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Translational immunologic safety evaluation: A perspective

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Abstract

Although translational research is a rapidly evolving area of biomedical sciences, translational immunologic safety evaluation has so far attracted only very limited attention. Assays and animal models have been developed to identify immunotoxic hazards related to immunosuppression, but less attention has been paid to immunostimulation, hypersensitivity, and autoimmunity. Some of these assays and models are recommended by regulatory bodies, even though it is as yet unsure to what extent they can predict the potential of, or lack of, new chemical entities and drug candidates for inducing significant immunotoxic effects. A translational approach should attempt to standardize and validate those models, assays, and biomarkers that could be used in regulatory non-clinical safety studies as well as clinical studies. Beyond translational immunologic safety, immune monitoring during clinical studies is intended to identify and evaluate potential immune safety issues not seen in non-clinical studies. Based on this overview of the current knowledge, it can be concluded that much remains to be done to conduct translational studies helpful to enhance the immunologic safety of drugs and chemicals.

Keywords: Translational research, immunotoxicology, in vitro assays, animal models, biomarkers, safety evaluation

Introduction

The term 'translation' refers to the transformation of knowledge through a continuum of successive fields of research from a basic science discovery to public health improvement (Drolet and Lorenzi, 2011). Translational research can be viewed as a paradigm for research alternatives to reduce the dichotomy of basic research—the main objective of which is the mere acquisition of knowledge—and applied (patient-oriented or population-based) research (Rubio et al., 2010). Alternatively, translational research can be considered as a 'bench to bedside' discipline designed to direct the findings of basic research to the production of new medications (Woolf, 2008), or as a process for ensuring the bidirectional flow of information from the research laboratory to the clinic and vice versa (Sung et al., 2003). A major contributor to the confusion around translational research may be the concept of 'translational blocks'. The first block represents the core of the translational component and is defined as 'the transfer of new understandings of disease mechanisms gained in the laboratory into the development of new methods for diagnosis, therapy, and preven tion and their first testing in humans'. The second block involves translation in the context of the community and ambulatory care setting and is described as 'the translation of results from clinical studies into everyday clinical practice and health decision-making' (Woolf, 2008).

Does the term translational research merely reflect another fashionable and fairly empty concept? The question may be worth asking: indeed, a Pubmed® search conducted on May 6th, 2012 using the keyword 'translational research' retrieved 67,307 references, of which only a fraction obviously addressed genuinely translational issues. Obviously, the desire of scientists

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to have their work used for the benefit of mankind is a major driver of translational research. Other critical aspects deserving consideration include the search for improvements in drug discovery/development through better-defined disease mechanisms and drug targets, the reduction of drug attrition rate, more effective inclusion within the 'knowledge economy', and a greater engagement of academia to instrumentalize knowledge, i.e. to obtain research funding (Morgan et al., 2011).

Translational immunology is seemingly an active area (7068 entries retrieved in Pubmed® using keyword 'translational immunology') in contrast to translational toxicology (500 entries), translational immunotoxicology (two entries both published in 1992), translational immunotoxicity (three entries) or translational immune safety (50 entries). The claim that 'toxicology has always been translational' (Mattes and Walker, 2009) is somewhat misleading. Indeed, although Orfila (1815), considered the father of modern toxicology by many, and subsequently quite a few toxicologists throughout the 19th century explored and compared the effects of poisons in animal experiments and intoxicated human patients from clinical, laboratory, and histopathological perspectives, the gap between fundamental research and clinical needs in the area of toxicology has been steadily widening since then.

Based on the available experience, four categories of immunotoxic effects must be differentiated, namely those related to: (i) immunosuppression, (ii) immunostimulation, (iii) hypersensitivity, and (iv) autoimmunity. Importantly, not only clinical manifestations of adverse events related to each of these four categories, but also laboratory tools to predict or diagnose these adverse events either in animals or humans, are usually very different. As reflected by current guidelines, immunosuppression and to a much lesser extent immunostimulation is the major focus of non-clinical immunotoxicity evaluation. Basic research tools and findings related to immunosuppressive agents, either pharmaceuticals or environmental chemicals, have long been transferred to the non-clinical immunotoxicity evaluation of new molecular entities and drug candidates. Nevertheless, the question of whether experimental studies on immunosuppression predict for man (Vos and van Loveren, 1995) is still pending. In addition, very limited experience has so far been gained on non-clinical evaluation and even more so on translational aspects in the area of hypersensitivity and autoimmunity.

