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A Review on Preclinical *In Vitro* and *In Vivo* Studies for Biologics.

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ABSTRACT

Biologics have emerged as a prominent class of therapeutics, encompassing a wide range of products derived from living organisms or their components. Prior to clinical trials and regulatory approval, rigorous preclinical evaluation is essential to assess their safety, efficacy, and pharmacological properties. *In vitro* binding studies, functional assays, toxicity screening, stability and formulation studies, enhances the translational relevance of preclinical research. *In vivo* studies complement *in vitro* findings by evaluating the pharmacokinetics, biodistribution, and pharmacodynamics of biologics in whole organisms. Animal models, including rodents, non-human primates, and genetically engineered organisms, serve as indispensable tools for assessing efficacy and safety profiles, as well as immunogenicity and off-target effects. The choice of pertinent species for nonclinical research, such as toxicological studies, is crucial in order to enable the correct translation of animal data into human studies, given the specificity of the target for the majority of biologics. Integration of preclinical *in vitro* and *in vivo* data is crucial for informing clinical trial design and regulatory decision-making.

Keywords: Biologics, Preclinical, In vitro, In vivo, Toxicity, Immunogenicity, Pharmacokinetics, Pharmacodynamics

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INTRODUCTION

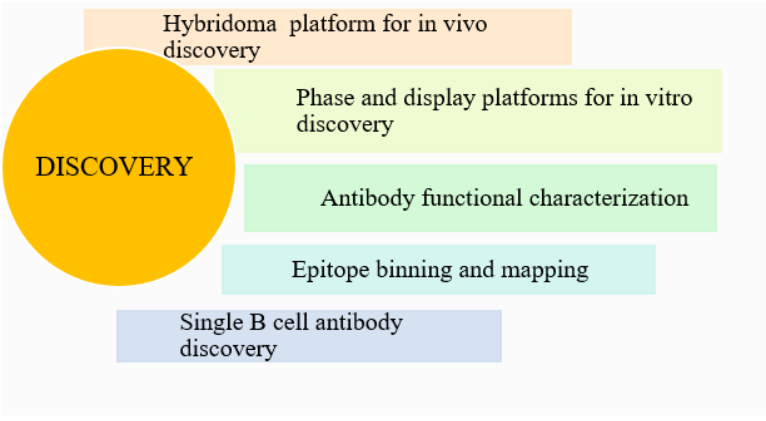
Biological products are used to diagnose, prevent, treat, and cure diseases and disorders. They are governed by the Food and Drug Administration (FDA). Biological products are a broad class of goods that are often large, complex molecules. Frequently more challenging to define than small molecule medications, these compounds can be made using biotechnology in a living system, such a microbe, plant cell, or animal cell. Therapeutic proteins like filgrastim, monoclonal antibodies like adalimumab, and vaccinations like tetanus and influenza are just a few of the numerous biological products that are authorized for use in the US. The characterisation and manufacture of biological products might provide obstacles due to their intrinsic variability and the manufacturing process, which are not often encountered in the development of small molecule medications [1]. At every stage of the protein synthesis process, new biologic (and pharmacological) targets become possible with the expansion of our understanding of genetics and cellular mechanisms. This results in novel treatments, which then lead to fresh insights on diseases. In order to treat anemia, cystic fibrosis, growth deficit, diabetes, hemophilia, hepatitis, genital warts, transplant rejection, and malignancies, biologics have discovered novel targets. Biologics forecast hereditary susceptibility to diseases like Parkinson's disease. Cultured tissues, immune system suppressants for transplantation, and growth factors for tissue reconstitution are examples of non-pharmaceutical biologics that are used to treat ailments including diabetic foot ulcers [2].

Table 1: Difference between small molecule drugs and biologics [3]

Small Molecule Drugs	Biological Products
Generally low molecular weight	Generally high molecular weight
Usually organic or chemical synthesis	Made with/from live cells organisms→ inherent & contamination risk
Fewer critical process steps	Many critical process steps
Well characterized	Less easily characterized
Known structure	Structure may or may not be completely defined or known
Homogenous drug substance	Heterogenous mixtures → may include variants
Usually not immunogenic	Often immunogenic

DISCOVERY OF BIOLOGICS

Figure 1: Discovery of biological products



FUNDAMENTAL ISSUES AND CONCERNS WITH BIOLOGICS

Biologics may not respond well to traditional methods of pharmaceutical toxicity assessment. Standard toxicity testing methodologies in regularly used species, such as rats and dogs, are frequently precluded by the biological activity, species, and/or tissue specificity of many biologics. Immunogenicity (i.e., generation of an antibody response) and immunotoxicity (agents intended to activate or repress the immune system may cause cell-mediated alterations) are two unique concerns that biologics may present that require attention in nonclinical investigations.

