity antibodies and the generation of immune memory require the participation of T lymphocytes (TL). In addition, in the case of biotherapeutics, the Td pathway is the most likely to occur. This explains the number of studies focused on the characterization of biologic-induced T-cell responses [12–15].

Initially, dendritic cells internalize the therapeutic protein *via* vesicles. As occurs mainly with extracellular antigens, the biologic is degraded by lysosomal enzymes and the resulting peptides are presented in the context of the major histocompatibility complex class II (in humans, known as HLA-II, for human leukocyte antigen) [33]. Then, naïve TL, *via* their receptor (TCR), specifically recognize the presented peptide. From here two scenarios can be observed; if the dendritic cells express sufficient amounts of co-stimulatory molecules, the TL will be activated and differentiated toward a specific effector profile. This effector profile is driven by the dendritic cell through the cytokines secreted. Conversely, in the absence of co-stimulation, the TL becomes anergic or undergoes apoptosis [34].

B lymphocytes (BL) are also professional antigen-presenting cells (APC) and can recognize the biotherapeutic through their membrane immunoglobulin (Ig). When this occurs, internalization, processing, and antigen presentation take place similarly to as described for dendritic cells. The biologic-derived peptides are then presented to effector TL and, in the presence of appropriate co-stimulation; the TL is activated and secretes cytokines that promote BL differentiation into memory BL and ADA-producing plasma cells [35]. These antibodies can bind to the therapeutic protein (binding antibodies) without affecting its biological activity or, on the contrary, neutralizing its function (Nab). Similarly, TL also respond to this interaction producing cytokines characteristic of the acquired profile. For instance, Th1 TL secrete IFN-γ, which induces an undesired inflammatory scenario in the patient. Conversely, in the absence of specific TL responses against the biologic, the reactive BL becomes anergic or undergoes apoptosis. Therefore, the presence of biologic-derived TL epitopes not only influences the formation of high-affinity ADA, but also the generation of a pro-inflammatory response. This highlights the need for immunogenicity assays based on the detection of TL-mediated responses.

3.1 Experimental platforms for the analysis of TL-mediated immunogenicity of biosimilar products

The immune response involving high affinity and long-lasting ADA requires specific TL help. Therefore, it is recommended to perform the analysis of the immune response mediated by these cells in a preclinical stage.

Currently, there are several experimental strategies to study the TL-driven immunogenicity of biosimilar products and they are grouped into *ex vivo* and *in vivo* assays. Both experimental platforms should be carried out by including the reference product and the biosimilar candidate in the same assay, under the same experimental conditions. Thus, this head-to-head comparison will allow distinguishing differences in immunogenicity and potential risks to the patient.

3.1.1 Ex vivo assays

These experimental platforms mainly consist of collecting cells, tissues, or organs from a living organism and then performing tests based on these biological samples [36]. For instance, in assays using PBMCs, the cells can be used fresh or thawed; however, it is recommended to perform these assays from freshly extracted cells since after the freezing/thawing process the intensity of the response may be diminished. Experimental protocols may present subtle differences but in general consist of incubating PBMC cultures with the innovator product and the biosimilar candidate at a specific concentration in single or multiple antigenic stimulations. At the end of the assay, culture supernatants are extracted and various cytokines that characterize the most relevant TL response profiles are assayed [37]. A more refined experimental platform involves the prior separation of monocytes from the PBMC sample, which are then differentiated into dendritic cells (DC) using a cytokine cocktail. The DCs are then treated with the biologic and undergo a maturation process with TNF-α or LPS. Finally, the biologic-derived peptide-pulsed-DCs are co-cultured with autologous TL and the induced TL response profile is identified by cytokine profile analysis in the culture supernatant [7, 38, 39]. This experimental approach is particularly useful when the therapeutic protein has direct antiproliferative action on TL, as is the case with IFN-type I.

