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A Comparison of the Humoral Immune Response Induced by a Recombinant Human Protein in Wild Type Mice and in Transgenic Mice Expressing the Protein

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Authors' contributions

This work was carried out in collaboration between all authors. Authors BG and NB designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors BG, NB, JH, KC and AL managed the analyses of the study. Authors BG, NB and KC managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this work was to investigate the correlation between anti drug antibody (ADA) induction and how different manufacturing processes of biopharmaceuticals affect the immunogenicity of the protein. This was done by testing four different batches of the same recombinant human protein in transgenic (Tg) mice.
Methodology: Wild type (Wt) and human protein-transgenic (Tg) mice were challenged by repeated subcutaneous injections of four batches of a drug candidate protein, obtained by different purification methods. Differences between drug-specific IgG1, IgG2a, IgG2b, IgG3 and IgM antibody patterns produced in Tg vs. Wt mice were investigated and compared to the plasma cytokine profiles. A conventional ELISA was used as a reference method for ADA detection.
Results: ADA responses detected in Tg mice were mainly of the IgG1 subclass and occurred only in significant response to the batch containing the highest level of proteins originating from the recombinant host cells. Wt mice, on the other hand, showed a combined IgG1/IgG2b response to all drug batches, except to the batch with the highest purity. The most pure batch failed to induce significant ADA in both Wt and Tg animals, suggesting host cell derived impurities to be a strong contributing factor to the antibody responses observed.
Conclusion: Thus, an isolated IgG1 response in drug-tolerant Tg mice may serve as a potential biomarker of an immunological reaction to process-related impurities of the protein drug. In contrast, a combined IgG1/IgG2b-profile, as observed in immunoreactive Wt mice, more likely reflects a xeno-response.

Keywords: ADA; biopharmaceuticals; manufacturing process; tg mice; Ig subclasses; cytokine profile; batch variation.

1. INTRODUCTION

Many pharmaceutical companies all over the world expand their pipeline with biopharmaceuticals as a result of their many advantages. High molecular weight drugs, *e.g.* monoclonal antibodies and other therapeutic proteins are highly target specific, with no toxic metabolites and therefore cause fewer side effects compared to chemically synthesized low molecular weight (LMW) drugs [1]. Further, the specific binding properties of biopharmaceuticals often exclude off-target interactions that are commonly observed with LMW drugs. However, recombinant proteins may carry species-specific epitopes or post-translational modifications that can trigger an immune response in the host [2] through one of several potential mechanisms [3]. These include, but are not limited to, increased uptake by antigen-presenting cells, antibody binding and increased uptake by B cells, or the generation of novel T cell epitopes [4]. As a consequence ADA may be generated. Presence of these antibodies can perturb the pharmacology the drug and even inhibit its efficacy [5]. ADA may even disturb normal function of the endogenous protein counterpart leading to autoimmunity [6]. Moreover, ADA-mediated hypersensitivity reactions can also occur [7,8].

Class- or subclass determination of ADA is not compulsory in pre-clinical drug development studies today [9], while clinical studies indeed require these analyses as part of the characterization package required for drug approval [10]. Filling this gap by identifying potential ADA- subclass responses already at the preclinical stage of drug development may permit an understanding of immunogenicity issues for a therapeutic protein before entering

the clinical development phase. In fact, a combination of defining structural elements of the drug making it immunogenic as well as understanding biological function and pathogenic effects of ADA [11,12] may more efficiently aid safer drug development.

Human IgG1 and IgG3 fix complement and respond to protein antigens; the corresponding antibody subclasses in mice are IgG2a and IgG2b [13], induced by Th1 cells [14]. IgG4 responses in humans are mainly seen as ADA responses in patients treated for chronic diseases [15,16], *e.g.* Hemophilia a patients treated with human recombinant FVIII [17]. Human IgG4 is functionally similar to murine IgG1 [18] and is induced by Th2 cells [19]. To our knowledge an attempt to correlate various structural modifications of a biopharmaceutical to ADA subtype profiles has previously not been reported.

