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Introduction

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Animal Models in Biomedical Research

Modern biomedical research relies heavily on the use of laboratory animals, particularly mice, rats, and fish, which, according to UK data for 2013, accounted for 94% of animals used in research. The research included fundamental studies aimed at understanding biological processes, the preclinical testing of potential new drugs and therapies, the development of diagnostic reagents and, in the case of monoclonal antibodies (mAbs), the production of therapeutic agents themselves.

In all developed countries the use of animals should (and probably is) strictly regulated in order to minimize pain and distress. All research workers should be familiar with the "Three Rs," Replacement, Refinement, and Reduction described in the book *The Principles Of Humane Experimental Technique* [1]. Thus, where possible, non-sentient alternatives to the use of animals should be used as a "Replacement," but if animals must be used, then "Refinements," such as anesthesia and analgesia as well as enriched housing conditions, should be used to minimize pain, distress, or lasting harm, and, finally, the number of animals used should be "Reduced" to the minimum necessary to meet the objectives of the study.

There are continued, successful, efforts to develop alternatives to the use of animals. For example, large numbers of animals were once used for assaying many biological reagents such as hormones and vaccines. These have now largely been replaced by *in vitro* methods such as direct immunological or chemical assays. Fundamental research uses large numbers of mice but also makes extensive use of cell cultures and tissues from animals, which have been humanely euthanized. An important "Refinement" has been the development of disease-free or so-called "specific pathogen free" mice, rats, guinea pigs, rabbits, cats, and a few other species. These are free of clinical and sub-clinical infections that can cause problems if the animals are stressed by an experimental treatment. "Reduction" is achieved by good experimental design in which neither too many animals are used, which would be wasteful, nor too few, which might mean that important reactions are missed.

In vivo research plays an important role in life science, particularly in preclinical drug development. The standard drug development process nowadays consists of several sub-phases (research phase, preclinical development, clinical phases I – III), which take several years and give rise to costs between US\$ 50 million and 2 billion [2-4].

This development process is highly prone to attrition. One critical step is the translation of preclinical animal research results to the clinic. In the last decade, it has been frequently revealed in many research fields that translation rates are minimal, non-existent, or generally shrinking [5-7]. A literature investigation by Thomson-Reuters revealed that the success rates of development projects in phase II clinical trials fell from 28% to 18% between 2009 and 2010 [7]. In more than half of the cases the reason for attrition identified was insufficient efficacy.

1.2 Animals in the Drug Development Process: Historic Background

Animals were already used as surrogate organisms for humans in the nineteenth century for the purpose of understanding chemistry-based drug effects on physiological function. The first Pure Food and Drugs Act in the USA (1906) described official standards for drugs and proper labeling and prohibited the interstate commerce of unsafe drugs. In 1938 the Food, Drug, and Cosmetic Act further required proof of safety and authorized inspections as a consequence of the sulfanilamidedisaster in 1937 that killed over a hundred people in the USA. The reason for the toxicity was that the cough syrup containing the antibiotic sulfanilamide also contained the toxic solvent diethylene glycol, which made the syrup very popular with children because of its sweet taste.

The first privately financed, nationally supervised, and evaluated drug was streptomycin, which was approved in 1945. However, the use of regulatory authorities and the movement toward supervised drug development was still limited to the Anglo-Saxon world. At that time in continental Europe the view persisted that diseases were non-comparable processes, not suitable for statistical evaluation "since the treatment never concerns populations but only individual patients" [8]. In 1954, the German company Grünenthal patented the sedative thalidomide and in 1957 launched it as Contergan in West Germany. Since the company considered the drug particularly safe, they marketed it as a sleeping pill for pregnant women, as well as a morning sickness preventative in early pregnancy. However 2 years later the first reports of nerve damage related to Contergan appeared, and in 1961 Grünenthal had to withdraw the drug from the market when thousands of babies were born with extremity abnormalities. This tragedy, known as the thalidomide-disaster, led to the first drug law in Germany (1961). The USA revised its existing drug law a year later to require proof of efficacy and sufficient pharmacological and toxicological results from animal trials before granting a license for market authorization. This provided the basis for the drug development process, as it is known today in developed countries. To date, it has not been possible to fully replace live animals as human surrogates, and therefore it is of great concern that such experiments should be performed in the most ethical way possible [9-12].

In various official pronouncements, for example, of the Royal Society in the UK, the UK Department of Health or the US Department of Public Health, it is stated that "Virtually every medical achievement of the last century has depended directly or indirectly on research with animals." Whether or not this claim can be backed by any proven evidence was unclear until 2008, when Robert Matthews investigated it. In his article, he came to the conclusion that even though the statement does not generally hold true, "animal models can and have provided many crucial insights that have led to major advances in medicine and surgery" [13].

