Development of immunogenicity models in mice for improved risk assessment of biopharmaceuticals

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ABSTRACT

The development of anti-drug-antibodies (ADA) to biopharmaceuticals, *e.g.* recombinant proteins including monoclonal antibodies (mAb) can lead to adverse events and clinical complications. These include reduced effect of the drug and autoimmune conditions if the administered drug is analogous to endogenous proteins. Immunogenicity assessment is critical during biopharmaceutical development and evidence for possible immunogenicity is required before drug approval.

In this thesis, we have focused on improving the assessment of immunogenicity to human recombinant protein drugs using *in vivo* mouse models in order to I) develop an *ex vivo* screening-method that can detect, characterize and semi-quantify specific ADA in one single plasma sample; II) characterize the differences in immune responses to a human recombinant protein drug-candidate with respect to ADA class- and subclass profiles, between wild type (Wt) and transgenic (Tg) mice expressing the human protein; III) investigate subclass profiling of ADA responses in Wt- and Tg-mice and to correlate the drug-specific subclasses with mechanisms for ADA production; IV) generate an immune-tolerant mouse model expressing human coagulation factors II, VII and X to be used for process optimization of biopharmaceuticals; V) correlate the ADA response in mouse models with the quality of batch formulations, *e.g.* presence of degradation products, drug fragments, endotoxins and host-cell-proteins, known as potential contributors to increased immunogenicity; VI) work towards the principles of "3R".

The results indicate that the developed *ex vivo* immunogenicity assay can detect immunoglobulin (Ig) subclasses of ADA with high specificity and sensitivity (125 ng/ml) in one single sample. Further, we saw a connection between low batch purity and high ADA levels. The least pure batch induced a significant increase in ADA of subclass IgG1 in both Wt- and Tg-mice. Since the Tg-mice were supposed to be tolerant to immunization with the human protein itself, the impurities (fragments, degradation products, endotoxins and more), included in the formulation, likely caused broken tolerance and subsequent ADA-formation in these animals. Wt-mice also showed IgG2b responses in a majority of the animals compared to none of the Tg-mice. It is suggested that the IgG2b response in Wt-mice is an expression of a xeno-response to the human protein. The combination of IgG1 and IgG2b in Wt-mice was reflected by a Th2-related cytokine repertoire in plasma. Finally, the developed triple transgenic mouse model expressing human coagulation factors II, VII and X, showed only low titers of ADA after immunization with pure drug formulation. Therefore, this model will be valuable during process optimization in order to monitor a potential ADA response.

By developing an assay for detection of subclasses of ADA, we have enabled the monitoring of immunogenicity in pre-clinical studies in a new way. By implementing the use of immune tolerant mouse models, commonly used for product quality assessment, we have contributed to reduce the use of animals and at the same time added tools for better risk assessment of immunogenicity.

Keywords: anti-drug antibodies, immunogenicity, biopharmaceuticals, immunoglobulin, 3R

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SAMMANFATTNING PÅ SVENSKA

Biologiskt framställda läkemedel (t.ex. proteiner inklusive monoklonala antikroppar) möjliggör behandling av sjukdomar såsom cancer och blödarsjuka. Trots dessa läkemedels enorma terapeutiska potential så utgör dess storlek och struktur ett potentiellt problem. Våra immunförsvar är skapade för att skydda oss mot främmande ämnen för att säkerställa vår överlevnad. Vid administrering av biologiska läkemedel kan immunförsvaret reagera på strukturella element hos proteinet och antikroppar specifika mot drogen kan utvecklas via ett s.k. immunogenicitetsrespons. I den här avhandlingen har vi utvecklat och förfinat *ex vivo* och *in vivo* metoder i mus i syfte att I) utveckla en metod som möjliggör detektion av drogspecifika antikroppar av olika klasser och subklasser i ett enkelt prov; II) undersöka eventuella skillnader i immunologiskt svar mot läkemedel i vildtypsmöss och transgena möss med avseende på antikroppsklass, subklass och cytokinprofil; III) undersöka sambandet mellan subklassprofilering och immunologiska mekanismer; IV) generera en immuntolerant musmodell som uttrycker humana koagulationsfaktorer (II, VII och X) för att kringgå det artspecifika svaret och möjliggöra en mer humanlik djurmodell för immunogenicitetsstudier; V) koppla antikroppssvar i musmodeller till olika faktorer/strukturella element i drogen som kan orsaka immunogenicitet; VI) uppfylla målen för ”3R” genom att minska behovet av försöksdjur. Resultaten visar att vi har utvecklat en immunogenicitetsmetod som, i ett enda prov, kan detektera olika klasser och subklasser av drogspecifika antikroppar. Detta bidrar till en enklare monitorering av immunogenicitet jämfört med konventionellt använda metoder. Två olika transgenmodeller har använts; den första för att jämföra immunologiskt svar med motsvarande i icke genetiskt modifierade (vildtyps-) möss. Resultaten visade att vildtypsmössen reagerar med ett kraftigare och bredare svar än de transgena mössen, vilket talar för att dessa uppvisar ett immunologiskt svar mot delar av proteinet som är artfrämmande. De transgena mössen reagerade med ett svagare och mer begränsat svar. Vi fann att den proteinformulering som innehöll mest proteinfragment och degraderingsprodukter gav upphov till det starkaste svaret, samtidigt som den renaste proteinpreparationen gav lägst respons i både transgena möss och vildtypsmöss. Genom att följa det specifika antikroppssvaret mot olika formuleringar av ett protein, kan de farmakologiska och biologiska mekanismer som ligger bakom utvecklandet av ett immunsvar mot biologiska läkemedel utredas och bidra till bättre riskbedömningar under läkemedelsutvecklingen. I den andra transgena modellen uttrycker musen tre olika humana proteiner och resultaten visar på att den här modellen fördelaktigt kan användas under optimering av

tillverkningsprocessen av biologiska läkemedel samtidigt som behovet av antalet försöksdjur minskar.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Granath, B**. Holgersson, J. Brenden, N. Refined analysis of antigen-specific antibody responses- A new one-step tool in immunogenicity studies. European Journal of Pharmaceutical Sciences 44 (2011) 187-193.

II. Brenden, N. Madeyski-Bengtson, K. Martinsson, K. Svärd, R. Albery-Larsdotter, S. **Granath, B**. Lundgren, H. Lövgren, A. A triple Transgenic Immunotolerant Mouse Model. Journal of Pharmaceutical Sciences, Vol. 102, No. 3, March 2013.

III. **Granath, B**. Holgersson, J. Cederbrant, K. Brenden, N. A Comparison of the humoral immune response induced by a recombinant human protein in wild type mice and in transgenic mice expressing the protein. (Manuscript).

CONTENT

ABBREVIATIONS

Ab	antibody
ABT	antigen binding test
ADA	anti-drug antibody
APC	antigen presenting cell
BAb	binding antibody
CI	confidence interval
CMV	cytomegalovirus
ELISA	enzyme-linked immunosorbent assay
EMA	european medicines agency
EPO	erythropoetin
FACS	fluorescence-activated cell sorting
FDA	food and drug administration (US agency)
FI	fluorescence intensity
GH	growth hormone
HCP	host cell protein
HLA	human leukocyte antigen
ICH	International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (Europe, Japan and the US)

Ig	immunoglobulin
i.v	intravenously
K-EDTA	potassium-ethylenediaminetetraacetic acid
LMW	low-molecular weight
mAb	monoclonal antibody
MABEL	minimum anticipated biological effect level
MHC	major histocompatibility complex
MS	multiple sclerosis
Nab	neutralizing antibody
NOAEL	no observed adverse effect level
OD	optical density
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PD	pharmacodynamic
PK	pharmacokinetic
PRCA	pure red cell aplasia
PRR	pattern recognition receptor
PTM	post translational modification
QC	quality control
Rh	recombinant human

RIA	radio immunoassay
SA-PE	streptavidine-phycoerythrin
SAR	structure activity relationship
S.c	subcutaneous
SD	standard deviation
SEC	size exclusion chromatography
SNP	single-nucleotide polymorphism
SPR	surface plasmon resonance
TD	T-cell dependent
TLR	toll-like receptor
Tg	transgenic
TI	T-cell independent or thymus-independent
Wt	Wild-type

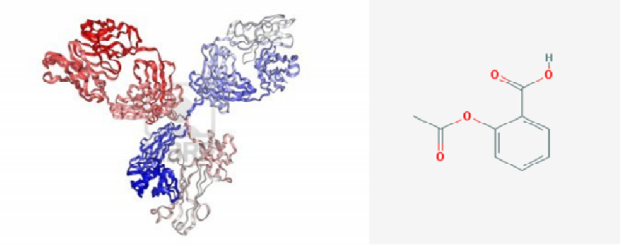
1 INTRODUCTION

1.1 Biopharmaceuticals

Biopharmaceuticals, including protein therapeutics, are medical drugs derived using biotechnology. Protein drugs (*e.g* endogenous proteins, monoclonal antibodies (mAb), cytokines or enzymes) can be naturally derived or recombinantly engineered. These molecules have during the last 30 years gone through a massive evolution and today the overall pharmaceutical market and pipelines consist of about 30% biopharmaceuticals *(Brinks, 2011).*

The first recombinant protein drug, human insulin, was approved by Food and Drug Administration (FDA) in 1982 *(Nagle, 2008),* and during the same decade the first commercial biotech companies were established. The mapping of the human genome together with progress in high-throughput screening technologies for drug discovery have created an explosion of growth in the development of biological therapeutic agents. Biopharmaceuticals are extremely large in size compared to conventional inorganic compound drugs and much more complex in their structure as demonstrated in Figure 1. Their complicated structures suggest that their biological function is based on advanced structure activity relationships (SAR), but also that variations in their structure, including post-translational modifications, increase the risk of side-effects including unwanted immune reactions.

Figure 1. Structural differences of a monoclonal antibody and a classical low-molecular weight drug.



A biopharmaceutical drug and its complex structure (left) compared to a chemically synthesized low-molecular weight drug (right). An Immunoglobulin G molecule consists of ~25000 atoms (150 kDa), while acetylsalicylic acid/aspirin consists of only 21 atoms (180 Da).

Biopharmaceuticals have successfully served patients with treatments for diseases that lacked effective therapy prior to the biotechnology revolution. There are several advantages with the use of biopharmaceuticals. The first is the high specificity for the target, because of binding of the biopharmaceutical to naturally existing receptors in the body. The second is the low frequency of toxicological side effects of these drugs. During treatment with chemically synthesized low-molecular weight (LMW) drugs, the biggest challenge is to avoid toxic metabolites (off-target effects) responsible for most of the side effects after administration of the drug *(Bussiere, 2009).* However, toxic metabolites are generally not seen after administration of a protein drug, because its natural catabolism leaves only amino acids.

The use of biopharmaceuticals in treatments of human disease can still be a challenge due to drug recognition by the immune system of the host if the drug has antigenic properties. As a consequence, there is a growing concern regarding the development of adverse effects like autoimmunity and hypersensitivity after treatment with biologics. Not to mention the loss of pharmacological activity caused by neutralizing antibodies.

1.2 Immune responses against biotechnology derived biopharmaceuticals

Due to the complexity of biological molecules and their sensitivity to environmental effects, the manufacturing process is important for the potential immune reaction against the drug.

The immune system is comprised of cells and molecules utilized by the body to defend itself against infection in order to prevent morbidity and mortality. This is possible by the involvement of various complex molecular interactions by which cellular entities interact in synergy to target invading microorganisms. Immunogenicity (or antigenicity) is a structural feature of a molecule that triggers an immune response.

The immune system consists of two arms; the innate and the adaptive immune system, and both of them can play a role in reactions against biopharmaceuticals.