Despite growing awareness of the need to improve the immunologic safety of xenobiotics, particularly pharmaceuticals, the translation of non-clinical tools and findings to the clinical setting is still in its infancy. The aim of this article is to overview those tools that can be considered for use in the timely implementation of translational immunologic safety evaluation and delineate perspectives for further development.

Immune function tools for translational immunologic safety evaluation

The evaluation of the immunologic safety of small-molecular-weight pharmaceuticals is mainly regulated by the ICH S8 guideline (ICH, 2005), which requires the systematic incorporation of immunotoxicity evaluation into standard drug development. Importantly, for the purpose of this guideline, immunotoxicity is restricted to unintended immunosuppression or enhancement, thus excluding hypersensitivity and autoimmunity. This guideline describes a weight-of-evidence decision-making approach primarily based on the findings of standard toxicity studies including clinical signs (especially infections), changes in standard hematology and clinical chemistry parameters, the development of tumors when no other cause can be identified, and histological examination of the main lymphoid organs (bone marrow, thymus, spleen, lymph nodes, and mucosa-associated lymphoid tissue or MALT). Other factors to be considered include the pharmacological properties of the drug candidate (i.e. on- and off-target effects on the immune system), the intended patient population (e.g. immunocompromised patients), structural similarities with a known immunotoxicant, disposition of the test article (in particular, high concentrations within cells of the immune system), and finally adverse events during early clinical trials. At this stage (prior to Phase III trials at the latest), focused (additional) immunotoxicity studies should only be considered if the weight of evidence review concludes to a possible cause for concern defined as one finding of sufficient magnitude, or two or more milder findings among the items listed above.

Additional immunotoxicity studies most commonly consist of 28-day rodent studies, even though any relevant animal species or study duration can be selected. Immunologic end-points, especially immune function parameters, to be measured during such studies are considered case-by-case depending on previous findings in standard toxicity studies, the known or suspected mechanism of action of the test article, and other potentially meaningful factors. If the results of these studies confirm the potential for the drug candidate to induce immunotoxic effects, it may be deemed necessary to conduct further studies in non-rodent species and subsequently during clinical trials or even post-marketing drug surveillance. It should be mentioned that other regulatory bodies consider that an immune function assay should be performed during the first step of the immunologic safety evaluation of non-pharmaceutical products, e.g. pesticides (US EPA, 2007) or medical devices (ISO, 2006). While abiding to confidentiality, the author of this article can tell that several test articles, which induced absolutely no changes in standard hematology parameters as well as the histology of the main lymphoid organs, have nevertheless been found to induce a statistically significant decrease in T-dependent antibody responses (TDAR). Finally, the recently revised ICH S6R1 guideline

(ICH, 2011) confirmed the importance of non-clinical immunologic safety evaluation of biotechnology-derived pharmaceuticals ('biologics'). Surprisingly, no reference to the weight of evidence review approach is made in this revised guideline, although it could be rather easily adapted to the specificities of biologics. However, the role of mechanistic studies in addition to screening immunotoxicity studies is highlighted, which could serve as an impetus to develop translational immunologic safety strategies.

A number of immune function assays and animal models are available, at least as far as immunosuppression is concerned (Descotes, 2006), but the ongoing development of immuno-modulatory drugs is increasingly a trigger to adapt these assays and models to the area of immunostimulation (Kawabata and Evans, 2012).

TDAR assays

Immune responses are divided into innate (antigen-non-specific) and adaptive (antigen-specific) responses, but until recently much more attention has been paid to adaptive immune responses, in particular humoral responses with the use of T-dependent antibody response (TDAR) assays.

Two main types of TDAR assays can assess humoral responses from a different perspective: (i) TDAR assays that measure the number of cells producing antibodies, such as the plaque-forming cell (PFC) assay where sheep red blood cells (SRBC) are the selected antigens (Ladics, 2007); and (ii) TDAR assays that measure the levels of antigen-specific antibodies in the sera of previously sensitized animals. The latter typically rely on ELISA methods and, although Keyhole Limpet Hemocyanin (KLH) is the preferred antigen nowadays (Plitnick and Herzyk, 2010), other antigens such as tetanus toxoid (Vos et al., 1979) or BSA (Henningsen et al., 1984) have sometimes been utilized. Direct comparison of effects obtained with the same reference products in the same laboratory and the same animal species using the PFC assay and the anti-KLH ELISA is rather scarce (Bugelski and Kim, 2007; White et al., 2007). Nevertheless, the PFC assay is considered to be sensitive, especially for detection of IgM antibodies. In contrast, ELISA is thought to be sensitive for detection of IgG antibodies. Whether a more sensitive assay is more appropriate for hazard identification and a less sensitive assay more appropriate for risk assessment remains an open question.