While the use of cell lines provides valuable, reproducible, and validatable data, primary cell cultures are still an attractive tool as they present a diversity of cellular components that correlates in composition with the *in vivo* context. For this reason, primary cell cultures derived from human peripheral blood and murine splenic cells are an interesting strategy in the preclinical stage of immunogenicity testing of innovator and biosimilar products. The most important advantages compared to established cell lines are the lower limit of detection and, as mentioned, the possibility to evaluate the immune response of multiple cellular protagonists simultaneously. The main disadvantages are the inconveniences in obtaining blood samples (availability), low reproducibility, difficulty to validate, and the safety requirements in cell culture handling [23].

3.1.2 In vivo assays

In vitro and *ex vivo* assays provide relevant information on the pattern of immune responses and allow comparison between innovative products and biosimilar candidates. However, they are still far from the *in vivo* context in clinical trials. For this reason, *in vivo* assays in model animals provide a bridge between both scenarios and allow prediction with certain accuracy of immunogenicity risks in the clinic [23]. The animal models most commonly used for immunogenicity assessments are transgenic mice tolerant to therapeutic proteins and HLA-DR mice strains [40].

3.1.2.1 Tolerant mice strains

Mice tolerant to human proteins allow mimicking human-like responses to the therapeutic version of that protein. Thus, these animal models allow identifying neo-epitopes and performing comparative immunogenicity studies of a biosimilar product and the corresponding innovator. In both cases, this animal model makes it possible to evaluate the potential breakdown of immunological tolerance toward the biologic, as a consequence of changes in the sequence, structure, and formulation of the product. The first strains developed were the human tissue plasminogen activator (htPA)-tolerant mouse [41] and the human insulin-producing animal model [42]. Both animal models proved to be valuable because of the sensitivity to detect changes of only one amino acid in the protein sequence. Later, the development of a mouse strain tolerant to human IFN-β was also useful for immunogenicity assessments of different formulations of this therapeutic protein [43].

Currently, numerous animal models tolerant to human proteins, such as growth hormone (GH) [44] and IFN-alpha [45], are available. Although these animal models are of significant utility, they have limitations associated with the murine antigenic presentation (H-2), which differs from the human system (HLA). For this reason, the use of transgenic mice expressing HLA molecules has gained great participation in preclinical immunogenicity studies.

3.1.2.2 HLA-DR-expressing transgenic mice

These mouse strains were deleted in the murine H-2 genes and instead express HLA alleles. The wide variety of HLA-DR mouse strains has allowed the extensive study of protein immunogenicity, including the identification of HLA-DR alleles with a major incidence of immunogenicity to a given biologic, as well as

susceptibility or resistance to certain autoimmune diseases, infectious diseases, and vaccine efficacy [46] In addition, the utility of these transgenic mice has been confirmed by the high correlation of observed TL-mediated immune responses with results obtained later in clinical trials [47].

4. Identification and immunogenicity of HCPs in biotherapeutics

Host cell proteins (HCPs) are process-related impurities that can affect the quality and safety of biotherapeutics. Despite the efforts made during the downstream process, a little number of host endogenous proteins may co-purify with the drug substance. HCPs could be released during the fermentation process due to cell death or come from the cell lysate when the biologic is produced intracellularly. The reasons why HCPs could be present in the final product include specific or unspecific drug interaction, co-elution in chromatography steps, or due to high homology with the protein of interest [4].

4.1 Challenges and relevance of HCPs characterization

To guarantee drug purity, patient safety, and manufacturing process consistency, biotherapeutics manufacturers must demonstrate impurities and contaminants clearance. HCPs are considered a critical quality attribute [48] and regulatory guidelines [49, 50] establish that HCP levels must be monitored. Typically, 100 ppm or ng HCP/mg of therapeutic protein quantified by ELISA (see below) is the upper limit adopted in the industry [51]. However, because HCPs quantification assays for different biotherapeutics cannot be compared, it is not possible to establish a unique HCP safe threshold level.