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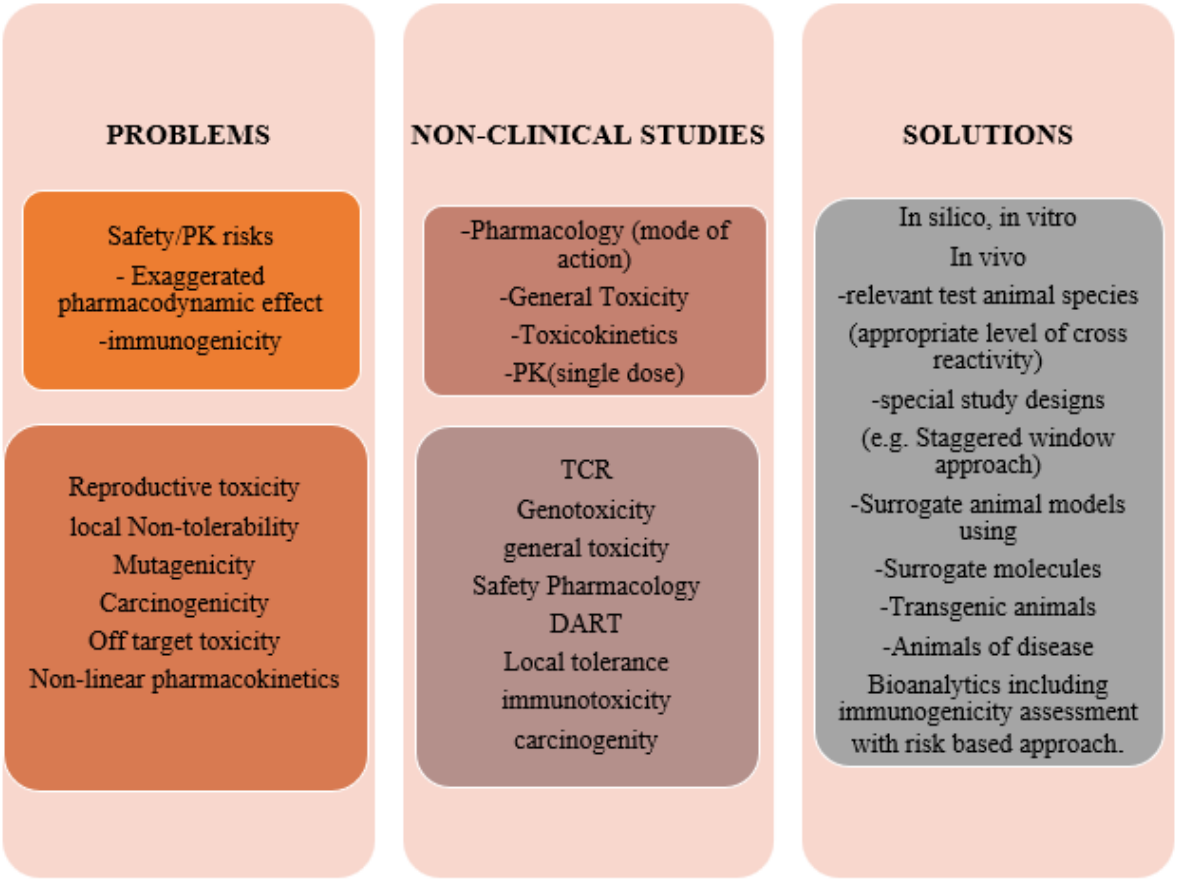
Challenges in pre-clinical development of biologics

Preclinical phase success is critical to the continued use of biologics as a primary formulation technology. Preclinical biologics have an already difficult task success rate of only 31.8%, which presents a number of particular difficulties for drug development teams. Biologics are challenging in part because of their inherent huge size and complexity. It is important to choose the right formula for these reasons. Nearly half of all newly approved drugs in the pharmaceutical industry are biotherapeutics, a segment that is expanding quickly. Monoclonal antibodies account for a significant fraction of these approvals each year (mAbs). Since mAbs are derived from a biological source, their non-clinical pharmacology and toxicology testing during development is different from that of chemical entities. This is because, in order to elicit a pharmacological response, animal models must share the same epitopes (targets) as humans. Although mAbs include both pharmacological and non-pharmacological toxicity mechanisms, these biotherapeutics are not now subject to the conventional in silico predictive toxicological techniques employed in the study and development of chemical entities. There are obstacles and chances for improved approaches to offer a more predictive program to evaluate and track any negative medication responses of monoclonal antibodies (mAbs) for particular patients before to, during, and following market approval [4].

Figure 2: Challenges in development of biologics.



GUIDANCE FOR SAFETY TESTING OF BIOLOGICS

S6(R1) Preclinical Safety Evaluation of Biologics

In 1997, the harmonized ICH S6 was completed. The standards for non-clinical safety assessment of biologics are covered in this document. The Guideline was renamed S6(R1) when an amendment was produced in 2011 that updated the subjects of immunogenicity, reproductive and developmental toxicity, species selection, research design, and evaluation of carcinogenic risk in addition to providing clarification on S6.

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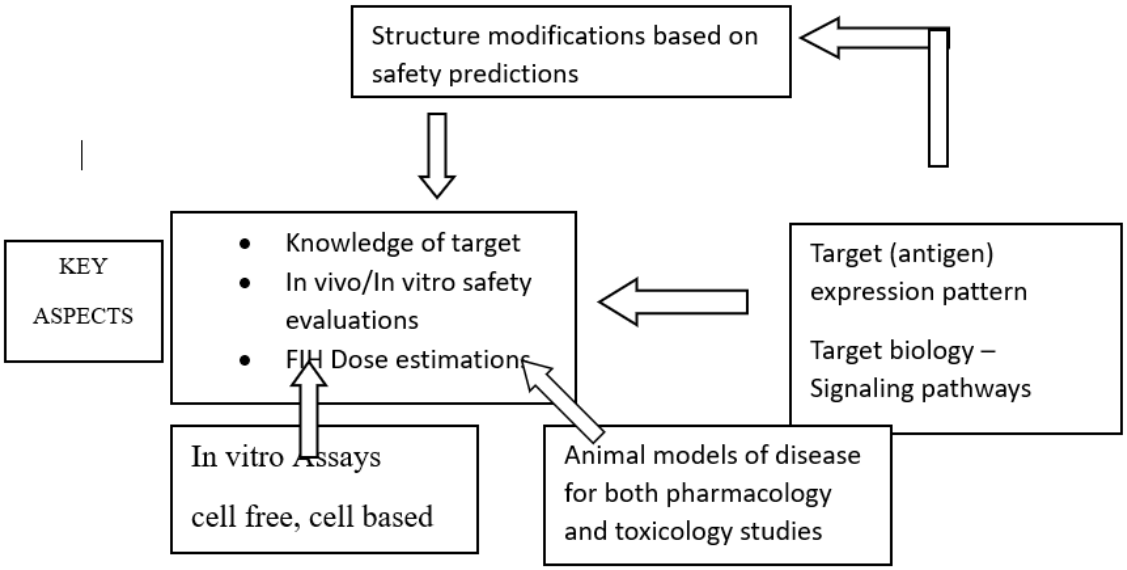
Figure 3: Approach for Predictive Toxicology [5]



***In Vitro* Studies**

Cell culture studies

In vitro cell culture studies constitute a fundamental component of preclinical evaluation for biologics and vaccines. These studies are indispensable for assessing the safety and purity of the final product. Tests are conducted on cell substrates, control cell cultures, viral seeds, and vaccine harvests to detect the presence of adventitious agents. Diverse assays, including those for cytopathic, heamadsorbing, and hemagglutinating viruses, are employed to identify potential contaminants. The selection of appropriate cell types is pivotal, ensuring relevance to potential exposures during production. Specialized testing may be necessary to detect specific viruses, such as insect viruses or arboviruses. Importantly, testing is performed at different production stages, with a focus on stages where adventitious agents are most likely to be present. Emphasis is placed on testing for mycoplasma contamination and the necessity of testing control cell cultures to verify the absence of adventitious agents. Overall, in vitro cell culture studies play a critical role in characterizing and qualifying cell substrates and other biological materials essential for the production of viral vaccines and biologics [6].

