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REVIEW

Immunogenicity to Biologics: Mechanisms, Prediction and Reduction

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Abstract Currently, there is a significant rise in the development and clinical use of a unique class of pharmaceuticals termed as *Biopharmaceuticals* or *Biologics,* in the management of a range of disease conditions with, remarkable therapeutic benefits. However, there is an equally growing concern regarding development of adverse effects like immunogenicity in the form of anti-drug antibodies (ADA) production and hypersensitivity. Immunogenicity to biologics represents a significant hurdle in the continuing therapy of patients in a number of disease settings. Efforts focussed on the identification of factors that contribute towards the onset of immunogenic response to biologics have led to reductions in the incidence of immunogenicity. An in-depth understanding of the cellular and molecular mechanism underpinning immunogenic responses will likely improve the safety profile of biologics. This review addresses the mechanistic basis of ADA generation to biologics, with emphasis on the role of antigen processing and presentation in this process. The article also addresses the potential contribution of complement system in augmenting or modulating this response. Identifying specific factors that influences processing and presentation of biologic-derived antigens in different genotype and disease background may offer additional options for intervention in the immunogenic process and consequently, the management of immunogenicity to biologics.

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Abbreviations

ADA	Anti-drug antibody
APC	Antigen-presenting cells
BAb	Binding antibody
BCR	B cell receptor
BMP7	Bone morphogenetic protein-7
C1q	Complement component 1q
C3a	Complement factor 3a
C3aR	Complement 3a receptor
C3d	Complement factor 3d
C5a	Complement factor 5a
CD	Cluster of differentiation
CpG	Deoxy-cytidylate-phosphate-deoxy-guanylate
DCs	Dendritic cells
EGFR	Epidermal growth factor receptor
FcγR	Fc gamma receptor
GH	Growth hormone
GHRH	Growth-hormone-releasing hormone
GM-CSF	Granulocyte macrophage colony stimulating factor
GnRH	Gonadotrophin-releasing hormone
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papilloma virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LFA	Lymphocyte function-associated antigen
LPS	Lipopolysaccharides
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
NAb	Neutralizing antibody

PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PSA	Prostate-specific antigen
Rh	Rhesus factor
Td	T cell-dependent
Th	T helper cells
Ti	T cell-independent
TAG-72	Tumor-associated glycoprotein 72
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
Tregs	T regulatory cells
VEGF	Vascular endothelial growth factor

Introduction

The last few decades has witnessed a significant expansion in the development by the pharmaceutical industry of a unique class of drugs, termed as *Biopharmaceuticals* or *Biologics*. Biologics are substances whose active component is derived from a biological source by being produced in microorganisms and cells (humans and animals) using biotechnology (Giezen et al. 2008; Rader 2008) and represents more than 30 % of licensed pharmaceutical products (DiMasi et al. 2010; Swinney and Anthony 2011). Biologics have expanded the range of options available for the treatment and management of complex diseases such as diabetes, cancer and autoimmune diseases (Schellekens 2008). Biologics consist of hormones (e.g. insulin, growth hormone, somatotropin), growth factors (e.g. erythropoietin), cytokines (e.g. interferons (IFNs), interleukin (IL)-2, granulo cyte macrophage colony stimulating factor (GM-CSF), vaccines, enzymes, antibodies (e.g. monoclonal antibodies (mAbs) against tumor necrosis factor (TNF)α, IL-2 receptor, lymphocyte function-associated antigen 1, epidermal growth factor receptor), fusion proteins (soluble receptors and cellular ligands) and hybrid proteins (e.g. diphtheria toxin: IL-2) (Giezen et al. 2008; L Revers 2010; Scherer et al. 2010). Based on their pharmacological action and therapeutic application, biologics have been categorized by Leader et al. (2008) into those with regulating activity (e.g. recombinant proteins, cytokines), specific targeting capability (e.g. mAbs), vaccines and diagnostics (Table 1).

Adverse Effects of Biologics: The Problem of Immunogenicity

Despite its therapeutic success, the incidence of adverse drug reactions to biologics is becoming increasingly evident (Weber 2004). These reactions can be grouped into those arising from either pharmacological or from non-pharmacological effects. The pharmacological associated adverse reactions are those which arise due to the interaction of the biologics with the intended target and are most often predictable, whereas the non-pharmacological are those which are not associated with the pharmacological action of the biologics (Clarke 2010). The latter includes immunotoxicity which comprises both immune

Table 1 Classification of biologics

Categories	Examples[a]
Group I: Biologics with enzymatic or regulatory activity	
Ia: Replacing a protein that is deficient or abnormal	Insulin, GH/Somatotropin, factor VIII, factor IX, protein C, β-glucocerbrosidase, α-1-protienase, adenosine deaminase, human albumin
Ib: Augmenting an existing pathway	Erythropoietin, IL-11, IFNs, factor VIIa, BMP7, GnRH, PDGF
Ic: Providing a novel function or activity	Botulinum toxin, Collagenase, Hyaluroindase, L-asparaginase, Streptokinase
Group II: Biologics with special targeting activity	
IIa: Interfering with a molecule or organism	mAb targeting VEGF, EGFR, CD3, CD52, TNFα, IL-2R, IL-1R, C5. Fusion proteins that bind to CD2 and blocks the interaction of lymphocytes with LFA
IIb: Delivering other compounds or proteins	Diphtheria toxin conjugated with IL-2, anti-CD33 conjugated to calicheamicin
Group III: Vaccines	
IIIa: Protecting against a deleterious foreign agent	Vaccines against hepatitis B virus, HPV
IIIb: Treating an autoimmune disease	anti-Rh IgG
IIIc: Treating cancer	Vaccine for B cell Non-Hodgkins lymphoma
Group IV: Diagnostic biologics	
	Glucagon, GHRH, Secretin
	Imaging agent labelled anti-PSA, anti-TAG-72, GPIIb/IIIa receptors
	Antibodies against HIV and hepatitis C virus

