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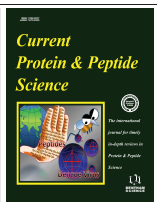
REVIEW ARTICLE

Immunogenicity in Protein and Peptide Based-Therapeutics: An Overview



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Abstract: Currently it is well known that all biological drugs, including those with a fully human structure, are capable of inducing a host immune response known as immunogenicity [1]. The presence of ADAs can condition the drug´s level and action, thus modifying the therapeutic effect and even the safety profile by its mechanism of action - neutralizing or non-neutralizing - and / or an increase in its clearance. Immunogenicity is a dynamic factor to be taken into account in biological therapy, especially in long-term treatments, and as a relevant aspect in the assessment of secondary response loss [2]. With the above, not only the knowledge but also the management of the immunogenicity of the different biological treatments, represent a useful instrument for optimization of the strategies of use for each drug, and in the design of predictive models of response, which finally permits a significant improvement in the efficacy and safety profile, aiming to a personalization of the therapies, especially in patients with autoimmune diseases, genetic disorders and cancer [3]. This review summarizes the events of immunogenicity that produce the biological drug, the factor that influence to immunogenicity and the assessment of immunogenicity.

Keywords: Immunogenicity, biosensor, peptide, biological drugs, anti-drugs antibodies, ADAs.

1. INTRODUCTION

Biological drugs are known as those produced by biotechnology, through living organisms; some of these are hormones, cytokines, growth factor (GF), antibodies (Ab), and Ab fragments, among others; and have been used for the treatment of multiple pathologies in medical fields such as rheumatology, dermatology and cancer [4]. Biological products are partially or completely chemically constituted by amino acids that are linked to each other forming proteins. Although they were initially conceived to supply failures of some endogenous proteins such as insulin or erythropoietin [5, 6] and for diagnostic uses currently, its potential for therapeutic use is enormous given the unlimited repertoire to create protein structures that interact in a specific way with receptors and target molecules in the body, thus modulating the body's response to various pathologies.

The human body consists of a large number of proteins, each of which has the potential ability to stimulate the immune system; however, through tolerance generation mechanisms during the early stages of life, the immune system learns to recognize such molecules as their own, and only when these mechanisms fail, autoimmune pathologies are produced and can have serious consequences for health. An example of this is the severe anemia that occurs when the immune system attacks the body's own erythropoietin [4, 7].

1.1. Events that Trigger Immunogenicity

Immunogenicity is understood as the ability of a molecule (usually exogenous) to trigger a reaction by the patient's immune system, be it humoral or cellular [8, 9]. The use of proteins and peptides in the treatment of disease is justified by its high specificity to recognize a particular receptor, a characteristic given in large part by its three-dimensional structure, molecular size and charge. However, the protein nature and chemical composition of a biological product can, potentially, trigger a response by the host's immune system to eliminate it, when it recognizes it as a foreign body [7]. Given the above, it is considered that the greater the identity with own molecules, the lower the risk of presenting immunogenicity, an inverse proportion. However, the intrinsic structural properties of the biological molecule such as the protein sequence, the different post-transductional modifications and the possible presence of neo-epitopes [10, 11] may latently give rise to particular responses, eventually undesirable, with potential affectation of the safety / efficacy parameters of a specific formulation.

In general terms, there is great uncertainty about the incidence of immunogenicity according to its origin and / or class (humanized or chimeric drugs, plasma derivatives or recombinant); therefore, the approach of its study must be

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carried out in an integral manner, taking into account three relevant aspects: immunochemical characterization, related clinical research and population characteristics. It is estimated that other factors related to the development of immunogenicity are the characteristics of the patient, the therapeutic regimen (dosage) and associated immunosuppressive treatments [9, 12].

Antigen-presenting cells (APC) such as Dendritic Cells (DC), Macrophages and B Cells, play a very important role in the formation of immunogenicity or tolerance (allergy) towards the therapeutic protein in the body, since they are the ones which in the presence of the Major Histocompatibility Complex Type II (MHC II) present antigens to $CD4^+$ T lymphocytes by activation of these [13-15]. These last orchestrate the sustained immune response, including the proliferation of B cells and their differentiation to Plasma B cells, for the production of the various isotypes of Ig, especially IgG, the most abundant in the blood. It should also be mentioned that the production of Ab by B cells can be independent from T cell stimulation, in which case IgM is produced, an Ab of early onset in the immune response [7].

All biological drugs, given their generally protein nature, have the ability to produce immunogenicity, that is to say, they have the ability to stimulate the patient's immune system [4, 16], allowing for example, the appearance of Antidrug Antibodies (ADAs) [9, 17]. This situation may lead to a decrease in the efficacy of the active ingredient, in between 30% and 50% of the patients [18] and to modify their safety profile making it necessary to adjust the dosage, to include or exclude other drugs, and even completely change the treatment, thus impacting the quality and costs of therapy [7, 8]. In addition, immunogenicity may be manifested by severe acute allergies (immunoglobulin E (IgE)-mediated type I severe reactions) including hypotension, bronchospasm, laryngeal or pharyngeal edema, wheezing, urticaria and even anaphylactic reaction and generation of autoimmunity in the patient by predisposing the body to not recognize the endogenous proteins as their own [4, 5, 7, 19]. In addition, may be manifested by complement activation, tissue inflammation and leukocyte hypersensitivity [20].

