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COMMENTARY

Primate testing of TGN1412: right target, wrong cell

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The failure of toxicity studies in non-human primates to predict the cytokine release syndrome during a first-in-man study of the CD28-specific monoclonal antibody TGN1412 has remained unexplained so far. In this issue of the *BJP*, work from the NIBSC first identifies the effector memory subset of human T-lymphocytes as the most likely source of the pro-inflammatory cytokines released during the study, and goes on to show that in cynomolgus monkeys, this subset lacks CD28, the target molecule of TGN1412. We discuss the implications for the TGN1412 catastrophe and for preclinical evaluation of biologicals in animal models in general.

Abbreviations

CD28SA, CD28 superagonist; FIH, first-in-human; MABEL, minimal anticipated biological effect level; NOAEL, no observed adverse effect level; PBMC, peripheral blood mononuclear cells; TCR, T-cell antigen receptor; TNF, tumour necrosis factor

On 13 March, 2006, the catastrophic outcome of the 'London trial' not only rocked the world of biomedical research and drug development but also the public, receiving intense and long-lasting media coverage. What happened was an unexpected cytokine release syndrome encountered during a first-in-man trial of TGN1412, a fully humanized monoclonal antibody (mAb) specific for CD28 (Suntharalingam *et al.*, 2006). CD28 is the key co-stimulatory molecule on T-cells, that is, it provides an essential 'second signal' during T-cell activation, via the T-cell antigen receptor (TCR).

The two signal requirement for T-cell activation is also apparent when mAb are used instead of natural ligands. Thus, a TCR-specific mAb (signal 1) complemented by a CD28-specific mAb (signal 2) allows full T-cell activation, a technique widely used to expand human T-cells for experimental and even therapeutic purposes. The potency of TGN1412, however, goes beyond providing such a co-stimulus. This mAb belongs to a functionally distinct class called CD28 superagonists (CD28SA), which are able to stimulate T-cells without the need to ligate the TCR (Hunig, 2007).

In rodents, *in vivo* application of CD28SA leads to lymphocytosis with a marked preference for the numeric expansion and functional activation of an important type of suppressor T-cell, the 'natural' regulatory T-cell (nTreg cell). Indeed, the power of mouse and rat CD28SA to polyclonally activate nTreg cells has been established as a possible therapeutic principle in a broad spectrum of rodent

autoimmune and inflammatory disease models (Hunig, 2007). Based on such preclinical findings, a human CD28-specific superagonistic mAb was identified, and developed as a fully humanized CD28SA of the IgG4 subclass by TeGenero AG, the sponsor of the London trial. Of this mAb, 100 $\mu g \cdot kg^{-1}$ was administered as a bolus i.v. injection to healthy young men with the known deleterious consequences. Preclinical data supplied by TeGenero had convinced both British and German regulatory authorities that the proposed phase I study was safe. Where, then, did the preclinical safety testing fail?

In summary, three distinct experimental systems with three distinct sets of data had failed to predict the cytokine storm generated in the human volunteers: these systems comprised the *in vivo* rodent experiments using surrogate antibodies to TG1412, the *in vitro* experiments using human peripheral blood mononuclear cells (PBMC) and the *in vivo* primate (cynomolgus monkey) experiments in which TGN1412 was applied at up to 50 $mg \cdot kg^{-1}$ without detectable toxicity. The latter experiments formed the basis for the dose calculation performed by the then valid no observed adverse effect level (NOAEL) method. After the catastrophe, PBMC culture and monkey experiments were repeated by the NIBSC acting on behalf of the scientific expert group on phase I trials convened by the UK Ministry of Health (Duff, 2006). They yielded the same inconspicuous results submitted by the trial's sponsor.

With regard to rodents, it is now, some years later, clear that it is the very efficient activation of Treg cells that prevents a CD28SA cytokine storm. If this cell type is experimentally deleted in mice, serious levels of pro-inflammatory cytokines, including tumour necrosis factor (TNF)-α, are observed after application of a mouse-specific CD28SA (Gogishvili *et al.*, 2009).

But what about the monkeys? In the days after the TGN1412 catastrophe, explanations came a dime a dozen. The most popular, and most spectacularly wrong was the repeated claim that due to a sequence difference in the extracellular domain of CD28 between humans and cynomolgus monkeys, TGN1412 would bind poorly or not at all in the monkey, thereby making it resistant to its stimulatory effects (Kenter and Cohen, 2006). Such speculations were based on sequencing errors of rhesus CD28 (no published cynomolgus sequence existed at that time). However, the sponsor had not only determined the relevant extracellular sequence (which is 100% identical to the human one) but also the binding affinities of the mAb, which were identical for human and monkey (Hanke, 2006).