Table adapted from Leader et al. (2008)

[a] Only few examples have been stated. More detailed information on the examples for each group has been discussed by Leader et al. (2008)

Table 2 Classification of immunotoxicity

Type α	Immunostimulation
Type β	Immunogenicity (ADA → neutralization or hypersensitivity reactions)
Type γ	Immune deviation
Type δ	Cross reactivity
Type ε	Non immunological based reactions

response-mediated (immunogenicity, hypersensitivity and autoimmunity) and non immune response-mediated reactions like acute phase reactions (Clarke 2010). Adverse immunological reactions to Group I and II types (refer Table 1 for groups) of biologics are predominantly associated with long-term treatment regimens, and represent a growing concern to both regulatory bodies and the pharmaceutical industry. The immunotoxicity spectrum includes biologic-induced or biologic-associated infectious complications (Bongartz et al. 2006; Rychly and DiPiro 2005), unwanted immunostimulation (Suntharalingam et al. 2006), anti-drug antibody (ADA) generation (Aarskog et al. 2009; Li et al. 2001; Sorensen et al. 2003) and hypersensitivity reactions (Corona et al. 1999; Shopnick et al. 1996). To better understand, predict and manage immunotoxicity, an immunological classification of these reactions based on the pathologic mechanism was proposed (Pichler 2006; Scherer et al. 2010) and is listed in Table 2. In this article, we will be focussing on immunogenicity—Type β reaction which includes the production of ADA and subsequent neutralization and hypersensitivity reactions to Group I and II types of (non vaccine based) biologics. In particular, we emphasise the role of antigen processing and presentation in this misdirected immune response and consider approaches to predict and reduce such responses.

Anti-Drug Antibodies: Types, Subclasses and Clinical Outcomes

The development of ADA against biologics like IFNs (Janson et al. 1992; Kivisakk et al. 2000; Ronnblom et al. 1992; Scagnolari et al. 2002; Steis et al. 1991), erythropoietin (Casadevall et al. 2002; Weber et al. 2002), factor VIII (Hay et al. 2006b), factor IX (Warrier et al. 1997), insulin (Hirsch 2005), GM-CSF (Wadhwa et al. 2000) and anti-TNFα (Radstake et al. 2009; Svenson et al. 2007) results in compromised therapeutic efficacy and safety. An overt immune reaction to an exogenous version of an endogenous human protein or the failure of immune tolerance to self antigens could be the underlying triggers for ADA development (Goodnow 2001; Schellekens 2003). ADAs can be either binding antibodies (BAb) or neutralizing antibodies (NAb) and can alter pharmacokinetics, decrease the efficacy of the biologic and in some instances induce allergic reactions (Pedotti et al. 2001; Rosenberg 2003, 2006). BAb can either expedite the clearance of the biologic, termed clearing antibodies or they can prolong bioavailability, called sustaining antibodies (Ponce et al. 2009). The difference in effects between BAb and NAb is attributable to sites or epitopes on the therapeutic protein to which they bind. BAbs bind to epitopes that lie within regions of the biologic that do not participate in the interaction between the biologic and its respective receptor/target, whereas NAbs interact with the biologic by binding to epitope(s) that are functionally relevant for ligand–receptor interaction thus rendering the biologic inactive and compromising therapeutic efficacy (Bertolotto et al. 2002, 2004). As seen in response to IFNβ therapy, BAb titres are higher than Nab titres, tend to be produced much earlier during treatment (Scagnolari et al. 2002) and persist longer than NAbs (Bellomi et al. 2003). Based on a few studies, in approximately 40–75 % of positive cases, the NAb tends to disappear or is markedly reduced on continued and prolonged treatment (Hegen et al. 2012; Pungor et al. 1998; Rice et al. 1999). The generation of NAb, primarily of immunoglobulin (Ig)G isotype can cause life-threatening conditions as in the case of pure red cell aplasia by ADAs against erythropoietin (Casadevall et al. 2002) and thrombocytopenia by ADAs against thrombopoietin (Li et al. 2001). ADAs consist of low titre, transient IgM; high titre, persistent IgG (IgG1–IgG4) or IgE immunoglobulin isotypes (Baker et al. 2010; Baker and Jones 2007; Jefferis 2007; Singh 2011). Protein antigens predominantly trigger IgG1 and IgG3, whereas IgG2 antibodies are induced by carbohydrate-based antigens (Jefferis 2007). IgG4 is usually in response to chronic antigen stimulation (Jefferis 2007), and hence is commonly observed in response to long-term treatment with biologics. It is reported that the neutralizing property of IgG4 is higher compared to IgG1 and IgG2 ADA (Baker et al. 2010; Reding et al. 2002; Reding 2006). IgG-ADAs can also induce IgE-independent anaphylactic reactions (Finkelman 2007; Weber et al. 2002) and can include the activation of the complement system (Vultaggio et al. 2011). It is well known that IgE mediates lethal hypersensitivity reactions (Purcell and Lockey 2008). Despite the rarity of the incidence, IgE ADA-mediated anaphylactic response with recombinant human insulin (Chng et al. 1995; Kumar 1997) and various therapeutic mAbs (Stubenrauch et al. 2010; Vultaggio et al. 2010) clearly indicates its clinical significance. Previous exposure to substances within biologic formulations has also been reported to contribute to IgE-mediated immune reactions (Price and Hamilton 2007; Steele et al. 2005). IgM-based ADAs have also been reported with anti-TNFα mAbs (Vultaggio et al. 2010) and possibly during IFNα therapy (von Wussow et al. 1989).