Binding studies

In vitro binding studies are pivotal for understanding the interaction between biological products and binding proteins. These studies provide insights into binding affinities with proteins like albumin and α-1 acid glycoprotein, thereby guiding pharmacokinetic assessments. Understanding protein binding dynamics is crucial for optimizing dosing, ensuring safety, and comprehending potential adverse reactions. Moreover, binding studies aid in tailoring treatment strategies based on individual patient needs, particularly in complex scenarios involving concomitant medications or medical conditions affecting protein binding kinetics. Identification of potential competition for binding sites is essential for accurate interpretation of drug interactions [7].

Functional Assays

Functional assays are indispensable tools for evaluating the efficacy of biological products. They encompass various methodologies, including cell-based, ligand binding, and neutralization assays, selected based on the therapeutic agent's mechanism of action. Different assays are required for therapeutics targeting distinct cellular components or disrupting specific interactions. Meticulous optimization and validation processes are imperative to ensure the precision and reproducibility of results, particularly for antibody assays. Stringent validation protocols empower researchers and clinicians to interpret assay outcomes confidently, facilitating effective decision-making in both clinical and research settings [8].

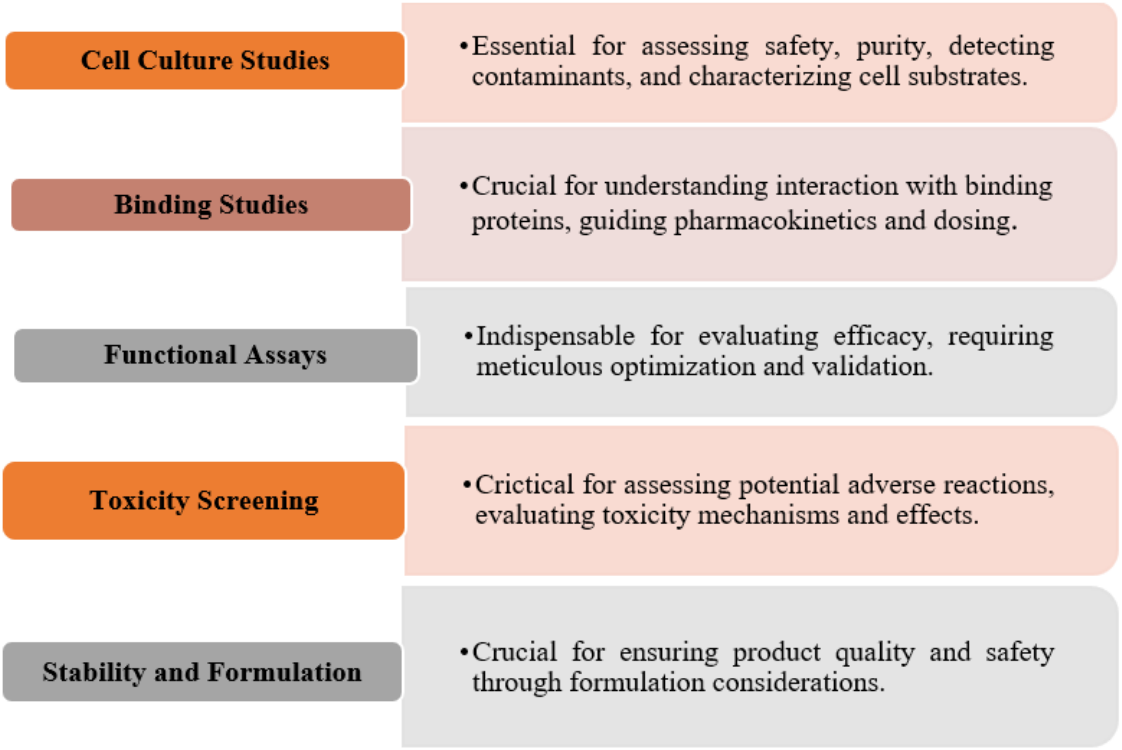
Toxicity Screening

In vitro toxicity screening serves as a crucial step in the preclinical assessment of biologics. While biologics generally exhibit fewer adverse reactions compared to conventional drugs, potential toxicities must be thoroughly evaluated. Toxicities may arise from pharmacological or nonpharmacological mechanisms, often associated with the interaction of the agent with its intended target. Unforeseen toxicities may result from previously unknown biology or manufacturing-related issues. Manufacturing quality significantly influences biologics toxicity, with advancements in technology minimizing contaminants. However, manufacturing processes can alter protein properties, necessitating rigorous screening for potential toxicities [9].

Stability and Formulation Studies

Developing stable and effective formulations for biologics is a complex endeavor requiring careful consideration of various factors. Contamination with biological impurities and conformational changes during production must be addressed to ensure product quality and safety. Utilizing well-documented and validated biological, physical, and chemical methods is essential in this process. Given the inherent instability of proteins in the gastrointestinal tract, systemic protein administration typically occurs parenterally. Efforts to enhance bioavailability through alternative delivery methods, such as controlled chemical modifications and lipid-based formulations, are ongoing. Collaboration between academia, the pharmaceutical industry, and regulatory authorities is crucial in developing safe and effective biologic formulations for the benefit of patients and society [10].