The explanation for the failure of cynomolgus monkeys to respond to TGN1412 with a cytokine storm given in this issue of the *BJP* by Stebbings and colleagues (Eastwood *et al.*, 2010) is simple, convincing, and, most probably, correct. The cell type that responds with the release of pro-inflammatory cytokines in humans, simply does not express the target molecule CD28 in cynomolgus monkeys! First, the authors identified the source of TGN1412-induced toxic cytokines such as TNF-α and interferon-γ in humans. In a modified *in vitro* assay using human PBMC, the antibody was immobilized on the plastic surface of microculture plates, a technique traditionally used in cellular immunology to check for stimulatory properties of mAb directed at cell surface receptors. While this is no direct reflection of the *in vivo* situation, it endows mAb with maximum potency by allowing them to densely cluster their target receptors on T-cells. Using this technique, Eastwood *et al*. found that one cell type, almost exclusively, released pro-inflammatory cytokines when confronted with immobilized TGN1412: the CD4 effector memory cells (CD4em). CD4 T-cells can be phenotypically distinguished as being naive or antigen-experienced by their expression of two different CD45 isoforms, CD45RA (naive) or CD45RO (memory). Furthermore, CD4 memory T-cells can be subdivided into those homing to lymph nodes where they await the opportunity to make a secondary response (central memory cells) and those homing to tissues where they can be instantaneously reactivated as effector cells to produce high levels of the pro-inflammatory cytokines (effector memory cells). The cell surface marker used for this distinction is CCR7, a chemokine receptor directing migration to lymph nodes. From this observation, it was only one step to ask whether this cell type expresses CD28 in cynomolgus monkeys. Indeed, previous work done in Rhesus macaques had indicated that in that species, differentiation to CD4 effector memory cells is associated with a loss of CD28 (Pitcher *et al.*, 2002). It is now shown that cynomolgus monkeys have a CD4+ CD45RO+, CCR7-negative subset that fails to express CD28, but can be induced by pharmacological triggering, bypassing cell surface receptors, to release the relevant toxic cytokines. For obvious reasons, however, this subset cannot be triggered by anti-CD28, explaining the failure to provoke cytokine release in cynomolgus monkeys.

What are the lessons to be drawn from these results in terms of risk-assessment for first-in-human (FIH) administration of new biological agents? For 'conventional' drugs, guidelines from regulatory agencies recommend carrying out toxicology and safety pharmacology in two relevant animal species

(one rodent and one non-rodent) to identify the target organs. From these studies, the NOAEL is then determined using these non-clinical safety studies performed in the most sensitive and relevant animal species, adjusted with allometric factors or on the basis of pharmacokinetics. The FIH dose is then adjusted using appropriate safety factors. In the case of 'chemical' drugs, 'relevant species' is mostly linked to differences in metabolism and pharmacokinetics interspecies. However, biological agents and especially therapeutic monoclonal antibodies, generally exhibit exclusive species specificity for the target antigen. So, in this case the most difficult task for non-clinical safety studies is to find a 'relevant species'. During the recent era of therapeutic monoclonal antibody development, a relevant animal species has been often defined as a species showing antibody binding to the animal homologous target and also comparable pharmacological effects. The problem is how the pharmacological effect is defined and if effects on other biological system are assessed during preclinical development. This point is central to the NIBSC work and for the development of therapeutic monoclonal antibodies.

The first lesson provided by the work from NIBSC is that investigations based on a strong scientific rationale have identified the cynomolgus monkey as a wrong species for non-clinical safety assessment of TGN1412 despite similar binding. This point reinforces the usefulness of the new guidelines published by the EMEA in 2007 where 'factors of risk' are now defined (EMA, European Medicines Agency, 2007).

The second lesson is that dose calculation was not the main problem in the TGN1412 incident. Following the TeGenero incident, much emphasis had been put on the NOAEL approach versus the MABEL (minimal anticipated biological effect level) approach. However, as shown by Eastwood *et al.* (2010), for TGN1412 it was not a problem of dose calculation, it was simply the fact that hazard identification cannot be done using monkeys, a fact not predictable for investigators and regulators alike, during preclinical development at the time. This point should be kept in mind for the future, that is, trying to set a FIH dose using a wrong model for hazard identification will always yield wrong results.

The third lesson is that a cytokine release syndrome may occur by different mechanisms with different clinical outcomes. Mabthera® and Campath-1H® are two mAb stimulating cytokine release from accessory cells; Orthoclone® induces cytokine release from CD3-positive T-cells and TGN1412 from CD4+ effector memory T-cells, all with very different results.

Finally, cytokine release assays have a place in predicting the potential for a product to trigger cytokine release syndrome in humans and its clinical consequences. However, these assays are not yet sufficiently developed and validated to provide an accurate and reliable tool to estimate and set up FIH doses.

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