A distinguishing feature of HCPs from other impurities is their complexity and the impossibility of predicting their profile in different product batches. Thousands of host cell proteins with high heterogeneity (molecular weight, PI, hydrophobicity, and isoforms) may accompany the biotherapeutic in the downstream process. Notably, more than 6000 HCPs have been identified in the CHO cell proteome [52] but only a few proteins are found in final products [53–55]. Surprisingly, biotherapeutics expressed at low levels as human coagulation factors may represent only 3% of the starting material for purification, being HCPs the remaining 97% [4]. As a result, HCP monitoring and clearance represent an analytical challenge for the industry.

HCP complexity also provides a spectrum of risks associated with its presence in the final product. A four-category classification of high-risk HCPs based on their impacts was proposed by the Biophorum Development Group (BPDG): (1) product quality, (2) formulation, (3) direct biological function in humans, and (4) immunogenicity [56]. They have also published a collection of high-risks CHO HCPs on mAb production and developed an online platform with updatable content (https://www.biophorum.com/host-cell-proteins/). Furthermore, an industrial examples review of problematic HCPs was reported [4]. In addition, De Zafra et al. provided a risk assessment tool for residual HCPs identified in biotherapeutics, and factors, such as HCP identity, are discussed [51]. Homolog HCP may be active in humans or induce the formation of antibodies that cross-react with endogenous proteins. In contrast, if the HCP is highly divergent to human ortholog, that is, nonmammalian hosts, such as *Escherichia coli* or yeast; they could induce a robust immune reaction in humans.

Challenges in HCP monitoring and clearance are relevant not only for innovative biotherapeutics but also for biosimilar products. Advances in proteomics provided evidence about significant changes in HCP profile according to upstream parameters (i.e., feeding, temperature, cell line, viability, and media formulation) and factors associated with the downstream process [57–59]. Consequently, it is not possible to predict biosimilar HCP content and its potential risk.

The relevance of HCP-related impact in biotherapeutics development and production highlights the necessity of analytical tools, which provide accurate and critical information on these impurities.

4.2 Analytical methodologies for HCP monitoring

Monitoring HCP in biotherapeutics development assesses product purity and process consistency [53]. In the early process, a broad population of HCPs is present, but only a small amount of residual HCP remains in the final product. Manufacturers must demonstrate the reduction of HCPs during the downstream process. Therefore, HCPs assays are employed to measure proteins present in the final product. Detection of HCP profile changes in case of process failure or modification is also desired.

4.2.1 HCP-ELISA

There is vast evidence about analytical methodologies to monitor HCP in biotherapeutics [59–62].

Currently, the anti-HCP enzyme-linked immunosorbent assay (HCP-ELISA) is the most widely used in the biopharmaceutical industry [63]. These experimental platforms are based on the use of polyclonal anti-HCP antibodies. Antibody-producing species, such as goats, sheep, or rabbits, are immunized with cell lysate or partially purified material from a null cell line fermentation. As a result, values obtained from ELISA assays are expressed as ppm of HCPs and constitute a measure of immune-equivalent nanograms of HCPs per milligram of drug substance [53]. This is a semiquantitative assay as ELISA quantification depends on the coverage and reactivity of the anti-HCP polyclonal antibodies used and the reference antigen used as standard. ELISA-based methodologies are routinely used in industry and R&D laboratories. HCP-ELISAs have several advantages, such as sensitivity (1–100 ppm) [60], selectivity, high availability, and throughput. However, no information about HCP identity or individual amount is provided. In addition, the detection of low immunogenic HCPs is compromised due to a bias in polyclonal antibody reagent development [63].

