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feature

Current challenges and opportunities in nonclinical safety testing of biologics

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Nonclinical safety testing of new biotherapeutic entities represents its own challenges and opportunities in drug development. Hot topics in this field have been discussed recently at the 2nd Annual BioSafe European General Membership Meeting. In this feature article, discussions on the challenges surrounding the use of PEGylated therapeutic proteins, selection of cynomolgus monkey as preclinical species, unexpected pharmacokinetics of biologics and the safety implications thereof are summarized. In addition, new developments in immunosafety testing of biologics, the use of transgenic mouse models and PK and safety implications of multispecific targeting approaches are discussed. Overall, the increasing complexity of new biologic modalities and formats warrants tailor-made nonclinical development strategies and experimental testing.

Introduction

Industry experts gathered on 3–4 December 2012 in Basel for the 2nd Annual BioSafe European General Membership Meeting, where they shared experiences and insights into the nonclinical safety assessment of new biotherapeutic entities. The 135 scientists – mostly from a pharmacokinetics (PK), toxicology or pathology background – represented Europe-based biopharmaceutical companies such as Roche, Novartis, Pfizer, UCB Pharma, Amgen, MedImmune, Bayer, GSK and many more. The meeting was organized by the world's largest biotechnology trade association: Biotechnology Industry Organization (BIO), and hosted by Roche, Basel. Contact details of presenters can be found in Box 1. Delegates emphasized the value of being able to connect and exchange information on nonclinical safety assessment strategies for the development of biotherapeutics. The meeting covered several nonclinical safety issues from explaining the potential accumulation of polyethylene glycol (PEG)ylated proteins in tissues to dose setting for first-time-in-human clinical trials.

PEGylated therapeutic proteins and cellular vacuolization: defining the relevant disposition and safety questions and potential solutions

Conjugation of PEG to therapeutic proteins (TP) is widely used to improve PK properties of proteins [1]. PEGylated entities have a long and extensive safe use in consumer products and medicines and available data indicate toxicity only at very high parenteral doses essentially restricted to the kidney, which is the main excretory route for PEG [2]. Rob Webster (Pfizer) gave an overview of the analytical challenges faced in determining the biological distribution and fate of PEG from biotherapeutics. PEG characteristics generally make radiolabeling and mass spectroscopic techniques unsuitable, although gel electrophoresis has been useful [3]. Ted Parton (UCB) described the application of NMR to study PEG tissue and plasma concentration profiles and urine elimination in humans and rats with PEGylated TPs. This demonstrated that PEGylation of a molecule can fundamentally alter its biodistribution. An excretion study (mass balance) performed in rats using NMR techniques showed 83% measured and 91% extrapolated PEG recovery after 12 weeks.

PEG-associated vacuolization in macrophages (foam cells), predominantly within tissues comprising the reticuloendothelial system, is well documented and is without apparent toxicologic significance [4]. Following high PEG TP exposure,

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BOX 1

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Immunosafety

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vacuolated macrophages are seen in other organs and/or tissues, including the liver, kidney, urinary bladder and brain choroid plexus (CP), and are considered to reflect normal physiologic processing of foreign material by scavenger phagocytic cells, producing no apparent effect on cell function or viability (Fig. 1). Other cell types can display vacuolization after high PEG TP exposure including hepatocytes, urinary bladder, epididymis, adrenal cortex, synoviocytes, ciliary bodies of the eyes and CP of the brain.

Anna Brändli-Baiocco (Roche) presented a pathology view of cytoplasmic vacuolizations, which can result from abnormal catabolism, transport or secretion and/or uptake of indigestible or slowly digestible materials. For PEG TPs, dose- and duration-dependent PEG accumulation and cytoplasmic vacuolization can be nonspecific or frequently target-associated as described for renal tubular epithelial cells [5] and neurons without cellular damage or functional impairment. Following administration of 40 kDa PEG TPs cellular vacuolizations appear to show only intact (40 kDa) PEG as demonstrated using immunohistochemistry and confocal microscopy. PEG vacuolization was only partially reversible and not reversible in neurons but without an apparent effect on neuronal function (*i.e.* for nerve conduction velocity and Fluro-Jade® stain to detect degenerating neurons, manuscript in preparation).