Today, several of the biopharmaceutical drugs tested in preclinical studies are fully human with respect to amino acid sequences, e.g. interferons [20], erythropoetin [21], monoclonal antibodies [22] and coagulation factors [23]. As expected, animals will mount an immune response against species-specific determinants on human proteins – a so called xeno-response. However, human protein sequences may be immunogenic in humans as well, and give rise to ADA suggesting that additional structural determinants besides the peptide sequence determine immunogenicity [24]. By using Tg animal models some of the species-specific xeno responses may be avoided and thereby facilitate the drug development process. Tg mouse models expressing the human protein developed as a drug candidate, could potentially allow for studying ADA in a more subtle environment. Additionally, such a Tg model could also be a useful tool when investigating relative immunogenicity during batch process development/optimization. In these cases, Tg models could help monitor and assess factors that could potentially promote development of ADA later in clinical trials. Today, data on immunogenicity is not part of the criteria for clinical batch selection in regulatory studies. ADA data is only used to help explaining observed toxicities and deviating pharmacological effects of the drug.

Production of recombinant therapeutic proteins is highly complex and various factors during the upstream and downstream processes (Table 1) can affect the structure of the drug and thereby its immunogenicity. Further, factors hitherto unknown may also affect immunologic properties of the drug.

Table 1. Chemistry, manufacturing and control (CMC)-related factors contributing to the increased risk of immunogenicity of biopharmaceuticals

Upstream process (Expression systems)
• Selection of host cell and strain types → various *glycosylation* patterns
• Culture conditions, temperature, pH
Downstream processing and purification
• pH, salt concentration, extraction steps, purification and concentrations steps, impurities, host cell proteins, endotoxins → *Aggregation* → *Oxidation* (Loss of activity) → *Deamidation* (Loss of activity) → *Loss of glycosylations*
• Storage

Results from a previously published methodological paper described the development of the current multiparametric bead analysis assay using the same protein but in Wt mice only [25].

Based on this analytical method we have for the first time compared the immune response against a recombinant human plasma protein in Tg and Wt mice with regard to immunoglobulin class and subclass profiles produced. Batch variations due to differences in manufacturing methods as well as strain-specific responses were reflected by unique immunoglobulin expression and suggest antibody profiles as potential biomarkers for improved risk assessment of immunogenicity.

2. MATERIALS AND METHODS

2.1 Assays and Reagents

2.1.1 Batches of recombinant human protein candidate drug

The recombinant protein was produced in Chinese Hamster Ovarian (CHO) cells. Four different batches of a recombinant human plasma protein were obtained from AstraZeneca's early process development. The recombinant human product was not aggregation prone. Impurities detected by size exclusion chromatography (SEC) were mainly fragments and/or degradation products (Table 2). Endotoxins were measured using the Limulus Amebocyte Lysate (LAL)-method [26], host cell protein content was determined by an immunoenzymetric method (Cygnus, Southport, North Carolina) and DNA content was determined by a PicoGreen assay.

2.1.2 Wild type and immune-tolerant transgenic mice

The human protein expression construct was injected into the pronucleus of the one cell stage embryo of B6CBA mice and was randomly integrated. DNA encoding the human plasma protein was expressed under the phosphoglyceryl kinas promotor, a constitutive promoter. In the chosen mouse line, expression of the recombinant human protein was confirmed by analysis of plasma samples, and the expression level was 0,1-1 µg/ml plasma.

Further breeding of the chosen mouse strain was done in C57BL/6. Tg mice used in the study were identified by genotyping and their Wt litter mates were the source of Wt animals.

Table 2. Characteristics of the recombinant protein batch 1-4 used in this study

Batch	Endotoxin (endotoxin units/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

2.1.3 Immunization

The mice were approximately 8 weeks at the start of dosing (Table 3). A total of 4 subcutaneous injections were given, distributed as single doses (1 mg/kg) every second

week in individualized dose volumes based on body weight. All animal experiments described in this paper were performed in accordance with Swedish law regulating animal experimentation.