1.2.1 Immunogenicity and the innate immune system

The innate immune system is responsible for the first line of defense against invasion by microbes characterized by an immediate recognition and response. The innate immune system can identify highly conserved repetitive patterns, so called pathogen-associated molecular patterns (PAMP´s), through pattern recognition receptors (PRR´s). Examples of such receptors are the Toll-like receptors (TLR) that recognize bacterial cell wall components. Several factors, including bacterial products, appearing during production and formulation of biopharmaceuticals, may potentially activate the innate immune system. Aggregates due to modifications of the drug and formed during processing or handling of the drug can mimic the structure of PAMP´s and act as ligands for PRR´s, thus activating the innate immune system *(Foged, 2008)*.

1.2.2 Adaptive responses against biopharmaceuticals

Adverse immune reactions against biopharmaceuticals, caused as an effect of immunization, generally occur with low incidence. Still, most of these drug products have shown immunogenic properties in man even when fully humanized *(Hermeling, 2004)*.

The adaptive immune system constitutes the second line of defense and will be triggered by all compounds perceived as non-self. Antigen activation of T- and B-cell lymphocytes initiates specific cell-mediated and/or humoral immune responses, respectively, resulting in effector cell action and/or antibody formation as well as generation of memory lymphocytes. Immune tolerance is a state during which the immune system does not respond to a particular antigen.

In this thesis I will discuss the adaptive humoral immune responses against biopharmaceuticals, as well as conditions for breaking of tolerance against protein pharmaceuticals expressed as endogenous proteins in transgenic mice.

1.3 Recommendations regarding immunogenicity in pre-clinical studies of biopharmaceuticals

Guidelines from international drug agencies *e.g.* European Medicines Agency (EMA) and FDA, together with the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) S6 guideline, summarizing the recommendations regarding immunogenicity testing of anti-drug antibodies (ADA), are accessible to the industry. Several White papers have been created, stating detailed strategies and recommendations concerning assay development. The most accurate guidelines and White papers are listed in Table 1.

Table 1. Guidance documents and industrial white papers including recommendation of immunogenicity risk assessment of biopharmaceuticals.

Guideline documents and industrial White papers*	
ICH S6 Guideline	Preclinical safety evaluation of biotechnology-derived pharmaceuticals
FDA Guideline	Guidance for Industry: Immunogenicity Assessment for therapeutic Protein Products
EMA Guideline	Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins
FDA Guideline	Guidance for Industry: Early Clinical Trial with Live Biotechnology Products: Chemistry, Manufacturing, and Control information
White Paper by Mire-Sluis, AR et al.	Design parameters for anti-drug antibody immunoassays
White paper by Gupta, S et al.	Neutralizing antibody assay validation
White paper by Shankar, G et al.	Validation of anti-drug antibody immunoassays
White paper by Koren, E et al.	Immunogenicity testing strategy

*) all documents in this table are referred to in the reference list.

For clinical studies, the agencies recommend a multi-tiered, risk-based approach that is clinically driven and takes into account pharmacokinetic data *(Torvey, 2011)* for sample-testing. The appropriate screening assay should be capable of detecting both IgM and IgG ADAs. Samples should be tested in a screening assay and the samples potentially positive will further be tested in a specificity assay, usually by competition with unlabeled drug, using the same assay format as that used for the screening assay. Further, the samples should be tested for neutralizing antibodies using a cell-based assay whenever possible *(FDA, 2013 and EMA, 2007 Guidelines)*. Recommendations regarding experimental design of screening assays, validation and calculation of positive samples are described in the industrial White papers listed in Table 1.

However, in the preclinical setting characterization of ADA is not required unless there are obvious changes in the pharmacokinetics reflecting loss of drug activity or evidence of immune-mediated reactions, such as anaphylaxis.

1.4 Biosimilar products and immunogenicity

In LMW-pharmaceutical development the concept of generics is a well known term for copies of an already known and licensed product. However, in protein therapeutics development, generics is not the proper term to be used since creating a copy of a specific protein is challenging or even impossible in new facilities due to complex structural build-ups and post-translational additions. Instead we use the term biosimilar or follow-on biologics, when explaining a copy version of a licensed original product. According to the latest European guideline regarding biosimilars *(EMA, 2005)*, a biosimilar should go through comprehensive comparability exercises, including physico-chemical and *in vitro* biological tests, non-clinical and clinical studies in which reference substance data should be bioequivalent to biosimilar data. The biosimilar structure should be identical on the amino acid level and post-translational modifications shall be adjusted to be as similar to the original product as possible.

Production of the protein in new facilities by a new manufacturer brings with it the highest risk of introducing new structural features to the protein that may make it immunogenic. However, changes in the process or formulation in the same facility can also be accompanied by structural changes to the protein. Post-translational modifications are affected by product-specific factors such as new formulations, new processes and new containers, (see

more contributing factors under section 1.7). However, patient-specific factors are usually known from the original product trials and seem not to be an additional problem during biosimilar development *(Buttler, 2011).*

1.5 Anti-drug antibodies

Most therapeutic protein products are known to be immunogenic, even though the generated ADAs, in most cases, have no significant impact on the safety and efficacy of the drug *(Koren, 2008).* In the industrial setting, *immunogenicity* is a definition used for many years to describe immune activation against the administered drug resulting in ADA formation. Since the response can lead to clinical manifestations, *e.g.* reduced effect of the drug, these responses must be carefully monitored during treatment. Various biopharmaceuticals used for treatment of different diseases are associated with the generation of ADAs (Table 2). However, there are still biopharmaceuticals on the market, *e.g.* IFNγ that do not show any signs of immunogenicity despite many years in clinical use *(Sathish, 2013).*

Table 2. Examples of recombinant biopharmaceutical drugs used in the clinic.

Drug	Indication(s)	Safety warning(s)	Reference
Rh IL-2	Metastatic renal cell carcinoma, metastatic melanoma	Risk of hypersensitivity reported	Abraham, 2003
IFNα	Chronic hepatitis C, leukaemia,	Autoimmune reactions and infections Risk of immunogenicity	Strayer, 2012
IFNβ1a	Multiple sclerosis	Immunogenicity reported Anaphylaxis reported post-approval	Strayer, 2012
IFNβ1b	Multiple sclerosis	Immunogenicity reported	Strayer, 2012
Natalizumab; Humanized mAb	Multiple sclerosis, Crohn´s disease	Serious infections Immunogenicity reported	Sorensen, 2011
FVIII	Hemophilia A	Immunogenicity reported	Lusher, 1993
Epoetin	Chronic kidney disease, cancer	Immunogenicity reported	Bennett, 2004
Thrombopoietin	Chemotherapy patients, thrombocytopenia	Immunogenicity reported	Li, 2001

1.5.1 Binding- and neutralizing ADA

ADAs are classified as being either binding antibodies (BAbs) or neutralizing antibodies (NAbs). BAbs bind to the protein and can enhance clearance or prolong systemic exposure, without neutralizing the drug *(Ponce, 2009).* However, BAbs also have the potential of triggering the generation of NAbs through epitope spreading *(Singh, 2011).* NAbs, on the other hand, bind to the drug and disturb its ability to bind to the intended target and by that they neutralize the function of the drug. The NAbs can disturb the biological activity by either binding to epitopes within the active site or blocking the active site through binding to epitopes close to the active site *(Gupta, 2007).* NAbs will, in high titers, inhibit efficacy and biological activity of the drug. The most serious effect of NAbs is when they act by cross-binding to endogenous proteins and subsequently neutralize important biological functions *(Rosenberg, 2004).* One example is increased titers of IFN-β1b specific IgG4-ADA in multiple sclerosis (MS) patients treated with this cytokine *(Deisenhammer, 2001).*

Another well studied example is neutralizing ADA responses seen in Hemophilia A patients treated with recombinant human FVIII *(Verbruggen, 2009).* However, neutralizing ADAs only represent part of the overall spectrum of ADAs directed against FVIII. Binding ADA, altering the pharmacokinetic or pharmacodynamic profile of the drug, also exist *(Whelan, 2013).* FVIII specific ADAs, directed against functional or non-functional epitopes on FVIII, occur as polyclonal high affinity IgG antibodies *(Tellier, 2009).* The severity of the underlying disease is classified according to level of normal FVIII function (mild 5-50%, moderate 1-5% and severe < 1%), impacting on the potential ADA response *(Zhang, 2009).* The risk of ADA development is highest in severe Hemophilia A, most likely due to the lowest self-tolerance to FVIII among patients in this group.

1.5.2 Hypersensitivity reactions involving ADA

The production of ADAs can lead to hypersensitivity reactions of type I-III. The most serious reactions include substance-specific reactions leading to production of IgE, IgM and IgG antibodies resulting in immediate type reactions *e.g.* urticaria and anaphylaxis, or serum sickness *(Scherer, 2010).*

1.5.3 Classes and subclasses of murine and human ADA

The ADA responses against biopharmaceuticals in humans are mainly characterized by transient IgM in low titers, persistent IgG1-IgG4 or IgE antibodies *(Sethu, 2012)*.

The murine (m) and human (h) counterparts of IgG subclasses are shown in Table 3, in which the common characteristics, based on biological and functional activity, of the antibodies are explained. A broad generalization can be made that protein antigens induce hIgG1/mIgG2a and hIgG3/mIgG2b responses; carbohydrate antigens induce hIgG2/mIgG3, and hIgG4/mIgG1 may be induced by chronic antigen stimulation *(Jefferis, 2007 and Hussain, 1995)* as demonstrated in Hemophilia A patients treated with FVIII *(Whelan, 2013).*

The anti-FVIII antibodies in Hemophilia A patients are primarily of the IgG4 subclass (equivalent to mouse IgG1) together with lower levels of drug-specific IgG1 (equivalent to mouse IgG2b) *(Wu, 2001).* Analysis of IgG isotypes in Hemophilia A patients concluded that successful immune tolerance induction and low titers of ADA correlated with Th1 mediated IgG1 and IgG2 responses, while patients with high anti-FVIII titers had Th2 driven IgG4 antibodies *(André, 2009).* The IgG4 dominant response in Hemophilia A patients can be explained by a chronic antigen stimulation in these patients, resulting in a shift from an IgG1 to an IgG4 response following repeated administrations (equivalent with repeated exposure to bee-venoms) *(Fulcher, 1987).* Anti-FVIII autoantibodies are not only found in alloimmunized Hemophilia A patients and in patients with acquired Hemophilia A, but also in healthy individuals and in elderly people with various autoimmune conditions *(Wootla, 2009).*

Table 3. Murine IgG antibody subclasses and their human counterparts based on similarities in biological and functional activities.

Murine	Human	Characteristics	References
IgG1	IgG4	Bind to mast cells Produced after chronic treatment of antigen. Class switch from IgG1.	Jefferis, 2007; Hussain, 1995
IgG2a	IgG1	Fix complement and bind to protein antigens Sensitize B cells and induce of apoptosis *in vitro*	Jefferis, 2007; Hussain, 1995
IgG2b	IgG3	Similar biological activities as mIgG2a/hIgG1	Jefferis, 2007; Hussain, 1995
IgG3	IgG2	Recognize carbohydrate epitopes. Resistant to proteolysis	Jefferis, 2007; Hussain, 1995

As mentioned in section 1.3, immunogenicity studies aiming at class- and subclass profiling are not included in the pre-clinical test package required by the authorities today. Still, some pharmaceutical companies recommend immune-tolerant transgenic mouse models for studying ADA from a product quality perspective (own observations). This type of data is usually kept as in-house reports by the companies and is rarely published. We believe that not publishing results of ADA-screening, including subclass profiling, for quality purposes in pre-clinical development may prevent the scientific community from gathering additional know-how on the immunogenicity of biopharmaceuticals.