As regards the relevance and predictability of the triggered immune response for human situations, the role played by the nature of the selected antigen, i.e., a particulate antigen (such as SRBC) in the PFC assay vs a foreign protein as in the anti-KLH ELISA assay is a matter of debate. In addition to being poorly standardized antigens, which tends to increase the inherent variability of immune responses, SRBC are widely used in rodents, but neither in non-rodents nor in humans. In contrast, foreign proteins such as KLH or tetanus toxoid can be used in all mammal species including man. It remains, however, to be shown that immunotoxic effects on the humoral responses to foreign proteins in humans do compare to those seen in laboratory animals, both qualitatively and quantitatively.

From a translational perspective, another critical issue is to determine whether testing primary or secondary humoral responses or both should be preferred. Indeed, in the PFC assay, rodents are typically injected once with sheep erythrocytes so that the measured response is a primary response. Although the PFC assay can also be used to measure secondary response, the database is fairly small. In contrast, KLH or other T-dependent proteinic antigens can be injected on two occasions to the same animals and, if an adequate study design is used, both primary and secondary humoral responses can be measured. It may be assumed that primary immune responses are more relevant as regards children, whereas adults tend to develop secondary immune responses more frequently.

Cellular immune responses

Cellular immune responses are being increasingly considered as potentially useful tools for evaluating the immunologic safety of drug candidates. Currently available tools include in *vivo* models and *in vitro* assays.

Delayed-type hypersensitivity models can be used in rodents as well as non-rodents. In rodents, DTH responses are typically induced by a challenging injection of a T-dependent antigen (e.g., BSA, KLH, or SRBC) into one footpad (Henningsen et al., 1984) or by a topical application of a contact sensitizer (e.g., DNCB, oxazolone, picryl chloride) on one ear (Descotes et al., 1985) in animals previously sensitized with the same antigen. DTH responses manifest by local swelling, i.e. increased thickness of the footpad or ear. The use of these models is restricted to rodents. In non-rodents, DTH responses can be induced using an experimental design close to skin tests, as performed in human subjects (Miyamoto et al., 1995; Cordoba et al., 2008). Although skin tests are invaluable tools to diagnose prior sensitization in human subjects with a history of hypersensitivity reaction (Brockow and Romano, 2008), the *de novo* induction of sensitization with a potent contact sensitizer such as dinitrochlorobenzene (Friedmann et al., 1983) or the use of recall antigens (Kniker et al., 1979) to induce DTH responses in the skin of human subjects for assessing cellular immunity, are no longer performed due to wide inter- and intra-individual variability. Thus, currently available DTH models do not seem to be suitable for translational immunologic safety evaluation.

Lymphocyte proliferation assays have long been used to assess cellular immunity. Typically, proliferation of cultured lymphocytes is triggered non-specifically by mitogens, such as concanavalin A, LPS, or anti-CD3. A mixed lymphocyte reaction (MLR) is more rarely used nowadays

in the context of non-clinical immunotoxicity evaluation. Antigen-specific proliferation can also be measured when lymphocytes from a previously sensitized host (ovalbumin, tetanus toxoid, influenza, …) are used. Lymphocyte proliferation is most often measured from the incorporation of tritiated thymidine, but alternative methods such as Elispot to measure cytokine release, e.g., interferon-γ (Cox et al., 2006) or flow cytometry using fluorescent dyes (Quah and Parish, 2012) have been proposed. Interestingly, the results of lymphocyte proliferation assays have long been shown to correlate with DTH responses in rodents (Luster et al., 1982). The use of these assays in translational immunologic safety evaluation is currently limited by the very small database comparing results across species and from animal to man. It is also noteworthy that lymphocyte proliferation assays are still poorly reliable tools to monitor the level of immunosuppression achieved in transplant patients (Nickel et al., 2009).