Table 3. Number of animals included in each dose group in the immunogenicity assay and the ELISA

	Multi-parametic assay		ELISA	
	Wt	Tg	Wt	Tg
Batch 1	4	4	10	10
Batch 2	4	4	10	10
Batch 3	4	4	4	7
Batch 4	4	4	10	10

2.1.4 Plasma samples

Blood samples were collected in K-EDTA tubes pre-dose and two weeks after the last injection from the orbital plexus of Wt and Tg mice under isoflurane and oxygen (O_2) anaesthesia. The cells were spun down, the plasma collected and stored at -70℃ until testing.

2.1.5 Multiparametric bead analysis

ADAs were measured by a previously refined and validated multiparametric bead analysis assay that allows detection of multiple antibody classes and subclasses in a single sample using the Luminex-100® platform [25,27]. Shortly, polystyrene beads, dyed with various ratios of fluorophores, are conjugated with anti-antibodies specific for IgG1, IgG2a, IgG2b, IgG3, IgA and IgM, making identification of a specific anti-drug antibody class possible. Biotinylated drug is then added for the assay to distinguish between drug-specific and non-specific antibodies after addition of streptavidin-phycoerythrin (PE) The readout is fluorescence intensity (FI) allowing anti-drug antibodies of various subclasses and isotypes to be semi-quantified in one single sample.

2.1.5.1 Positive and negative control

A commercial solution containing a known concentration of mouse monoclonal IgG1 antibodies specific for the protein was used as positive control. A pre-dose sample was used as a negative control for each individual animal.

2.1.5.2 Verification of specificity by pre-incubation

Potentially positive samples having values above the cut point (described in section 2.3) were further characterized by confirming the specificity of the reaction. This was done by competitive inhibition of antibody binding to the drug by potentially positive serum samples. A representative number of positive plasma samples from Tg animals immunized with protein of all four different batches were diluted to their IgG1 cut-off titres, meaning the dilution required to reach cut point. Plasma samples adjusted to the optimal titre were pre-incubated with an optimal concentration of drug (200 µg/ml) as previously established [25]. True positive samples showed a reduction in signal strength or became negative, *i.e.* below the cut point.

2.1.6 ELISA

ADA titers in pre-/post-dose samples from the same Wt and Tg mice treated with the recombinant human plasma protein have previously been measured with ELISA and the antibody-responses to the different batches ranked according to relative titers. A commercial screening kit (Organtec Diagnostica GmbH, Germany) specific for the drug was used, and the procedure carried out according to the instructions of the manufacturer. Since the kit was designed for human samples, the provided anti-human secondary antibody was substituted with a secondary HRP-conjugated polyclonal rabbit anti-mouse antibody recognizing mouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM (cat.no P0260; DakoCytomation, Copenhagen, Denmark). The assay was performed on the ELI-8 system supplied by Tecan Nordic AB (Mölndal, Sweden). Serum was diluted 1:100 and thereafter 1:5 until the concentration was within the detection range of the kit. A standard curve with a range between 6.3 and 100 U/mL was used on each plate. The mean value (U/mL) of a total ADA response (all IgG, IgA and IgM subclasses) from each dose group was ranked according to the response against the different batches of protein drug, and compared with equivalent data generated from the Luminex®-based assay described in 2.1.5.

2.1.7 Cytokine assay

A commercial mouse cytokine-specific analysis kit from Millipore (Solna, Sweden) was used and included IL-12, IL-2, IFN-γ for Th1 and IL-4, IL-5 and IL-10 for Th2 responses. The procedure was carried out according to the instructions provided by the manufacturer.