Ian Wakefield (UCB) described CP epithelial cell vacuolization in primates following high, sustained PEG TP exposure. The vacuolization appears as an inert finding, probably representing engulfed nonmetabolized material with no pathology indicative of any toxicologic significance, and can represent physiologic clearance by cells having endocytic capability. 'No effect' exposure levels, exceeding clinical exposure, suggest a potential threshold effect for PEG-induced CP vacuolization. Resolution could be partly dependent on cell turnover. Overall, the absence of pathology associated with PEG-related vacuolization in animals and extensive clinical use over many patient years provides evidence of safety to support the use of PEG for PEGylated TPs. The genesis of vacuolization and the potential impact of slow or no reversibility requires further understanding.

The cynomolgus monkey as a preclinical species

Jonathan Moggs (Novartis) started the session describing how the recent paradigm shift in the genetic characterization of the cynomolgus monkey *via* deep DNA sequencing technologies [6–9] provides a comprehensive picture of

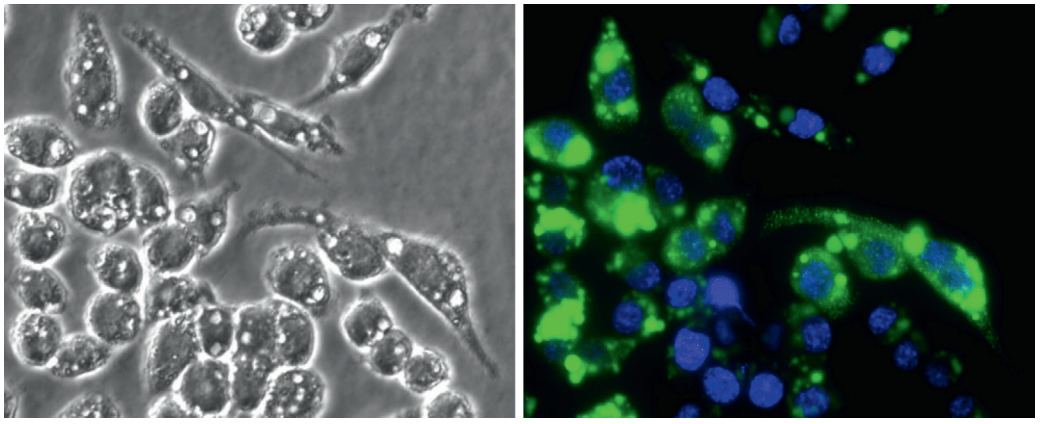

FIGURE 1

In vitro accumulation and vacuolization of PEG–fluorescein in murine macrophage cell line RAW264.7 following 24 hour exposure. PEG-associated vacuolization in macrophages is well documented and considered to reflect normal physiologic processing of foreign material by scavenger phagocytic cells, producing no apparent effect on cell function or viability. The pictures show cells using a light microscope **(a)** and fluorescence light microscopy **(b)**. Data source: UCB–Celltech, Slough, UK.

genetic variation *versus* the rhesus and human genomes. Furthermore, emerging cynomolgus monkey exome and transcriptome data reveal striking intrastrain genetic variations in a subset of genomic loci that could contribute to previously documented immune system and pathophysiologic differences [10,11]. In particular, cynomologus monkey geographic-origin-dependent genetic variations raise important questions regarding the optimal strategy for therapeutic monoclonal antibody (mAb) safety assessment and can impact target binding and Fc effector functions. Systematic genotyping of non-human primates (NHPs), combined with known human target genetic variation in the intended patient population, can facilitate prospective and retrospective early assessment of intrastrain target genetic variation. This is crucial for ensuring mAb specificity and therefore binding and potency assays should be compatible with the target genetic variation present in the animals used for toxicology studies. Characterizing which genetic variants give rise to functional differences in target and pathway biology can, however, become a significant challenge. Furthermore, the increasing complexity of biologic drug modalities (*e.g.* bispecific mAbs) is likely to confound optimal toxicology species selection further, where distinct targets exhibit different degrees of intrastrain variation.