IgM antibodies are of low affinity, high avidity, transient, induced by multivalent antigens (Boyer et al. 1977; Minuk et al. 1983) and are capable of activating the complement system (Richard and Prang 2010). While the evidence regarding IgM-ADAs is rather limited, IgM antibodies against polyethylene glycol (PEG)—a multivalent modifying agent tagged to biologics to improve bioavailability has been reported (Richter and Akerblom 1984).

Immunological Processes that Underlie Development of ADAs

Anti-drug antibodies can be generated by both T cell-dependent (Td) and T cell-independent (Ti) pathways, which involves the production of antibodies by B cells with and without the assistance of T cells (De Groot and Scott 2007). In the Td pathway, T cells are activated by the recognition of the antigenic peptides derived from the biologic and presented by antigen-presenting cells (APCs) via the major histocompatibility complex (MHC) II complex. Activated T cells then stimulate B cells to generate antibodies against the biologic. An immune response to a biologic can involve rapid induction of a Td response leading to expansion of epitope-specific B cells (Bachmann et al. 1994). The Td pathway of antibody production results in a long lasting, high antibody titre response to foreign or exogenous therapeutic proteins. T cell subset polarization also determines therapeutic outcome to the ADA generated, where a Th2 response drives neutralizing IgG4 ADA compared to Th1 which mounts an IgG1 and IgG2-based ADA, which may in some instances be non-neutralizing in nature (Baker et al. 2010; Reding et al. 2002; Reding 2006).

The production of ADAs through the Ti pathway involves polyvalent antigens that bind to B cell receptors (BCRs) and induce receptor clustering (Vos et al. 2000). It is conceivable that an aggregated biologic that displays repeating epitopes can cluster BCRs, cause B cell activation and result in a Ti response (Batista and Harwood 2009; Depoil et al. 2009). Biologics can also be engulfed by blood-borne peripheral dendritic cells (DCs) which then migrate to the spleen. Here, these DCs present biologic-derived antigens to B cells in the splenic marginal zone (Balazs et al. 2002). Since, there is no T cell help in this mechanism, ADAs generated by this process will be of IgM isotype or low-affinity IgGs. The presence of additional signals either from danger signals (Toll-like receptors: TLRs) or antigen-specific T helper (Th) cells can lead to affinity maturation, class switching and a more potent IgG response (Bachmann and Zinkernagel 1997; Batista and Harwood 2009). This second signal or danger signal can be provided by impurities in the biologic formulations and inflammatory milieu associated with the disease condition. While each pathway is distinct, there is crosstalk or interactions between the pathways as illustrated in Fig. 1. Antigen laden marginal zone B cells (from the Ti pathway) can also function as APCs by presenting the biologic to T cells (Fehr et al. 1998), and is an example of how the Td and Ti pathways interact and synergise. Another event which can represent interaction between the pathways is B cell epitope spreading, which may play a role in the evolution of the specificity of the ADA during the treatment period. It is known that the presence of ADA-biologic immune complex (the ADAs generated from primary immune response to the biologic) can activate naive B cells to mount a secondary response (Goins et al. 2010). Improved uptake of the biologic by B cells due to the formation of ADA-biologic complex may induce potent Td antibody production directed against epitopes different from those of the primary response.

A body of evidence accrued over recent years has clearly implicated a variety of factors in ADA generation (either singly or in combination) and is listed in Table 3 (Kromminga and Schellekens 2005; Pichler 2006; Schellekens 2002). While it is generally accepted that the immune system selectively determines, predisposes and plays a pivotal role in initiating and propagating an immunogenic response to various biologics, the role of antigen processing and presentation processes as critical drivers of immunogenicity has not been fully appreciated and merits consideration.

Antigen Processing and Presentation as Key Events in Immunogenicity

Antigen processing and presentation of biologics are performed by professional APCs such as DCs, macrophages and B cells. Antigen processing and presentation involves two key events: (1) antigen capture that delivers antigens to the cellular antigen processing machinery and (2) antigen processing and presentation that generates antigenic peptides bound to MHC molecules for presentation to adaptive immune cells. The various factors associated with immunogenicity as listed in Table 3 could potentially exercise their influence by modulating antigen processing and presentation as shown schematically in Fig. 2.