Appearance of allergic reactions has been associated with two fundamental factors: the first one is the therapeutic use of drugs whose purification or storage processes have been deficient, giving way to the possible formation of protein aggregates by losing their secondary, tertiary or quaternary structure [18]. Aggregate formation can also occur during the administration of the drug, when it is exposed to liquid-air or liquid-solid interfaces, very strong agitation or heating by the patient or health team, and even waste of rubber or silicone from the stoppers of the vials or from the infusion pumps, can favor the development of immunogenicity [16]; The formation of amyloid-like aggregates in proteinaceous pharmaceutical products due to conformational changes, has been described. It may be of importance in the development of immunogenicity because of the stimulation of receptors in dendritic cells such as: scavenger receptor (SR) class B member 1 (SR-B1) and class A member 1 SR-A1, receptor for advanced glycation end products (RAGE) , low density lipoprotein receptor-related protein 1 (LRP1) and cluster of differentiation 36 (CD36) [17].

The second factor refers to the use of proteins from species other than human [17]. However, it is important to point out that currently, the incidence of these factors at the therapeutic level has been reduced, due, on one hand, to technological progress in the production and purification of proteins from living beings, as well as correct storage and transport practices; and on the other hand, to the increasingly wide range of proteins with an increasingly higher proportion of similarity to the human species, which include their protein conformation and in some cases glycosylation patterns. However, it is found that the presence of ADAs favors the appearance of allergic reactions at the time of administration, where for Infliximab, 50% of the suspension of treatment is due to reactions at the time of perfusion when there is ADAs present, compared to 2% when it is not present [5, 9, 12, 18].

Since the drug-receptor interaction (which triggers the pharmacotherapeutic action) may be affected by the immune system's response to the drug, it becomes necessary to know more in depth the mechanisms of immunogenicity that may occur during the various treatments, allowing an adequate adjustment of these, and finally leading to an optimal outcome in the management of the disease by the health team. Fig. (**1**) shows some factors that influence the immunogenicity in biological drugs.

1.2. Therapeutic Antibodies

Ab (Immunoglobulins- Ig) are part of the humoral and adaptive immune response of mammals, chemically described as glycoproteins, composed of two identical heavy chains between 50 and 70 KDa, and of two light chains of 25 KDa; disulfide bonds mediate the binding of these chains. The enzymatic degradation of Ig with papain produces two fragments capable of recognizing antigens, called the Antigen Binding Fragment (Fab) and a fragment called crystallizable (Fc); In the Fab portion are found the hypervariable regions (HVR) which determine the specificity of antigen binding that are also called Complementarity-Determining Regions (CDRs), while the Fc portion fulfills functions such as complement fixation and binding to immune cell receptors [9]. And under normal conditions, they have the ability to recognize and bind to exogenous substances (foreign to the body), with the aim to eliminate them through several mechanisms such as direct neutralization, signaling to facilitate their phagocytosis, or complement activation. There are multiple Ab types, with IgG being the most abundant [8].

The first Ab used with therapeutic purposes was of murine origin (-momab) and about 90% of the patients treated with it developed Ab to these human antimouse antibodies (HAMA). Later technological developments allowed the appearance of molecules such as Rituximab, in which the constant regions of these Ab were replaced with human fragments and were called chimeric monoclonal antibodies (cAb) (-ximab). However, Ab against these drugs human antichimeric antibodies (HACA) were also identified, although to a lesser extent than in the case of Murine Ab [24]. It is now known that even humanized (-zumab) and fully human (-[m]umab) Ab, can also stimulate the patient's immune response due to variability at the idiotypes level to produce human antihuman antibodies (HAHA). This demon-