Max Warncke (Novartis) described an example where comparative NHP–human IgG–FcγR structure–function relationships have provided potentially important novel insights for toxicology species selection for therapeutic mAb safety assessment. A range of differences in expression and function of immunologic co-stimulatory molecules and factors in NHPs and humans have been described, including a difference in CD28 expression on memory T cells [12], differences in complement pathway components [13,14] and differences in IgG–FcγR interactions [15–17]. In the highlighted example, IgG1 was shown to have identical FcγR interaction and effector function profile in cynomolgus monkeys and humans, whereas fundamental differences in the IgG2 and IgG4 Ab subclasses were found between these two species. To balance this shift, the cynomolgus inhibitory FcγRIIb receptor shows strongly increased affinity for IgG2. In view of these findings, *in vitro* and *in vivo* results for human IgG2 and IgG4 obtained in the cynomolgus monkey have to be cautiously interpreted, whereas effector-function-related effects of human IgG1 Abs are expected to be predictable for humans.

In a subsequent panel discussion, the potential for genotyping animals before inclusion in nonclinical safety studies was discussed and feedback from the audience was that this should be feasible and at a reasonable cost in the not too distant future, although the advantages of such a strategy for drug development are not yet clear. There was consensus on the importance of genotyping as a resource for target characterization and as an investigative tool for retrospective mechanistic investigations. Furthermore, it was commented that the current main geographic origins of cynomolgus monkeys – Chinese, Mauritian and others – should probably be seen as distinct subgroups exhibiting potentially important genetic variability.

Unexpected fast clearing mAbs: mechanisms, risk assessment and development implications

This session covered recent observations of mAbs with unexpected fast clearance potentially caused by off-target binding [18]. The presentations in this session as well as the roundtable discussion that followed were focused on the current knowledge around the mechanisms behind unexpectedly fast clearing mAbs. The case examples presented, however, are clearly indicative of off-target binding deviating from the exquisite binding selectivity of mAbs with consequent rapid elimination, which in some cases resulted in termination of mAb development.

Wolfgang Richter (Roche) introduced the concept of typical PK behavior of mAbs, which is common for the nonspecific elimination of mAbs *via* proteolysis and salvage effect by the neonatal Fc receptor (FcRn). The first case example of highly specific off-target binding was published for an anti-FGFR4 (fibroblast growth factor receptor 4), which binds unexpectedly to rodent complement factor resulting in rapid elimination [19]. In contrast to binding rodent complement, this mAb did not bind to cynomolgus monkey and human complement factor *in vitro*. Consequently, the PK profile observed in cynomolgus monkeys was in line with expectations, and consistent with the lack of off-target binding in this species. Other case examples of highly specific off-target binding have been published for an anti-amyloid-beta (Aβ) mAb [20] and another mAb directed against an undisclosed target [21]. The unexpected fast clearing mAbs suggest low affinity and high capacity binding as a result of the fast and most often dose linear PK.

Stewart Jones (Novartis) presented applications of immunohistochemistry (IHC) as a tool to characterize unexpected and expected binding of mAbs because it could help in the process of species selection for nonclinical safety testing per ICH guidance S6 [ICH Topic S6 Addendum to Preclinical Evaluation of Biotechnology-derived Pharmaceuticals (June 2011)]. An additional application of IHC as an early screening tool to identify unexpected binding behavior as part of the clinical candidate selection was discussed using case examples to characterize early candidates linking their binding behavior to the respective PK of those candidates. In addition to IHC, protein chips could be considered as screening tools for untypical binding of mAbs.