1.5.4 Immunoglobulin class-switching

Mature and naïve $IgM^{+}IgD^{+}$ B cells will undergo antibody class switching in response to antigen stimulation and costimulatory signals. T cell cytokines will direct the class switch, making it possible for naïve B cells to switch to any isotype (IgG, IgA, and IgE) *(Stavnezer, 2008).* During immunoglobulin class

switching, initiated by CD40-CD40L cell associations *(McHeyzer-Williams, 2005),* the B cells will undergo maturation involving replacement of the constant μ region by a downstream constant *x* region from another class of Ig. These events will result in various isotypes with different effector functions but with the same variable region and thus the same antigen specificity and affinity *(Durandy, 2012)*. Th1 cytokines promote synthesis of subclasses that bind complement, *e.g.* hIgG1/mIgG2b and hIgG2/mIgG3, while Th2 cytokines promote synthesis of classes and subclasses that do not fix complement such as hIgG4/mIgG1 *(Reding, 2002).*

Combinations of IL-2, -4 and -5 have been shown to regulate the secretion of murine IgG1. In the presence of IL-5 and IL-2, B cells showed increased sensitivity to IL-4 and its IgG1-inducing effect. High levels of IgG1 production was seen with as little as 1 U/ml of IL-4 when acting in synergy with IL-5 and IL-2. When acting alone, a concentration of 1000 U/ml of IL-4 was required to achieve the same amount of IgG1 *(McHeyzer-Williams, 1989)*.

1.6 Mechanisms of immunogenicity

Immune responses against therapeutic proteins can be divided into those elicited by a typical response to a protein recognized as foreign and those arising as a consequence of breaking B- and T cell tolerance to endogenous proteins *(De Groot, 2007).*

1.6.1 Classical humoral immune response against foreign antigens

A classical immune reaction occurs when the protein is of foreign nature to the host. This phenomenon was for the first time observed after administration of animal proteins to humans, *e.g.* anti-serum from horses or insulin from pigs. The same type of exogenous response will be initiated after administration of drug products derived from microbes or plants that often results in the production of NAbs *(Kessler, 2006)*.

T cell dependent ADA-production

A classical immune response is initiated by uptake of foreign proteins by antigen-presenting cells (APC), which can be professional dendritic cells (DC) or B cells capable of recognizing foreign structures via B cell receptors (BCR). The APCs further presents the peptides/fragments of the protein on its surface in the context of major histocompatibility complex (MHC) class II

molecules. After antigen encounter, (in the periphery for DC and in secondary lymphoid organs for the B cells), APCs migrate to the T cells zone of the lymphoid organs in which T cells are scanning the surface of APCs to find a match for its T cell receptor. Interactions between the receptor of T cells and the MHC class II complex takes place in a so called "immunological synapse" *(Tsourkas, 2007).* Antigen-primed T cells will leave the T cell zone and migrate to the B cell follicles in which B cells are activated, in a T cell-dependent (TD) manner, to form antigen-specific antibodies. Cytokines formed by T cells and other surrounding cell types will enhance and contribute to the B cell response. A TD immune response against a foreign structure is dependent on three signals to induce proliferation, and differentiation of naïve B-cells, and isotype switching of antibodies. The first signal is cross linking of the B cell receptor by antigen, the second signal occurs when the cognate interaction with T helper cells is completed in the immunological synapse, and the third, and most recently found signal, occurs when Toll-like receptors (TLR) on the surface of B cells are triggered by a ligand *(Ruprecht, 2006).* There are several examples of TD antibody responses to biopharmaceuticals *(Yeung, 2004 and Jacquemin 2003)*. For example, HIV-infected hemophiliac patients with ADA to FVIII post-drug treatment show a decrease in ADA titers as HIV progresses. Simultaneously, a decline in CD4+ T cell counts is observed (*Bray, 1993).*

T cell independent ADA-responses

B cells can also be activated without the help from T cells. This is the case when thymus-independent (TI) antigens are activating B cells. Examples of TI antigens include polysaccharides and lipids. TI responses can occur if the protein drug forms a multimeric complex (aggregate) that crosslink B-cell receptors to an extent where co-stimulation from T-helper cells is not required for an ADA response to occur *(Baker, 2010)*. The mechanism behind TI responses, including the ability of TI antigens to trigger the adaptive immune response to produce antibodies (mainly low-affinity IgM) without the help of T cells, is still not fully understood. *(Sauerborn, 2009).*

1.6.2 T cells and cytokines in ADA responses

Cytokines are responsible for driving the immune response from antigen presentation to antibody class switching, which in turn determines the Ab effector function and the type of ADA response (neutralization or allergic reaction). The naïve form of CD4+ T cells leaving the thymus must be provided with activation signals in order to differentiate into various subsets of effector cells capable of mediating inflammatory or humoral responses. This is demonstrated in both Hemophilia A patients and in equivalent mouse

models, since both species develop Th1 and Th2 responses leading to production of FVIII-ADA *(Reding, 2002).*

We will in this thesis focus on the Th1 and Th2 cytokines involved in immunogenicity responses to a therapeutic protein drug-candidate. Th1 differentiation from naïve CD4+ T helper cells is promoted by IL-12 and IFN-γ produced by NK-cells. Basophils and/or mast cells are producing IL-4 promoting Th2 differentiation together with IL-10 *(Constant, 1997)*. The cytokines produced by Th1 cells are IFN-γ, IL-2, IL-12 and TNF-β, while Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 *(Romagnani, 2000)*. Both Th1 and Th2 cells favor differentiation of B cells to produce antibodies *(Balasa, 2000).*

1.6.3 Breaking of tolerance

Tolerance is a state of non-reactivity of the immune system against autologous (self) proteins. Maintaining tolerance against self-proteins is essential in order to avoid attacks on cells and tissues by autoreactive antibodies and T cells. CD4+ helper T cells control most of the immune responses against protein antigens. Therefore can CD4+ T cell tolerance be enough to control both cell-mediated and humoral immune responses against self-protein antigens. Central tolerance occurs in the primary lymphoid organs, *i.e.* the bone-marrow and thymus *(Kyewski, 2006).* Immature lymphocytes that interact with a self-protein presented as a peptide bound to self MHC molecules are negatively selected and deleted through apoptosis *(Bluestone, 2011).* Recognition of self-antigens by B cells can lead to receptor editing and T lymphocytes binding self-antigens can develop into regulatory T cells (CD4+ cells only). Central tolerance may be incomplete such that lymphocytes escape the control mechanism and migrate into the periphery. Therefore, to further protect against autoimmunity, there is a backup mechanism of peripheral tolerance in the tissues. Mature T cells recognizing self-proteins or regulatory T cells suppressing self-reactive lymphocytes induce peripheral tolerance resulting in anergy (functional inactivation) or cell death. *(Kindt, 2007 and Abbas, 2011)*. However, the tolerogenic mechanisms are not complete and 25-40 % of auto-reactive T cells escape clonal deletion into the periphery *(Bouneaud, 2000)*. Therefore, breaking of tolerance to endogenous proteins do happen despite the control mechanisms, and administration of drugs based on endogenous proteins can lead to production of BAbs. The response develops slowly and is mediated by B cells through breakdown of tolerance. An ADA response can be clinically apparent years

after initial drug treatment and may often disappear after treatment withdrawal *(Kessler, 2006).*

Several studies have reported that tolerogenic dendritic cells, and their role as APCs, are crucial for maintaining immune tolerance against self-antigens *(Mueller, 2010).* Breaking of tolerance against FVIIa in a human FVII transgenic mouse model was suggested to be caused by pro-inflammatory dendritic cells presenting FVIIa peptides to the immune system. These DC may trigger auto-reactive CD4+ T cells that have the capacity to activate auto-reactive B cells, which in turn differentiate into plasma cells producing FVIIa-specific auto-antibodies *(Lenk, 2013).* Very little is known about the mechanism(s) that breaks tolerance of self-proteins, but data supports the idea of T cell dependent mechanisms and the involvement of innate immunity.

1.7 Factors contributing to immunogenicity

Various known and so far unknown parameters contribute to the immunogenicity of a biopharmaceutical drug. Several factors, ranging from the cell line used for recombinant protein production to storage conditions for the final formulation, may affect the structure of a protein drug and thereby its immunogenicity. Some of the known factors influencing biopharmaceutical drug immunogenicity are listed in Table 4.

Table 4. Factors contributing to immunogenicity of protein drugs.

Patient related	Treatment related	Product related
Disease state and immune status	Dose	Protein structure (species, PTM[c], T cell epitopes)
	Number of doses	
Genetic background (MHC[a] genotype, HLA[b] phenotype)	Route of administration	Contaminants and impurities
	Frequency of dosing	
Concomitant therapy	Length of treatment	

Table modified from *Singh, 2011.*
[a] Major histocompatibility complex
[b] Human leukocyte antigen
[c] Post translational modifications

1.7.1 Patient- and treatment related factors

Patient-related factors such as genetic predisposition and age can influence the immune response to biopharmaceuticals *(De Groot, 2007).* Genetic factors can contribute to inter-patient variability due to allelic polymorphisms in the MHC, impacting the interaction between HLA and peptides derived from the antigen *(Kessler, 2006).* Further, genes encoding the T-cell receptor polypeptides on T-helper cells may influence the outcome of the response and whether immunological tolerance is induced or not *(EMA Guideline 2007).*

Single-nucleotide polymorphisms (SNPs) is the most common source of genetic variation in the human population and also a good predictor of whether inhibitory anti-FVIII Abs will develop or not. Large differences in frequency of ADA have been seen between distinct populations associated with the distribution of specific SNPs. *(Yanover, 2011).*

Treatment-related factors such as dose, number of injections, treatment frequency and route of administration have all been proven to affect the ADA response, as demonstrated in rhIFNβ immune tolerant mice *(Kijanka, 2013).* The degree of immunogenicity has been shown to correlate with the route of administration as follows *(Koren, 2008):*

Inhalation > subcutaneous > intraperitoneal > intramuscular > intravenous

Another factor influencing the potential immunogenicity of a protein drug, but not yet well explored, is the risk of administration of a specific allotypic therapeutic protein to patients homozygous for the alternative allotype (s). So far, all therapeutics have been developed as a single allotypic form *(Jefferis, 2009).*

1.7.2 Chemistry, manufacturing and control (CMC) related factors

The origin and nature of the protein *i.e.* the primary structure is clearly influencing the immunogenicity. However, fully human protein drugs may also trigger an immune reaction in patients. Various factors originating from the complex manufacturing process can influence the structure. For example, the expression system including the host cell line in which the genetic sequence is translated into the protein product may affect its structure. Species-specific post-translational modifications (PTM) such as glycosylation, phosphorylation, acetylation and methylation, may activate the immune system resulting in ADA. The most common post-translational modification is glycosylation which can be both species- and cell-specific,

and may differ in sequence, chain length and branching *(Hermeling, 2004).* The choice of expression system determines the presence or absence of glycosylation *(Singh, 2011).* Higher eukaryotic cells produce glycosylation patterns more similar to human cells than prokaryotic cells, which more or less lack glycosylation. Therefore eukaryotic expression systems [*e.g.* Yeast or Chinese hamster ovary (CHO) cells] are often chosen *(Rodney, 2003).* However, there are differences in glycosylation between human cells and other eukaryotic cells that may give rise to immune responses and even hypersensitivity reactions *(Commins, 2009).* Variations in protein structure incurred by PTMs, chemical or enzymatic degradation and/or modifications such as deamidation and/or oxidation are other structural alterations arising from the development process that may contribute to increased immunogenicity *(EMA Guideline, 2007)*

During the manufacturing process large amounts of proteins are produced and drug-producing cells and/or cell fractions are continuously present in cultivation media. To meet purity and sterility standards, required for human injection, several purification steps are required in the down-stream process. Starting with a cell suspension, the down-stream process follows with a solids-liquid separation or clarification, concentration, purification and finally quality control and assurance *(Rodney, 2003 and Desai, 2000)*. All steps during the manufacturing process include high concentration and high temperature steps, a wide range of pH, exposure to air and light among others, which can be stressful for the protein, lead to aggregation *(Cromwell, 2006)* and potentially increased immunogenicity.

