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Short communication

HAHA – nothing to laugh about. Measuring the immunogenicity (human anti-human antibody response) induced by humanized monoclonal antibodies applying ELISA and SPR technology

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ABSTRACT

Immunogenicity induced by passively applied proteins is a serious issue because it is directly related to the patient's safety. The out-come of an immune reaction to a therapeutic protein can range from transient appearance of antibodies without any clinical significance to severe life threatening conditions. Within this article, enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) methodology to measure immunogenicity are compared and the pros and cons are discussed.

1. Introduction: the importance of human anti-human antibody (HAHA) response

Following the success of recombinant proteins, therapeutic monoclonal antibodies (mAbs) represent the second wave of innovation created by biotechnology during the past 20 years. Immunogenicity induced by passively applied proteins [1] is a serious issue because it is directly related to the patient's safety. The out-come of an immune reaction to a therapeutic protein can range from transient appearance of antibodies without any clinical significance to severe life threatening conditions [2]. Potential clinical consequences are severe hypersensitivity-type reactions, decrease in efficacy and induction of autoimmunity [3]. Patient-related factors that might predispose an individual to an immune response include: underlying disease, genetic background, immune status, including immuno-modulating therapy.

Today, most therapeutic mAbs in the clinic are at least humanized meaning that, theoretically, only a minimal immunogenic potential remains (as compared to murine mAbs) [4,5]. Nevertheless, also antibody responses against humanized mAbs have been observed suggesting that other factors are contributing to the immunogenicity [6] such as the nature of the antigen, the disease process treated and the schedule of administration. An induced antibody response against such humanized therapeutic mAbs is called human anti-human antibody (HAHA) response [7]. The EMEA has recognized the seriousness of this topic and addressed it in its draft version of 'Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins [8] that '*it is essential to adopt an appropriate strategy for the development of adequate screening and confirmatory assays to measure an immune response against a therapeutic protein.*' Furthermore detailed requirements for such assays are given – mainly reflecting parameters that are also required during method validation according to the ICH guidelines [9] like linear responses to relevant analytes as well as appropriate accuracy, precision, sensitivity, specificity and robustness. Also the FDA makes specific recommendations on monitoring the development of HAHA responses [10] further stressing the importance of this issue. Furthermore authorities [8] recommend that '*the screening assays should be sensitive enough to detect low titre antibodies as well as Abs to conformational and linear epitopes.*' If the result of the initial HAHA assay is positive, one has to implement a second assay for further characterization of the neutralizing potential of these induced HAHA's [11]. An immune response comprised of neutralizing antibodies can lead to loss of efficacy or potentially more serious clinical sequelae. Therefore, it is important to monitor the immunogenicity of biological therapeutics throughout the drug product development cycle [12].

The aforementioned requirements are scientifically sound but are neglecting the main issue regarding such HAHA assays: the

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appropriate calibrator. Many novel biotherapeutics do not have true standards or reference materials. In this case, the use of appropriate positive controls is recommended. Ideally, the calibrator is a purified HAHA response from a patient with a defined IgG content allowing reporting, e.g. 'μg/ml HAHA IgG equivalents'. Practically, such calibrator is hardly available; therefore HAHA mimics have to be generated. Immunization with drug substance of humans is ethically not possible; the raise of antibodies in experimental animals is costly and time-consuming and hampered by the fact that animal models often do not reliably predict the immune response in humans. The remaining calibrator options are (i) purified polyclonal anti-drug substance sera with a defined IgG concentration, (ii) a mixture of anti-drug substance mAbs (generating a pseudo-polyclonal preparation) or (iii) anti-idiotypic antibodies that are directed against the potential immunogenic region in the therapeutic mAb. Finally, after all the efforts concerning the calibrator the question remains if and how the measured 'HAHA equivalents' relate to clinical relevance [13]. Although anti-drug antibodies (ADA's) of the IgG isotype are of main concern due to their potential of inducing memory and mediating effector functions via Fc-receptors [14], IgM antibodies should also be considered.

Regarding the quality of the induced HAHA response measured in serum samples of patients treated with therapeutic mAbs, two types have been described by Ritter et al. [7]. This group measured the HAHA response after repeated administration of the human EpCAM specific Ab A33 in patients suffering from colon cancer using surface plasmon resonance (SPR) technology. Onset of all measured HAHA responses was observed after day 7 and reached peak values at day 14; upon subsequent administration, the HAHA reactivity decreased indicative for the HAHA response designated 'Type I'. In contrast, HAHA response 'Type II' is characterized by delayed onset and a steadily increasing reactivity upon repeated human Ab administration. In summary, the HAHA response Type I is transient, consist of IgG only and is not related to side effects. In contrast, Type II resembles the feature of a classical immune response: first IgM then IgG ('isotype switch'), boostable by further application ('memory') and sometimes related to side effects.

2. Methodological aspects. SPR vs. ELISA

Historically, clinical trials applying humanized mAbs have used enzyme-linked immunosorbent assay (ELISA)-based methods for the assessment of HAHA reactivity [15–20]. ELISA, however, has several inherent problems: in the case of direct ELISA, the use of an anti-human detection reagent is not applicable for drugs such as humanized antibodies. For double antigen ELISA, significant challenges include the careful optimization of the reagent concentration, the availability of the labelled antibody drug with comparable affinity and the potential interaction with excess of therapeutic antibody resulting in a delayed monitoring of HAHA responses [21,22]. Moreover, the method is considerably complex the cross-reactivity issue of catching/detecting reagent and time-consuming, e.g. regarding the coating procedure, the incubation times and/or the several washing steps. Besides ELISA, the use of surface plasmon resonance (SPR)-based assays for the determination of the induced HAHA response has been reported [7,23,24]. SPR measures binding to an immobilized ligand in real time without the need of a secondary antibody and has been effectively used for the characterization of antigen–antibody interactions. The main issues to be investigated upon an SPR-based assay are the procedure of covalent immobilization of the drug product and the regeneration conditions because both might affect HAHA detection. The use of SPR-based assays to support clinical Phase I studies was described for IGN311 (anti-Lewis Y mAb) [24] and for the humanized anti-EpCAM mAb A33 measuring induced HAHA response following administration [7,23].