Thereafter, Frank-Peter Theil (UCB Pharma) showed several case examples presented recently at the National Biotech Conference in San Diego by Genentech (2012). These data suggest that mAbs with atypical PK can be divided into two subsets. Subset one shows highly specific off-target binding to a specific biological structure (or 'target') often resulting in profound species differences. Most often, when the off-target binding site is identified it is very specific to certain proteins and shows pronounced species and even strain differences. The other subset of mAbs with potential off-target binding shows consistently fast elimination across several animal species and also in humans. In all these cases, specific target binding sites could not be identified. Some evidence suggests that charge and hydrophobic patches in the complementarity-determining region (CDR) might cause promiscuous binding of a potentially 'sticky' mAb. The roundtable discussion focused on the consequences of atypical PK of mAbs, which can lead to termination of mAb development; because the projected therapeutic dose could become too high, dosing would need to be too frequent to maintain therapeutic levels, or off-target binding could lead to an unacceptable safety profile. A recent publication by Amgen [21] demonstrated pronounced acute thrombocytopenia in NHPs owing to off-target binding, which was also associated with fast clearance. Only a few such case examples are currently in the public domain.

In the discussion it was also highlighted that there is a lack of mechanistic knowledge about the fast clearance of those mAb candidates, often no rational mechanism-based strategy for candidate selection can be used. Therefore, several companies seem to use empirical screening paradigms to find clinical mAb candidates, which exhibit the expected PK behavior of an IgG. Main components of the screening strategy are T-cell receptor (TCR), empirical nonspecific binding assays (*e.g.* using baculovirus particles) [22] and extensive PK characterization trying to explore potential deviations of the PK from the expectations of a classical mAb. At least one company uses the rat to screen for antibodies with abnormal PK behavior, regardless of whether or not this species is pharmacologically relevant. It is important that these case examples of atypical mAb PK are published to enable collective learning.

Multispecific targeting: PK and safety implications

The increased functionality of bispecific antibodies, which bind to two different targets or potentially two epitopes on the same target, compared with classical IgGs makes them attractive for development as therapeutic products. Today there is a notable revival of interest in bispecific antibody formats, which formerly had many clinical failures in the early 1990s, mainly as a result of poor safety and efficacy profiles as well as manufacturing problems. A total of ten candidates were undergoing evaluation in clinical studies in 2012 [23].

Benno Rattel (Amgen) presented on nonclinical testing strategies of bispecific T cell engaging (BiTE®) antibodies. BiTEs® comprise two flexibly linked single-chain antibodies, one directed against a tumor antigen and one targeting CD3. BiTEs® can therefore transiently link tumor cells with resting CD3+ polyclonal T cells for induction of a surface target antigen-dependent redirected lysis of tumor cells, closely mimicking a natural cytotoxic T cell response. *In vitro*, BiTEs® activate T cells in a highly conditional manner that is dependent on the presence of target cells. First-generation BiTEs® only crossreact with the respective antigens from chimpanzees [24]. To facilitate *in vivo* safety testing, surrogate BiTEs® were generated that were crossreactive with murine antigens. The pharmacologic characterization of BiTEs® includes in-depth analysis of their effects on tumor as well as T cells. Various xenograft models are available for *in vivo* efficacy testing. The second-generation BiTEs® are fully human in sequence and crossreact with NHPs [25]. Strategies for nonclinical assessment and defining a safe clinical starting dose were presented for BiTEs® with specificity for various tumor-associated antigens.

In the next presentation, Andreas Baumann (Bayer) presented a case example on PK of a BiTE® antibody. BAY2010112, in development for the treatment of patients with prostate cancer, is bispecific for prostate-specific membrane antigen (PSMA) and the CD3 epsilon subunit of the TCR complex. BAY 2010112 binds PSMA and CD3 of human and macaque origin enabling assessment of safety, PK and pharmacodynamics (PD) in a relevant animal species [25]. PK/PD data were generated in xenograft mouse models used for pharmacologic characterization. Cynomolgus monkey PK/toxicokinetics (TK) studies were performed with single and repeated subcutaneous (SC) as well as intravenous (IV) administration of BAY 2010112. Single-species-based allometric scaling was used to estimate the human exposure at First-in-man (FIM) doses. Distribution studies with biologics are not routinely required as Investigational New Drug (IND) enabling, but they can generate a mechanistic understanding to aid internal decision making. Such studies can provide information on the major tissue distribution compartments and underlying mechanisms of disposition kinetics, elucidate on- and off-target binding kinetics in tissues of interest, and quantify the drug entity and/or its relevant parts. Initial results were presented showing that ^{14}C-labeled BAY 2010112 accumulates in SC-implanted LNCaP PCa tumors of mice after tail vein injection [26].