Figure 4: *In Vitro* studies involved in biologics



***In Vivo* Studies**

Considerations in the selection of the animal species

Given the specificity of the target for most biologics, selecting relevant species for nonclinical research (e.g., toxicological investigations) is essential to allow accurate translation of animal data into human studies. The main factor in ICH S6(R1) for choosing an animal species for toxicity testing is the species' pharmacologic relevance. According to ICH S6, comparing target sequence homology between species may be a good place to start. Next, comparable target binding affinities, receptor/ligand occupancy, and kinetics may be quantitatively and qualitatively compared between species utilizing cell-based

experiments. Assessments of functional activities are also recommended. Therefore, a species needs to meet three requirements in order to be deemed pharmacologically relevant: it needs to express the target protein or amino acids; it needs to be recognized by therapeutic biologics with sufficient affinity to allow for the evaluation of the pharmacological activity; and it needs to have pharmacologic functions that are similar to those of humans. In humans and animals, the biologic should likewise possess a similar degree of functional efficacy. The target's tissue distribution in the selected animal species is one of the other elements that should resemble those in humans.

Vaccines represent a unique class of biologics. For example, immunizations given prophylactically usually target infectious illnesses; hence, they may not exhibit sequence homology with mammals. Vaccine programs typically use a single species, which must be demonstrated to be an adequate animal model based on the vaccine's immunogenicity or efficacy in the selected animal species. This is in contrast to toxicology studies for SMs and other biologics. Nonrodent animals such as rats, mice, or rabbits are frequently used as study subjects in toxicology investigations. Nonhuman primates (NHPs) may be used in therapeutic immunization trials if they are the only species that has a homology with the human antigen [11].

Sequence Homology

Two toxicity species—one rodent and one nonrodent—are still often required for nonclinical toxicology investigations. One should use publicly accessible databases or existing literature as a basis for analyzing the homology of DNA, RNA, or amino acid sequences between humans and other animal species. Although sequence similarity on its own is inadequate to produce a viable species, sequence homology may be directly related to the biological function of the target. The facts, however, may be taken into serious account when choosing animal species for toxicological research. The hematopoietic cytokine erythropoietin (Epo) is one that regulates the generation of red blood cells. The sequences of different species are quite similar. (human Epo is 91% identical to monkey, 85% to cat and dog, and 80 to 82% to pig, sheep, mouse, and rat) [11]. The observed biological and immunologic cross-reactivity in different animals may be explained in part by this homology. Therefore, sequencing analysis would be the first step in selecting relevant toxicological species.

Target Affinity, Distribution, Biology, and Biochemical Pathways

Nonclinical in vivo safety studies for new chemical entities (NCEs) seek to identify a broad range of potential adverse effects that may or may not be associated with the drug's pharmacologic efficacy. Biological products, such monoclonal antibodies (mAbs), have a high target specificity as long as the pharmacologic activities of various species are equal, and their toxicities are frequently brought on by excessive pharmacology. This might aid in more accurate side effect prediction. It is desirable for the target to be altered in a way that is similar to what occurs in humans, for as by activating downstream signaling pathways or effector function, in order for a species to be deemed pharmacologically relevant. Therefore, a thorough analysis of the target's biological activity and a comparison of its expression profile in animal and human species would provide valuable information on the selection of species that are relevant to toxicology. Prior experiences with TGN1412 (anti-CD28) and natalizumab (a4-intergrin), two therapeutic mAbs, have raised issues related to target biology-associated toxicities. After receiving TGN1412, a super-agonist mAb, six healthy individuals had excruciating adverse effects from T cell activation. Natalizumab was removed off the market after two patients had progressive multifocal leukoencephalopathy, a rare and fatal viral demyelinating condition. Natalizumab was used to treat multiple sclerosis. These cases imply that if the distribution and activity of mAbs with immunomodulatory characteristics are not well known, they may result in unexpected toxicities [11].

Target Binding and Specificity Assay

Based on surface plasmon resonance (SPR) technology, the Biacore test is a great instrument for carrying out qualitative investigations to confirm the specificity of target contacts in addition to quantitative measures for measuring affinity, kinetics, and concentration. The optical biosensors evaluate binding events using materials such as complex mixtures, lipid vesicles, viruses, bacteria, eukaryotic cells, proteins, nucleic acids, and SMs. It is simple to test a tiny quantity of analyte for selective binding to 200–400 targets at simultaneously, depending on the equipment platform chosen. Therefore, using biacore assays is one of the finest ways to ascertain the biologics' target binding affinity and specificity. Alternatively, a sensitive and widely used technique for evaluating antibody binding to cells in the creation

of biologics is flow-activated cell sorting, or FACS. Other available and authorized in vitro methods for binding and specificity testing include the enzyme-linked immunosorbent assay (ELISA). Biacore and FACS analysis can be used to determine if the biologic binds at the same location as the endogenous ligand or at a different one. With these techniques, the association or disassociation rate constants may be determined, providing information on the binding affinity [11].

Biodistribution

In humans and related animal species, the target antigen's biodistribution in vivo should be comparable to facilitate the evaluation of on-target toxicity, which is the phrase used to describe the damage that arises from binding to the target antigen. Understanding how various tissues express various RNA or proteins will help achieve this. Research on tissue cross-reactivity may also provide information regarding the distribution of the target [11].

Target Functional Activity Assay

According to ICH recommendation S6, a functional analysis of a biologic binding to the target is necessary to determine if the selected species is pharmacologically relevant. Using cell lines and primary cell cultures, researchers have examined the direct effects of the biologics on cellular phenotype, live cell activities, such as the production of cytokines and chemokines, and proliferation. The data may also be used to predict certain aspects of in vivo activities and statistically assess the relative sensitivity of various species, including humans. An in vitro cell-based assay may be used to determine if the therapeutic mAb has the desired pharmacologic effects. For example, T-cell proliferation is linked to the agonist mAb CD28. On the other hand, an antagonist monoclonal antibody (like Remicade) would functionally block the effects of a specific human tumor necrosis factor a (TNF-a) in an in vitro cell-based assay. It may not be possible to do an in vitro test for every target in order to fully understand the functional effects of binding to the target; instead, detailed target characterisation may be required [11].