Commercial-, platform- or -product-specific HCP-ELISA could be chosen to monitor HCP levels. When using either commercial or specific ELISA assays, experiment validation is needed. In addition, coverage analysis must be done to characterize the critical reagents. Of note, differences in total HCP amounts were detected between them [53]. For instance, platform-HCP-ELISA reported 6 to 20-fold more HCP content than generic commercial ELISA. Therefore, the choice of HCP-ELISA must be carefully analyzed. In general, generic assays are widely applied in early biopharmaceutical development stages, while validated assays may be used for licensed pharmaceuticals with process-specific performance.

For antibody coverage analysis, the most common methods are Western blotting, 2D-PAGE, 2D-DIGE, and immunocapture followed by LC-MS.

4.2.2 LC-MS as an orthogonal method to HCP profiling

The lack of HCPs identity information and potential underestimation of HCP-ELISA assays bring out the relevance to apply an orthogonal approach to monitoring HCP in biopharmaceuticals. These techniques should ideally be able to detect HCPs across a wide dynamic range, follow the profile and concentration of HCPs during a bioprocess and measure small amounts of HCPs in the presence of high levels of the drug in the final product [61]. Regulators recommend the use of LC-MS as an orthogonal method [49, 50]. LC-MS has high sensitivity and allows the identification of HCPs through the downstream process [62, 64]. However, some disadvantages of this methodology include technical complexity, big data processing, and qualified staff are usually required. A recent report showed that mass spectrometry and 2D gels/PAGE for total HCP characterization and ELISAs (commercial-, process-, or platform-specific) as release tests for total HCP quantification are mainly used among 26 surveyed biopharmaceutical companies [Jonas 2021]. The authors also proposed an analytical strategy for monitoring HCPs and a workflow if high-risk HCPs were identified. In addition, HCP profiling in CHO cells [54, 56, 65, 66] and E. coli [53, 55, 67] were made taking advantage of current advances in proteomic technology.

In conclusion, knowing the exact HCP profile of a biopharmaceutical product allows the improvement of downstream processes, especially for difficult-to-remove HCPs. Additionally, analytical methods, such as specific ELISAs, may be developed to monitor the presence of high-risk HCP impurities. Proteomics studies also provide central information to implement quality-by-design (QbD) approaches to reduce HCP content in the final product [59]. Improved HCP monitoring will result in a biologic with the lowest risk related to HCP impurities, with benefits to the patient and the pharmaceutical industry [53].

Studies of the comparability of biosimilars and the innovative drug must take into consideration the monitoring and impact of the HCP profile in order to guarantee safety and product stability.

4.3 Impact of HCPs on the overall product immunogenicity

The presence of immunogenic or immunomodulatory HCPs poses a safety hazard for biopharmaceutical products. HCPs from nonhuman expression platforms can be detected as foreign to the human immune system [68]. As a result, HCPs can act as adjuvant and modulate an undesired immune response in the patient, characterized by the generation of a pro-inflammatory response and subsequently by the development of antidrug antibodies (ADAs). Additionally, cross-reactive anti-HCP antibodies may interact with endogenous human homolog proteins, neutralizing their biological activity or developing the formation of immune complexes and inducing an inflammatory scenario in the patient. ADA responses may also lead to other clinical adverse effects, such as immunotoxicity, which includes hypersensitivity reactions, cytokine release, infusion reactions, anaphylaxis, or immune complex diseases [17]. Moreover, ADAs can neutralize the biologic's efficacy and consequently affect its pharmacokinetic parameters [69].

Some examples of immunogenic HCPs are summarized in **Table 1**. Evidence linking patient safety risk to HCP exposure is limited and lacking in detail [51]. This is relevant as underestimated immune adverse effects triggered by impurities, such as

HCP identity/ information	Product/drug-related	Effect	References
E. coli ribose phosphate isomerase	Recombinant human growth hormone	Immunogenic, adjuvant	[4]
E. coli Flagellin	Recombinant human apolipoprotein A-I Milano	Adjuvant, TL5 response	[4, 70, 71]
CHO HCP	Coagulation factor IX	Immunogenic	[4]
E. coli HCPs of 20 and 30 Kda	Recombinant granulocyte-macrophage colony-stimulating factor	Immunogenic, adjuvant	[72]
CHO PBL2	Lebrikizumab	Immunogenic	[73, 74]
E. coli DnaK	scFv aggregates	adjuvant	[75]
Concentrate of *E. coli* HCPs	Ranibizumab	ocular inflammation, adjuvant	[76]

Table 1.
Summary of HCPs with reported immunogenicity.

