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Immunotoxicity, Immunogenicity and Immunopharmacology of Therapeutic Proteins (13-Nov-2004)

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Introduction

Immunotoxicity testing for biotechnology products such as human proteins and monoclonal antibodies is often conducted in nonhuman primates. These human proteins may not be pharmacologically active in rodents or may be immunogenic, such that rodents would develop neutralizing antibodies to the drug. These don't appear to be very significant points!!! There are certain advantages to using nonhuman primates for immunotoxicity testing in that blood volume is not as limited as in rodents and many of the immune tests in nonhuman primates can then be used in the clinical trials. The major disadvantage of nonhuman primates is that animal numbers are limited and the variability is large; similar to the variability seen in humans. Many of the standard immunotoxicity tests are not well characterized in the nonhuman primate and further development of these assays is needed. The cynomolgus or rhesus macaque is the most common nonhuman primate species used in toxicology studies and are currently the most used species for immunotoxicity testing. Marmosets may also be useful in immunotoxicity testing and have an advantage due to their small size, although the historical database is not as extensive for this species. Some human proteins are only pharmacologically active in chimpanzees and although immune testing can be done in this species, the number of animals is so small that they are unlikely to provide much useful information.

Fundamentals of Study Design

Immunotoxicity testing guidelines exist for small molecules where the toxicology is largely unpredictable and rodent species are typically used. For human therapeutic proteins, the immune system is often the intended target of the therapy and the immunotoxicity observed is often exaggerated pharmacology. As mentioned previously, nonhuman primates are generally used and the immune tests conducted need to be selected based on the known immunomodulatory properties of the drug. It is important to distinguish between immunopharmacology; where the immune system is the target organ of the therapeutic effect, immunotoxicity; where non-target immune effects such as autoimmunity or immunosuppression may be observed; and immunogenicity, which represents an immune response to the drug.

Immunogenicity is typically measured in GLP toxicology studies to determine the immunogenic potential of the therapeutic protein. This immunogenic response can be manifested as binding antibodies, neutralizing antibodies, cross-reactive antibodies or sustaining antibodies. Binding antibodies are generally not a significant concern, as they do not generally cause toxicity nor decrease the activity of the drug. These are often seen in animal studies and generally do not correlate with the immunogenic potential of the drug in humans. Neutralizing antibodies are of concern as they often decrease the efficacy and increase the clearance of the drug. These neutralizing antibodies may interfere with the binding of the drug with its target, interfere with the pharmacologic activity of the drug or may alter the pharmacokinetics of the drug. The presence of neutralizing antibodies in the animal studies is generally not predictive of the response in humans, except to compare routes of administration. For example, in preclinical studies, a humanized monoclonal antibody given by the IV or SC route that produced minor binding antibodies, failed to illicit neutralizing antibodies in cynomolgus monkeys. When cynomolgus monkeys were administered the same drug by the inhalation route, a strong neutralizing antibody response was seen (Bussiere et al., 1997). In clinical studies, no antibodies were observed following IV or SC administration of the drug. However, following inhalation exposure, one patient developed neutralizing antibodies (Sweeney et. al., 2001).

Cross-reactive antibodies are of the greatest concern due to their potential for clinical toxicity. These antibodies, which develop to the drug, can cross-react with the endogenous protein and lead to a toxic response. These may be predicted by testing the homologous protein in the appropriate animal species (i.e., the cynomolgus protein tested in cynomolgus monkeys). Sustaining antibodies can decrease the clearance of the drug, thus increasing exposure and leading to toxicity.

Immune tests that are typically incorporated into standard GLP toxicology studies include: hematology, lymphoid organ weights, and immunopathology. Other immune tests that can be easily added include: immunophenotyping by flow cytometry, functional immune tests such as T-dependent antibody response (primary and secondary) and measurement of cell-mediated immunity (CMI) by delayed-type hypersensitivity (DTH) testing. It is important and sometimes difficult to understand what moderate changes in any of these immune parameters mean to the overall immune competence of the host and susceptibility to disease.

There are several important considerations when including immunotoxicity testing into standard GLP toxicology studies. These include whether to use the main study animals or a satellite group and the timing of these tests within the context of the GLP toxicology study. The advantage of using the main study animals for immunotoxicity testing is the animal use is reduced and you can correlate any immunotoxicity findings with other toxicities seen in those animals. The disadvantage of using main study animals is that the additional manipulations for immune testing (e.g., injection of an antigen for determining antibody response or for assessing a DTH response) may influence the toxicity or immunogenicity of the therapeutic agent. Immunotoxicity testing is generally included in the one-month nonhuman primate toxicology studies. It is very important to include several baseline measurements due to the variability seen between animals, and even in the same animal over time. Due to the small number of nonhuman primates per group, it is important to reduce the variability in the assays as much as possible with regards to antigen source, technique, etc.