Animal Studies

Non-human primates (NHPs) and rodents (rats and mice) are the most commonly employed species for nonclinical safety assessment of biologics, while any species suitable for use in nonclinical toxicity studies can be taken into consideration. The Cynomolgus monkey is the most commonly utilized nonhuman primate (NHP), mostly due to the high level of biologic cross-reactivity observed in this species. They also have the benefit of being smaller, which means they require less compound; they also require a high degree of background data and are easier to handle manually, while rhesus monkeys can also be employed [12]. Other non-rodent animals that might be taken into consideration are dogs, mini-pigs, and rabbits, among others.

The biological activity and species- or tissue-specific activity of many biologics preclude testing them in widely used animal species like rats and dogs. Rather, in order to determine a "relevant species," or "one in which the test material is pharmacologically active due to the expression of the receptor or an epitope (in the case of monoclonal antibodies)," sponsors must employ a range of procedures, including in vitro binding assays and functional testing [13].

Figure 5: Test animals used in *In Vivo* studies for biologics

It is possible to test biologics on the human target antigen using transgenic mice that express the relevant human gene, such as knockout or human knock-in mouse models. A transgenic mouse model for biologics does have several drawbacks, though. In the event that a rodent lacks the target naturally, information from illness models, human mutations and polymorphisms, genetically engineered animals

(transgenic or knockout); sequence analysis; information from competitors on chemicals intended to impact related pathways; information on the conservation of function between species may frequently be used to pinpoint major risks, facilitate the understanding of safety-related information, and provide value to conversations about target selection or de-selection.[12] Surrogate biologics, or homologous proteins, must be thoroughly described in terms of their resemblance to their human counterparts, specificity, yield, and bioanalytical potential. Furthermore, its use would only be partially predictive in the absence of functional homology (e.g., when a rodent model with a distinct biology from humans uses a murine homologue). While using species-homologous equivalents of the human protein can help better understand the target's physiological role, it is not always possible to predict the human reaction [14].

Alternately, the biologic may be examined in an animal model of disease, which is also employed to assess effectiveness. These models frequently have short lifespans, which can reduce the amount of exposure time available for evaluating safety outcomes. It is crucial that disease models be thoroughly defined in order to evaluate possible toxicity against the backdrop of disease pathology when using them to evaluate safety outcomes. Any translation of safety endpoints should take into account the model to the human disease scenario. Immunogenicity is a possibility after administering a humanized chemical to a nonclinical species, as is the case with most toxicity investigations. Despite the numerous obstacles, the only chance to look at safety before administering the molecule to humans may be through the use of disease models to identify hazards. These methods need a lot of money, time, and resources to develop and are not without major obstacles.

Route of Administration

The desired clinical regimen determines the biologics' delivery route. In general, a biologic cannot be administered orally. Clinical delivery of most anticancer biologics, such as ADCs and mAbs, especially for advanced cancer, involves intravenous (IV) administration. SC is usually the route of administration for the majority of biologics used for chronic indications. Although the oral and intranasal routes are being actively employed, historically, the IM, SC, and intradermal routes have been used for vaccinations. When possible, nonclinical trials should provide drugs via the anticipated clinical route [15].

Selection of Doses for Toxicity Studies

As stated in ICH S6, the exposure-response relationship should be considered when choosing a dosage. Dosage selection can be supported by knowledge on pharmacology, PK, and PD. The selection of dose in all studies should be based on the relationship between exposure and response (area under the concentration time curve, efficacious concentration, etc.), rather than just the dose (mg/kg). This is because the exposure or response in one species at a given dose might not always correspond to a similar exposure and response in another species, or in humans [15].

There is no scientific evidence to suggest that animals cannot be given large protein dosages for extended periods of time. There are cases when high doses of protein have been given repeatedly without causing any harm, and the idea of "protein burden toxicity" has not been validated. Thus, based on these justifications, there isn't currently a need to restrict the high dosage. The most current version of the ICH S6 offers several recommendations for choosing the high dose, which one must take into account: Two types of doses are available: (1) one that yields the greatest desired pharmacologic impact through exposure, and (2) one that yields an exposure that is roughly ten times greater than the maximum dosage allowed in the clinic (note that this is not the minimum effective dose). Unless there is scientific evidence to justify a lower dose, the larger of these two doses should be chosen as the high dose in the repeat dose trials.

In the absence of in vivo/ex vivo PD endpoints, PK data, as well as accessible in vitro binding or pharmacology data, may be used to guide the high dosage selection process. To accomplish these goals, several firms have set default dosage levels for novel biologics. For NHPs and rodents, respectively, the maximum dosage is typically set at 100 mg/kg and 200 mg/kg, unless it involves ADCs, oligos, or vaccines. Using the above-discussed assumptions, high dosage levels for ADCs may be much higher than their maximum tolerated dose. To guarantee that the toxicity tests are successful, a smaller dosage should be employed. It is normally permissible to set the high dosage of vaccinations to match the highest dose that is clinically tolerable for humans [15].

Safety Pharmacology

Specific guidelines on Safety Pharmacology investigations for human medicines are represented by the ICH S7a and ICH S7b, in addition to the ICH S6, S6(R1), and S9 guidelines. Assessments of the central nervous system (CNS), pulmonary function, and cardiovascular (CV) must be completed before to FIH in compliance with regulatory criteria [15].

For the most part, standard biopharmaceuticals are safe to include evaluations in single-dose or repeat-dose toxicity studies; however, some biologics—such as those for diabetes, cardiovascular disease, or metabolic indications, or those with targets expressed on cardiac tissues—carry a higher level of risk for safety pharmacology issues based on target tissue expression, mechanism of action, therapeutic indication, or patient population.

Single-Dose Toxicity Studies

Studies using single and repeated ascending doses are carried out to assess safety and acceptability, ascertain the highest dose that can be tolerated, and describe side effects that are dose-limiting [16]. The goal of single-dose toxicity studies with biologics is not to determine the no-observed-adverse-event level (NOAEL), but rather to describe the connection between dosage and systemic and local effects as well as to identify dose levels for use in repeat-dose studies. These investigations ought to be carried out in rodents (usually rats) and nonhuman primates (usually Cynomolgus monkeys), two species that are significant to pharmacology. A single species ought to be adequate for the majority of biologics, for which NHP is the sole pertinent species. To screen for off-target related toxicities, it is therefore advised to do a single- or two-dose non-GLP toxicity study in rodents (usually rats) in addition to NHPs. Single-dose toxicity studies are often not carried out for preventive vaccinations.