The three-dimensional structure of a protein can be degraded during production and purification, but also during improper storage or handling of the protein. Aggregation of the drug may reveal new epitopes, normally hidden, and by that stimulate the immune system, as demonstrated by antibody formation against aggregated human insulin *(Schernthaner, 1993).*

Product quality factors related to production such as impurities, fragments, aggregates and degradation products, are associated with the generation of immune responses against the drug *(Barbosa, 2012).* Impurities and degradation products can alter the drug structure and by that present novel epitopes, while contaminants *e.g*. host cell proteins (HCP), endotoxins, DNA and leaches can serve as adjuvants for the immune system *(Singh, 2011).*

ADA production has been associated with the development of pure red cell aplasia (PRCA) after administration of recombinant erythropoietin (EPO). Investigations have shown that organic compounds from uncoated rubber

stoppers had leached into the pre-filled syringes and it was concluded that Polysorbate-80 induced the immunogenicity response in patients leading to autoimmune reactions. Polysorbate-80 was later replaced by a fluororesin, which together with an altered route of administration (*s.c* to *i.v*) lead to decrease incidence of PRCA *(Boven, 2005)*.

1.8 Clinical aspects of immunogenicity by biopharmaceuticals

The main concern when evaluating new biological drugs is the potential clinical impacts. Adverse events caused by biologics can generally be divided into two main groups; exaggerated pharmacology and immunogenicity.

Exaggerated pharmacology is an effect by the drug and can result in life-threatening responses. One known example is the cytokine storm seen in the Tegenero disaster in London 2006 when six healthy male volunteers, included in a first-time-in-man phase I trial with a super agonist anti-human CD28 monoclonal antibody (mAb), all fell victims for life-threatening acute inflammatory responses *(Suntharalingam, 2006).* The drug (TGN1412), intended for treatment of leukemia and rheumatoid arthritis, had previously been tested in pre-clinical trials including rodents and non-human primates. No serious adverse effects had been noted and the administered dose to the volunteers was 500 times lower than the No Observed Adverse Effect Level (NOAEL) dose tested in the animal models. Reports afterwards concluded that the adverse events were unique unforeseen biological effects in man. Some of the mechanisms behind the strong responses seen in man, but not observed in animals, are still unknown. However, inter-species differences in CD28 expression on CD4+ T cells could be one explanation for the different immune responses between animals and man *(Eastwood, 2010).* Differences in signal transduction pathways between man and non-human primates may also explain the differences in the response following TGN1412 administration *(Stebbings, 2009)*. Many lessons regarding evaluation of new biological drugs were learnt from this experience and the European Guideline was revised after this particular incident. Exaggerated pharmacology is often mistaken for immunogenicity, and it is important to be aware of the different aspects of clinical manifestation. In the following section immunogenicity responses to a selection of different therapeutic proteins will be discussed in more detail.

The consequences of immunogenicity can be alterations of the drug´s pharmacokinetics, reduction/loss in efficacy or cross-reactions of ADA with

endogenous proteins causing autoimmune conditions. In some cases, however, ADA may not influence drug potency. One example of this is growth hormone (GH), for which no effect on clinical efficacy was noted as a consequence of GH-specific ADA. This can be explained by the fact that the ADA and the GH receptor bind to different epitopes on the GH molecule *(Shellekens and Casadevall, 2004)*.

Alterations of the pharmacokinetics of the drug have been observed in the treatment of diabetes mellitus with rh-Insulin. Insulin molecules exist as monomers in its physiological state, but when stored under therapeutic dose concentrations dimers and hexamers are formed, creating an insulin depot after *s.c* administration. The individual molecules must dissociate before entering the blood stream and the plasma levels peak after 90-120 minutes explaining why the dose should be taken one hour prior to food consumption. New fast-acting insulin analogues have been developed with increased steric hindrance *(Walsh, 2005)*, thus preventing aggregation and alterations of the pharmacokinetics. Importantly, drug aggregates also have the potential to increase immunogenicity influencing pharmacokinetics *(Chirmule, 2012).*

NAbs directed against interferon-β (IFNβ) are associated with loss of clinical efficacy in patients with multiple sclerosis, and have been demonstrated in several studies and with various drugs (*e.g.* Avonex® and Betaferon®) *(Malucchi, 2008).* In contrast, ADA formation to aggregates of IFN-β has been shown to result in absence of neutralizing antibodies along with lack of immunological memory *(Van Beers, 2010).*

Several studies show evidence of cross-reactivity of ADA with endogenous proteins after treatment with recombinant human proteins, resulting in the most severe type of adverse event known after treatment with biopharmaceuticals- autoimmunity. Patients treated with EPO for chronic kidney disease developed pure red cell aplasias (PRCA) that lead to anemia *(Casadevall, 2002).* Autoimmune syndromes have also been observed after administration of thrombopoietin, causing thrombocytopenia *(Koren, 2008).* These autoimmune conditions are seen in patients with ADA capable of neutralizing the biological effect of both the drug and the endogenous counterpart.

Hemophilia A is a life-threatening congenital X-linked bleeding disorder and is caused by absence or abnormal functional of coagulation factor VIII (FVIII), resulting in incomplete coagulation. The classical treatment of Hemophilia A is *i.v.* administration of rhFVIII to replace the missing or defect coagulation factor. Administration of rhFVIII can lead to adverse

immune responses such as allergic reactions, anaphylactic shock, or most commonly production of ADA against antigenic epitopes on FVIII. The development of inhibitory ADA against infused FVIII is one of the most serious complications in the treatment of Hemophilia A patients. About 30% of patients receiving FVIII develop neutralizing anti-FVIII antibodies *(Kaveri, 2009).* FVIII-specific ADA (of the inhibitor type) causes replacement therapy resistance *(Lacroix-Desmazes, 2002)* and because the ADA neutralizes the haemostatic effect of FVIII, the patients continue to bleed from tissues and joints *(Lavigne-Lissade, 2008).*

1.9 Current analytical methods and predictive tests for immunogenicity

1.9.1 Assays used to analyze an immune response against biopharmaceuticals

Various assays can be used for measuring immune responses to biopharmaceuticals including end-points for detection of *e.g.* ADA, drug-specific T cell responses, and cytokine release (Table 5).

Monitoring of ADA

Methods used for measuring specific ADA have evolved and the recommendations for assay design and validation can be found in two industry White papers *(Sluis, 2004, and Gupta, 2007).* See section 1.3.

In vitro models

In vitro assays used for studying immunogenicity are mostly T-cell based and aims at assessing the potential of whole proteins to challenge memory-cell activation in already drug-sensitized individuals *(Delluc, 2011).* Lymphocyte proliferation is a common end-point most often measured by ^{3}H-thymidine incorporation or flow cytometric analysis (FACS) that may include activation markers.

Table 5. List of available assays used in immunogenicity research.

End-point	Assay	References
ADA	ELISA	Liu, 2011
	Radioimmunoassay (RIA)	Pijpe, 2005
	Multiparametric bead- analysis (Luminex®)	McCutcheon, 2005
	Surface Plasmon Resonance (SPR) (BiaCore®)	Lewis, 2013 Scott, 2005
	Antigen binding test (ABT)	Van Schouwenburg, 2010
Neutralization	Bioassays	Finco, 2011
T cell proliferation	Thymidine incorporation	Naisbitt, 2001
Cytokine release	ELISA	
	ELISpot	Anthony, 2003
T cell responses	Flow Cytometry; FACS	James, 2009

1.9.2 In vivo models

Animal models are used in pre-clinical drug-safety studies of biopharmaceuticals and sometimes employed as tools to study mechanisms underlying an ADA response. More importantly, animal models are not used to predict immunogenicity in man due to the large difficulties regarding extrapolation of results between species. Still, guidelines sometimes suggest the use of drug-tolerant Tg-mice as a way forward *(EMA, 2007 and ICH S6, 2009).* One advantage of using animal models is the ability to study the reaction in an intact immune system, which is an advantage as compared to *in vitro* and *in silico* assays. However, due to the high costs and time-consuming

experiments using animals, *in vivo* models are not used until after drug-candidate selection using *in silico* and *in vitro* models.

Today, most biopharmaceuticals are fully human proteins, with regard to their amino acid sequence, because of the risk of evoking a xeno-response after administration to patients. This can therefore be a problem in pre-clinical studies in which animal models are used. The animals will generally induce immune responses when given the foreign (*i.e.* limited sequence homology) human proteins. Even though the animal response may not be identical to the response in man, animal models can be useful immunogenicity models provided the results are critically evaluated. The predictive value is limited and, due to the species differences in the immune response, so is the value of mechanistic studies. In Table 6, examples of *in vivo* models used in immunogenicity studies are presented.

Table 6. Examples of in vivo models used in immunogenicity studies.

Biologic	Indication	References
Human insulin	Predict neo-epitopes	Ottesen, 1994
IFN-β	Relative immunogenicity Breaking of B cell tolerance	Sauerborn, 2013 and Van Beers, 2010
IFN-αb	Aggregation	Hermeling, 2008
Tissue plasminogen activator	Predict neo-epitopes	Stewart, 1989
Human growth hormone	Breaking of tolerance	Lee, 1997
FVIII	Inhibitor formation Mechanism of ADA production	Wu, 2001

Conventional mouse models

The use of Wt-mice in immunogenicity studies has limitations because human proteins will be experienced as foreign. Even when a protein drug is exogenous for both mice and humans (*e.g.* plant-derived proteins), species

differences in immune function makes it difficult to extrapolate results between species. Also, genetic restrictions in inbred mouse models constitute a limitation and can introduce results that have no value for making predictions about the human situation *(Brinks, 2011).*

Immune-tolerant mice

One way to prevent ADA responses in mice is to use transgenic models, immune-tolerant for the human protein they express. Immune-tolerant transgenic mouse models have been shown to be useful in immunogenicity studies, *e.g.* the effect of aggregation, prediction of neo-epitopes and breaking of immune tolerance (Table 6). These models can be used to determine relative immunogenicity between product batches and formulations. However, there are some limitations in the use of transgenic models, as well as for wild type animals, since the response against the protein will be via a rodent immune system. The absence of human MHC class II, in a T- cell dependent response, together with differences in T- and B-cell receptor repertoires will limit the usefulness of these models *(Brinks, 2011).*

Transgenic mice expressing human HLA

Yet another way to overcome the problem with species-differences leading to immune responses is the development of new animal models that potentially can be used in immunogenicity studies of therapeutic proteins. A transgenic knock-out mouse model expressing human leukocyte antigen (HLA) allotypes and lacking the murine MHC class II, has been generated *(Black, 2002).* This model could be useful when investigating TD responses and could be improved further when crossed with transgenic mice tolerant for a specific drug, for example human FVIII *(Madoiwa, 2009).*

Additional animal models

Proteins that are highly conserved between species, such as insulin and human growth hormone, have been tested in rats and monkeys without any induced immune responses in subchronic and chronic studies *(Zwickl, 1995 and 1996)*. It has also been shown that non-human primates can be used for predicting relative immunogenicity of different forms of human growth hormone in humans and the autoimmune reaction to thrombopoietin *(Wierda, 2001).*

The consensus so far is that animal models can be used for assessment of relative immunogenicity between protein products, but their predictive value for estimating immunogenicity in humans is still limited/absent. Likewise,

the ability of animal models to predict induction of NAbs is also limited *(Brinks, 2011).*

1.9.3 Predictive tools

In general, amino-acid sequences and their ability to act as T cell epitopes are good starting points to predict if there is going to be a human immune response against non-human proteins. Further, for therapeutic human proteins, immunogenicity is mainly determined by the presence of impurities, aggregates and protein degradation *(De Groot, 2007).*

Table 7. Examples of in silico assays used for prediction of immunogenicity.