Antigen Uptake

The first step in antigen processing is the acquisition of extracellular antigens. APCs internalise antigen through phagocytosis, macropinocytosis and receptor-mediated endocytosis (Conner and Schmid 2003; Lanzavecchia 1990). Injection site of the biologic will determine the APC type that will be involved in antigen capture. Following

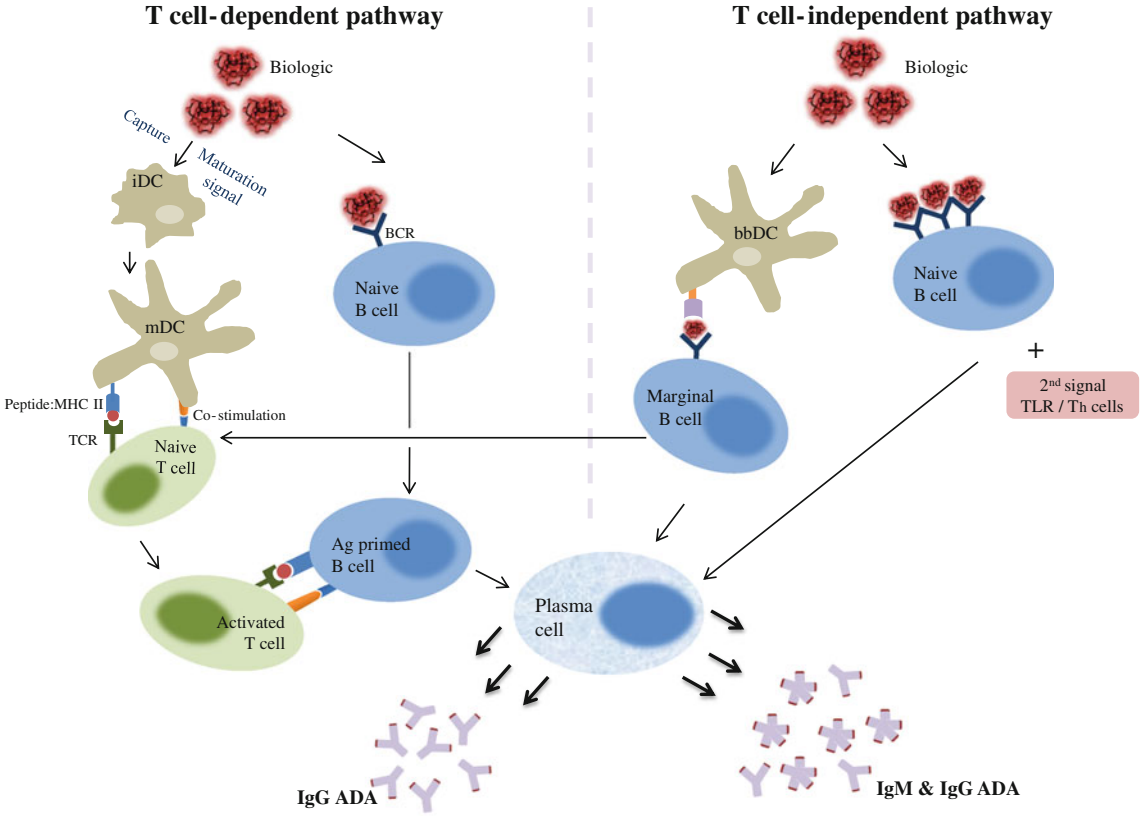

Fig. 1 T cell-dependent and independent pathway involved in an immunogenic response. T cell-dependent pathway involves the uptake of biologics by antigen-presenting cells like immature dendritic cells (iDCs) and B cells. DCs process the biologic into peptides, mature and migrate to the T cell zone of the draining lymph nodes where they present the antigenic peptides to naive T cells expressing antigen-specific T cell receptors. This leads to T cell activation and proliferation. B cells can also take up biologic through their B cell receptor, process and present biologic-derived peptides to activated T cells that have migrated to the B cell zones. Activated T cells stimulate B cells resulting in the generation of antigen-specific antibody secreting plasma cells. T cell-independent pathway involves the direct stimulation of B cells by aggregated form of biologic. Marginal zone B cells can be stimulated by biologic bearing blood borne peripheral DCs. This pathway leads to generation of plasma cells that predominantly secrete IgM antibodies. Cross talk between these pathways contributes significantly towards the immunogenic response. *ADA* anti-drug antibodies, *Ag* antigen, *bb*DC – blood-borne peripheral dendritic cells, *mDC* mature dendritic cell, *TCR* T cell receptor, *Th cell* T helper cell, *TLR* Toll-like receptor

Table 3 Factors contributing to immunogenicity of biologics

Biologic specific	Patient specific
Molecular structure or amino acid sequence differences between native and therapeutic protein (degree of humanization)	Age
Protein aggregation	Other concurrent medication
Protein degradation-oxidation, deamidation, glycosylation	Dose
Impurities/cofactors/adjuvants	Frequency of therapy
Formulation	Route of administration
Subclass of therapeutic IgGs	Genetic predisposition (HLA class and gene defects)
Nature of target protein (endogenous/redundant/unique)	Immune status and competence
Manufacturing process	Disease status (acute/chronic)
	Disease type (immune mediated/non-immune mediated disorders)

subcutaneous injection of biologics, immature DCs in the epidermis phagocytose and process the protein via the MHC class II processing pathway. Antigens can be captured by a number of receptors such as Fc receptors (FcγR and FcεR), TLRs, members of the C-type lectin family (DEC-205, DCIR), Ig superfamily and heat shock protein receptors. The presence of pre-existing antibodies and circulating IgM or IgG facilitates uptake of therapeutic