HCPs, may result in delays and even suspension of clinical trials [70]. Moreover, even when the clinical consequences elicited by HCPs are restricted to anti-HCPs antibody formation, the economic impact of this delay in drug development may be significant. For instance, Inspiration Biopharmaceuticals had to sell IB1001's rights to another company because the clinical trial was placed on hold due to immune responses to HCPs detected in the product [4].

Semiquantitative data provided by HCP-ELISA assays is not sufficient to monitor the risk of immunogenicity in the clinic. However, a comprehensive HCP profiling, including HCP identification along with *in vitro* and *in vivo* immunogenicity assays, during product development could anticipate immunogenicity risks and thus prevent delays in clinical trials. In particular, individual risk assessments are relevant to difficult-to-remove and "repeated" HCPs, which may affect more than one biotherapeutic.

So far, only a handful of reports have addressed immunogenicity studies of individual HCPs [75, 77]. However, neither of these works addressed the immunogenicity issue by exhaustively characterizing the immune responses induced by HCPs. On the other hand, recent reports combining *in vivo* and *ex vivo* or *in vitro* assays tested biologics spiked with HCPs pools [76] or samples with more than one individual HCP [78]. As a result, this makes it difficult to correlate an observed immune response in the clinic with a specific HCP.

In addition, the advent of immune-informatics tools has allowed the development of algorithms for predicting immunogenicity risks associated with the presence of CHO cell-derived HCPs [79]. However, data obtained from this predictive analysis should be confirmed using the available experimental platforms addressed in this chapter.

Finally, it is important to note that even when most biotherapeutics contain detectable HCPs levels, the overall impact of HCPs on biologic's safety is rare. However, although there is no clear evidence of the clinical effects of HCPs, they should be monitored during the development and production of biopharmaceuticals.

5. Admissibility criteria established by regulatory agencies

The expiration of patents protecting innovative products has made possible the arrival of numerous similar biological products in the biopharmaceutical industry. However, before reaching the market, biosimilars must go through rigorous stages of supervision and meet the requirements of the regulatory agencies of the countries where the manufacturer is seeking to market its product. In this section, we will explore some of the requirements of the US and European regulatory agencies about the admissibility criteria for biosimilar products, addressing the suggestions of both institutions at the time of carrying out an application for approval.

5.1 EMA guidelines on immunogenicity assessment of biotherapeutics

The following information was obtained from "Guideline on Immunogenicity assessment of therapeutic Proteins", published by the European Medicines Agency on May 18, 2017 [80].

For nonclinical immunogenicity assessments, human and humanized therapeutic proteins will be recognized as foreign by animals; therefore, the predictability of these studies is low. In addition, nonclinical *in vitro* and *in vivo* studies to predict immunogenicity in humans are not normally required. However, according to this guideline, it is recommended to consider emerging technologies (*in vitro*, *in vivo*, and *in silico*) during development or as a first approximation of immunogenicity risk in the clinic. *In vitro* cell-based assays for testing innate and adaptive immune responses may be useful in elucidating cell-mediated responses. In addition, if the administered therapeutic protein has an endogenous counterpart, cross-reactivity reactions may also occur. In these cases, prior knowledge of the biological functions of the endogenous protein will be useful in predicting the therapy's safety risks.

Cell-mediated responses may have a major impact in those cases where unwanted effects or the biologic's pharmacodynamics may be mediated by cellular immune responses, for example, cytotoxic T-cell-mediated responses or delayed hypersensitivity reactions.