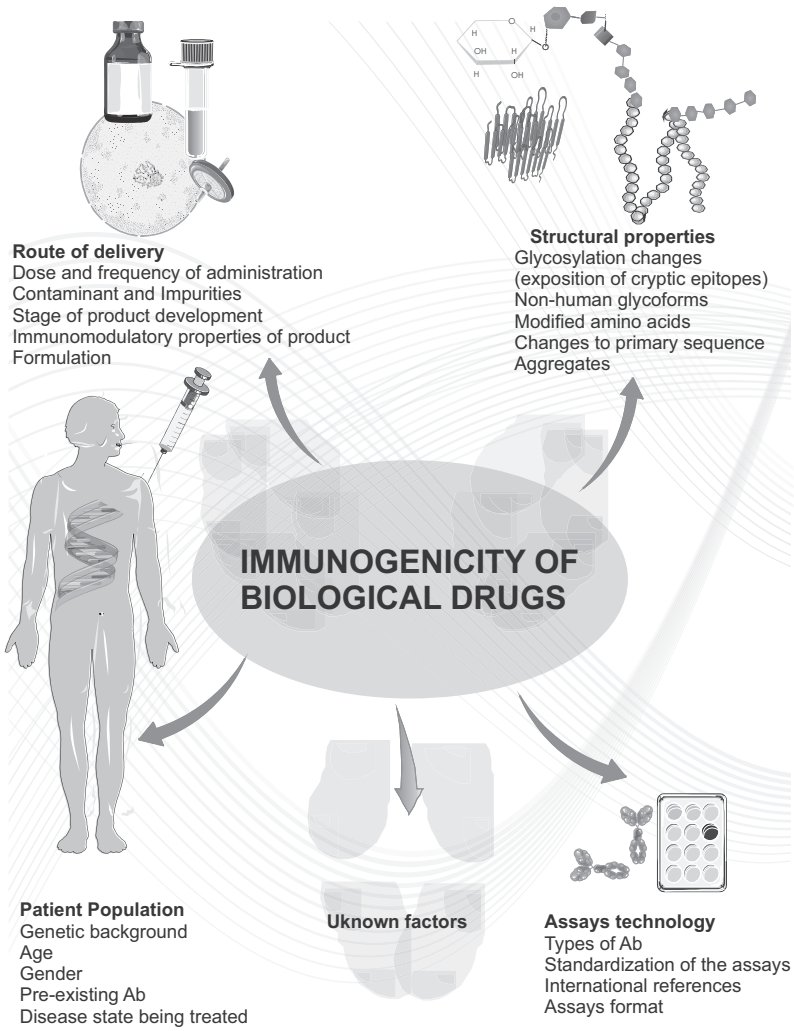

Fig. (1). Factors that influence the immunogenicity of biopharmaceuticals [21, 22]. Vector graphics is licensed under Creative Commons Attribution 3.0 Unported License [23].

strates the capability of the immune system to recognize the self from not self, even at the same species level, and that the most immunogenic epitopes are the ones carried in the CDRs of Fab regions [4, 7, 9].

Proteins analogous to endogenous proteins, Ig, fusion proteins, conjugated Ab with active principles and PEGylated proteins, have been developed by means of biotechnological processes. The most used Ab in therapy are IgG, especially IgG1 subclass, and to a lesser extent IgG2 (Denosumab) and IgG4 (Natalizumab). Other types of proteins have also been designed as the fusion proteins in which the Fc portion of IgG is bound to receptor´s parts, in order to trap their respective ligands so that they do not act with the true receptors; examples of these are Etanercept: Recombinant tumor necrosis factor receptor p75 fusion protein (TNFR:Fc), Abatacept: Cytotoxic T-lymphocyte-associated protein 4 (CTLA4-Fc) chimera and Rinolacept: Recombinant Human Interleukin-*1* receptor type 1 (IL-1R-Fc) chimera; Among the group of receptor antagonists, is Anakinra (receptor ligand for IL-1) [9].

In order to reduce the patient's immune response to exogenous molecules, it has been tried to emulate, each time with greater fidelity, the conformation of the body´s molecules (endogenous); An example to be cited in the treatment of rheumatoid arthritis (RA), is the one provided by the molecules Adalimumab, which is a fully human monoclonal antibody (mAb), that has shown to generate less immunogenicity than Infliximab, an chimeric monoclonal Antibody (cAb) type ([25, 26]. Another case is Etanercept, a fusion protein in which less immunogenicity has been described than its therapeutic analogues Infliximab and Adalimumab, and even non-detectable Ab levels [27].

The occurrence of immunogenicity during the administration of the biological products is determined by drug related factors, such as their peptide sequence (*i.e*., in the case of Ab may vary depending on their origin being cAb, humanized or fully human), the glycosylation degree, the presence of aggregates [28], formulation, impurities, formation of immune complexes, action of the immune system, and factors related to the patient or therapeutic scheme such as tolerance to the molecule antigens, pathophysiology of the disease, associated drugs, dose, administration frequency, route of administration, individual pharmacokinetics and genetic characteristics [7-9, 16, 29, 30].

ADAs generation in the patient treated with biological products can lead to two types of mechanisms of action, one that runs by neutralizing (NAb), and another by non-neutralizing (non-NAb); the neutralizing mechanism implies that ADAs recognize as antigens the drug domains related to receptors, by inhibition of the drug-receptor interaction, so the therapeutic effect decreases. On the other hand, non-NAb have the capability to bind to the drug in domains that are not important for the development of their therapeutic activity, and appear to have no biological effect on the patient beyond the reduction of the therapeutic effect [5]. Both mechanisms tend to accelerate the drug elimination in the Reticuloendothelial system (RES) once the drug-ADAs complex is formed, thus decreasing the half-life and serum concentration of the drug [9]; IgG1 has a half-life of 3 weeks, while immune complexes are eliminated much faster from the body [4]. The formation of ADAs has been described even in cases in which the peptidic drugs are completely human; that is possibly due to the polymorphisms of these molecules [8].

ADAs have a deep impact on the efficacy and safety of the drug: In terms of safety, this is especially important when working with therapeutic protein products with endogenous analogues in a patient, because the risk of appearance of cross-reactivity is possible. For example, administering erythropoietin-analog proteins may generate autoimmunity against the latter, potentially leading to erythroblastopenia (pure red cell aplasia) [17]. Similar problems can be expected when administering proteins analogous to Granulocyte Colony Stimulating Factor (GCSF), Thrombopoietin (THPO) and Growth Hormone (GH), among others. In therapeutic proteins that do not have an endogenous part, the risk of autoimmune generation is lower, and in these the loss of efficacy by the formation of ADAs is the main problem [16]. Loss of efficacy in the treatment of relapsing-remitting multiple sclerosis (RRMS) has been related with recombinant human interferon beta (rHuIFN-beta), by ADAs formation, and that the intensity in the formation of these depends on the used brand of the product (in example CinnoVex® , Rebif®, Avonex®, or Betaferon®), possibly due to differences in glycosidation and amino acid sequence [28, 30].