Researchers often use research-grade drug substances (DS) for single-dose toxicity studies, which are carried out early in the drug development process. Selecting a large dose may be practically restricted by resource limitations, such as the quantity of DS that is accessible. Blood samples should be provided for assessments of plasma drug concentrations in NHP animals studied for single-dose toxicity, and the animals should be monitored for at least two weeks following treatment. An examination under a microscope of a restricted major organ panel and probable target organs is recommended if necropsy is needed. Heart, testicles or ovaries, eyes, liver, lung, kidneys, spleen, bone or bone marrow, and injection site should all be represented on the panel. To get valid data and enable dosage selection for lengthier repeat dose studies, the dose level and study period should be modified based on the kind of biological modality. A 2-week observation period with serum collection for drug level assessments and a necropsy following might be necessary if the biologic cross-reacts with rodents. The major organs should be evaluated under a microscope, following the guidelines described for non-rodents. To support dosage in early clinical trials, a more thorough GLP single-dose toxicity assessment, as described in the ICH M3(R2), may be worthwhile if a biologic is to be delivered just once in the clinic.

Repeat-Dose Toxicity Studies

Since many biologics are meant to be administered again in the clinic, repeat-dose toxicity studies are typically necessary to substantiate the safety of the medication before the first human clinical trial can begin. In terms of manufacturing procedures and analytical quality, the DP utilized for repeat-dose toxicity studies should be very similar to, if not the same as, the DP to be used for human investigations. During the development of biologics, many repeat-dose studies with varying durations are probably going to be carried out. According to ICH S6 and ICH M3(R2), the dose period in these investigations should be enough to facilitate clinical development. For chronic toxicological studies that support biological product registration, the longest dose length is typically six months, with the exception of late-stage cancer indications, for which a three-month toxicology study is deemed sufficient. This does not apply to vaccinations, as the total number of doses given usually exceeds the maximum clinical dosage (N) by at least one administration (N+1). IM injection is usually the method of administration for vaccines, and the dose frequency—typically biweekly—should be adequate to elicit the appropriate immunological response. The repeat-dose toxicity studies are often carried out in compliance with GLP guidelines. If suitable, two relevant species—rodents, usually rats—and non-rodents, usually non-human primates—should be used in the investigations. Usually, animals receive four doses at least once every half-life, plus an extra dosage two to three days prior to termination. The look of the injection site, clinical symptoms,

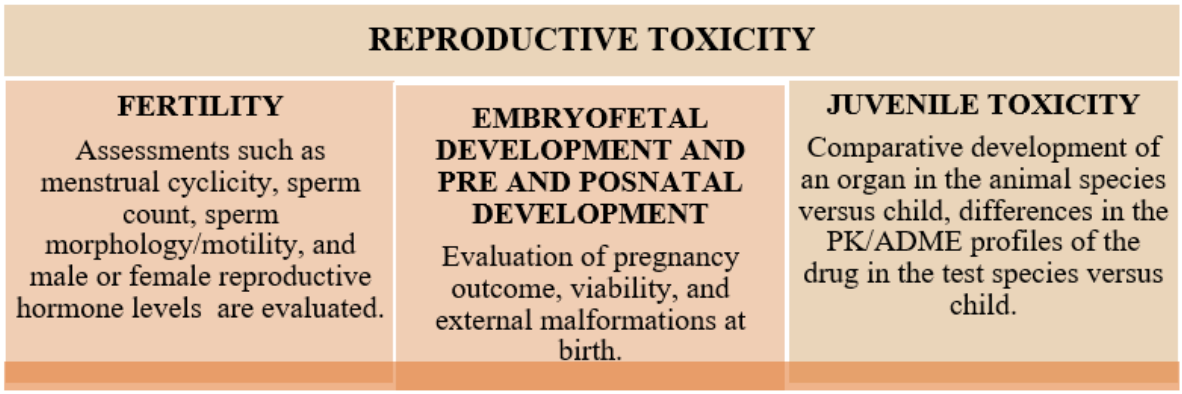
hematology, clinical chemistry, urinalysis, and microscopic and macroscopic examination of every organ can all be included in the analysis. Depending on the suggested clinical dosage frequency, animals in the ADC studies are normally administered two to four doses every three weeks or every other week. For pathology examination, animals in the dosing phase are put down three days following the final dosage.

Doses should be chosen to cover the range from a no effect level to one that generates toxicity in repeat-dose toxicity studies, which normally consist of three dosing groups and a vehicle control group. All suggested dosage levels for a biologic, however, may be higher than the maximum pharmacologically active dose, making dose selection challenging. In order to better characterize the dosage response of any observed impact, the mid-dose is included. The low dose should be representative of a level that offers an analogous exposure to at least an effective exposure.

When the repeat-dose toxicity studies are started in many development programs, the final clinical route of administration has not yet been determined. As a result, a fourth dosage group may be added to the trial, this time utilizing an alternate method of administration for large doses. The repeat dosage trial would thus address two possible clinic administration routes. To investigate the reversibility of any harmful effects noted during the dosing phase research, a recovery time has to be incorporated. If, on the other hand, there are no side effects at the conclusion of the dosage period or adequate scientific support is available, then the evaluation of recovery is not necessary. According to ICH guidelines S6, a full reversal of harmful effects is not required.

Reproductive Toxicity

Figure 6: Types of reproductive toxicity considered in biologics



Genotoxicity

According to ICH guideline S6, biological products are not expected to interact directly with DNA or other chromosomal material, hence routine genotoxicity tests like the Ames assay, in vitro micronucleus, and in vivo micronucleus assays are not need to be performed.[15] Research on pertinent and accessible systems, including those that have recently been created, has to be carried out in situations when the product is reason for worry (for instance, when an organic linker molecule is present in a conjugated protein product). It is not thought suitable to evaluate the genotoxic potential of process pollutants using traditional genotoxicity tests. But if done for this reason, a justification should be given.