2.2 Statistical Analyses

Fluorescence intensity (FI) was the read-out from the multi-parameter (Luminex®) assay and data from the ELISA was generated as U/mL derived from optical density (OD) values. Log transformed data showed normal distribution and the paired *t*-test was used to statistically verify the differences between pre- and post-dose samples in the antibody subclass screening and cytokine profiles. Differences were considered statistically significant if p-values were <0.05.

The screening cut point was calculated based on background values of pre-dose samples and set to 22.1 (equal to $10^{1.34}$) for Tg animals according to ref [28]. By using 95% confidence interval when calculating the cut points, at least 5% false positive samples are expected, thereby reducing the risk of missing false negative samples. Further, a second cut point, known as the specificity cut point, is used to statistically confirm true positive samples. The specificity cut point was set to 65.5% in Tg mice based on competitive binding of negative plasma. Thus, 65.5% decrease in signal from potentially positive samples will be required after pre-incubation with drug to be deemed as true positives. For details see ref [28].

Tg Screening cut point= mean (12.9) + 1.645 x Standard Deviation (5.6)
Screening cut point= 22.1

Tg Specificity cut point was calculated accordingly;
y= mean of log transformed ratio (0.0767) – 3.09 x SD (0.174)
Specificity Cut point = 100 x (1- antilog value y), y= -0.462
Specificity cut point = 65.5%

3. RESULTS

3.1 Antibody Subclass Distribution of ADA in Transgenic Mice Expressing the Human Drug Protein Used for Immunization

Pre- and post-dose samples from Tg mouse plasma, in 100x dilution, were analysed for drug-specific IgG1, IgG2a, IgG2b, IgG3 and IgM antibodies. IgG1 was significantly increased (p=0,05) as an effect of immunization with batch 4 (Fig. 1a and Table 4). A majority of the animals, 11/15 (73%), were scored as positive for IgG1 to any of the batches used for immunization. The proportion of animals with a positive response to a given batch is shown in (Table 4). All positive samples were further pre-incubated with excess of drug and drug-specificity was confirmed. No significant changes or positive responses were observed with respect to IgG2a, IgG2b, IgG3 or IgM.

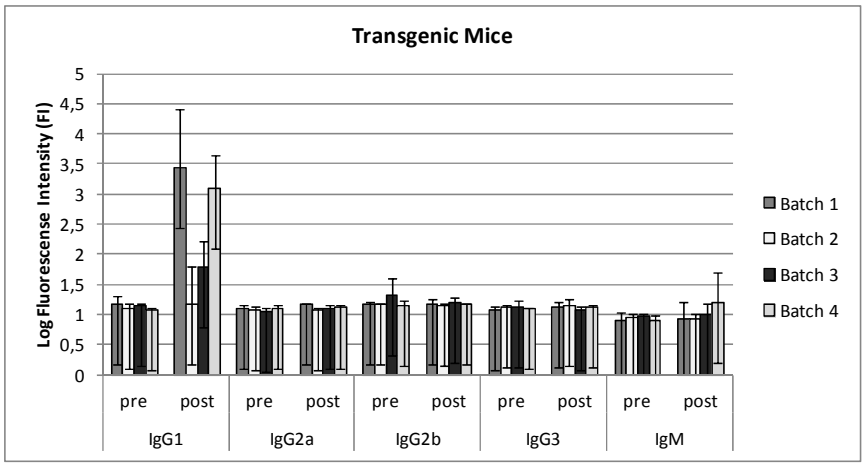

Fig. 1a. ADA response in Tg mice. Logarithmic mean value ±SD from each dose group are shown. IgG1 was significantly increased (p=0,05) after administration of batch 4

3.2 ADA in Wild Type Mice Immunized with the Human Drug Protein, Multi-parametric Bead-analysis

In the same way as for Tg mice, pre- and post-dosing samples, in x100 dilution, were analysed for drug specific IgG1, IgG2a, IgG2b, IgG3 and IgM antibodies in Wt mice. A significant increase in IgG1 was observed against batch 1 ($p<0,05$), batch 2 ($p<0,05$) and batch 4 ($p<0,0001$) of the administered drug (Fig. 1b and Table 4). A majority of Wt animals, 13/14 (93%), were scored as positive for IgG1 and 9/14 (64%) were scored as positive for IgG2b. The proportion of animals with positive results to a given batch is shown in (Table 5). All positive samples were further pre-incubated with excess of drug and drug-specificity was confirmed. No significant changes or positive responses were observed with respect to IgG2a, IgG3 or IgM.