In the next presentation, Niels Jørgen Ø. Skartved (Symphogen) presented on Sym004, a mixture of two human mAbs directed against epidermal growth factor receptor (EGFR). To determine the optimal antibody mixture, an antibody screen was developed that showed that a mixture of two antibodies recognizing nonoverlapping epitopes on domain III of EGFR induces more-rapid and -effective EGFR internalization and degradation and superior growth inhibition of cancer cells *in vitro* and *in vivo* compared with cetuximab and panitumumab. On the basis of experimental data, it is thought this is achieved through more-efficient EGFR crosslinking at the cell surface. Given these properties, it is hypothesized that Sym004 would be able to be effective in a broader target patient population. Nonclinical safety studies in support of Sym004 clinical development have involved nonclinical safety studies with each of the Sym004 constituent antibodies, as well as the mixture, and showed enhanced PD effects (in the form of skin lesions) with the mixture.

Transgenic mouse models for specific questions in drug disposition and toxicology

In this session, the use of transgenic animals, either for assessment of nonclinical safety or for addressing specific questions on PK and immunogenicity, was described. For nonclinical

safety assessment, two transgenic models were described. In the first presentation, Lolke de Haan (MedImmune) described the use of knockin/knockout (KIKO) models, in which a human immune target is knocked in to replace the analogous murine receptor, to be able to test the safety of a human target-selective antibody. Although the KIKO mouse showed all the desired properties in a pharmacology model, the nonclinical safety assessment was hampered by immunogenicity of the human antibody in the mouse and consequent immune complex-mediated hypersensitivity reactions. In a second presentation from Rajni Fagg (GSK), a KI model was described in which the human soluble target was knocked in to enable assessment of PK/PD relationships and nonclinical safety. This proved to be a very successful approach with respect to regulatory acceptance, although the relevance of the risk assessment for humans was questioned, and it was felt this at best represented a useful PK/PD model for clinical dose setting because the human transgene was not functional in mice. Overall, the case examples emphasized some of the limitations when using transgenic mice in nonclinical safety testing: murine models, when administered with human proteins, can be prone to IgG-mediated hypersensitivity reactions [27] mediated by the release of platelet-activating factor [28], and differences in their target biology and expression pattern might limit the use of these models for hazard identification even further.

In the second half of the session, the use of transgenic animals for addressing PK- and immunogenicity-related questions was discussed. In particular, the use of human neonatal Fc receptor (huFcRn) transgenic animals to test novel IgG-based constructs was described by Michael Otteneder (Roche). Various transgenic lines available from JAX laboratories were explored [29]. The data derived from Tg huFcRn mice showed that a direct translation to humans is complicated by unknown expression levels of FcRn as well as by extremely low endogenous IgG levels (0.15 μM in transgenic *versus* 15 μM in wild-type mice) resulting in very low receptor occupancy. A pharmacometric approach is needed to allow a better quantitative understanding [30].