1.3. Assessment of Immunogenicity: Analytical Aspects

Currently the measurement of ADAs in biotherapeutic products, constitutes the pillar of the approach for the study of immunogenicity. Thus, the detection and detailed characterization of the above mentioned ADAs, will allow to optimize the understanding in relation to the potential impact as for the efficiency and safety of the molecule object of study [31]. There is no test at the moment that provides all the necessary information to define a specific immunogenicity profile [32]. Therefore, the conception of a bioanalytical strategy has been necessary, one that includes in a sequential way the accomplishment of a test panel. The most widely accepted methodology includes in the first place the accomplishment of a screening test that can assess the capacity of the Ab to binding biotherapeutic proteins. Thereafter, a test to determine the NAb capacity, data which will be analyzed in the light of pharmacokinetic / pharmacodynamic parameters, and magnitude of the biological effect in patients [31, 33]; as well as the risk profile that will allow to establish the rigor in the time parameters for the sampling.

With this in mind, a careful, detailed and well-defined sample collection plan, that includes initial sample, serial collection of samples during treatment and sample posterior to total clearance of the biotherapeutic drug, will allow access to measurement data whose values of sensitivity and specificity are sufficiently robust to recommend their serial use in the usual clinical practice, in accordance to the high quality standards defined in the World Health Organization WHO Guidelines for Biological Medicines [34]. Determination of the presence or absence of Ab directed to therapeutic proteins, based on the ability to recognize antigenic determinants, involves a great challenge given the difficulty in establishing the correlation between their levels and their clinical impact, as well as the enormous variability that these Ab can show in terms of isotypes and affinity between different patients, and even in the same patient, at different administration times [25].

Formation of ADAs is a measure of the immunogenicity produced by a drug, and therefore is of particular interest to regulatory authorities, becoming one of the main criteria to determine the immune response to biological products. Currently, due to the polyclonal characteristic of the immune response, there is no unified methodology for ADAs determination, and the drug presence increases the difficulty for detection, because being this last one the binding molecule of these Ab causes its free concentration in the samples to decrease, thus lessening their quantification. Immunological methods such as ELISA (Enzyme Linked Immunosorbent Assay), Bridging Immunogenicity Assays, radioimmunoassays and Electrochemilumenescense (ECLA), are used as detection techniques for ADAs [8, 35]. None of these techniques has the ability to recognize the various isotypes of Ab that the immune system can generate (in example IgA, IgD, IgE, IgG, and IgM) as well as allotypes. The comparability between techniques is low because of their differences in specificity and sensitivity [9]. Another factor that hinders the detection of immunogenicity, lies in the fact that this is a gradual process that develops and changes over time [4]. It is necessary to carry out treatment monitoring that includes the measurement of the decrease of the pathology, as well as verification of levels of the drug and ADAs, to be able to make the treatments more effective.

As an alternative technique to immunoassays, works with surface plasmon resonance (SPR) have also been done to determine ADAs, demonstrating specificity to distinguish between different HAHA types during treatment development [24, 36, 37]. Table **1** summarizes the main analytical methods used for the measurement of therapeutic levels of drugs of biological origin, ADAs and non-ADAs.

Immunogenicity is a problem of great importance for health agencies in such a way that they require, during clinical trials, verification of immune response development towards the product, and the monitoring of patients during the commercialization of the drug [29, 38, 39]. Several tools have been developed to predict the immunogenicity of therapeutic proteins before they are released to the market, including preclinical models *in silico, in vitro* and *in vivo*, but so far none of them has been able to completely predict un-

Table 1. Analytical methods for the measurement of therapeutic levels of biological drugs.

Technique	Advantage	Disadvantage	Biological Drugs
ELISA Direct / Indirect [32, 54, 55]	Easy to use / Automated High performance High therapeutic tolerance Low cost	Non-specific Binding Large (distortion) background Failure in detection of low affinity Ab Requires secondary reagent species	Adalimumab [56-59] Infliximab [57] Tocilizumab [60] Other drugs [55]
Bridging ELISA [61]	Easy to use / Automated High performance Low noise / High therapeutic tolerance in solution phase High specificity (double arm attachment)	Required Antigen labeling Failure in detection of low affinity Ab Highly susceptible to interference by drugs and serum components Failure in IgG4 detection	Adalimumab [62-64] Metuzumab [60, 65] Other Ab [63, 66]
Electrochemiluminiscence [67]	High performance High dynamic range Minimal affection for the matrix High drug tolerance in solution phase Consistent signal detection	Possible requirement of 2 antigenic conjugates Required Antigen Labeling Susceptible to interference by drugs and serum components Failure in IgG4 detection High price	Fulranumab [68] Beta-Interferon (IFN-ß) [69] Trastuzumab [70] Other Ab [71-73]
Radioimmuno-precipitation assay	Moderate performance High sensitivity Moderate / high specificity Low cost	Isotype specificity Failure detection of low affinity Ab Requirement of radiolabeled antigens Radiolabeled decline may affect antigenic stability	Other Ab [73-80]
Surface Plasmon Resonance [32]	Automated Determination of specificity, isotype and relative affinity of binding Easy High and Low Affinity Ab Detection Marker detection not required	Immobilization / Antigenic Regeneration may affect performance Decreased sensitivity to binding assays High price	Adalimumab [81] Trebananib [82] Tocilizumab [82] Other Ab [83-88]

wanted immune reactions, which is why it is necessary to combine them to obtain more reliable results [16, 18, 29, 40-42].