For biosimilar products, the comparative study of humoral immune responses (antibodies) as a comparability exercise is not recommended to be carried out in animals, because, as mentioned above, these studies show a low correlation with the potential immunogenicity in humans.

The analysis of antibody formation should be carried out using valid and sensitive experimental strategies. The response should therefore be studied using an experimental platform that includes a screening stage to differentiate those antibody-positive samples, a method to confirm the presence of those antibodies, and an assay to evaluate the specificity of those antibodies. Then, the guideline also recommends complementing the study by assessing the antibody-neutralizing capacity through cell-based or non-cell-based assays, depending on the biological effect exerted by the product. This experimental method should also be validated.

As mentioned above, it may also be required to determine the Ab cross-reactivity to endogenous proteins, especially in cases where the therapy safety and/or efficacy is compromised. In addition, due to the potential clinical consequences, for Ab-positive samples, the applicant should include further characterization, including the kinetic study of the Ab response, the intensity, and response duration. Also, the study should address the following aspects: Ab titer, Ab-neutralizing function, characterization of

Ab class and subclass, specificity, and affinity. In addition, other case-specific aspects may also be required.

On the other hand, other important aspects to be considered are product-related and process-related impurities, for example, HCPs. These entities can also induce the development of antibodies in the patient and should therefore be kept to the minimum level as possible. In this case, assays should be developed and validated to allow the detection of antibodies against these impurities in patient samples.

The guideline also suggests taking into consideration aspects related to the experimental platform chosen to detect antibodies in plasma and serum samples, to minimize the number of false positives cases and avoid false negatives, the epitope masking effect, matrix effect, and sample collection time, among other aspects. In addition, it also addresses aspects related to assay controls and reagents used, assay validation, and interpretation of the results.

Concerning the immunogenicity assessment of a biosimilar candidate, the EMA guideline states the following:

> *"Comparative immunogenicity studies are always needed in the development of biosimilars. Immunogenicity testing of the biosimilar and the reference product should be conducted within the biosimilar comparability exercise by using the same assay format and sampling schedule. The assays should preferably be capable of detecting antibodies against all epitopes of both biosimilar and reference molecules. If separate assays are used for the biosimilar and the reference product, this two-antigen assay approach requires careful validation to exclude any bias due to differences in sensitivity and drug tolerance. Demonstration of similar incidence of ADAs and a good concordance between the assays provides good evidence for comparable immunogenicity."*

5.2 FDA's considerations in demonstrating biosimilarity

The following is a summary of some contents extracted from the guideline for the industry: "Scientific Considerations in Demonstrating Biosimilarity to a Reference Product" published in April 2015 by the Food and Drug Administration (FDA) [81].

When demonstrating the biosimilarity of a therapeutic protein, the guideline suggests following a stepwise approach that includes, among several studies, the characterization of the clinical immunogenicity of the biosimilar candidate.

In this regard, the applicant should consider conducting a comparative analysis of the clinical immunogenicity, including the reference product and the biosimilar candidate, in an appropriate study population.

Similarly to the guidance published by EMA, the FDA guidance also indicates that immunogenicity studies conducted in animals may not predict the potential immunogenicity of therapeutic proteins in humans. However, the FDA guidance also acknowledges that antidrug antibody responses from animal studies may provide useful information on differences in immunogenicity between the reference product and the biosimilar candidate when both products are produced by different manufacturing processes.

Therefore, the clinical immunogenicity comparative study of a biosimilar product and the reference product should allow identifying potential differences as well as assessing the incidence and severity of immunogenicity events. The impact of immune responses in humans may have a direct incidence on therapy efficacy and safety. The observed effect may be variable and include alterations in the product pharmacokinetics and the development of neutralizing antibodies to both the

administered product and the endogenous counterpart if present. In more severe cases, anaphylactic reactions may occur. Thus, demonstrating no clinically significant differences in the immune responses induced by the two products is key evidence for the biosimilarity of the proposed biologic.