NK cell activity

Because of the suspected, but not fully elucidated role of NK cells in the immunosurveillance of cancer, NK cell activity is often measured in standard as well as additional immunotoxicity studies. The ^{51}Cr release assay is definitely the gold standard assay (Li, 2010). Interestingly, this assay can be used in all mammal species including man, one major difference being the source of target cells, namely YAC-1 cells in rodents, L5178Y cells in non-human primates and humans, and K562 cells in mini-pigs. Published data, however, did not evidence overt differences in the effects of reference compounds on NK cell activity across species, despite the use of different target cells. NK cell activity can also be measured by flow cytometry (Cederbrant et al., 2003). However, data using this technique have been generated mainly in rats and monkeys.

Neutrophil and macrophage function assays

The function of phagocytes (i.e., neutrophils and macrophages) has been a matter of very limited interest until recently. A variety of methods have been used in research laboratories (Bilitewski, 2008), but so far very few have been used in regulatory safety studies. Interestingly, commercially available kits can be used to measure phagocytosis, oxidative burst, and chemo-taxis by flow cytometry. These methods are routinely used in the clinic for the diagnosis of inborn defects in neutrophil/macrophage function. Limited data suggest they could be straight-forwardly used in animal species (Horand et al., 2003; Freebern et al., 2012), but further studies are warranted to demonstrate to what extent animal data correlate with human data.

Immunologic biomarkers

Since the publication of recommendations by the Subcommittee of the US National Research Council (National Research Council, 1992) and the Agency for Toxic Substances and Disease Registry (Straight et al., 1994) on immunological markers to be used in clinical studies, relatively limited attention has so far been paid to the development and use of biomarkers of immunotoxicity in clinical trials/epidemiological studies (Descotes et al., 1996; Gennari et al., 2005; Dietert, 2010; Duramad and Holland, 2011). Indeed, despite tremendous progress in the performance of available techniques, most biomarkers in use today for immunologic safety evaluation were in use two decades ago.

Lymphocyte immunophenotyping

Well-standardized flow cytometry techniques are available in all relevant animal species used in non-clinical immunotoxicity evaluation. The most commonly used parameters include B-cells, total T-cells, and both $CD4^+$ and $CD8^+$ T-cells. Technical and/or research efforts are still needed regarding the flow cytometry analysis of other important cells of the immune system such as NK cells, monocytes, or regulatory T (T_{reg})-cells, especially due to overt variations across animal species and from animal to man.

The relevance of lymphocyte immunophenotyping in immunotoxicity evaluation has been debated (Immunotoxicology Technical Committee, 2001). It is fair to say that, more than 10 years later, the conclusions of these expert panelists that 'immunophenotyping has not been sufficiently validated for routine use and is unlikely to be used by itself to predict the immunotoxic potential of a previously uncharacterized chemical' are still largely valid. Indeed, observed changes in commonly analyzed lymphocyte sub-sets are rarely useful from an immunotoxicologic perspective, except in thoserare instances where the mechanism of action of the test article can provide clues for the sake of data interpretation.

It would be probably more valuable to analyze markers of lymphocyte activation. Although the need has long been identified (Burchiel et al., 1999), and despite a wealth of research papers devoted to the identification and validation of such markers, no reliable (i.e. standardized and validated) markers are still available for immunologic safety evaluation.

Cytokine profiling

Cytokine assays are increasingly used in non-clinical immunotoxicity studies (Corsini and House, 2010). Although the data obtained may support previous information related to the mechanism of action of a well-characterized immunomodulatory drug, they are nowadays rarely useful to predict or monitor immunotoxic adverse events, except for the potential of therapeutic proteins to induce cytokine release syndrome, at least to some extent (Walker et al., 2010).

The immunotoxicologic significance of changes in cytokine profiling is questionable due to the obvious, but too often overlooked pleiotropic effects of cytokines.

It may be worth reminding that changes in one or a few immunologic parameters do not necessarily mean a test article is an immunotoxicant. It is also important to keep in mind differences in cytokine effects or profiles across species including man (Tarrant, 2010). Last but not least, differences in measured cytokine responses can also be seen, depending on selected time points or assay techniques (from ELISA, Multiplex° to PCR).

Serum immunoglobulins

Although assays to measure serum immunoglobulin levels are fairly simple and inexpensive, extremely limited information can be expected from results obtained during animal as well as human studies.

Complement activation

Despite its critical role in many facets of the immune system, extremely limited attention has been paid to the complement system. One exception, however, is complement activation that can be triggered in a non-antigen-specific manner by various pharmaceutical products, including monoclonal antibodies such as rituximab (van der Kolk et al., 2001), nanomedicines such as liposomes (Szebeni et al., 2011), and pharmaceutical solvents such as Cremophor El° (Weiszhár et al., 2012). Activation of the complement cascade can result in acute hypersensitivity reactions somewhat mimicking anaphylaxis and in the generation of the anaphylatoxins C3a and C5a that can be measured *in vitro* or *in vivo* in the serum of rats, dogs, pigs, and humans.