Indication	Method	References
T cell epitopes	In silico tools	Dönnes, 2006, De Groot, 1997
B cell epitopes	In vivo, in vitro, in silico	Roggen, 2008

In Silico methods

Immunogenicity studies have gone from hypothesis driven to data driven research. The genomic revolution together with the ability to analyze high through put data sets with informatics tools has opened new doors for investigations into immunogenicity. The informatics tools include the more common forms of bioinformatics tools such as information on the human and microbial genome sequences, and the newer immunoinformatics tools that are based on computer science and integrating biophysics, structural biology and protein homology modeling *(Flower, 2007)*. Identification of immunogenic epitopes and predictions on the immunogenicity of whole proteins can be accomplished by immunoinformatics (Table 7). Another type of *in silico* tools is referred to as immunomics. This is the interface between the host immune system and the proteins derived from pathogens or self, and includes mapping the epitopes and searching for the antigens that stimulate an immune response *(De Groot, 2006).* T-cell epitope algorithms have emerged into validated powerful tools used in drug discovery for a range of biomedical applications such as design of new vaccines and therapeutic proteins, antigen discovery, autoimmunity among others *(De Groot, 2006).* The T-cell epitope content is contributing to the antigenicity of a drug and the binding strength of T cell epitopes by MHC molecule is a key determinant in the resulting immune response. The drug epitopes with high binding affinity to MHC are more likely to be displayed on the surface of APC and presented to the T cell

receptor *(Weber, 2009)*. This makes the T cell epitopes a determining factor of immunogenicity and can also be used as potential biomarkers for immunogenicity. *In silico* tools for predicting MHC class II-binding epitopes have been developed *(Dönnes, 2006, De Groot, 1997)* making it possible to identify sequences in the protein that bind to MHC class II in a rapid and relative low cost analysis. However, *in silico* methods have the disadvantage of over-predicting immunogenicity responses and do not predict the overall ability to activate T cells or other immune components, only the interaction between the peptide sequence of the drug and MHC class II can be predicted. Therefore, *in silico* methods cannot be used as stand-alone assays and in the end combinations of *in silico, in vitro* and *in vivo* studies will be needed.

In vitro methods

After the Tegenero incidence in which six healthy male volunteers developed an exaggerated pharmacological response after treatment with TGN1412, *in vitro* testing using human material was brought further into light. *In vitro* testing using TGN1412 and human PBMC indicated that the volunteers had actually been given a near-maximum immune stimulatory dose of the drug *(Stebbings, 2009).* The starting dose had been calculated using the traditional toxicological-based NOAEL, including *in vivo* data from animal models only. After Tegenero, the EMA guideline included the use of the minimum anticipated biological effect level (MABEL) approach (*EMA Guideline, 2007) and Milton, 2009)*, summarizing all available data from both *in vitro* and *in vivo* tests. Today, many regulatory agencies request the assessment of cytokine release using co-cultured *in vitro* systems *(Stebbings, 2007).*

HLA-associations in risk management

HLA has a central role in the potential development of ADA since these molecules are the critical antigen-presenters of the immune system. HLA-typing can be used as a tool for identifying individuals at risk for ADA-development.

Associations between specific HLA-alleles (DRB1*07:01, *04:01, *04:08) and the development of ADA after treatment with IFN-β have been identified *(Barbosa, 2006 and Hoffmann, 2008)* suggesting that HLA typing in MS patients should be done pre-treatment and in those patients with a high risk to develop ADA, alternative treatments should be considered.

MHC Class II proteins are highly polymorphic and their distribution differs between ethnicities *(Mayer, 2008).* Thus, even identical peptides will interact differently among patients because they carry different alleles of MHC-class II.

1.10 The "Three Rs"

The 3R´s is a concept grounded by W.M.S. Russell and R.L. Burch in 1959. These two men were about 25 years ahead of their time when introducing and questioning the humane use of animals in scientific research. In 1978, David Smyth introduced the word alternatives to define the principles *(Smyth, 1978)*. The R´s stands for refining, reducing and replacing the use of animals in research, testing and education.

Since the publication of Russell and Burch in 1959, relatively little attention was paid to the concept of 3R. During the 1980s laws and guidelines were introduced, which did not only highlight the concept of three Rs, but also placed legal and moral obligations to reduce, refine and/or replace the use of laboratory animals wherever possible. The *Bologna declaration in 1999* concluded that "the only acceptable animal experiment is one which has been approved by an ethical review committee, used the smallest possible number of animals, and caused the least possible suffering which is consistent with the achievement of its scientific purpose". The current legalization is to some extent different between countries, but the main objectives are that all proposed use of animals in laboratory research should be subject to review to determine whether such use appears to be scientific and ethically justifiable *(Zurlo, 1996)*. The concrete facts included in the following sections (1.10.1-1.10.4) will be referred to using *Russell and Burch's "The Principles of Humane Experimental Technique"* from 1959 if not anything else is stated.

1.10.1 Refinement

The term refinement is used to describe those methods which enhance animal well-being by alleviate or minimize potential pain and distress.

1.10.2 Reduction

The goal with reduction alternatives is to obtain the same level of information from the use of fewer animals in laboratory procedures, or obtaining more information from the same number of animals, which in the long run can result in fewer animals needed to implement a given project or test.

1.10.3 Replacement

Russell and Burch distinguished between relative replacement, *e.g.* the humane killing of a vertebrate to provide tissues, cells and organs for *in vitro* testing, and absolute replacement in which animals would not be used at all *i.e.* the use of invertebrate and/or human cells and tissues. However, in the

Bologna declaration, a range of replacement alternative approaches are suggested:

- Improved storage, exchange and use of already performed animal experiments, and through that avoiding unnecessary repetition.
- The use of physical and chemical techniques.
- The use of mathematical and computer modeling.
- The use of lower species *e.g.* invertebrate animals, plants and microorganisms.
- The use of *in vitro* methods.
- Human studies.

1.10.4 Why should we do it?

Reduction, refinement and possible replacement of animal use in scientific research, testing and education have a political as well as a scientific value. By calculating and optimizing the correct number of animals and studies needed for a significant result, higher quality will be gained and a reduced number of animals will be needed for the same results. Further, refinement of methods and analyses can result in less material needed to perform the test and/or the possibility to combine several analyses in one study leading to less pain and distress for the animals. The most challenging issue in the 3R´s principles is the replacement of animals in scientific research. First, knowledge about the alternatives are not yet understood or even invented. Second, tradition is a strong power and many facilities, both industrial and academic, are not willing to change a working concept, even though it means less animals needed. However, in some cases animal studies have been successfully replaced by cell-based methods *(European Commission, 2008)* showing better and more predictive results.

The greatest scientific achievements have always been the most humane and the most aesthetically attractive, conveying that sense of beauty and elegance which is the essence of science at its most successful.

Russell & Burch, 1959.

2 AIM

The emphasis of this project was to investigate the value of class and subclass profiling of anti-drug antibodies (ADA) in immunogenicity studies, and further link these profiles to factors that potentially contribute to biopharmaceutical drug-specific responses using transgenic- and wild type mouse models. Knowledge from these studies was intended to improve risk assessment of ADA-production in early pharmaceutical development.

2.1 Objectives in paper I

Develop an ex-vivo immunogenicity assay that can detect ADA of various classes and subclasses in a single plasma sample.
Investigate the value of antibody class, subclass and cytokine profiles as biomarkers for immunogenicity of biopharmaceuticals.

2.2 Objectives in paper II

Investigate ADA titers in a newly developed triple-transgenic mouse model expressing human coagulation factors II, VII and X, and validate the potential of this model for drug-induced immunogenicity screening.

2.3 Objectives in paper III

Investigate the differences between ADA-profiles in wild-type (Wt) mice vs. transgenic (Tg) mice encoding a human recombinant protein drug.
Investigate how these antibody responses can be linked to different immunological mechanisms.
Investigate how presence of impurities in different batches of the selected drug can contribute to different ADA responses against the drug in Wt- and Tg-mice.

2.4 General objectives

Exploit the use of Tg-mouse models, commonly used for product quality assessment, as a tool for solving issues and gaining more knowledge on immunogenicity – a current problem in development of biopharmaceutical drugs.

Meet the requirements of 3R´s (reduce, refine and replace) by developing effective and sensitive assays that requires less material compared to conventionally used methods.

3 MATERIALS AND METHODS

3.1 Recombinant human protein

Various batches of a candidate drug based on a recombinant human protein, were used in Paper I and III, and described in more detail in Table 8. The protein solutions were prepared using 2 mM trisodium citrate, 10 mM histidine and 140 mM NaCl, in a pH 7.4 buffer. The protein formulations were obtained from AstraZeneca early process development. Due to trade secrets regarding the protein and the risk of complicating patent registration, the drug cannot be identified by its complete name. The concentrations of this test compound were determined using UV spectroscopy. The noted impurities seen in the different batches were detected by Size Exclusion Chromatography (SEC) and were mainly fragments or degradation products. The protein product was not aggregation prone.

Table 8. Characteristics of various batches of the recombinant human protein candidate-drug used in Paper I and III.

Batch	Endotoxin (EU/mg)	Host cell protein (ppm)	DNA (ppm)	Purity by SEC (%)	No of chromatography steps in purification
1	0.1	20	5	97.9	2
2	3	6	6	98.6	2
3	0.3	1	5	99.5	2
4	0.3	8	<0.05	97.7	3

In Paper II, recombinant human coagulation factors (AstraZeneca, Sweden); FII, FVII and FX were used in combination. The protein solution was prepared in a 2 mM trisodium citrate, 10 mM histidine, and 140 mM NaCl, in a pH 7.4 buffer containing <0.001 EU/mL endotoxin. The concentration was determined using UV spectroscopy and the purity of each test compound was assessed using SEC. Coagulation factor assays were used to assure the bioactivity and the potency of the proteins. A prothrombinase assay (*Kirchhof et al, 1978),* commercially available as Rox Prothrombin, (Art No. 200040, Rossix, Mölndal, Sweden) was used for FII. FVII potency was tested using the Biophen FVII assay (Aniara, Mason, Ohio, cat No. A221304), and the Biophen FX assay (Aniara, Cat No. A221705) was used for FX potency assessment. These assays were also used for determining the concentration of mouse coagulation factors in mouse plasma.

All recombinant human proteins used in Paper I-III were produced in Chinese Hamster Ovary (CHO) cells.

3.2 Animal models

Wt mice and immune-tolerant (Tg) mice expressing human proteins have been used in this thesis.

3.2.1 Wild type mice

The Wt mice used in Paper I-III all had C57BL/6 background.

3.2.2 Transgenic mouse model encoding a human protein drug candidate

The immune tolerant mouse model used in Paper I and III had previously been generated in the facilities of AstraZeneca, Södertälje, Sweden. The human DNA construct was injected into the pronucleus of the one cell stage embryo of B6CBA mice. The offspring was backcrossed to C57BL/6 two times resulting in 87.5% C57BL/6.

3.2.3 Transgenic mouse model encoding human coagulation factors II, VII and X

A triple-transgenic mouse model was constructed in Paper II, expressing the human coagulation factors II, VII and X. The vector was designed to harbor all three factors in tandem, although separate expression units and a cytomegalovirus (CMV) promoter controlled the expression of the human coagulation factors. For more details regarding the transgenic DNA construct, see Paper II. The transgenic DNA construct was injected into the pronucleus of fertilized C57BL/6 (Charles River Laboratories, Sulzfeld, Germany) eggs and implanted into pseudo-pregnant foster mothers. Eight founder lines were generated and two of them; line E and H, proved to express all of the three coagulation factors. Genotyping of the mice was done using PCR amplification of genomic DNA, derived from ear biopsies, to detect sequences encoding the human coagulation factors and by that confirm the transgenic expression in the mice.

3.3 Immunization

In Paper I and III, both Wt- and Tg-mice were immunized following the same study design. The mice were approximately 8 weeks at the start of dosing and were given a total of 4 *s.c* injections of rh-protein candidate-drug

(AstraZeneca, Sweden) on the back just below the neck. The injections were distributed as single doses every second week (Day 1, 15, 29 and 43), at a dose level of 1 mg/kg, using individual dose volumes based on body weight values.

In Paper II, transgenic mice encoding for the human coagulation factors -II, -VII and -X, from two different lines (E and H), and their Wt littermates were approximately 12 weeks of age when included in the immunization study. A total of 48 animals, six Wt- and six Tg-mice from each line and gender, were included and dosed with 1 mg/kg of a combination of human FII, FVII and FX. The mice were given 4 *s.c* injections just below the neck, distributed as single doses every second week.