Such immunogenicity studies, which are mostly predictive, require a rigorous validation process to establish an adequate correlation level with clinical results, and also determine feasibility to extrapolate the findings [43, 44]. In this sense, some studies have shown a good correlation (*i.e.,* different formulations of beta interferon) [45]. However, others have not achieved the desired goal [3, 11]. Among these three models, *in vivo* has special acceptance since animals such as mice and primates have a complete immune system, and additionally allow the evaluation of the impact of the administration route, among other uses. For example, the existing mouse strains, including the transgenic ones, allow to diversify these tests such as the use of C57Bl/6, FVB/N mice and crosses of these to study the development of tolerance to various rhIFNβ [30]. However, this technique´s limitation lies in the phylogenetic distance that mediates between the human being and the animal models of experimentation, which does not allow to draw definitive conclusions about the behavior of the immune system, due to specific-species differences in antigen recognition, reactivity of lymphoid and non-lymphoid tissues, and systemic coordination of immunity at the level of the whole body [16, 18, 20]. An example of the difference in immune response between species was found during the development of TGN1412 protein, a humanized mAb with possible pharmacological activity in the treatment of chronic B cell lymphocytic leukemia, which showed preclinical efficacy in mice, but when administered in the first study in humans, triggered an adverse reaction known as a cytokine storm that was not evidenced with experimental animals. Among the developed symptoms were systemic inflammatory response, headache, myalgia, nausea, diarrhea, erythema, hypotension, pulmonary infiltration, renal failure and disseminated intravascular coagulation, making necessary the use of mechanical ventilation, dialysis and plasma infusion [46]. Table **2** shows the associated protein and metabolism of biological drug/peptide using different models.

Several software programs have been developed with the aim to perform *in silico* studies of proteins with therapeutic potential, which try to determine the amount and position of their possible epitopes that can bind to the type II Human Leukocyte Antigen (HLA), and thus stimulate the immune system through T cells. Examples of these are: iTopeTM, TCEDTM, EpibaseTM and EpiMatrixTM. *In vitro* assays also seek to identify potential immunological responses, and as prediction tools, products such as EpiScreen ™, Epibase ™ and REVEAL® can be found [16, 29].

1.4. Clinical Aspects of Immunogenicity

Given the potential decrease in the pharmacotherapeutic effect due to ADA production, it is considered important to

Table 2. The clinical manifestation regarding biological drugs/peptide.

Pathophysiology	Peptide	Associated Protein / Metabolism	Model	Refs.
Bladder cancer	Hybrid Peptide Bld-1-KLA	Bld-1 (CSNRDARRC peptide): Selective binding to HT1376 bladder tumor cells KLA (peptide D -KLAKLAKKLAKLAK): Mitochondrial membrane disruption and induces apoptosis	HT1376 (human bladder tumor)	[89]
Acute myocardial infarction	Atrial Natriuretic Peptide (ANP)	The modification of Renin-Angiotensin-Aldosterone System (RAAS), involved with myocardial remodeling, inhibits the release of Renin in the kidney and the synthesis of Aldosterone in the adrenal gland. Inhibits apoptosis in cardiac myocytes of rat. ↑ cyclic guanosine 3′,5′-monophosphate (cGMP) levels and activation of the phosphatidyl inositol 3′-hydroxy kinase /AKt complex (PI3K/ Akt1) pathway.	Cardiac myocytes of rat Rat heart Retrospective study of cohorts in humans	[90, 91]
Cancer Treatment	P39	Inhibits the interaction between the serine/threonine specific protein kinase (CK1δ) and α/β-tubulin, modifying the mitosis process	HT29 Human colorectal adenocarcinoma cell line. HeLa Cervical cancer cell line. CV-1	[92]
Doxorubicin release system	RHD/p53	RHD: reducible branched cationic polypeptides (RBCP) crosslinked with the cationic peptides $(CRR)_2KRRC$ and $(CHH)_2KHHC$ and fixed Doxorubicin by hydrazone bridges. Antitumor effect of p53: tumor suppression gene. The $(CRR)_2KRRC$ can penetrate the cell, the RHD complex, upon entering the endosome releases Doxorubicin	NIH3T3: Mouse Embryo Fibroblasts HeLa: human cervix adenocarcinoma	[93]
Antimicrobial action against *Mycobacterium tuberculosis*	$1\text{-}C13_{4mer}$	Surfactant character. Disruption of the outer membrane rich in waxes	RAW 264.7 and J774: Mouse macrophages cell lines	[94]
Broad spectrum antibiotic against pathogens Gram +	Daptomycin	13-amino acid (AA) lipopeptide: the C-terminal portion consists of a ring of 10 AA and an exocyclic 3 AA chain whose terminal AA (Tryptophan) is attached to an acyl decanoate molecule. Interaction with Ca^{++}-dependent cell membrane phospholipids; Formation of pores releasing K^+ and depolarizing the membrane. Bactericidal activity against Gram positive bacteria. Example: Use in *Staphylococcus aureus* bacteremia and Vancomycin-resistant *Enterococcus* infections.	*Enterococcus faecium*	[95-97]
Chronic pain management in cancer and AIDS	Ziconotide (Prialt®)	Synthetic equivalent of *Conus magus* venom ω-MVIIA; Blockade of voltage-sensitive N-type calcium channels (N-VSCCs) by inhibiting pain-sensitive primary nonceptors. At the level of the posterior horn of the spinal cord ↓ the release of pronociceptive neurotransmitters.	Behavior models in acute and chronic pain in rats (intrathecal). Intranasal for the treatment of chronic pain in Sprague-Dawley rats	[98-101]
Regulation of the response to acute inflammation	EAK16-I plus methionine and ethanol 2%	Peptides and amino acids self-assembling platform. EAK16-I (EAKAEAKAEAKAEAK). Used to intravenously release PP2, Src inhibitor of Family of Proteins Tyrosine Kinases (PTK) related to acute inflammation response regulation	In situ pulmonary ischemia-reperfusion model in male Sprague-Dawley rats	[102]