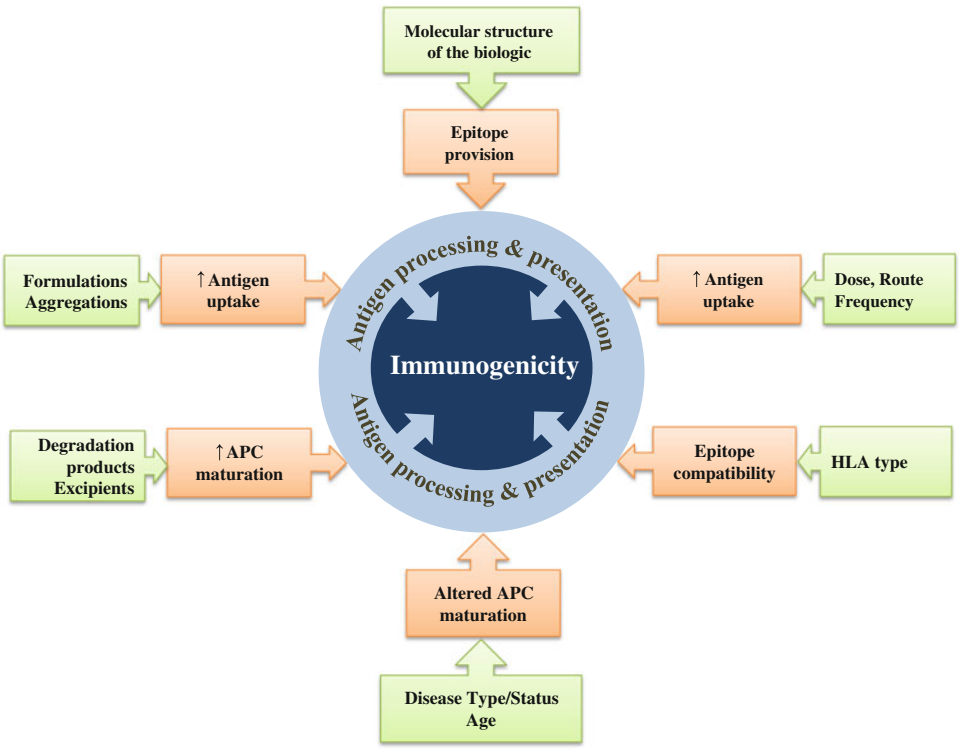

Fig. 2 Antigen processing and presentation central to immunogenic response. A variety of biologic-specific and patient-specific factors are associated with the onset and progression of immunogenicity to biologics. These factors exert their influence by modulating antigen processing and presentation events through altering antigen uptake, co-stimulatory molecule expression, maturation status and provision of immunogenic epitopes by APCs. *APC* antigen-presenting cell, *HLA* human leukocyte antigen

protein antigen by FcγRs. Furthermore, complement receptors might also enhance such endocytic processes (Bajtay et al. 2006). The binding of antibody–antigen complex to receptors on DCs or macrophages will also aid in antigen processing and presentation (Regnault et al. 1999). Engagement of receptors like TLRs enhance internalization of the complex thus facilitating antigen processing (Hayashi et al. 2001; Lankar et al. 2002). Formulation buffers can affect protein conformation of the biologic and thereby predisposing it to be internalised and processed by DCs (Jaber and Baker 2007; Jaber et al. 2007).

Physical modification/degradation including misfolding, unfolding, aggregation, oxidation and deamidation of biologics caused during purification, production, storage or formulation can predispose to immunogenic response by facilitating increased antigen uptake. It is clear that aggregates are a significant factor for immunogenic response as it is associated with increases in the incidence of immunogenicity (Antonelli and Dianzani 1999; Hermeling et al. 2004). This is probably due to their multiple epitopes attribute and/or changes to the structural conformation of the individual aggregated protein molecule (Kumar et al. 2011; Medzhitov and Janeway 2002; Rosenberg 2006; van Beers et al. 2010). The presence of antigenic epitopes in aggregates but not in monomer molecules (Kumar et al. 2011; Robbins et al. 1987) can directly stimulate B cells or can enhance its uptake by APCs (Jones et al. 2011). Aggregated biologics with multimeric structures can be also captured by blood-borne DCs and presented to marginal B cells (Fehr et al. 1997; Martin et al. 2001). Though the mechanism behind the predisposition of aggregated protein for capture is not completely defined, it is likely to be due to the presentation of B cell epitopes in a repetitive manner by the aggregated proteins. Aggregates could also induce immunogenicity through breaking existing immune tolerance towards monomeric version of the biologic (Braun et al. 1997; Moore and Leppert 1980). Oxidation contributes to immunogenicity by facilitating aggregate formation (Wang 2005). Several studies have shown that the oxidised form of the biologic was more immunogenic than non-oxidized form (Hochuli 1997; van Beers et al. 2011). The route of administration (in the case of an injected biologic) has been shown to have a profound effect on the onset of immunogenicity. Subcutaneous route was found to cause immunogenic response more frequently than other routes (Mohanan et al. 2010; Peng et al. 2009; Perini et al. 2001; Ross et al. 2000). Localization, prolonged presence, increased concentration and proximity to APCs when the biologic is delivered by the subcutaneous route could enable enhanced capture, processing and presentation of biologic-derived antigens by APCs thus leading to immunogenicity.