3.4 Plasma sampling

In Paper I and III, blood samples were taken from Wt- and Tg-mice pre-dose (negative control) and two weeks after the last injection. Blood was taken from orbital plexus, under isoflurane and oxide (O_2) anesthesia, and collected in K-EDTA tubes. Plasma was prepared and stored at -70ºC until testing.

In Paper II, pre-and post-dose blood samples were taken from vena saphena, without anesthesia, and collected and stored under the same conditions as in Paper I and III.

3.5 Ethical consideration

All animal experiments described in this thesis were performed in accordance with Swedish law regulating animal experimentation and approved by animal ethics committee.

3.6 ADA determination

In Paper I, multiparametric bead-analysis was used for determination of ADA. In Paper III, both multiparametric ADA-assay and enzyme-linked immunosorbent assay (ELISA) was used. Distribution of samples analyzed in Paper III is shown in Table 9.

Table 9. Number of animals included in each dose group tested with a new multiparametric ADA-assay and with ELISA, Paper III.

	Multiparametric assay		**ELISA**	
	Wt	***Tg***	***Wt***	***Tg***
Batch 1	4* (3)	4* (3)	10	10
Batch 2	4*(3)	4	10	10
Batch 3	4	4	4	7
Batch 4	4	4	10	10

*Groups marked with *, denotes animals excluded due to methodological error and therefore results from these groups were calculated on three animals instead of four.*

3.6.1 Multiparametric ADA-assay

The immunogenicity assay refined and validated in Paper I, and used for detection and measurements of ADA in plasma samples from Tg- and Wt mice in Paper I and III, is based on the Luminex Technology, which is a multiparametric bead analysis instrument (Luminex-100®) that enable detection of multiple analytes in one single sample. General information about the read-out system can be found in *Bio-Plex™ User Guide*. Details of the specific assay and validation of the same can be found in Paper I. Shortly, polystyrene beads were conjugated with various ratios of fluorophores and conjugated with anti-antibodies specific for IgG1, IgG2a, IgG2b, IgG3, IgA and IgM. This makes identification of specific ADA classes and subclasses possible. By adding biotinylated drug, which will bind strongly to streptavidin-phycoerythrin (SA-PE), used as detection signal, the assay can distinguish between drug-specific and non-specific antibodies. The signal from PE will be correlated with the signal from the dyed bead, and the ratio will be correlated to the specific ADA, when the sample is analyzed in the array reader. The readout is fluorescence intensity (FI) and ADA of various classes and subclasses can be semi-quantified in one single sample.

The Luminex-technology was chosen for the intended purpose due to its low sample volume requirements, its multiparametric potential and also for the

pedagogical layout, reducing the risk of methodological errors, which improves reproducibility.

3.6.2 Enzyme-linked immunosorbent assay

In Paper II, ELISA was used for measuring ADA (IgG, IgA and IgM) titers against FII, FVII and FX in plasma samples from Tg- and Wt-mice using pre-washed streptavidine-coated plates (Nunc, Roskilde, Denmark).

In Paper III, ELISA also served as a reference method in validating the multiparametric bead assay for detection and measurement of ADA titers against rh-protein drug-candidate.

ELISA is a conventionally used method for detecting antibodies of various classes and from different species, and the most frequently used assay for detection of ADA *(Mikulskis, 2011).* The wide battery of assays and detection antibodies accessible for everyone makes the ELISA a useful and easy-to-use tool in immunological testing.

3.7 Cytokine profiling

Cytokine screening in mouse plasma in pre-and post-dose samples were done in Paper I and III not only to verify the ADA response, but also to investigate the cellular response against administered human drug.

There are some limitations in the scientific value using frozen plasma samples for cytokine analysis. Cytokine levels can be extremely variable between different time points and the sustainable levels in frozen plasma samples can be discussed. However, in clinical trials, the only possible way of detecting and measuring cytokines (sometimes recommended by the regulatory agencies) are by the use of blood samples. We therefore chose to include cytokine profiling in these studies to mimic the clinical sample testing.

A commercial mouse-specific cytokine analysis kit was used, produced and purchased from Millipore (Solna, Sweden), including IL-12, IL-2, and IFN-γ for detection of Th1- and IL-4, IL-5 and IL-10 for Th2-responses. The procedure was carried out according to instructions provided by the supplier and analyzed using Luminex-100®.

3.8 Statistical methods

To discriminate positive samples from background, a screening cut-point was used (Papers I, II and III) to determine positive samples based on calculated threshold limit of non-specific background (pre-dose values) above which samples are considered positive and below which they are considered negative. The screening cut-point is based on the 95% confidence interval (CI) of the normal distribution, allowing 5% of the positive samples to be false positive, and thus, minimizing the risk of missing true low positive responders *(Shankar, 2008).*

Screening cut point was calculated accordingly:

Cut-point = mean of pre-dose samples[1] + 1.645[2] x Standard Deviation (SD)

1) Optical density (OD) values for ELISA and FI values for multiparametic assay
2) Where 1.645 is the 95th percentile of the normal distribution according to *Mire-Sluis, AR, 2004.*

In Paper I, cut-off was calculated to an FI of 16.3 for Wt mice. In Paper II, the cut-off was calculate to an OD_{450} of 0.438 (FII), 0.103 (FVII), and 0.043 (FX), and for Paper III the cut-off was calculated to an FI of 22.1 for Tg-mice. Values above this cut-point were regarded as positive.

In Papers I and III, additional statistical analyses were used to confirm the positive samples and a second cut-point was calculated. A specificity cut-point was calculated and used to confirm true-positives samples. Background values from pre-dose samples were used to calculate a magnitude of signal inhibition in percent required for a sample to be deemed as true positive *i.e.* containing ADA.

Specificity cut-point was calculated accordingly:

y = mean of log transformed ration – 3.09 x SD

Specificity cut-point = 100 x (1- antilog value y)

The specificity cut-point was calculated to a reduction in FI of 28.8% in Wt-(Paper I) and 65.5% in Tg-mice (Paper III).

Data from positive responders (according to previously described cut-point) in Paper II were generated as concentrations (ng/mL) obtained from OD

values correlated to standard curve from ELISA data. Log-transformed data showed normal distribution and possible significant differences in ADA responses were calculated using one-way ANOVA. Differences between pre- and post dose samples were analyzed using OD values and a paired *t*-test. A p-value <0.05 was considered to be statistically significant for both ANOVA and the paired *t*-test.

In Paper III, Log transformed data (OD for ELISA and FI for multiparametric ADA-assay) showed normal distribution and the paired *t*-test was used to statistically verify the differences in pre- and post-dose samples in the antibody subclass screening and cytokine analysis. Differences were considered statistically significant if p-values <0.05.

4 RESULTS

4.1 Development of a multiparametric immunogenicity assay (Paper I)

A schematic presentation of the assay is demonstrated in Figure 2. During assay development, the included parameters were optimized and titrated to be within the range of the assay and for obtaining the optimal concentrations of each of the four parameters; anti-mouse coupled beads, biotinylated human protein, ADAs and the detection complex SA-PE. Additionally, incubation conditions and various Luminex settings including volume and calibration conditions were optimized.

Validation according to regulatory recommendations regarding immunogenicity testing was done to investigate the stability of the assay between plates and days. A sigmoid shaped standard curve including 8 concentrations and quality control (QC) values were included in the validation for measurement of ADA of subclasses IgG1, IgG2a, IgG2b, IgG3 and IgM. The results showed that the assay is stable between different plates and days, with no observed drift over time.

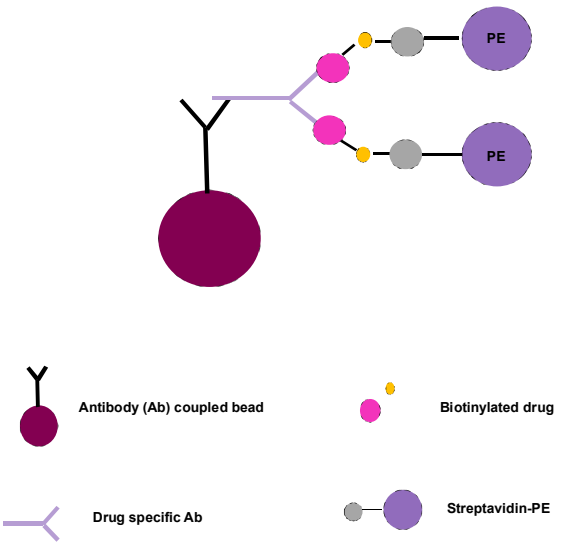
Sensitivity of an assay is the lowest possible concentration detected over background *i.e.* negative control plasma. The sensitivity of this assay showed to be 125 ng/mL in non-diluted plasma and thereby highly sensitive and with good marginal in line with the regulatory recommendations for pre-clinical samples (500-1000 ng/mL).

Assay interference can be a problem in ADA testing due to presence of drug in the samples that can compete for binding to product-specific antibodies, and thus disturb the binding of positive samples in the assay leading to false negative results. In our assay the potential problem was presence of free drug in the plasma samples and the risk of blocking ADA for further binding. To investigate this type of interference, increasing concentrations of excess drug was added to selected plasma samples, with positive ADA-titers, prior to confirmatory analysis. The results showed that despite excess drug candidate (5 μg/mL) in plasma samples, the assay was still able to detect ADA.

After successful optimization and validation of the assay, pre- and post-dose samples from Wt- and Tg-mice (Paper III) were analyzed and tested for

production of ADA after treatment of rh-protein candidate-drug. Results are presented in section 4.2.

Figure 2. Schematic representation of the assay format developed in Paper I.



4.2 Class- and subclass profiling of ADA (Paper I and III)

Both Wt- and immune tolerant Tg-mice were treated with rh-protein candidate-drug using four different batches (1-4) with various degree of purity. The pre-and post-dose samples were analyzed using the assay described in Paper I and by that, classes as well as subclasses of ADA were measured in one single sample.

The results showed that IgG1-titres were significantly increased ($p=0.05$) in Tg-mice after immunization with batch 4. No other significant changes, in response to batch 1, 2 or 3, were detected when titers were used as a measure of ADA presence.

Studying proportions of Tg-mice a majority of these animals, 11/15 (73%), were scored as positive for IgG1 to any of the given batches, Table 10. No significant change or positive responders were observed with respect to IgG2a, IgG2b, IgG3 or IgM.

Wt-mice showed a significant increase in IgG1 to batch 1 ($p<0.05$), 2 ($p<0.05$) and 4 ($p<0.0001$) but not to batch 3 when ADA-titers were used as a measure.

Studying proportions of Wt-mice, a majority of the animals, 13/14 (93%), were scored positive for IgG1 and 9/14 (64%) were scored positive for IgG2b, Table 10. No significant changes or positive responders were observed with IgG2a, IgG3 or IgM.

Table 10. Number of positive responders after administration of recombinant human protein (candidate drug) Batch 1-4, determined by cut-point calculations.

	Tg	Wt	
	IgG1	IgG1	IgG2b
Batch 1	3/3*	3/3*	3/3*
Batch 2	3/4	3/3*	1/3*
Batch 3	1/4	3/4	1/4
Batch 4	4/4	4/4	4/4
Total	11/15 (73%)	13/14 (93%)	9/14 (64%)

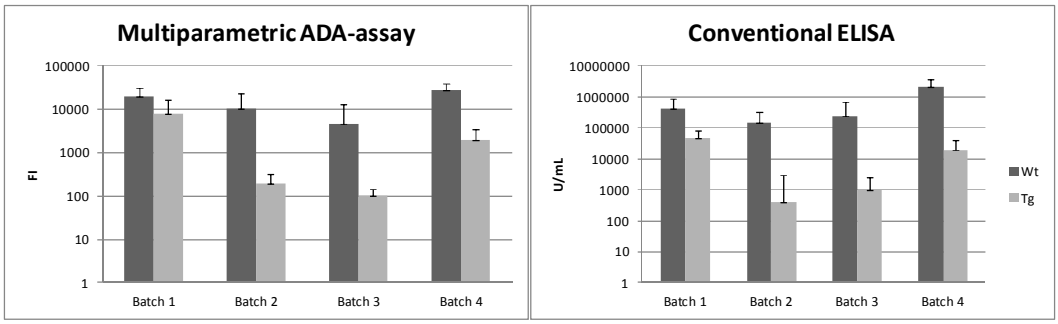
*Groups marked with *, denote animals excluded due to methodological error and therefore results from these groups were calculated on three animals instead of four.*

4.3 Comparative ELISA data (Paper III)

ELISA results generated from a previously performed study, in which determination of total Ig ADA-titers was done, have been used as reference data for the multiparametric bead-analysis method. Identical plasma samples were analyzed with both detection systems, to detect ADA in plasma samples from Wt- and Tg-mice. The results in Paper III demonstrate that the ADA patterns are similar between the assays irrespective of mouse strain tested. Both assays identified Batch 4 as being most immunogenic in Wt–mice and Batch 1 as being most immunogenic in Tg-mice when post-dose titers were used as a measure of ADA, Figure 3.