Nonhuman Primate Immunotoxicology Endpoints

Immunopathology - The current regulatory guidelines recommend that immunopathology be used as the initial screen to detect immunotoxicity. As is the case with rodents, standard hematology and histopathology may often be sufficient to detect immune system alterations. This can include total and differential white blood cell counts, and evaluation of the histopathology of lymphoid organs such as the thymus, spleen, lymph nodes, gut-associated lymphoid tissue (GALT) and the bone marrow. In addition, more detailed measurements of any change in size and cellularity of immune cells, germinal center development, cortex:medulla ratio of the thymus, and immunohistochemistry of the lymphoid organs may be included.

Flow cytometry can be easily included in a GLP toxicology study to evaluate changes in lymphocyte subsets, including T cells (CD4+, CD8+), B cells (CD20+), NK cells (CD16+) and monocytes (CD14+). This is typically conducted using peripheral blood that allows repeat sampling over time within the same animal. However, immunophenotyping can also be conducted on tissues to determine if there are effects on lymphocyte trafficking, although time points are limited to sacrifice unless serial biopsies can be performed (i.e., on lymph nodes). Flow cytometry can also be used for more functional endpoints of immune competence including lymphocyte activation, cytokine release, phagocytosis, apoptosis, oxidative burst, natural killer cell activity, etc. These could be added if the mechanism of action of the drug targets suggests involvement of a particular function of immune cells.

Humoral Immunity - In nonhuman primates, the assay most commonly used to assess the ability to mount a T dependent antibody response is immunization with keyhole limpet hemocyanin (KLH) or tetanus toxoid (TT), and measurement of circulating antigen-specific antibody levels by ELISA methods. Immunization with KLH or TT should occur prior to drug treatment to assess the effects on the secondary antibody response (i.e., first immunization SC on Day -3 and second immunization 14 days later), and the other antigen can be injected after two weeks of treatment to determine the effect on the primary immune response 7 to 10 days later. This allows for the assessment of both the primary and the secondary T-dependent antibody response within the one-month GLP toxicology study. For studies of longer duration, a booster immunization can be given at a later time point to assess the effect on the memory response.

Cell-Mediated Immunity - Two different assays may be used to assess CMI in cynomolgus monkeys, the local lymph node assay (LLNA) and the DTH assay. For the LLNA, 3 daily doses of oxazolone are applied to the ear and the draining lymph node is then harvested on Day 5. The cells are cultured *ex vivo* with tritiated thymidine or by staining with a marker of proliferation and assessing by flow cytometry. Determination of the number of cells, the percentage of B lymphocytes, or proliferation is increased following treatment with oxazolone. A known immunosuppressant, cyclosporine A, has been shown to suppress this response. Thus, in this case, the LLNA is not used to identify a drug as a contact sensitizer, but to evaluate

the ability of the drug to modulate the immune response to oxazolone. Use of the DTH assay in cynomolgus monkeys was first published by Blevins and de la Iglesia (1995), but modifications have continued to be made. The advantages of DTH testing are that it is noninvasive and can be repeated during the treatment period, special equipment is not required and minimal expertise is needed. In addition, this type of testing can be included in the clinical trials to compare the immunomodulatory effects of a compound.

Multiple studies had been conducted with a combination of DTH antigens including *Candida albicans*, Diphtheria toxoid, and Trichophyton toxoid (Tetanus toxoid can also be added to this cocktail). The antigens are injected SC on Days -6, -2 and 6, and animals are then challenged with an intradermal injection of the antigens on Day 20. There is high variability in the both the magnitude of the response and in the number of animals responding. However, in general most animals responded to *Candida albicans* and approximately 50% respond to Diphtheria toxoid, although few respond to Trichophyton toxoid (Bussiere, et. al., 2001). The maximal response was generally seen at 24 hr post challenge. In an effort to improve the response and reduce the variability of the response to the antigens, the response to serial dilutions of *Candida albicans* was tested in cynomolgus monkeys. In this study, 50% of control animals responded to all four dilutions of *Candida* (75-25000 PNU), while only 33% of animals treated with 15 mg methotrexate responded to all dilutions, and 17% of animals treated with 25 mg methotrexate responded (Bussiere, unpublished data).