3.5 Multi-parametric Vs. ELISA Data

Multi-parameter bead analysis data was further validated using ELISA as a reference method. The results, as demonstrated in (Fig. 2 and Table 6), show that the antibody

response patterns are similar irrespective of which assay is used. Both assays identified batch 4 as being most immunogenic in Wt mice and batch 1 as being most immunogenic in Tg mice in the validation study.

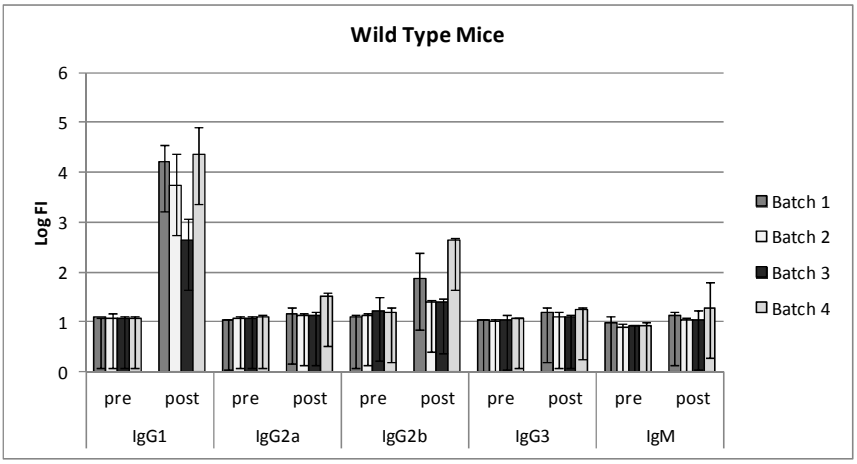

Fig. 1b. ADA response in Wt mice. Logarithmic mean values ±SD of each dose-group are represented. IgG1 was significantly increased after administration of batch 1 (p<0,05), 2 (p<0,05) and 4 (p<0,0001)

Table 4. Statistical evaluation (p-values derived by student´s paired t-test) of increases in ADA as an effect of repeated *s.c* immunization (n=4) with four different batches of a human recombinant protein in Tg and Wt mice, respectively. Significant increases are shown in bold text

	Tg					Wt				
	IgG1	**IgG2a**	**IgG2b**	**IgG3**	**IgM**	**IgG1**	**IgG2a**	**IgG2b**	**IgG3**	**IgM**
Batch 1	0,06	0,23	0,91	0,25	0,83	**<0,05**	0,22	0,14	0,17	0,33
Batch 2	0,09	0,65	0,55	0,75	0,27	**<0,05**	0,07	0,43	0,28	0,16
Batch 3	0,40	0,27	0,28	0,37	0,77	0,10	0,08	0,45	0,43	0,11
Batch 4	**0,05**	0,78	0,78	0,47	0,31	**<0,0001**	0,30	0,06	0,09	0,14

Table 5. Proportion of positive animals per each batch of protein

	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

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

3.6 Cytokine Profile

To examine whether the drug-induced response was Th1- or Th2-mediated, and also to investigate potential differences in cytokine patterns and correlate these with the different antibody responses in Wt and Tg animals, cytokine analyses were performed. The

expression levels of individual cytokines differed between Wt and Tg mice as well as between different batches of the protein drug (Table 7). Cytokine levels were in general higher in Wt mice, both pre- and post-dosing. This correlated with the higher levels of induced drug-specific antibodies seen in Wt mice compared to Tg mice post-dosing. Wt-mice responded with significant down-regulation of IL-12 and up-regulation of IL-4 and IL-5 when injected with batch 4; the same batch inducing a significant ADA response in these mice. Tg mice also responded with significant down-regulation of IL-12 after treatment with batch 4.