Indeed, research using laboratory and domestic animals has underpinned many major advances in human medicine. Perhaps Louis Pasteur in the nineteenth century should be credited with the first use of scientific methods to develop new treatments for infectious disease. He used dogs and rabbits to develop methods for immunizing dogs and humans against rabies, a viral disease (although viruses were not known at that time), and sheep to immunize sheep against anthrax, a bacterial disease. His methods laid the groundwork for the development of vaccines used today to control diseases such as polio, measles, mumps, and rubella. Two infectious viral diseases, smallpox and rinderpest, a serious disease of cattle, have even been entirely eliminated from the wild and polio has nearly been eliminated. The first oncogenic retrovirus was discovered in 1911 by Peyton Rous, who found that cancer can be induced in chickens by injecting them with a cell-free extract from a chicken tumor. Further studies of the biology of murine retroviruses, such as the Bittner mammary tumor virus and murine leukemia virus, meant that when the human immunodeficiency virus (HIV) appeared, at least the biology of retroviruses was largely understood. Although infectious diseases have now largely been controlled in developed countries, new zoonotic diseases such as that caused by the Ebola virus, which is maintained in wild animals in West Africa, and various strains of the influenza virus present in wild and domestic birds remain a constant threat, especially in view of the rapidity with which diseases can be transmitted throughout the world. Moreover, antibiotic-resistant bacteria also remain a constant threat.

Transplantation of kidneys, hearts, and other organs has saved many lives. This was made possible by the discovery of immunological tolerance by Peter Medawar in the 1950s. At that time it was known that skin grafts between two individuals would be rejected, but it was assumed that this was a physiological problem. Medawar showed that reciprocal skin grafts between two different strains of mice are rejected. However, if lymphocytes of a donor strain were injected into baby mice of a recipient strain, treated adult mice of the recipient strain would then permanently tolerate grafts from the donor strain. This showed for the first time that graft rejection is an immunological rather than a physiological phenomenon, and that it can be controlled by immunological methods. The development of drugs such as cephalosporin to dampen the immune system, again using laboratory animals, has made organ transplantation possible.

The first chemotherapy was developed by Paul Ehrlich, who, in 1909 screened 606 chemicals for activity against the spirochete causing syphilis, using rabbits infected with the organism, and found one, salvarsan, that was effective. For a time it was the most widely prescribed drug in the world. The development of new drugs now depends on an understanding of the biology of the disease, the identification of possible drug targets and the screening of large numbers of chemicals likely to interact with the target, using in vitro and in vivo methods involving research animals. Any potential new drugs will be tested in animals for safety and efficacy, usually in mice, rats, and dogs before proceeding to clinical trials.

The discovery of insulin in the early 1920s has saved many millions of human lives. Banting and Best ligated the pancreatic duct of dogs and found that cells associated with the production of digestive enzymes degenerated, leaving islands of cells. These secreted the hormone later designated as insulin, and they showed that it could be used to maintain diabetic dogs. The biochemist Collip developed methods of purifying it from porcine and bovine pancreases, using several thousand rabbits to assay it. Fortunately, although these insulins are different from human insulin, they are sufficiently similar to be effective in humans. Before that time, type I diabetes was usually fatal. For many years, batches of porcine or boyine insulin had to be assayed using mice or rabbits. Frederick Sanger sequenced the insulin protein in 1955 and genetically modified human insulin is now produced in bacterial cultures and assayed chemically.

Antibiotics have probably saved more lives than any other medical intervention. Penicillin was discovered by Alexander Fleming in 1928, but he was unable to isolate it and verify that it was effective. This was done by Ernst Chain and Howard Florey, who were able to show that it was both effective and non-toxic in mice. They went on to develop a method for producing it on a large scale. Many other antibiotics have been discovered since then. For example, Selman Waksman discovered streptomycin in research involving mice, guinea pigs, and chickens.

Nutritional deficiency diseases are fortunately now rare in developed countries, but are still a problem in some underdeveloped ones. Frederick Gowland Hopkins showed that young rats given diets of purified protein, carbohydrate, minerals, and fat stopped growing, but when they were given a small amount of milk they grew. He postulated the existence of substances required in the diet in minute amounts, which were later called vitamins. The vitamin that he discovered was designated vitamin A. His work coincided with that of Christiaan Eijkman, who was attempting to find the cause of beriberi, a disease characterized by loss of feeling in the feet and difficulties in breathing. He injected the blood of soldiers hospitalized with beriberi into chickens, but also noticed that the chickens fed on scraps of the same polished rice diet as the soldiers also got sick, whereas those receiving unpolished rice remained healthy. The disease was caused by a deficiency of what we now call vitamin B1 (thiamine). Hopkins and Eijkman shared the 1929 Nobel Prize for their work.