(Table 2) contd….

Pathophysiology	Peptide	Associated Protein / Metabolism	Model	Refs.
Microenvironments generation in the myocardium (cell recruitment)	RAD16-II	RAD16-II (AcN-RADADDRAND-CNH2). Upon injection of the peptide solution, a nanofibers microenvironment is formed which allows the recruitment of progenitor cells expressing endothelial markers as well as vascular smooth muscle cells and cells expressing sarcomeric α-actin and transcription factor Nkx2.5. Injecting the solution of self-assayable peptides with cardiomyocytes, it was reported that these survived and ↑ endogenous cell recruitment	Male adult C57BL/6 mice aged 8 to 10 weeks	[103]
Irritable bowel syndrome with constipation	Linaclotide	Synthetic peptide of 14 amino acids; Inhibitor of cystine knots; Belongs to a structural family of ultra-stable peptides generally between 30 and 50 amino acids with a tertiary structure with at least three disulfide bridges. Agonist of guanylate cyclase C; ↑ the level of cGMP, ↑ fluid secretion and ↓ visceral hypersensitivity, thereby accelerating gastrointestinal transit	Phase 3, randomized placebo-controlled, double-blind, parallel-group study. 804 patients, mean age: 44 years; Female: 90%; White: 78%	[104-106]
Treatment for Chronic Myeloid Leukemia	MTAbl06, MTAbl07 and MTAbl13	Modifications of the cyclotide peptide oMCoTI-II (*Momordica cochinchinensis* trypsin inhibitor II, extracted from the seeds of *Momordica cochinchinensis*) Incorporating bioactive molecules such as those against ABL kinase (substrate-based kinase inhibitors) B	HeLa Cervical Human Cancer Cells	[104, 107]
Angiogenesis Inductor	SFTI-1	Sunflower Trypsin Inhibitor-1 (SFTI-1); Cyclic peptide Cyclotide of 14 amino acids to which peptides with potent angiogenic activity were incorporated as an heptapeptide of Osteoponine (OPN) which interacts with α9β1 and α4β1 integrins by regulating hematopoietic stem cells and progenitor cells. The SFTI-OPN binding was shown to be stable in human serum and active *in vivo* at nanomolar concentrations.	*In vivo* test with chorioallantoic membrane of fertilized quail eggs	[108]
Cervical Cancer Vaccine (Human Papilloma Virus HPV)	E6 43–57 and E7 44–57	Vaccine based on peptide subunits E43-57 (QLLRREVYDFAFRDL) and E744-57 (QAEPDRAHYNIVTF): epitopes for $CD4^+$ T and $CD8^+$ T from HPV E6 and E7 oncoproteins which are conjugated to a polymeric release system (tert-butyl acrylate polymer). Stimulation of the individual's immune response via $CD4^+$ helper and $TCD8^+$ T lymphocytes to kill cancer cells.	C57B1 / 6 (6-8 weeks old) female mice inoculated with TC-1 cells (C57B1 / 6 murine lung epithelial cells transformed with HPV-16 E6 / E7 oncogens and ras)	[109]
Treatment of Alzheimer's disease	$TxA\beta_{36-42}$	TxAβ36-42: C-terminal amyloid-β motif conjugate peptide (VGGVVIA) with the antioxidant Trolox (aromatic portion of vitamin E). Therapeutic action of two indicators of Alzheimer's disease (AD) at the cerebral level: formation of peptide aggregates of variants amyloid-β (cytotoxic) and oxidative damage (antioxidant effect against reactive oxygen species of Trolox).	Human neuroblastoma cell line SHSY5Y	[110]
Antibiotic against Gram positive bacteria resistant to other drugs	Vancomycin	Glycopeptide (C66H75Cl2N9O24). Hydrogen bonding to the terminal portion D-Ala-D-Ala (positions 4 and 5) of the peptide chain of lipid II by avoiding coupling to Penicillin-binding Proteins (PBPs) by inhibiting transpeptidation reactions and Transglicylation required for the formation of the bacterial cell wall	*Enterococcus spp; Staphylococcus spp*	[111]

(Table 2) contd….