Table 6. Ranking of antibody subclass response; based on total drug-specific (ADA) Ig titers. Grading goes from the least to the most immunogenic batch in each mouse strain as measured by the multi-parametic assay and ELISA, respectively

	Multiparametric assay	Conventional ELISA
	Batch no	Batch no
Wild type mice	3<2<1<4	2<3<1<4
Transgenic mice	3<2<4<1	2<3<4<1

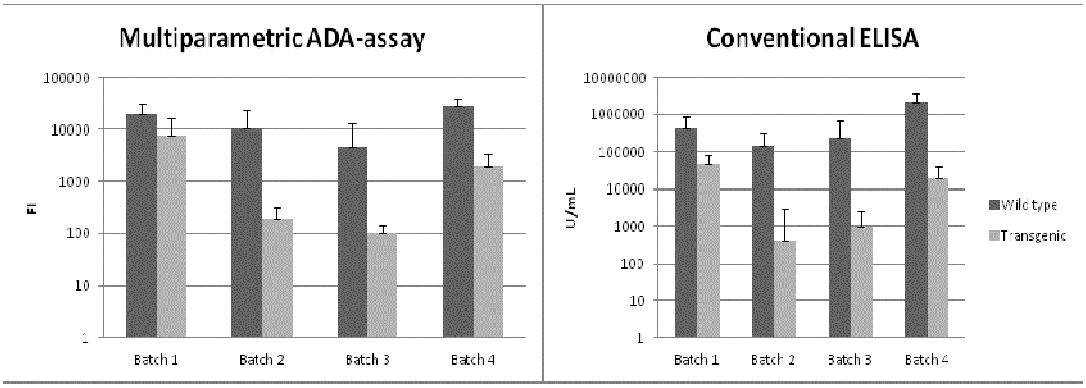

Fig. 2. Mean protein-specific (ADA) total Ig responses in wild type and transgenic mice as determined using multi-parametric bead analysis and ELISA

4. DISCUSSION

Responses against therapeutic proteins are in most cases not translational between species, and hence the predictive value of pre-clinical immunogenicity studies for relevance in humans is considered low [29]. However, we think transgenic mouse models, in which human proteins are expressed, can be part of a solution to overcome problems, *e.g.* xeno-responses, with translating data obtained in the mouse to the human situation [30]. Tg mouse models, expressing human therapeutic proteins, may allow detection of drug-specific antibody responses induced by similar or identical mechanisms as expected in patients receiving the drug [31]. This may be so because in both cases, an ADA-response requires breaking tolerance against self [32]. It should be emphasized though, that also a human protein expressed in the mouse may carry post-translational modifications that are not seen on the protein endogenously expressed in man. Thus, there may still be species-specific differences in the antibody response towards a human protein in man and a Tg mouse expressing the human protein (e.g. Toll-like receptors [33] and major histocompatibility complex (MHC)). Nevertheless, transgenic models may help elucidate mechanisms of human protein immunogenicity such that improved predictive tools can be developed.

Table 7. Cytokine expression in wild type and transgenic mice after a total of four *s.c* injections with a recombinant human plasma protein. 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

		Tg				Wt			
		Batch 1	**Batch 2**	**Batch 3**	**Batch 4**	**Batch 1**	**Batch 2**	**Batch 3**	**Batch 4**
TH1	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
	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**
TH2	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**
	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

We have previously developed an assay that allows identification and semi-quantification of several classes and subclasses of ADAs in a small volume of mouse plasma [25]. Today regulatory bodies require that antibody subclass identification is done as part of immunogenicity studies in clinical trials when ADA reactions are identified, but not in pre-clinical studies. To investigate the value of filling this gap, we set out to validate the use of our assay in Wt and Tg mice subcutaneously challenged with a human protein drug.