Autoantibodies

Autoantibodies are well-recognized hallmarks of autoimmune phenomena. The statement made in 1997 (Verdier et al., 1997), that the search for autoantibodies during standard toxicity studies was useless for predicting the potential of drugs and chemicals to induce autoimmune diseases, is still valid. Indeed, drugs that have been unequivocally shown to induce autoimmune diseases in humans have consistently been negative when administered to animals. In addition, the induction of autoantibodies does not necessarily mean that the affected humans will develop a full-blown clinical disease (Lleo et al., 2010).

Current pitfalls and perspectives

Until recently, translational research in immunotoxicology has been largely overlooked. Translational obviously does not mean merely extrapolating data from animal to man. Preferably, a translational approach should attempt to standardize and validate those models, assays, and biomarkers that could be used in regulatory non-clinical safety studies as well as clinical studies. Beyond translational immunologic safety, immune monitoring during clinical studies is intended to identify and evaluate potential immune safety issues not seen in non-clinical studies. Immunotoxicity hazard identification using standard animal models or assays remains the first and inescapable step in this long process intended to determine the immunologic safety of new molecular entities and drug candidates.

How can immune findings in animals be transferred to clinical studies and beyond, to ensure the immune safety of drug candidates and new molecular entities? As briefly overviewed above, a number of assays and animal models are currently available. Most of these have proven appropriate for hazard identification, but relatively few are expected to be good candidates for translational research. With the exception of TDAR assays that measure specific antibodies in treated humans previously sensitized with a proteinic antigen, most candidate tools are either *in vitro* or *ex vivo* assays.

For translational purposes, it seems more appropriate to use the same or very close experimental conditions in animals and humans. As briefly overviewed above, nearly all these models and assay have not been extensively validated from rodents to non-rodents, and from animals to man. This is undoubtedly one urgent goal to be accomplished by translational researchers. Validation cannot be restricted to a few reference positive products, but include a decent set of positive as well as negative products. In contrast to the PFC assay, this extensive validation remains to be done with the anti-KLH assay.

Another urgent goal of translational immune safety is the development, standardization, and validation of markers of immunotoxicity. It is noteworthy that most assays and measured end-points during non-clinical studies have been developed many years ago. Despite obvious progress in available technical tools, such as flow cytometry, ELISA, or EliSpot, the array of measured end-points has not evolved significantly over the last few decades. Immunological markers that are being progressively introduced in clinical trials of novel immunomodulatory drugs are primarily intended to assess and understand efficacy in patients with cancer (Callahan et al., 2010) or organ transplant (Millán et al., 2009). It is, however, uncertain whether immunological markers of efficacy can also serve as markers of immunotoxicity. The search for a correlation between changes in selected immunological markers and the risk of immunotoxic complications, e.g. infections (Krause et al., 2011), especially in patients treated with potent immunomodulatory patients, is deemed to be essential. Indeed, the results of such studies could prove instrumental to identify those immunological markers that are predictive of adverse clinical consequences involving the immune system—i.e., genuine biomarkers of immunotoxicity—and those that are not predictive reliably enough.

In this setting, the search for novel biomarkers of immunotoxicity is considered essential. Immunotoxicogenomics is emerging as a promising area (Frawley et al., 2011; van

Kol et al., 2012). Somewhat surprisingly, efforts have so far essentially focused on the immunotoxicologic potential of environmental chemicals. The rapid development of new animal models such as transgenic and human immunized mice has been instrumental to accelerate our understanding of the immune system (Shultz et al., 2007). These models have also been helpful to predict the efficacy of a host of novel therapeutics, but they have seldom been used in the setting of immunologic safety. The use of new markers of immunotoxicity in such models may be worth consideration.

Translational research in the area of immunologic safety evaluation should primarily be aimed at defining and validating models, assays, and biomarkers that could help transfer non-clinical findings to the clinic. Clinical immunotoxicology has traditionally been the missing link of immunotoxicity risk assessment (Descotes, 2004). With the timely development of translational immunologic safety, the gap between findings in non-clinical studies and post-marketing surveillance may hopefully diminish.

Declaration of interest

The author reports no conflict of interest. The author alone is responsible for the content and writing of the paper.

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