Pathophysiology	Peptide	Associated Protein / Metabolism	Model	Refs.
Antibiotic against Gram-positive bacteria resistant to other drugs; Food preservative; Oral hygiene; Treatment against bovine mastitis.Antibiotic against Gram positive bacteria	Nisin	Lantibiotic. (I-Dhb-AI-Dha-LA-Abu-PGAK-Abu-GALMGANMK-Abu-A-Abu-AHASIHV-Dha-K) Cyclic peptide produced by Lactococcus lactis (Gram-positive bacteria) at ribosomal level with post-translational modifications such as formation of thioether linkages and proteolytic processes that activate it. They contain sites of attachment to Lipid II (important precursor in the synthesis of the bacterial cell wall) avoiding the formation of the cell wall; Also forms lipid pores of II-peptide in the cellular membrane causing death by lysis.	*Streptococcus pneumoniae, Staphilococcus aureus, Enterococcus faecium, Enterococcus faecalis, Mycobacterium tuberculosis* H37Ra, *Mycobacterium avium, Mycobacterium kansasii* CIT11/06 , *Listeria monocytogenes*	[111-116]
Antibiotic against Gram positive bacteria resistant to other drugs	Plectasin	Defensin of fungal origin (Pseudoplectania nigrella); Contains an α-β motif consisting of a helix α and two antiparallel β strands stabilized by three disufuro bridges (Cys4-Cys30, Cys15-Cys37 and Cys19-Cys39); Sharing these characteristics as well as the amphipathic cationic character. They contain sites of attachment to Lipid II (a major precursor in the synthesis of the bacterial cell wall) avoiding the formation of the cell wall	*Streptococcus pneumoniae, Staphylococcus aureus, Streptococcus suis*	[111, 117, 118]
Melanoma Treatment (Skin Cancer) Testicular cancer treatment	Bleomycin (BLM)	Glycopeptide. It has endonuclease activity. The active form of BLM requires a reduced transition metal (Fe (II) or Cu (I)), oxygen and a one-electron reductant; The active BLM is inserted into the DNA strand or attached to the minor strand of the strand and removes the hydrogen atom from the 4 'carbon of the deoxyribose linked to a pyrimidine causing the DNA to break in a single strand or both. It exhibits low myelosuppression and low immunosuppression during the treatments.	A multi-institutional prospective study, 253 patients from 23 institutions in Australia and New Zealand with prognosis of germ cell carcinoma. 218 patients from 20 institutions in Australia and New Zealand with prognosis of germ cell carcinoma.	[119-122]
Colorectal Cancer Treatment Treatment against *Salmonella typhimurium* and *Mycobacterium tuberculosis* infection	Interleukin 24 (IL-24) MDA-7 (Melanoma differentiation-associated gen 7)	Glycoprotein, Cytokine belonging to the IL-10 family, secreted mainly by activated monocytes and T cells; contains three N-glycosylation consensus sites (N85IT, N99VS and N126RT). Media induction of Th-1 cytokines such as IFN-γ and TNF-α, as well as IL-6, IL-1, IL-2 and granulocyte and macrophage colony-stimulating factor (GM-SCF) in mononuclear cells of Peripheral blood (PBMC). It stimulates neutrophils to produce IL-12, IFN-γ by activating CD8 + T cells protecting against bacterial infection; Also stimulates NO production.	Male BALB / c mice; Inoculated with CT-26 mouse colon cancer cells. Male BALB / c and C57BL76 mice (in *Salmonella typhimurium* infection) BALB / c mice; *Mycobacterium tuberculosis* H37Rv	[66, 123, 124]
Mycobacterium tuberculosis vaccine	M72/AS01$_E$	System composed of the recombinant fusion protein M72, derived from the proteins Mtb32A and Mtb39A of M. tuberculosis and the adjuvant system AS01E. Development of humoral and polyfunctional immunity mediated by CD4 + T cells	Randomized controlled phase II study (n=80), adults previously treated for tuberculosis (n=49) and adults who completed the intensive phase for the treatment of tuberculosis (n=13)	[125]

take action to try to reduce immunogenicity in patients. Strategies to achieve this goal include: modifying drug administration, increasing the drug dose, or administration of immunosuppressive therapies to decrease the body's capability to produce ADAs [16].

Undoubtedly, biological drugs have revolutionized the standard of care for a large number of both chronic and acute clinical conditions with life-threatening potential to patients [47], such as blood neoplasms, solid tumors and autoimmune diseases such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and psoriasis (Ps). Despite the great clinical revolution they have imposed since their introduction to medical practice, they are not risk-free medicinal products, and immunogenicity should

be taken into account among the most relevant in terms of impact, understood as the ability to affect efficacy and / or safety. The presence of immunogenicity is given by the presence of ADAs in plasma, following the administration of a biological drug [48]. Within the ADAs should be considered those that bind to the site of biological therapeutic target or target, identified as NAbs, and those that do not interfere in their biological / physiological effect, known as non-Nabs. However, in spite of not interfering with the mechanism of action, it may compromise therapeutic efficacy by decreasing bioavailability, even leading to induce in some cases, therapeutic failure [22, 49].