Antigen Processing and Presentation

Antigen uptake is followed by antigen processing and the formation of peptide–MHC complexes. Antigens that have

been captured and internalised are trafficked into endosomal compartments, and processed into peptides and are presented by APCs for T cell recognition. Based on studies in vaccine technology, it is emerging that aggregated forms of antigens can increase antigen processing thereby contributing to a more potent immunogenic response (Jones et al. 2011). The quality of the antigen presentation depends on the quality of the peptide-MHC complexes and there is a direct relationship between peptide-MHC complex stability and the immunogenic response (Lazarski et al. 2005). Human leukocyte antigen (HLA) haplotype and T cell epitopes are among the major contributors towards an immunogenic response against biologics. Specific HLA types have been found to be implicated in an ADA immunogenic response to biologics as listed in Table 4 (Barbosa et al. 2006; Buck et al. 2011; Ettinger et al. 2010; Hay et al. 1997; Hoffmann et al. 2008; Ohta et al. 1999; Praditpornsilpa et al. 2009; Simonney et al. 1985; Stickler et al. 2004). This suggests that there may be particular MHCs that are more able to complex with biologic-derived antigenic peptides. The contribution by the T cell epitopes within the biologic is equally pivotal to the immunogenic response. T cell epitope profiling studies have identified specific sequences of amino acids in various biologics which contribute towards immunogenicity (Jones et al. 2005; Parker et al. 2011; Stickler et al. 2004; van Haren et al. 2011). Hence, the potency of the peptide-MHC II complex on the surface of the APC to activate the T cells to initiate an immunogenic response is determined by a combination of the type of HLA and a compatible antigenic peptide that provides the T cell epitope.

Antigen presentation to initiate a T cell response by APCs is influenced by external stimuli and signals. Immature DCs which are highly endocytic but not very efficient at processing and presenting antigens undergo a maturation process in response to external signals or "danger signals" like TLR ligands (LPS, CPG motifs), inflammatory cytokines and complement (De Smedt et al. 1996; Sparwasser et al. 1998). These signals increase their efficiency for sustained processing and presentation of antigens. APCs activate naïve T cells by the recognition of antigenic peptide:MHC class II complex on their cell surface by T cell receptors and by the ligation of co-stimulatory receptor, CD28 on the T cell surface to the B7 co-stimulatory molecule on the APCs. The expression of co-stimulatory molecules on APCs can be induced by various factors present in the formulation (like excipients) of the biologics. Degradation products of excipients present in the formulation can increase co-stimulatory molecule expression on the surface of DCs (Mueller et al. 2009). The presence of reactive oxygen species either due to degradation products in the formulation or disease-associated inflammation can also provide the danger signals and up-regulate co-stimulatory molecules on the DCs (Rutault et al. 1999). Patients being treated with a biologic for immune disorders like autoimmunity or inflammatory disorder may be prone for developing immunogenicity. Increased expression of co-stimulatory molecules on APCs in patients with immune-mediated diseases may underlie such a predisposition (Anderson 2005). Altered co-stimulatory molecule expression and function of APCs have been reported in the elderly and hence, an immunogenic response towards the biologic might vary with age (Guy 2010; Pereira et al. 2011; Rafi et al. 2003; Shurin et al. 2007).

Break in Tolerance Underlies Immunogenicity

Tolerance is a mechanism by which immune cells are prevented from mounting a response against self antigens. Response against biologics which are considered to be similar to their endogenous counterparts could be due to the breaking of such immune tolerance. The presence of impurities such as endotoxins or microbial DNA in the biologic may act as danger signals and activate autoreactive B cells to self-antigens. The presence of foreign T cell epitopes coupled with self-antigens can also break tolerance towards the self antigen. Another important mode by which tolerance is broken is by repeated presentation of

Table 4 HLA type implicated in immunogenic response to biologics

Biologics	HLA type	Study
IFNβ	HLA-DRB1*0401, HLA-DRB1*0408	Buck et al. (2011), Hoffmann et al. (2008)
	HLA-DRB1*1601	Buck et al. (2011)
	HLA-DRB1*0701	Barbosa et al. (2006)
	HLA-DR2, HLA-DQ6, DQB1*0602 and HLA-DR15	Stickler et al. (2004)
Factor VIII	HLA-DQA1*0102	Hay et al. (1997)
	HLA-DR4.1, DQ4 and DQA1*0301	Ohta et al. (1999), Simonney et al. (1985)
	HLA-DRA-DRB1*1104	Ettinger et al. (2010)
Erythropoietin	HLA-DRB1*09-DQB1*0309	Praditpornsilpa et al. (2009)

self-antigens (Chackerian et al. 2002) as is the case during biologic therapy. Aggregated antigens are also efficient in activating anergic B cells on repeated exposure (Kromminga and Schellekens 2005). In vivo experiments in transgenic mouse models have also indicated that the immunogenic response to aggregated proteins is due to the breaking of tolerance (Braun et al. 1997).

Does Complement Play a Role in Processing and Presentation of Biologic-Derived Antigens?

Complement—a group of plasma proteins can be activated by three pathways—classical, alternative and lectin pathways. Immune complexes are known to activate complement by the classical pathway. One of the primary effector responses following complement activation is the mediation of adaptive immune responses by anaphylatoxins (C3a and C5a). Activation of complement could occur as a result of the characteristics of the biologic such as the structure, aggregation ability and impurities in the formulation and the isotypes of the therapeutic antibody. Since, antibody–antigen complexes are known to activate the complement system by the classical pathway; the presence of ADA-biologic immune complexes may also activate complement pathways. Of all the immunoglobulins, only IgM and IgG can activate complement (Bindon et al. 1988). Immune complexes consisting of pentameric IgM are potent activators of complement and even low levels of IgM—as would be in the case of patients receiving their first doses of biologic—can bind to aggregated biologic and trigger complement activation. Of the IgG subclasses, IgG1 and IgG3 are potent activators of complement whereas IgG2 and IgG4 are weak activators (Bindon et al. 1988; Woof and Burton 2004). However, the presence of IgG4 along with IgG1 was reported to have amplified the immune complex-mediated complement activation response (Bergamaschini et al. 1996). IgG3 complexes can also activate complement potently by binding to C1q following spontaneous multimerization (Greenspan and Cooper 1992). Due to the potency of these isotypes, suboptimal levels of the biologic-immune complex aggregate can be sufficient to activate complement system. As ADAs are predominantly of the IgG isotype, it becomes increasingly relevant to explore the role of ADA immune complex-mediated complement activation and the role of the complement in the onset and propagation of immunogenicity. Our preliminary results suggest that such ADA-biologic immune complexes induce complement activation (manuscript in preparation). The presence of impurities of bacterial origin in the biologic can also activate complement system via the alternative pathway.