As earlier discussed in this thesis, ELISA is a conventionally used method for detecting antibodies in pre-clinical and clinical samples. However, the comparable results between the assays indicate a potentially more efficient analysis of subclass profiling of ADA using the multiparametric assay since a multitude of information can be gathered from a single sample of 25µl only.

Figure 3. Mean protein-specific (ADA) total Ig responses in Wt- and Tg- mice using multiparametric ADA-assay as compared to ELISA.



4.4 ADA response in immune tolerant Tg-mice expressing human coagulation factors (Paper II)

Wt- and tolerant triple Tg-mice, treated with a combination of recombinant human FII, FVII and FX, showed notable differences comparing post-treatment titers of coagulation factor-specific ADA. Most important, the Tg-animals responded with significantly lower titers of ADA compared to Wt-littermates (p-values varied between 0.05-0.001).

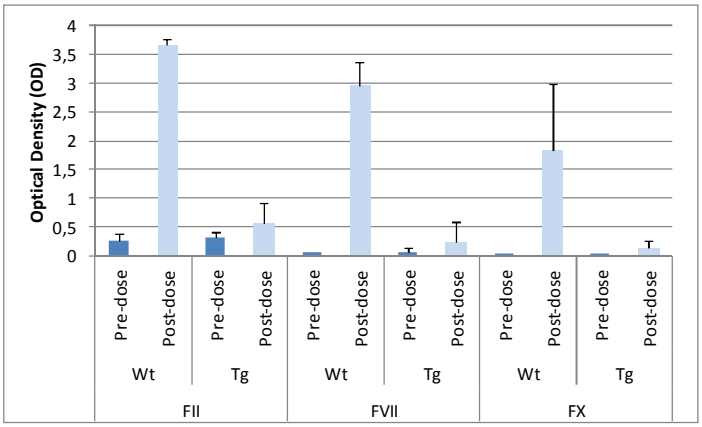
4.4.1 ADA-titers

Both male and female Wt-mice from line E and H showed a significant increase in ADA-titers (Ig) against FII ($p<0.0001$) post-treatment, while only female Tg-mice from line E showed a significant increase ($p= 0.03$). Male and female Wt-mice, from both lines, showed significant increase of FVII ADAs ($p \leq 0.0001$), while only Line E male Tg-mice and Line H female Tg-mice showed significant increases ($p=0.04$ and 0.0001 respectively). ADA-responses against FX were significantly increased in male Wt-mice line H ($p=0.04$) and in female Wt-mice line E and H ($p=0.001$ and $p<0.0001$

respectively). Tg-mice showed a similar pattern with significant ADA-increase in male Line H (p=0.003) and female line E (p=0.01) and line H (p=0.04). In conclusion, for Wt-mice a significant ADA-increase was detected at 11 out of 12 measurement occasions whilst the corresponding distribution regarding Tg-mice was 6 out of 12.

Post-dosing ADA-titers in Tg-mice were significantly lower than in Wt-mice regardless of which coagulation factor was used for immunization. An overview of mean ADA-titers in Wt- vs. Tg-mice are shown in Figure 4.

Figure 4. Pre-and post-dose ADA response in Wt- and Tg-mice after immunization of human FII, FVII and FX.



Mean OD-values+SD including both genders (F+M) and lines (E+H).

4.5 Cellular immune reactions to biopharmaceuticals (Paper III)

Cytokine profiling in pre-and post-dose samples from Tg- and Wt-mice was performed in Paper III to investigate the response to a rh-protein candidate-drug on a cellular basis. The cytokine analysis was performed to correlate the ADA response with cytokine patterns with respect to Th1 and/or Th2 subsets, and through that the potential involvement of T-cells.

The results (Table 11) showed generally higher levels of cytokines in Wt-mice, both pre-and post-dose, compared to Tg-mice. The higher cytokine levels detected post dosing reflects the increased levels of ADA in Wt-mice. Wt-mice responded with significant down-regulation of IL-12 post-dose, and up-regulation of IL-4 and IL-5, after administration of Batch 4, also inducing

IgG1 and IgG2b response in these mice. Significant decrease of IL-12 after administration of Batch 4 was also seen in Tg-mice.

Table 11. Cytokine profiles in plasma samples from Wt- and Tg-mice treated with rh-protein candidate-drug (Batch 1-4).

		Tg				Wt			
		Batch 1	Batch 2	Batch 3	Batch 4	Batch 1	Batch 2	Batch 3	Batch 4
	IFN-y	(0,0) 0,0	(0,0) 0,0	(0,0) 1,5	(1,3) 0,0	(13,2) 19,9	(47,8) 24,8	(10,0) 20,0	(23,7) 15,6
	IL-2	(0,0) 0,0	(0,3) 0,0	(0,6) 0,1	(1,1) 0,0	(5,1) 9,7	(12,1) 7,5	(4,38) 0,0	(6,57) 6,4
TH1	IL-12	(4,9) 0,7	(5,4) 6,7	(6,8) 4,1	(6,0) **3,0**	(29,1) 20,4	(29,6) 116,8	(22,5) 18,4	(36,1) **21,6**
	IL-4	(0,0) 1,0	(0,0) 2,2	(0,4) 1,9	(0,8) 0,0	(0,0) 1,5	(1,1) 62,7	(1,9) 1,4	(0,65) **11,8**
	IL-5	(9,5) 17,4	(8,6) 11,6	(15,0) 13,1	(8,9) 12,2	(9,2) 14,7	(14,0) 34,7	(12,7) 13,3	(9,21) **38,6**
TH2	IL-10	(1,0) 2,2	(2,6) 0,6	(1,7) 2,4	(2,4) 0,3	(38,1) 40,9	(45,2) 82,3	(36,0) 34,1	(56,8) 70,2

Results are presented as mean concentration (pg/ml) cytokine expression pre- (in brackets) and post-dose. Statistically significant changes (student´s paired t-test) are shown in bold text.

In summary, Tg-mice responded against Batch 4 through down-regulation of Th1 cytokine IL-12 and IgG1 antibodies, while Wt-mice responded with down regulation of Th1 and up-regulation of Th2 cytokines and the release of both IgG1 and IgG2b against Batch 4.

The release of cytokines from T-helper subsets are demonstrating involvement of T-cell help in the response to the rh-protein candidate-drug towards production of ADA.

4.6 Transgenic mouse models used in process optimization of biopharmaceuticals (Paper II and III)

The use of tolerant Tg-animals in immunogenicity studies have proven useful as a tool to overcome the xeno-response seen in Wt animals *(Bi, 2013)* enabling studies of mechanisms leading to breaking of tolerance. In this thesis we have used two Tg-mouse models to study the ADA response, both being immune tolerant to the administered protein(s). In paper II, a Tg-mouse expressing human coagulation FII, FVII and FX was used. The relatively lower titers of ADA obtained in these mice indicate that this Tg-model can be used in process optimization of the given biopharmaceuticals. That is, any structural changes of the products or presence of impurities in formulations would show as an unexpected up-regulation of ADA-titers in this in-vivo system.

Confirmatory, the tolerant Tg-model used in Paper III expressed an rh-protein candidate-drug. Same as in paper II, the post-immunization results showed

lower levels of ADA in Tg-mice compared to Wt littermates, indicating tolerance against the human protein in the Tg-model. The Tg-mice showed a significant ADA-response to the batch with the highest levels of impurities, suggesting it as a suitable model for process optimization *e.g.* purification improvement.

4.7 Contribution to the 3R-principle (Paper I-III)

We have through this thesis (Paper I-III) contributed to the 3R-principle in several aspects. First, an immunogenicity assay has been developed that requires smaller sample sizes compared to conventionally used methods, making it possible to exploit blood samples from other toxicological studies for the same drug. The assay uses 20 times less material compared to ELISA, contributing to both reduction and refinement of the use of animals in scientific research. Second, by designing a construct that harbors all three coagulation factors in tandem, immunological studies can be performed using one single mouse model compared to three. Another advantage of having all factors linked together is the more efficient breeding, as segregation of the factors is prevented, which dramatically reduces the number of animals needed.

5 DISCUSSION

5.1 Nature of the antigen

Biopharmaceuticals are highly target specific and have less toxic side effects than LMW drugs. However, because of their complex structure they are likely to induce immune responses. Their antigenic properties can trigger several immune cascades leading to ADA formation and subsequent neutralization of the drug. Sometimes they can induce autoimmune conditions as demonstrated during treatment with recombinant human erythropoietin *(Casadevall, 2005).* Besides the structure of the biopharmaceutical itself, the formulation can affect a drug's immunogenicity. In this thesis we have started to investigate the correlation between the nature of the drug in terms of purity and formulation, with the immune response induced after immunization. Associations between ADA (Ig) responses and drug impurities have been made for insulin and growth hormone *(Schellekens, 2005).* However, our goal was to not only investigate ADA titers as endpoint, but to use subclass profiling as an additional biomarker tool. We have used four different batches of an identical recombinant human protein with various degrees of purity. Our results suggest that a poor purity of the formulation, including drug fragments, degradation products, endotoxins and HCP, induces ADA to the rh-protein candidate-drug in both Wt and immune-tolerant Tg-mice. When the most pure formulation was used the proportion of ADA positive animals was 3 out of 4 among Wt-mice and 1 out of 4 among immune tolerant Tg-mice. Thus, Wt-mice likely develop ADA as a xenogenic response whilst tolerant Tg-mice are triggered by other antigenic epitopes *(Hermeling, 2005).*

5.2 Detection and measurements of ADA responses

The generation of an ADA response can in rare cases have clinical consequences and more frequently affect drug efficacy in human trials. In pre-clinical development, the ADA response can complicate the toxicity studies, or the PK and PD data. It is therefore important to have testing strategies that includes risk assessment of ADA responses during both pre-clinical and clinical development.

A White paper *(Mire-Sluis, 2004)* has been published in which recommendations for the development and validation processes of immunogenicity assays are

described. However, even though the recommendations from publishers and Guidelines are accessible, there are still some difficulties regarding assay development and how to value the results. The lack of standardization is a distinct problem. No international antibody assay standards are accessible, resulting in wide variations of the reported incidence of immune responses associated with a single product *(De Groot, 2007).*

The use of immune assays contributes to the understanding of immunogenicity. Our aim with the assay developed as a part of this thesis, was to meet the performance characteristics for immunogenicity assays and to set up an assay easily implemented in a typical laboratory. We have in this thesis (Paper I) developed an immunogenicity assay based on a multiparametric bead analysis that can detect several subclasses of ADA in one single sample. The data generated was compared to equivalent data obtained from a bridging-ELISA performed as part of a previous report. The bridging-ELISA was used in this thesis as a reference assay because it is the most commonly used assay for ADA detection *(Liang, 2007).*

The multiparametric ADA-assay presented in Paper I requires only 25 µl of diluted sample per well and thanks to its multiparametric nature, all analytes can be detected and measured in the same sample. Analyzing five different subclasses will need 20 times less material than if a traditional ELISA was to be used (4x more sample/well x 5 analytes). In a traditional ELISA each analyte has to be analyzed in separate wells. Because of the low volumes needed, test samples can be used in several assays, reducing the number of animals needed. Another advantage with the multiparametric ADA-assay is the reduced amount of reagents and material needed, resulting in an easy-to-use, less expensive assay. Five ADA subclasses can be analyzed in triplicate in pre- and post-dose samples of 12 animals together with a standard curve and two quality controls in duplicates using 96-plate well. Only five washes (200 µl x 2 and 200 µl x 3) are needed in the procedure, which lowers the risk of removing low-affinity antibodies, especially those with rapid dissociation rates *(Liang, 2007).* Another advantage with the multiparametric ADA-assay is the decreased risk of steric hindrance, because the molecules are mobile in solution and not coated on the bottom of the well as in the ELISA.