The immunogenicity study should include aspects related to the characteristics or profile of the immune response, as well as impact analysis of the immunogenicity event, that is, impact on the biologic's efficacy. In addition, the study should include an assessment of the incidence of such immunogenicity events and the population under study.

For a premarketing study, the guideline suggests performing a head-to-head analysis in patients who have not been previously treated with the biologic (treatment-naïve patients). However, depending on the clinical experience with the tested biologic, immunogenicity assessment in a subset of patients could also be requested. This type of study allows for obtaining more substantial results as well as it is useful to determine whether the administration of the biosimilar candidate leads to a higher risk of immunogenicity. This study population should be proposed by the applicant and accepted by the agency.

A relevant aspect to take into consideration is the selection of the clinical immunogenicity endpoint. For example, antibody development and cytokine levels should be monitored taking into account immunogenicity issues that have been observed during the use of the reference product. The applicant should define the criteria for measuring the potential immune response to the product and agree with the FDA on these criteria before starting the proposed study.

In addition, another issue to be agreed upon with the agency will be the length of the study, which will depend on the following factors:

- The time of the development of humoral immunogenicity (neutralizing antibodies) and cellular immunogenicity events, as well as the possible clinical consequences, which will be reported from the sequelae observed from the use of the reference product.

- The time until the disappearance of such immunogenicity events and the sequelae observed after completion of therapy.

- The treatment duration with the product.

Regarding the characteristics of the antibody-mediated immune response, the FDA guidance states the following:

- "Titer, specificity, relevant isotype distribution, time course of development, persistence, disappearance, impact on PK, and association with clinical sequelae.

- "Neutralization of product activity: neutralizing capacity to all relevant functions (e.g., uptake and catalytic activity, neutralization for replacement enzyme therapeutics)".

The assays proposed by the applicant should include the candidate biosimilar product and the reference product. Whenever possible, both products should be tested under the same conditions, that is, in the same assay and with the same patient sample.

Finally, as mentioned above, the development and validation of these assays should be performed at the early stages of development, consulting with the agency on the sufficiency of the study before starting the immunogenicity clinical trials.

6. Conclusion

Over the last decades, therapeutic proteins have been used for the treatment of numerous chronic and non-chronic diseases, such as cancer, autoimmune diseases and disorders, diabetes, and infectious diseases. Biologic's manufacturing process is complex and therefore requires rigorous checkpoints to ensure the required quality of the final product and batch-to-batch consistency. Among the quality control requirements, product immunogenicity stands out as a critical attribute. Thus, regulatory agencies have established guidelines that allow this analysis to be approached in a careful and concerted manner. The expiration of multiple patents protecting innovative products has allowed the arrival of numerous biosimilar candidates. Similarly to reference products, biosimilars must meet different requirements during the comparability exercise. Thus, the biosimilar immunogenicity assessments should be addressed comparatively with the reference product. This study should cover aspects, such as the presence of contaminants and impurities, that induce innate immune responses and the characterization of potential adaptive immune responses in the patient. To expedite this task, different experimental platforms are currently available and allow for predicting the potential product immunogenicity before reaching clinical trials. Finally, the advent of technologies, such as single-cell sequencing, the development of micro-organoids to mimic the human immune system, and the development of *in vitro* models of human diseases, will provide more precise tools to ensure more effective and safer biologics.

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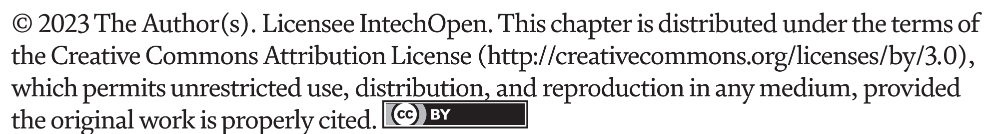
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