Activated complement factors such as C3a, C5a and C3d are potent factors that influence antibody responses by modulating DC, T cell and B cell function. APCs express a wide range of complement receptors, complement-regulatory proteins and complement are essential for optimal maturation and T cell activation by APCs (Hashimoto et al. 2010; Kerekes et al. 2001; Weaver et al. 2010; Zhou et al. 2006). Cyclic adenosine monophosphate production which is important for DC maturation, antigen presentation and cytokine synthesis is mediated by C3aR activation (Li et al. 2008). Complement also influences T cell responses by direct or indirect modulation of Th1/Th2 immunity (Kemper and Atkinson 2007). Local constitutive production of complement and its activated components are necessary for T cell viability, generation of IL-2 and for antigen-specific T cell priming (Kopf et al. 2002; Lalli et al. 2008; Strainic et al. 2008). Activated complement also has an immunomodulatory role in B cells by mediating antigen retention for B cell activation, antibody production and memory B cell formation (Carroll 2004; Fischer and Hugli 1997; Fleming et al. 2002; Ottonello et al. 1999; Reid et al. 2002). Complement system has also been implicated in the resolution of an immune response to prevent tissue damage and autoimmunity, and its role in T regulatory cells (Tregs) has also been described (O'Garra and Vieira 2004) further reiterating the potential role of complement in breaking tolerance. Owing to its multiple roles in adaptive immune response, it would be necessary to define the contribution of complement to the development of high affinity ADAs.

Predicting Immunogenicity by Exploring Factors Influencing Antigen Processing and Presentation

Characterisation and screening for physico-chemical determinants or formulation-based factors like impurities, heterogeneity, aggregate formation, oxidation and deamidation in the biologics will aid both in the prediction of immunogenicity and in the development of less immunogenic therapeutic agents. Moreover, predicting potential immunogenic epitopes in biologics will be an important and effective strategy to improve their safety and efficacy. A variety of preclinical immunogenicity screening strategies are being used during biologic development as listed in Table 5.

It is now well established that T cell epitopes within the protein sequence of the biologics contribute towards immunogenicity. Therefore, predicting the potential immunogenic T cell epitopes will lead to reductions in the incidence of immunogenicity. Prediction strategies used for designing effective vaccines and determining T cell epitopes in autoimmunity (De Groot and Berzofsky 2004; Inaba et al. 2006; Khan et al. 2006) can be adopted to predict immunogenicity to biologics. Screening for T cell

Table 5 Strategies in predicting and reducing immunogenicity to therapeutic proteins

Prediction	Reduction
Physiochemical characterization	Deimmunization (epitope modifications)
In silico	Humanization
T cell epitope predictions	
B cell epitope predictions	
Tregitopes predictions	
In vitro/ex vivo	Purity and formulations
T cell responses	Modifications
HLA binding assays	Fusion proteins
In vivo models	Combination biologics or combination therapy

epitopes in biologics early in drug development is being increasingly used by the pharmaceutical industry. A variety of in silico methods or computational tools to identify potential T cell epitopes within the biologic that have a higher propensity to bind to particular HLAs are being developed (De Groot and Moise 2007; De Groot and Martin 2009; Koren et al. 2007). An extensive discussion by Lafuente and Reche (2009) on various strategies practised in the prediction of peptide-MHC interaction clearly showcases the importance of T cell epitope mapping in predicting immunogenicity. Recently, a screening strategy aimed at harnessing the concept of neutral drift also shows promise for improved T cell epitope prediction (Cantor et al. 2011). A potential limitation of in silico prediction of immunogenic T cell epitopes is the lack of input from aspects of APC function such as antigen processing and eventual presentation of biologic-derived peptides to T cells. Therefore, in vitro/ex vivo assays with primary human cells that can integrate both APC function and T cell responses may uncover immunogenic epitopes more accurately and will be most relevant in predicting immunogenicity to biologics in humans. It is therefore crucial to integrate in silico predictive tools with in vitro and ex vivo testing using T cells and APCs from both primed and non-primed individuals across relevant MHC-II allotype cohorts to identify biologically meaningful epitopes and thereby improving prediction of immunogenicity to biologics.

Prediction of B cell epitopes has been very useful in the success of vaccine technology. This can be harnessed to identify B cell epitopes of biologics that represent targets for ADA. There are two types of B cell epitopes, linear and conformational. The latter type is of greater relevance constituting the majority of the B cell epitopes. Conformational epitopes consist of amino acids that are not contiguous in primary sequence, but are arranged together as an epitope by proximity arising through secondary/tertiary protein structure. In silico tools to predict B cell antigenic epitopes are available but are currently underexploited in immunogenicity prediction (El-Manzalawy et al. 2008; Kulkarni-Kale et al. 2005; Larsen et al. 2006; Saha et al. 2005; Schreiber et al. 2005; Sollner et al. 2008; Wang et al. 2011). Furthermore, antibody-specific epitope prediction methods with improved accuracy and biological relevance are becoming available (Zhao et al. 2011). A detailed report by El-Manzalawy and Honavar (2010) on B cell epitope prediction methods describes the technical nuances involved in this technology aimed at reducing immunogenic response to biologics.