However, such Ab presence does not strictly imply affections to the efficacy or safety in their use; in fact, hypersensitivity reactions within the use of this group of drugs are relatively uncommon, and in addition, those Ab may have cross-reactions with endogenous proteins and / or tissues, triggering deficit syndromes (cell aplasia, coagulation factors deficit and cytopenias) Cellular Defects, Coagulation Factor Deficiencies, and Cytopenias) [50]. In terms of immunogenicity determinants, multiple factors influence their development, considering 3 broad categories: associated to treatment, associated to the patient and associated with the medication itself, whose details are summarized in the following Table **3** [51].

All the factors listed above should be taken into account in the risk-based approach, when planning and designing studies to assess immunogenicity; all of which with particular relevance in molecules with multiple therapeutic domains [52], since it is of utmost importance, in order to adequately assess the risk of generating a host immune response, the potential severity of the induced response, and the risk / benefit balance in a previously determined study population [53]. Unfortunately, and with the currently available study models (*in silico, in vitro, in vivo*), the ability to assess, predict, and mitigate the risk of immunogenicity with broad sufficiency, is still remarkably limited [51].This explains why its clinical usefulness and the correlation with the physiological effect evident in the patient, still cannot be clearly defined.

It has been reported that the subcutaneous (SC) route of administration may produce greater immunogenicity than oral and intravenous (IV) routes [4]. Possibly because in the SC path there is a longer contact time between the drug and cells of the immune system such as DC, as well as greater probability of formation of protein aggregates that are concentrated at a specific place in the body; while IV route allows rapid dilution of drug contaminants into the bloodstream and may facilitate the dissociation of their aggregates [18]; In the same way, a low or disrupted dosage regimen may be more immunogenic than the high dosage regimen. Protein aggregation in currently considered to be one of the major risk factors for immunogenicity generation.

High doses of protein drugs have been reported to be less likely to produce immunogenicity than low doses [12]. On the other hand, when ADAs are detected, increasing the therapeutic dose in an attempt to counteract the plasma levels of ADAs affects the patient's exposure to high drug concentrations and increases treatment costs. Finally, the use of adjuvant drugs against the development of ADAs by interfering with the immune system, such as the use of Metrotexate, may be a viable alternative. However, it is important to consider the effects of these therapies on the patient at the adverse reactions level as product of polymedication [8]. On the other hand, the ability of proteins to develop response by the immune system has been used for a long time to generate immunity in the population; that is the reason d'être of vaccines and the developed schemes with these, in order to avoid diseases. But even in this field, a patient ´s too strong response is undesirable. A clear example is the development of recombinant vaccines against super antigens such as staphylococcal enterotoxin B (SEB), in which some domains are modified in order to diminish their pathogenicity, but maintaining their immunogenicity [126].

Given the importance of the immune system's response to pharmacotherapy with protein molecules, it is very important to consider this aspect from the very beginning of the drug development, based on a thorough analysis of the suitability (talent, skills and knowledge) of sponsors, researchers, clinical research organizations and regulatory authorities; as well as to know in depth, the mechanisms of action of the studied proteins, especially those that potentially involve new therapies, trying to detect as much as possible the probability of immunogenicity occurrence, before the drug gets to be marketed [46]. On the other hand, in the already approved for use product, the development of new treatment protocols or management guides that include the evaluation of the immunogenicity with techniques to detect levels of medication and ADAS (in case of not detecting the therapeutic molecule), can be a fundamental tool in treatments optimization, by allowing better therapeutic decisions that result in an efficacy and safety increase in the in the use of biological drugs [127].

Table 3. Factors that influence immunogenicity.

Category	Example	Refs.
Treatment	Mechanism of action Administration Route Frequency of administration Duration of treatment	[48, 128-136]
Patient	Type of disease Disease stage Immune system function Genetic factors Associated diseases Associated drugs Previous exhibition Previous sensitization	[4, 137-147]
Drug Properties	Recombinant system expression Post-transduction protein changes Impurities Contaminants Aggregates	[24, 27, 148-160]

CONCLUSION

Immunogenic response to therapeutic molecules can generate ADAs. Multiple factors can influence the immunogenicity in biological drugs as patients with impaired immune systems, genetic background of patient, route of administration of a therapeutic molecule, impurities, structure of the protein homologue and other unknown factors. The measurement and characterization of ADAs, Nab and non-NAb against biological therapeutics include conventional approaches such as ELISASs and radioimmunoprecipitation assays. Alternative technologies use instruments based in nanobiosensors as (SPR) and other biosensors, to detect and characterize antibodies against therapeutic proteins and other molecular interactions. The evaluation, safety and efficacy of biosimilars require more rigorous testing than conventional generic drugs. The profiles of biological drugs must demonstrate similar physicochemical and biological characteristics, efficacy and safety in accordance with the approval requirements of regulatory authorities

SOURCES

This review has been based on the relevant literature published during 1990-2016 and seminal prior material. MEDLINE, EMBASE, PUBMED and SCOPUS databases were searched. The key search terms were: immunogenicity, peptide, biological drugs, analytical methods, biosimilars, ADAs, immune system

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

All authors contributed towards writing the manuscript and have given their approval of the final version of the manuscript. The authors declare no competing financial interests.

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