In Table 1, published HAHA incidents are summarized and assigned to the method used for measuring.

Interestingly, the SPR method detects significantly higher HAHA cases raising two questions (i) is the SPR method more sensitive, or (ii) is this finding related to the two drug products, HuA33 and IGN311. While both mAbs have been humanized, IGN311 recognizes a carbohydrate (Lewis Y) and huA33 binds to a receptor (EpCAM). The EMEA has acknowledged that for HAHA detection various types of assays can be used, e.g. immunoassays (like ELISA), SPR or radio-immunoprecipitation [8]. All of them detect antigen/antibody interaction but differ in their underlying principle. From a regulatory perspective (regarding the patient's safety), the more sensitive method is preferable – ideally ELISA and SPR should be tested side-by-side and the results compared. This approach was taken by Lofgren et al. who tested the HAHA incidents induced by the fully human mAb Panitumumab which is binding to the EGF receptor [25]. The interesting finding was that SPR identified 4.1% positive patients in contrast to 0.3% by ELISA indicating that SPR is the more sensitive method. Noteworthy, of the five reference antibodies (with K_D values ranging from 8.1×10^{-10} M to 1.1×10^{-6} M) the highest affinity antibodies were detected with much better sensitivity by ELISA. In contrast, SPR detected the low affinity mAbs with a better sensitivity. The higher incidence of anti-Panitumumab Abs detected by the SPR assay is likely due to patients generating mostly low-affinity Abs, and although some had neutralizing activity, there was a lack of correlation between Ab development to clinical sequelae or loss of efficacy. The question which method provides the 'true' HAHA value is of relative importance because the question to be asked is which method guarantees the safety of the patients meaning which method is capable of detecting the on-set of such response earlier. Besides the two assay parameters sensitivity and specificity, a third – probably the most important – parameter comes into place: affinity. The ability of SPR to detect low affinity binders allows this platform to detect more antibodies than ELISA where the low affinity antibodies are lost during the repeated washing steps. With SPR, binding is detected in real

Table 1
HAHA incidents – ELISA vs. SPR. Except ABX-EGF (huIgG2) all mAbs are IgG1/κ.

Generic name	Target	Phase	Published	Author	Incidents	Method
M-195	CD33	I	1994	Caron [15]	0 of 13	ELISA
hOKT-3	CD3	I	1999	Richards [16]	6 of 24	ELISA
Trastuzumab	Her-2	I/II	1999	Cobleigh [17]	1 of 121	ELISA
huA33	EpCAM	I	2001	Ritter [7]	26 of 41	SPR
huA33	EpCAM	I	2003	Welt [23]	8 of 11	SPR
Alemtuzumab	CD52	II	2004	Hale [18]	0 of 30	ELISA
HuCC49delCH2	TAG-72	I	2004	Agnese [19]	0 of 5	ELISA
ABX-EGF	EGF	I	2004	Rowinsky [20]	0 of 21	ELISA
IGN311	Lewis Y	I	2006	Szolar [14]	6 of 12	SPR
Panitumumab	EGF-R	[a]	2007	Lofgren [25]	2 of 612	ELISA
					25 of 604	SPR

[a] Combined data of eight clinical studies.

Table 2
HAHA measurement by ELISA and SPR – pros and cons.

Parameter	ELISA	SPR
High through-put	+	–
Automatisation	+	+
Low affinity binding	–	+
Regulatory acceptance	+	+
On/off rate determination	–	+
Isotype determination	–	+

time by recording the association (K_a) and the dissociation rate (K_d) making it possible to calculate the affinity constant (K_D).

Regarding sensitivity, the early detection of on-set of a HAHA response is desirable with respect to intervention by the physician. A patient with a HAHA titer, but without any related clinical symptoms, will most likely receive a next application under close medical supervision. In rare cases, severe adverse events like pure red cell aplasia (in the case of erythropoietin) or thrombocytopenia (in the case of thrombopoietin) have been reported [26,27]. Specificity is defined according to ICH guidelines as the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. In the case of HAHA response anti-drug mAbs have to be determined in the present of the highly complex matrix human serum. By choosing running buffers with varying ionic strengths the specificity threshold of the interaction can be modulated [28]. Regarding the patient's safety, false positive results would be tolerable whereas false negative results – as in all bio-analytical methods – are not acceptable.

In Table 2, SPR and ELISA are compared based on parameters that are relevant for a reliable assay used for HAHA determination.

From the author's perspective, SPR is the method of choice for detecting HAHA responses because besides qualitative data (yes/no) also quantitative data (on/off rates) are generated. Additionally, within one SPR run the isotype (IgG or IgM) of bound antibodies can be determined [29] whereas in ELISA either IgG or IgM can be determined within one set-up. Most importantly, the capability of detecting induced low affinity antibodies is a major advantage over ELISA because these low affinity anti-drug antibodies bear the potential of evolving into high(er) affinity antibodies upon affinity maturation. The main argument against SPR (BIAcore) is the high price of the equipment compared to ELISA.

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