Antonio Iglesias (Roche) described the use of transgenic mice made tolerant to human IgG to investigate immunogenicity. These transgenic animals express an IgG1 repertoire with the most commonly used human V genes to assess immunogenicity of engineered mAbs, or the impact of certain impurities, such as protein aggregates, on the immune response. It was shown that the transgenic human germline V genes rearrange effectively to generate a human IgG repertoire and that the mice are immune-tolerant to repeat-dose administration of native hIgG1. Furthermore, the transgenic mice were immune-tolerant to a broad diversity of hIgG1 as demonstrated by the low antibody titer generated upon immunization with poly hIgG. Finally, the mice were used to assess the immunogenicity of hIgG1 dimer and oligomer aggregates generated either *via* UV light, pH stress or as byproducts. It was shown that, whereas dimers failed to break immune-tolerance, oligomeric material from UV-stressed hIgG1 preparations could break tolerance in the transgenic mice. A recent study also found differences in the immunogenic properties of hIgG aggregate preparations [31]. However, the model used in this study is transgenic for (and therefore tolerant to) human IgM rather than hIgG [32] making interpretation of these data difficult. The transgenic animals described here could therefore be a useful tool for studying the mechanisms of immunogenicity of IgG. They could help to improve our understanding of the role of different aggregates and particles in eliciting an immune response when present in drug products. Ongoing work of a third model on huFcγR mice was also presented. Human FcR genes inserted into the mouse genome could help predict FcR-dependent effects with human antibodies. Through gene replacement, the human FcR pattern of expression has been reproduced in experimental mice. Such humanized FcγR mice will be used to explore and assess FcR-dependent infusion-related reactions as caused by first infusion of certain monoclonal antibodies.

Immunosafety

A wide range of topics were covered spanning current and future challenges associated with the impact of immune modulation on drug safety. At the start of this session, Tobias Manigold (University Hospital, Basel) proposed a new classification system for immune-mediated adverse events (IAEs) to therapeutics to cover the varied mechanisms of reactions (unpublished). The different IAEs should be described according to time of onset and clinical signs and symptoms first, followed by appropriate workup to differentiate the various mechanisms. Next, Andrea Kiessling (Novartis) presented on cytokine-mediated reactions using an *in vitro* human whole blood assay with soluble mAb presentation [33], and Matthew Baker (Antitope) presented data using *in vitro* human dendritic cell (DC) and T cell assays to try and predict the immunogenicity of aggregated mAbs [34] (and unpublished).

The focus then shifted to the evaluation of novel and established *in vivo* models to assess immunosuppression. Andrea Kiessling summarized an internal Novartis study and International Life Sciences Institute–Health and Environmental Sciences Institute (ILSI–HESI) working group activities [35] relating to optimization of T-cell-dependent antibody response (TDAR) studies in NHPs. The internal analysis indicated that two–threefold differences in responses between a control and a treatment group can be statistically picked up with standard toxicity study animal numbers, if no gender differences exist and the analyses take into account several response days, not just the peak response day.

Christian Munz (University of Zürich) presented human immune system reconstituted mice infected with Ebstein-Barr-Virus (EBV) as a model to assess the risk of oncogenic virus activation and lymphoma induction by immunosuppressive mAbs. Humanized NOD-scid $\gamma c^{-/-}$ (NSG) mice were reconstituted with human $CD34^{+}$ cells followed by infection with EBV. High EBV titers, splenomegaly and EBNA2+ B cell lymphoma are observed in mice around eight weeks after low-dose EBV infection, which is exacerbated by immunosuppression with either tacrolimus or T cell depletion with antihuman CD4 and CD8 mAbs. Although this model shows promise for the assessment of immunosuppressive treatments, the limitation is that these mice are still relatively immunosuppressed compared with wild-type mice and humans, containing for example only low IgG levels, high frequencies of immature B cells and early differentiation stages of natural killer (NK) cells.

Concluding remarks

Nonclinical development strategies align nonclinical drug safety (toxicology and safety pharmacology), nonclinical PK and bioanalytics, including immunogenicity evaluation. Safety risks are generally directly related to uncertainty and are reduced through knowledge and best scientific practices. Because of the heterogeneity of biologics as a molecule class, as well as due to difference in the nonclinical safety strategies for biologics compared with small molecules, nonclinical programs must be considered largely on a case by case basis. Case examples were discussed to underline the need for tailor-made development strategies and experimental testing.

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