The few examples cited above demonstrate how, historically, animal research has contributed to the development of many areas of medicine. The development of mAbs is a relatively recent advance that has resulted in a limitless supply of highly specific diagnostic reagents as well as many promising new therapeutic agents. B-cell multiple myelomas have been recognized in humans for many years, and it was also known that they produced mAbs, known as Bence-Jones proteins. In the late 1960s it was found [14] that the BALB/c inbred strain of mice produced myelomas when injected i.p. with mineral oil. These myelomas were immortalized and could be maintained as permanent cell cultures. In 1975 Kohler and Millstein fused these myeloma cells with spleen cells from mice that had been immunized to sheep red blood cells and found that the "hybridomas" secreted antibodies to sheep red blood cells. They were able to select out individual hybridoma cells, each of which produced a monoclonal antibody. Subsequently, the immunoglobulin genes of the mice were replaced by the equivalent human genes by means of genetic engineering, so that human rather than murine mAbs could be produced. This avoids any possible problems associated with adverse reactions to mouse proteins. mAbs are now used to treat several diseases such as some forms of cancer and as anti-inflammatory agents to treat diseases like rheumatoid arthritis and Crohn's disease. Many more are being tested in clinical trials. Because of their high specificity they are also widely used in the diagnosis of disease.

Other examples where animals have made important contributions include blood transfusion, joint replacements, reproduction, and in vitro fertilization (allowing many otherwise infertile couples to have children), heart valve replacement, cancer, and stroke. Moreover, veterinary medicine and human medicine are converging. Dogs and humans get many of the same diseases, such as cancer, obesity, and type II diabetes. Dogs also get a number of hereditary diseases, in some cases as a result of many generations of selective breeding, which are inappropriate to or incompatible with good health.

13 Problems with Translation of Animal Data to the Clinic

Despite the impressive examples described above, many articles in scientific and non-scientific journals have criticized the quality and reporting of animal research in drug development in the last decades. For some diseases, animal models were found to have no predictive value for clinical applications. A mouse model developed to investigate cystic fibrosis, for example, turned out to show symptoms different from human patients, even though the same genetic modification was introduced [15]. Another example is the search for HIV vaccinations using nonhuman primates as a surrogate organism. Chimpanzees and macaques infected with the simian immunodeficiency virus (SIV), a virus similar to HIV and from which HIV is assumed to have developed, turned out to be responsive to various vaccination candidates, whereas none has been translated successfully to human patients so far [16-19].

Certainly, humans are not 70 kg mice and it is probably utopic to assume that the efficacy of any drug candidate can be predicted 100% reliably by the investigation of an animal surrogate; nevertheless, literature analyses in various fields

of research have revealed a variety of potential causes apart from pure genetics for the low predictive value of animal research data. Poor experimental planning, inappropriate statistical analysis, and insufficient reporting are keywords frequently summarized in the literature [6, 13, 16, 19-27] and it is highly conceivable that the predictive value of animal research can be increased substantially by eliminating such methodological shortcomings.

A disease field where considerable work has been done to detect potential reasons for translation failures is acute stroke. Literature analysis revealed that almost 500 intervention candidates have shown satisfactory efficacy in animal models, whereas only three interventions have been proven to be effective in patients suffering from acute stroke [5, 28]. For various interventions with positive outcome in animal models, meta-analyses were performed to investigate potential reasons for this high failure rate. Judging from a checklist with 10 quality criteria, researchers found that low-quality studies tended to overestimate effect sizes [5].

In analyzing the quality shortcomings of the studies, the investigators identified two main groups, which can be summarized as general, stroke-independent or stroke-specific shortcomings. The latter included the use of animal models (mostly mice) that did not reflect the general health state of an average stroke patient. Human patients are often elderly, suffering from additional health problems such as hypertension or diabetes [5], whereas mice are young and healthy apart from the artificially introduced lesion to trigger stroke symptoms. Furthermore, other researchers identified discrepancies in the administration schedule of a particular drug candidate. Treatment onset occurred much sooner in animals (median 10 min) than in patients (median 5 h) [29].