Carcinogenicity studies

Standard carcinogenicity bioassays are often unsuitable for biologics, as stated in the ICH S6 guidelines. However, depending on the patient demographic, length of clinical dosage, or biological action of the substance (growth factors, immunosuppressive medicines, etc.), an assessment of carcinogenic risk can still be required. A single rodent species may need to have its carcinogenic risk assessed if the substance is both immunogenic and physiologically active in rats. Alternative strategies need to be taken into account when the product is not immunologically relevant or is not physiologically active in rats. Before beginning the work, these alternative, scientifically supported methods should be reviewed with the regulatory agencies. These methods may involve the use of surrogates, which is generally not preferred, in vitro cell proliferation assays, knockout animals, or standard 2-year carcinogenicity studies.

Immunogenicity

In 2008, the European Medicines Agency (EMEA) approved a guideline on the immunogenicity evaluation of biologics; in 2009, more advice and a concept paper were scheduled. An EMEA guideline is one of the important factors for immunogenicity assay development and validation that has been extensively published. The performance parameters of immunogenicity tests, such as screening and specificity cut point, sensitivity, selectivity, accuracy, robustness, stability, and ruggedness appropriate for the assay's intended use, must be described (validated). These specifications will change based on the target population, product type, development stage, and assay application (clinical versus nonclinical).

The term "immunogenicity" describes a molecule's innate ability to elicit an immunological response. Protein structure, host immunological state, host genetics, the existence of circumstances that activate immunity, the mode and regimen of delivery, and other variables all affect how the immune system reacts to therapeutic administration. Large molecules, like proteins, are more likely to have immunogenicity issues, which need to be carefully thought out and evaluated. Large molecules make up the majority of biotechnology-based goods, therefore there's a chance they might cause the patient receiving them to mount an unwanted immune reaction.

Numerous elements, including those linked to the procedure, the product, and posttranslational alterations, have an impact on immunogenicity. Furthermore, it has been shown that some contaminants have suppressed immune responses rather than just increasing them (adjuvant effect, for example). Research on animal immune responses may yield information that is helpful in assessing quality characteristics, such as contaminants [17].

Animal models are not very predictive of human immunology, however screening and mechanistic investigations may be used in immunogenicity testing. Numerous biologics trigger immunological reactions, which may have an impact on the outcomes of preclinical research. While these outcomes are occasionally desired (as with vaccines), unintended immunogenicity may have negative impacts. Forming immunological complexes, extending the biologic's activity, or cross-reacting with endogenous chemicals are examples of possible undesirable consequences. Therefore, during repeat-dose toxicity studies, the sponsor should collect the required samples for antibody testing and, when interpreting the results, take into account the impact of antibody production on pharmacokinetics (PK), pharmacodynamics (PD), and adverse events. Preclinical research shouldn't end if antibodies are found unless the immune system counteracts the biologic's effects in "a large proportion" of the test animals. Additionally, PEGylation can decrease immunogenicity, lengthen the half-life in circulation, decrease clearance, and increase the solubility of the protein or peptide. Finally, sponsors need to understand that human immunological responses are not always the same as those of animals.

Since biologics are regarded as foreign substances, they frequently produce immunogenicity in animals, especially those of human origin or humanized products. However, this is usually not indicative of immunogenicity in humans. However, it is crucial to comprehend immunogenicity in the nonclinical trials in order to evaluate the results and, on occasion, to learn about prospective toxicities that might be observed in a clinic. Immunogenicity rates in patients are not predicted from nonclinical immunogenicity data. It should be mentioned that nonclinical data may be useful in "describing the consequences" of immunogenicity, according to FDA Guidance for Industry Immunogenicity Assessment for Therapeutic Protein Products.

Tissue Cross Reactivity

Studies of tissue cross-reactivity are often carried out using biopharmaceutical constructs with complimentary domain regions, such as Fabs, dAbs, and mAbs. Therefore, with targeted biologics like mAbs, tissue cross reactivity (TCR) investigations in a panel of human tissues evaluating the degree of cross recognition are required. It is therefore possible to identify possible binding to non-target tissues. A stand-alone safety pharmacology research should be taken into consideration if a biologic is known to have a direct physiological influence or if tissue cross reactivity investigations show binding to tissues such as heart, lung, or brain tissues. A TCR investigation should only be carried out with a panel of human tissues, following ICH S6(R1) guidelines. Evaluation should be done on appropriate positive control tissue or artificially generated tissue (such as antigen-absorbed beads embedded in resin).

Cytokine Storm Assay

A class of protein molecules known as cytokines is primarily generated by leukocytes and is involved in many other processes, including the regulation of cell development and differentiation, as well as the regulation of the immune and inflammatory responses. The way cytokines work is by activating cell surface receptors on their target. While leukocytes are the primary cells in the body that emit cytokines, other cells can also generate them. When it comes to the therapeutic uses of cytokines, two distinct types of cytokines—interleukins and interferons—are particularly interesting. Cytokines have a wide range of biological activities, making them excellent candidates for use as therapeutic agents in the treatment of a number of diseases, including viral infections and cancer.

It has long been known that specific mAb delivery has been linked to a widespread rise in a variety of cytokines (cytokine release syndrome). Biologics that excite certain immune cells or cancerous cells for immune-mediated death, or that engage the innate immune system, are becoming more widely recognized as possible targets for immunological activation. When these cells are stimulated, a large amount of cytokines can be released into the bloodstream, as was the case with the cytokine storm that happened after TGN1412 was administered. One must take into account the possibility of cytokine release syndrome if a biologic's target is likely to cause immunological activation that may lead to the production of cytokines. In order to assess this possibility, human cells as well as cells from the relevant animal species should be used for the toxicity tests.

Pharmacokinetics and Pharmacodynamics of Biologics

Particularly in contrast to small molecule drugs, biological drugs—such as monoclonal antibodies—have distinct pharmacokinetics and pharmacodynamic characteristics. Correlating PK with PD offers a model to guide clinical dosage-level selection, enabling modeling to anticipate at which dose levels efficacy may be detected, and give information on safety in the clinical studies, regardless of the kind of biologic (mAbs, dual dAbs, BiTEs) [18].