We have demonstrated that the new assay can detect drug-specific antibodies in the presence of pre-existing drug in the plasma sample, and by that overcoming the problem of drug interference. ELISAs have been shown to be susceptible to drug interference, and can only measure ADA in the absence of detectable drug levels *(Hart, 2011).*

However, the ELISA format is an easy-to-use assay that can be performed in most laboratories, without the need of technology platforms. This is one of the disadvantages with the assay we have chosen to develop. The assay is built on the Luminex-platform, requiring the access to expensive equipment and the use of commercially restricted reagents. Since the development process of a specific drug can proceed during decades, it is important to use assays that will last during this time period to be able to follow the incidence of ADA production from process optimization to clinical analyses post-approval.

Subclass profiling in pre-clinical studies are not required by the agencies. However, subclass identification of ADA responses is included in the characterization package recommended in human trials for establishment of clinical significance of ADA responses *(EMA Guideline, 2007)*. By this thesis we therefore want to highlight the value of identifying Ab subclasses and classes of ADA also in pre-clinical studies to be able to correlate these specific responses to various factors responsible for ADA generation. Determining classes and subclasses of ADA for the high risk drugs can help evaluating how far the drug-specific immune responses have advanced. However, the clinical relevance of subclass profiling in immunogenicity studies must be further explored.

A correlation between low batch purity and high ADA levels was shown in Paper III. The least pure batch induced a significant increase in ADA of subclass IgG1 in both Wt- and Tg-mice. Since the Tg-mice were supposed to be tolerant to immunization with the human protein itself, the impurities (fragments, degradation products, endotoxins and more) present in the formulation had likely contributed to breaking the tolerance. In Wt 9/14 animals had an IgG2b response compared to none of the Tg-mice. We suggest that the IgG2b response in Wt-mice reflects a xeno-response to the human protein. The purest batch was the only batch that did not induce significant levels of IgG1 in Wt-mice, reinforcing the hypothesis that impurities such as degradation products or drug fragments are responsible to the induction of ADA. The IgG2b response seen in Wt-mice may originate in a subclass switch from IgG1 to IgG2b. A new experiment including a prolonged treatment period could serve to confirm this hypothesis.

Different mechanisms are induced depending on the nature of the antigen. *(Schellekens, 2005)*.Our data suggests that an IgG1 response triggered by the impurities in the drug formulation is induced by breaking of tolerance, while the IgG2b response only seen in Wt-mice might be initiated through a classical immune response against non-self epitopes. However, additional

studies will be needed to clarify if the response is due to non-self *(Hermeling, 2005)* epitopes in Wt-mice and TLR-activating "danger-signals" *(Matzinger, 1994)* from the formulation impurities in both Wt- and Tg-mice.

Studies have been done in which ADA responses in Wt- and Tg-mice have been correlated to the structure of the protein drug *(Hermeling, 2005)*. To our knowledge no previous studies have correlated ADA subclass profiling to the level of impurities in a sample. Our results suggest that Ab subclass profiling may add information on the mechanism behind an immune response, and as such may add value as a screening tool in pre-clinical toxicological studies in general.

Th1-driven IgG2b responses are induced when the synthesis of ADA is slow, while Th2 driven IgG1 ADA responses in mice occurs when the ADA response is intense *(Reding, 2002)*. These previously published observations correlated well with the results seen in Paper III, in which high titer IgG1 responses were seen in combination with Th-2 cytokines, and the relatively lower IgG2b responses were seen together with Th-1 cytokines.

Measuring subclasses of ADA is not routinely performed in preclinical or clinical studies, but the ability to do so in an effective manner will help investigating specific clinical events, as demonstrated in the correlation between IgG subclasses and duration and outcome of immune tolerance induction in Hemophilia A patients *(van Helden, 2008)*.

Two different statistical analyses were used to discriminate positive ADA-responses from non-positive. First, paired t-test was used to statistically determine the differences in pre- and post-dose samples based on ADA-titers. This method was used for group comparisons only. Second, the more commonly used screening and specificity cut-point determination method were used *(Shankar, 2008 and Mire-Sluis, 2004)*. Using cut-points instead of p-values to determine a positive response enables discrimination of individual responses and ranking them as positive or not.

Using cut-points, background levels of the specific antibody is taken into account, which is an important aspect of immunogenicity testing. This is not done when using paired t-test only. Thus, cut-points should be used for determining positive samples during immunogenicity testing, but other statistical analyses, such as paired t-test, should be used for verifying the response. However, a non-statistically significant change ($p>0.05$) in a group should not be ignored if individual responses have been confirmed as truly positive, using competitive binding with the drug. A non-significant p-value

could be due to high variations in the dose group and low positive responders could be missed out if only group data is considered.

5.3 Use of transgenic animal models in immunogenicity studies

Immunogenicity of biopharmaceuticals to man cannot be predicted before entering clinical trials, due to the various unidentified factors responsible for immunogenicity induction *(Braun, 1997).* The use of well established Tg models in pre-clinical trials can help to identify and evaluate different drug modifications *(van Helden, 2010),* and also to identify modifications that could break immune tolerance to the protein *(Miyagawa, 2010).*

We generated a Tg-mouse model immune-tolerant to human coagulation factors II, VII and X (Paper II), and the results suggests that this mouse model can be used to study immunological responses to these factors given individually or in combination. The tolerant transgenic mouse model produced only low titers of ADA after immunization with the given drugs, suggesting that recipient mice were, at least in part, tolerant to the human coagulation factors. To be able to detect changes in immunogenicity as a result of alterations done do the manufacturing process, complete tolerance is not desired. This goal was fulfilled since only low titers of ADA were detected.

We have further investigated the value of Tg-mouse models for identifying factors contributing to the immunogenicity of protein drugs by comparing the Wt and Tg response of mice injected with different batches of an identical human recombinant protein (drug candidate) carrying different degrees of impurities. Our results show that using Tg models immune-tolerant to the protein of interest a better tool than Wt-mice, for studying immunogenicity, is available. Since Wt-mice will respond against human proteins through a classical immune activation probably involving T cells, they will not be suitable for mechanistic studies on how small modifications of the protein or impurities affect the immune response. Wt- mice were shown to respond with high ADA-titers likely to hide any minor modifications in an antigen response. Instead immune-tolerant mouse models should be used for such investigations.

After detection of an adverse immune event to a biopharmaceutical drug, cytokine profiles are regularly analyzed in clinical plasma/serum samples. This parameter is required by the regulatory agencies and therefore it would

be suitable to also include cytokine analysis in pre-clinical immunogenicity studies in mice. Plasma/serum samples constitute the only available material from clinical trials. Therefore the same source of material should be used in pre-clinical studies to enable extrapolation of data and gather further knowledge on conditions for prediction of immunogenicity in man.

6 CONCLUSION

The immune system is dependent on various molecules working in synergy towards a distinct goal: to defend the host from infections and prevent mortality and morbidity. To monitor and to fully understand the complexity of the immune response to an endogenous or/and exogenous protein is close to impossible. However, by contributing with new knowledge to the field of immunogenicity of biopharmaceuticals, a better understanding of the immune response in general can be obtained. I have through this thesis generated and refined *in vivo* and *ex vivo* methods for improving the risk assessment of immunogenicity of therapeutic proteins. By analyzing the response against various protein batches with different levels of purity, we have started to investigate the correlation between impurities and the ADA response with regard to class and subclass. In this thesis I have:

- Developed a new immunogenicity method that can be used for subclass profiling of ADA in one single sample.

- Generated a new immune transgenic mouse model tolerant for human coagulation factors II, VII and X, which can be used to study relative immunogenicity of these drugs during process optimization.

- Investigated the correlation between impurities, such as degradation products and drug fragments, and induction of specific subclass profiles of ADA. We suggest that there is a correlation between an IgG1 response and the breaking of self-tolerance in Tg-mice and that an IgG2b response arise after classical immune activation by non-self epitopes in Wt-mice.

7 FUTURE PERSPECTIVES

7.1 Paper I

Valid immunoassays are important for immunogenicity screening. To be able to perform relevant inter-study comparisons of ADA-data from identical or different biopharmaceutical drugs, international standardization protocols are needed.

The multiparametric bead analysis assay-protocol that we have developed for the preclinical setting could also be used for measuring classes- and subclasses of ADA in clinical samples. For this a validation procedure using human samples will be necessary.

7.2 Paper II

To be able to fully investigate the value of immune tolerant mouse models in the optimization of protein products, further studies will be needed. Future studies of interest would be to provoke the tolerance of the generated strains, tolerant for human coagulation factors II, VII and X, by using drug products of various quality in a stratified manner including *e.g.* aggregates and different glycosylation patterns.

Discussions of whether or not human immune responses can be predicted from animal models are frequently held. The literature from experts concludes that this is not the case today. Any animal model will always react against human proteins with anti-human antibodies. A different approach would be to develop prediction models consisting of human material *in vitro*. To be able to create an *in vitro* milieu that mimics the human situation, further knowledge about *in vivo* cell function, after drug exposure, needs to be gained (*Roggen, 2011).* By imitating the human situation in an *in vitro* system, xeno-responses would not be an issue. Overall, a combination of human *in vitro* models, animal *in vivo* models, and *in silico* and bioinformatic tools will be needed to be able to predict the human immunogenicity responses using pre-clinical studies.

7.3 Paper III

As in Paper II, future studies of interest would be to include immunization of tolerant mouse models with various biopharmaceutical drugs of different

quality and structures and by that continue to investigate the correlation of ADA subclasses with different cellular mechanisms and factors contributing to immunogenicity. Additional studies involving drug structure and formulation purity, (including *e.g.* fragments, degradation products and HCP), will be needed to further investigate the critical steps leading to ADA responses.

The mechanisms of action involved in immunogenicity responses against biopharmaceuticals are poorly understood. More studies are needed to be able to reveal the contributing factors leading to immunogenicity. One way to reduce the risk for susceptible patients is to gather relevant information including risk factors based on data from biopharmaceutical clinical trials, as initiated by the ABIRISK project. Work package 4a within this academia-industrial collaboration, aims at reducing the risk of immunogenicity after administration of biopharmaceuticals *(IMI- and/or ABIRISK-website*) by establishing a unique shared database for immunogenicity data.

7.4 General future perspectives

The interesting field of immunogenicity has several key-challenges to solve to be able to overcome the problems with adverse events after treatment with therapeutic proteins.

The main mission for drug researchers in the field of immunology, with specific focus on immunogenicity, will be to find the components involved in the initiation of an immune response against a therapeutic protein. Today, methods are accessible to detect ADA responses against protein products. However, these antibodies are the final result of the immunological cascade and the early biomarkers, produced during presentation and initiation need to be addressed and identified to be able to predict, monitor and minimize the production of ADA leading to clinical manifestations. We also need to monitor the response on a deeper level, including *e.g.* cytokine up- and down regulation and subclass profiling of ADA for further linking to specific adverse effects.

There are several ways to decrease potential immunogenicity of a product, *e.g* by blocking antibody-epitope binding via PEGylation *(Basu, 2006)* or by implementing glycosylation modifications *(von Delwig, 2006).*

Intelligent drug modifications together with more precise analysis systems and relevant prediction tools will improve the risk assessment of immunogenicity by biopharmaceuticals.

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