HLA binding assay is another reliable in vitro validation system for predicting immunogenic epitopes as there is a close association between HLA binding and immunogenic profiles (McMurry et al. 2005). This assay uses peptides from biologics to measure the binding affinity of epitopes to the various MHC II molecules (McMurry et al. 2007; Reijonen et al. 2002; Steere et al. 2006). The predicted epitopes can also be validated by measuring T cell responses, especially when blood samples are available from patients already exposed to the biologic (Barbosa et al. 2006; Hobeika et al. 2005; Jaber and Baker 2007; Kamate et al. 2007).

Reducing Immunogenicity: Intervening in Antigen Processing and Presentation

A variety of strategies designed to reduce immunogenicity have been tested and are listed in Table 5. Humanization is a process by which biologics of non-human origin are re-engineered to minimize the non-human component which can reduce immunogenicity. Modifications in the amino acid sequence of protein, and changes to the constant and variable regions of therapeutic antibodies have led to a marked decrease in the immunogenicity of biologics. The development of chimeric and humanized antibodies has helped in achieving decreased immunogenicity. The need to eliminate physico-chemical determinants that favour immunogenicity is extensively discussed in a recent review (Singh 2011). Deimmunization by depletion of potential immunogenic T cell epitopes through protein sequence modification is another effective strategy in reducing immunogenicity (De Groot et al. 2005; Hay et al. 2006a; Parker et al. 2011; Tangri et al. 2005; Yeung et al. 2004). This technique led to the development of many deimmunized biologics, especially mAbs which are in various stages of clinical trials with encouraging results. However, caution needs to be exercised in using this strategy as it could lead to the generation of new potentially immunogenic epitopes. Recently, a report utilizing a strategy with improved prediction methods along with saturation

mutagenesis was able to achieve protein sequences with decreased MHC II binding without compromising the function of the biologics (Cantor et al. 2011) represents a way forward in achieving improved safety in biologics. T cell epitopes associated with Tregs termed Tregitopes are being explored as a potential strategy to suppress immunogenicity (De Groot et al. 2008; De Groot and Martin 2009). Ex vivo and in vivo experiments using peptides with Tregitopes decreased the resulting immunogenic response to antigens (De Groot et al. 2008). Inclusion of Tregitopes can induce natural Tregs into a suppressive immune response to the biologic. This strategy paves a way for the generation of biologics with a less immunogenic adverse response. However, differentiating epitopes that are specific for Tregs from T helper cell epitopes may pose a significant hurdle and extensive studies in this developing area is warranted. Induction of tolerance can be another strategy to minimise immunogenicity. Administration of high concentration of biologics (antibodies), use of alternative routes like via the mucosal surfaces rather than subcutaneous route can decrease immunogenicity by the induction of peripheral tolerance through tolerizing DCs and expanding Tregs (Meritet et al. 2001a, b; Nagler-Anderson et al. 2001).

PEGylation and glycosylation are the two most common forms of modifications incorporated into the structure of biologics to reduce immunogenicity and improve therapeutic efficacy. The immunogenicity of large molecules like biologics can be minimized by modifying the therapeutic agent with PEG polymers. It is known that covalent attachment of PEG to biologics can reduce immunogenicity by interfering with processing and presentation and by masking immunogenic epitopes (Basu et al. 2006). Though PEG is generally non-immunogenic, there are reports which suggest that this is not always the case (Singh 2011). Anti-PEG antibodies have been detected in patients treated with PEGylated therapeutic enzymes (Armstrong et al. 2007; Ganson et al. 2006) and indicate the need to investigate the immunogenic mechanism triggered by PEG. There is growing evidence that such modification of biologics does not decrease the immunogenic potential (Jevsevar et al. 2010). Glycosylation is the most common form of post translation modification seen in half of all human proteins. Glycosylation of selective amino acid residues of the biologic interferes with MHC II restricted T cell recognition and through disrupting antigen processing by APCs (von Delwig et al. 2006). However, it can possibly increase immunogenicity by the generation of neopeptides as well (Singh 2011). There is a clear need for more detailed studies to validate these strategies and to develop new approaches that can target antigen processing/presentation for reduction of immunogenicity.

Concluding Remarks

The problem of immunogenicity has been under intense study but the contribution of antigen processing and presentation processes has not received enough attention. It is becoming clear that many of biologic-specific and patient-specific characteristics that are associated with higher incidence of immunogenicity have an impact on antigen processing and presentation mechanisms. Factors such as aggregate formation (either in vivo or in the formulation) or the presence of adjuvants in the formulation can enhance antigen capture, APC activation and lead to breaking of immune tolerance. The role of complement in augmenting or modulating immunogenicity through its effects on antigen processing is unexplored and merits detailed investigation. Much progress has been made in predicting and eliminating immunogenic epitopes contained within biologics. However, identifying factors that influence the processing and presentation of biologic-derived antigens including complement may offer additional options for intervention in the immunogenic process and consequently in the management of immunogenicity to biologics.

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