Our results showed that both Tg mice and Wt mice, injected with a recombinant human plasma protein, induced ADA of subclass IgG1. However, Wt mice presented a positive ADA response also of subclass IgG2b, according to the statistically calculated cut point, even though significance between pre- and post-dose samples could not be shown by evaluating the intensity of this response. These results suggest that the IgG2b response seen in Wt mice is more of a species-specific, mouse anti-human response, while the IgG1 response, seen in both Wt and Tg mice, more reflects a direct product-related and batch-specific response.

Immune-tolerant mouse models have continuously been generated [34,35,36] for studying immunogenicity against structural changes of recombinant human proteins. However, antibody-subclass profiling as a tool to estimate relative immunogenicity related to batch quality of an identical drug is not commonly, if at all, investigated. Subclass profiling is recommended in clinical trials of biopharmaceuticals, and for establishment of clinical significance. Since subclass discrimination is obviously of importance in human trials [10], we suggest that murine subclasses and their clinical relevance *In vivo* must be further explored. Improved knowledge on murine ADA-classes and -subclasses could reveal their possible value for prediction of drug immunogenicity in human.

When investigating ADA responses against the different batches of the recombinant human protein, results showed that the batch with the highest purity (No 3) was the only one that did not induce significant levels of ADAs in Wt mice, and this was even more pronounced in tg mice, suggesting that impurities in the remaining batches indeed were a contributing factor for induction of immunogenicity [37]. Interestingly, despite the extra purification step for batch 4, this particular formulation still induced relatively high levels of ADA. Specific components causing this immunogenicity may be host cell-related, since 8 ppm still was present in this formulation. Also, the 97.7% purity seen with this batch was the lowest among the tested batches allowing also other non-detected hitherto unknown impurities as candidates for causing immunogenicity.

Plasma cytokine profiles were determined in order to indirectly delineate the ADA response at the T cell level. Results showed a significant up-regulation of Th2 (IL-4 and IL-5) related cytokines in Wt mice, together with down-regulation of Th1 (IL-12) in both Wt and Tg mice after treatment with batch 4. Cytokine profile results together with the significant ADA response against batch 4, in all strains, further strengthen the hypothesis that host-related impurities in batch 4 are responsible for the immunogenicity. However, since the half-life of cytokines is short their potential for acting as biomarkers is weak and cytokine data is preferable used only to support other findings but not as stand-alone markers.

To verify results from the multi-parameter bead analyses, identical plasma samples were measured with regard to total IgG, IgA and IgM responses using ELISA. The results from the ELISA and the multiparametic-bead analysis were similar and both assays identified batch 4 as most immunogenic in Wt mice. However, the multiparametic-bead analysis requires 20 times less volume to screen for five classes of ADA compared to a conventional bridging

ELISA, making it possible to include immunogenicity screening with the Luminex®-based assay in preclinical toxicological and efficacy studies. Fewer animals can thus be used and more information extracted in immunogenicity studies relying on the detection and semi-quantification of ADA with the Luminex®-based assay.

5. CONCLUSION

In conclusion, different batches of a recombinant human plasma protein induced different patterns of ADA subclasses in Wt (IgG1 and IgG2b) as compared to immune-tolerant Tg mice (IgG1 only). The proportion of ADA-positive animals in each treated group could serve as a marker for batch impurities as both Tg and Wt animals correctly identified the same batch as least pure. The multi-parameter bead assay used in this study generated data that also could be confirmed by ELISA.

As a new approach for elucidating mechanisms associated with immunogenicity to biopharmaceuticals we have investigated ADA-subclasses and their potential as biomarkers for immunogenicity of human recombinant protein drugs, comparing Wt and immune-tolerant Tg mice as models.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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