Further work is needed to make the DTH assay more useful in detecting immunotoxicity of therapeutic drugs and to reduce the variability in the assay. There are several aspects of the assay that could improve the response including: the source of the antigen, concentration reported, the source of the animals, the emulsion technique and the consistency of the scoring. Additionally, further investigation is warranted to determine if response to serial dilutions of *Candida* will be more sensitive in detecting immunomodulators than response to just one concentration. It has not been determined if the cocktail of antigens is needed to get a good response to *Candida* or if *Candida* could be given by itself and would still elicit a strong and consistent response.

Innate Immune Function - Natural Killer (NK) cell activity can also be tested in nonhuman primates. Splenocytes or peripheral blood leukocytes are incubated *ex vivo* for 4 hours with 51Cr-labelled K562 tumor cells and the % lysis is determined. NK activity can also be measured by flow cytometry, which eliminates the use of radioactivity. As with other immune assays in nonhuman primates, the variability in NK activity between animals is high. Macrophage function can also be assessed using flow cytometry; to determine phagocytosis or oxidative burst capabilities.

Host Resistance - Host resistance assays in nonhuman primates have not been developed and present unusual challenges. The best way to determine if the therapeutic protein alters host resistance is to use a transgenic or knockout mouse (if available) in the host resistance models mentioned previously. Knockout (and transgenic) mice have been used to assess immunotoxicity of protein therapeutics. Particular emphasis in future pharmacology and toxicology studies will be directed toward conditional knockout mice (to evaluate the impact of chemically-mediated inhibition of a particular gene product at the relevant stage of life) and "humanized" knock-in animals (in which the endogenous mouse gene is replaced with the homologous human gene to examine its role in disease or drug metabolism).

With respect to the immune system, the physiological functions and pathways for many genes important to normal immune function have been investigated using knockout (and transgenic) mice (Ryffel, 1997). Again, "humanized" mice are of particular importance in modeling the human immunological response, as they have several advantages over conducting immunopharmacology and immunotoxicity studies in non-human primates (the only alternative if the human protein is not active in rodents). First, rodent studies are simple, relatively inexpensive, and can include enough experimental subjects to achieve suitable statistical power. More importantly, immunotoxicity assays are well characterized in the mouse, in contrast to the non-human primate. However, three caveats must be kept in mind when using genetically engineered mice for immunotoxicity assessment. First, the emphasis on morphologic assessment as the usual standard for phenotypic analysis means that the immune function of most genetically engineered mice is poorly characterized. Another important consideration is that conclusions reached using a standard knockout mouse (in which the gene is missing throughout gestation and postnatal life) may not accurately reflect disease or pharmacological interventions in which genetic function is nullified only during adulthood (the most likely clinical scenario). The most critical issue, however, is the background strain on which the null mutation is carried, as mice with different genetic backgrounds respond very differently to immune stimuli. The standard background of knockout mice is a mixture of C57BL/6 (the predominant component, derived from the blastocyst) and S129 (the major source of ES cells for gene targeting). Furthermore, not all S129 ES cell lines are comparable, and knockout mice often are not bred to achieve genetic homogeneity on a suitable genetic background. Therefore, it is critical to first assess the immunopharmacologic response using standard immune assays in the background

strain (C57BL/6) prior to testing knockout mice back-crossed to increase the C57BL/6 gene fraction, or to back-cross the null mutation onto mice with a genetic background relevant to immunotoxicity assessment (i.e., B6C3F1).

Data Interpretation

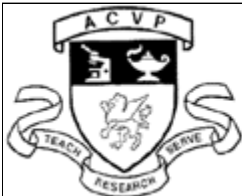
Although immunomodulation can be assessed in the nonhuman primate, the assays are less well characterized than those used in the rodent. One issue is that there has been no consistent protocol used, and the timing of incorporating these assays into standard GLP toxicology studies varies. More historical control data is needed and many assays have not been tested with an immunomodulatory control to confirm that the assay can detect a mild/moderate immune modulator (both immunoenhancing as well as immunosuppressive activity). There is naturally a greater variability in nonhuman primates than in in-bred rodents and the animal number per group is generally much smaller than in rodent studies. It is critical to find ways of reducing the variability in the assay to allow for more meaningful data interpretation. These can include decreasing the inter-animal variability (animal source, age, decreasing stress during the study, increasing the number of baseline samples, etc), as well as the assay variability (source of antigen, technique, standardizing the timing, etc.).

We are now at the stage where these assays can be conducted in nonhuman primates, but are lacking data as to which assays are the most useful in predicting immunomodulatory effects in humans. Assay methods need to be standardized so that we can truly compare the data to make that determination. It would also be useful to compare the data from the nonhuman primate with the immunotoxicity data in rodents to evaluate whether the nonhuman primate is more predictive of the human response.

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