Absorption

Because of their huge molecular size and the way proteins break down in the digestive system, oral administration is not feasible for biologics. Instead, parenteral administration—usually intravenous (IV), subcutaneous (SC), or intramuscular (IM)—is the preferred method of administering biologics. After SC and IM administration, the bioavailability—the percentage of the given dosage that enters the systemic circulation—can vary from 20 to 95%. Following these modes of administration, absorption usually happens via the lymphatic system and can also be quite slow with peak plasma concentrations recorded over one to eight days post-dose. Following SC dosing, the body experiences absorption rate-limited elimination due to this slow systematic absorption. Low flat concentration–time profiles of monoclonal antibodies following SC injection may result from this. Large therapeutic proteins (molecular weight more than 16 kDa) are an exception to this concept [18].

Distribution

Since the pharmacological target of therapeutic proteins is frequently an extracellular protein on the surface of cells inside tissues, distribution is a crucial pharmacokinetic property. The rates of extravasation, or the capacity to move from the circulation into tissue and partition into the interstitial space, determine how widely distributed therapeutic proteins are. Biologics are distributed and absorbed by tissues in extremely tiny amounts, usually between the extracellular space (0.23L/kg) and the volume of plasma (0.04L/kg), because of a large part to the molecular size and charge. Large proteins (molecular weight >30 kDa) move slowly through blood capillaries, but their distribution can be changed if they attach to certain binding proteins that are involved in their transport and control. Biologics can diffuse across a cell by passive diffusion (which mostly affects "smaller" proteins), convective transport, or transcytosis (a transcellular process that moves macromolecules throughout a cell).In order to assess mAbs' potential for use in cancer treatment, a thorough study of their transit across the blood-brain barrier has begun. With the exception of mAbs' nonlinear distribution, the plasma concentration–time (pharmacokinetic) profiles of mAbs after IV injection normally follow a biexponential drop. A two-compartment pharmacokinetic model, which is essentially a mathematical model that splits the body into fictitious central and peripheral compartments, best captures this [18].

Metabolism

Typically, biologics break down into smaller peptides or individual amino acids by processes that are well known for the endogenous substances. The endogenous pool would then utilise the various metabolites (amino acids) for the production of structural and functional proteins. Biologics do not require the standard metabolic research needed for new chemical entities due to the known metabolism to endogenous amino acids. Similar to endogenous proteins, the primary sites of metabolism for biologics are the liver, kidney, blood, and extravascular site of delivery. The reduced bioavailability seen following SC or IM treatment in comparison to IV administration is most likely explained by the extravascular metabolism. Nevertheless, there are specific situations in which the biologic's metabolism must be taken into account. ADCs, which consist of a mAb with a payload and an SM linker, must be assessed for payload release, metabolism, and the PKs of the released product, which includes dispersion and metabolism. When the released chemical is supplied alone, other investigations may be conducted to achieve a more thorough understanding of the metabolism of the released substance [18].

Excretion

Since most biologics are not eliminated as unmodified proteins, assessing excretion is usually not necessary for biologics, with the possible exception of ADCs, where evaluation of linker, payload, and metabolite excretion may be necessary. Proteins and peptides are examples of macromolecules that may be eliminated by the renal excretion process. The molecular size is the primary determinant of the molecule's level of elimination by glomerular filtration. Glomerular filtration will not remove large-sized molecules that are unable to pass through the glomeruli. Conversely, hydrophilic molecules that are tiny enough to fit through the glomeruli may be easily removed by glomerular filtration and excreted in the urine [18].

Selection of a Safe Starting Dose for First Time in Human Clinical Studies

All available information about the biologic (pharmacology, mode of action (agonist or antagonist), downstream signaling consequences, potency/affinity, and receptor occupancy), human PK profile and half-life, and relevant nonclinical species are taken into account when choosing the initial dose level for a FIH study. The first human administration of TGN1412, a mAb, on March 13, 2006, altered the criteria for determining the initial starting dose for biologics. This time, the initial starting doses were in accordance with FDA guidance, which called for using a fraction of the NOAEL dose level in nonclinical species. Thereafter, extremely dangerous toxicities developed.

The TGN1412 event served as a wake-up call for the regulatory bodies, the clinical trials community, and the pharmaceutical sector. Numerous organizations, including the Royal Statistical Society, the Early-Stage Clinical Trial Task-force, and the Expert Group on Phase One Clinical Trials (headed by Professor Gordon Duff), conducted in-depth investigations into the occurrence. In addition to outlining strategies for preventing similar adverse occurrences in future FIH trials, each of these organizations produced records that described the reasons behind the adverse events. The "Guideline on Strategies to Identify and Mitigate Risks for First-in-Human Clinical Trials with Investigational Medicinal Products" was released by the European Union as a result. The goal of this guideline is to help sponsors make the shift from nonclinical to early clinical research. It takes into account quality variables, nonclinical and clinical testing approaches, and designs for FIH clinical trials in addition to identifying risk factors for new investigational medicinal products.

The minimal anticipated biological effect level (MABEL) method is advised for experimental pharmaceuticals for which risk factors have been established, as the adoption of the NOAEL with the necessary modifications may not be acceptable. The dose at which people are expected to experience a minimum biological impact is known as the MABEL. As per the guidelines, all in vitro and in vivo data, including concentration-response curves in vitro in target cells from human and relevant animal species, dose/exposure-response in vivo in relevant animal species, and exposures at pharmacologic doses in relevant animal species, should be used in the calculation of MABEL. These data can include target binding and receptor occupancy studies in vitro in target cells from human and relevant animal species. The EMA guideline's computation of the safe beginning dosage is solely applicable to research using healthy, normal participants. In some circumstances, such as when studying traditional cytotoxic treatment in cancer patients, "other approaches may also be considered," according to the guideline.

CONCLUSION

Preclinical in vitro and in vivo studies are indispensable stages in the development pathway of biologics, playing a fundamental role in assessing their safety, efficacy, and pharmacological properties. These studies provide crucial insights into the mechanisms of action, pharmacokinetics, and potential adverse effects of biologic therapeutics before they are evaluated in clinical trials.

Integration of preclinical data from in vitro and in vivo studies is critical for informing key decisions in the drug development process, including dose selection, formulation optimization, and risk assessment. Moreover, these studies help identify potential safety concerns, such as immunogenicity and off-target effects, enabling researchers to mitigate risks early in the development process.

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