The other, more general, group of quality shortcomings concerns the frequent neglect of study design and performance concepts in animal research, which are standard for clinical trials. These include random allocation of animals to test and control groups, blinded performance and assessment of study outcome, and sample size calculation before study performance in order to guarantee a certain study power (which should by convention minimally be 80–90%) [5, 29, 30].

Similar issues with study quality were observed in amyotrophic lateral sclerosis research [31].

1.4 Animal Studies in Anti-cancer Drug Development

Failure rates of drug effects in the clinical test phase after successful animal experiments were reported to be highest in the field of oncology [32]. In 2011, the licensing success rate for anti-cancer drugs reached 5%, in contrast to 20% for that of cardiovascular diseases [32].

In a systematic review of 232 publications in this field, only 41% reported randomization, and only 2% reported blinded assessment of outcome. None reported allocation concealment and only one reported sample-size calculations [33]. Even though many articles have been published in the last decade emphasizing the importance of such study design features, there has been no increase in their reporting between the late 1990s and 2011 [33]. The only exception was the increased incorporation of conflict of interest statements in more recent articles than in older ones [33]. This phenomenon can easily be explained by more extensive author's guidelines of numerous scientific journals, which require a conflict of interest statement.

It seems that external enforcement, for example, by journal editors, is necessary to achieve an improvement in reporting quality—and, presumably, performance quality—of animal studies. There was also a tendency for higher quality studies to report more small or non-existent effects compared with low-quality studies [33].

Anti-angiogenic cancer drugs represent a striking example both of how clinically irrelevant animal models can mislead decision-making, and also how well clinically relevant animal models can provide important information not only on efficacy, but also on potential harmful side effects of drug candidates. After marketing of the drugs, evidence was found that certain anti-angiogenic drugs could trigger metastatic evasion of cancer cells in patients [34]. Retrospectively, it was found that this phenomenon could have been foreseen by investigating metastatic cancer models (highly clinically relevant) in mice. Primary cancer models did not show similar results [34].

Tumor location within the animal model can also play a crucial role in predictive value. The easiest and cheapest way of inoculating tumors into an animal is to use a subcutaneous injection into the shoulder or flank. It is then easy to follow tumor growth. However, tumor cells then grow in an area different from their naturally occurring stromal conditions, which might crucially influence their growth and reaction to cancer drugs. For preclinical drug testing, it would therefore make sense to use orthotopic tumor models where tumors are inoculated into the organ of tumor cell origin. It is also possible that the animal species has to be carefully considered; a spontaneous dog or cat tumor may be genetically and behaviorally closer to human tumors than a human tumor induced in mice, which would not occur naturally.

1.5 Toward Relevant Animal Data

Problems and shortcomings in animal research as described above for various fields of disease, and for cancer research in particular, need to be resolved in order to achieve maximal relevancy of animal data.

Choosing the most representative animal and disease model depends on the field of research, but methodological shortcomings of study design, evaluation, and reporting are found almost universally. Aspects of study design such as randomization, blinded assessment of experimental outcome, and sample size calculations have been mentioned in the previous sections, but there are further aspects to be added.

A clear research question and an appropriate strategy to answer that question need to be considered at the very beginning of a study's planning phase. Unfortunately, it is common for investigators to focus more on study logistics and technical aspects, before considering how the data will be analyzed. A significant proportion of biomedical investigators lack the necessary statistical expertise and it is difficult to estimate how frequently they receive appropriate support from others [35].

1.6 Aim of the Book

This book offers an in-depth discussion of all relevant aspects of animal models for cancer drug development in the particular context of preclinical efficacy studies. It elucidates the many parameters that have to be considered in order to generate reliable animal results with a highly predictive value for the translation of animal efficacy to clinical practice.

The introduction gives an overview of the history and the present state of animal experiments and the need to improve them. It is followed by a chapter about ethical aspects of animal experimentation as a basis for the use of animal models in general.

The next three chapters describe general concepts, which are not exclusive to cancer research, but which must be implemented in all animal studies to ensure the quality of the results. These chapters broach the issues of study design, proper reporting, reporting bias, and animal housing and handling.

Chapters 6-9 discuss in detail the very important issue of clinically relevant animal models for anti-cancer drug development. First, a comparative description of humanized, genetically engineered, and other mouse models is presented in respect to cancer drug development. Second, mouse models of advanced spontaneous metastasis and the discrepancies in efficacy between primary and metastasis models are compared. Then, two chapters focus on dog (and cat) models of spontaneously occurring cancer and their value additional to mouse models. Finally, the book finishes with a chapter on important lessons to be learned from human and canine trials to improve animal models in research through back translation.

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