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CHAPTER

19

Advantages and Limitations of Commonly Used Nonhuman Primate Species in Research and Development of Biopharmaceuticals

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OUTLINE

INTRODUCTION

Biopharmaceuticals, also known as a biologics or biologicals, differ from other pharmaceutical products, or small molecules, in that they are created using biological processes rather than being chemically synthesized. They include a wide variety of medicinal products such as vaccines, blood or blood components, somatic cells, gene therapy, tissue, recombinant

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therapeutic proteins, or living cells that are used as therapeutics to treat diseases. This chapter focuses on the main classes of biopharmaceuticals that are tested in nonhuman primates (NHPs), i.e., monoclonal antibodies (mAbs) and recombinant proteins/peptides.

The goal of biopharmaceutical development is to maximize therapeutic benefit while minimizing the risk of treatment-related toxicity. Animal testing is key in the prediction of toxicity; pharmacokinetics and pharmacodynamics of these drugs are a prerequisite for the drug approval process. To maximize the chances of detecting toxicity of biopharmaceuticals, including prediction of putative interpatient variability in the responsiveness to the test article, selecting a relevant species for conducting safety assessment studies is important; according the International Conference for Harmonization (ICH) S6 guidance [1], this is a species in which the test article is pharmacologically active due to the expression of the target or, in the case of antibodies, to the expression of an epitope. In addition, understanding the relative sensitivity of the species or the strain to the pharmacological effect, as well as to other effects that may be mediated by the nature of the test item, is important. Finally, the physiological similarity between humans and the test species considered for the pathway or system to be evaluated is key. Appropriate selection of the relevant species that is sensitive to the pharmacological effects of the biopharmaceutical of interest enables the identification of factors that most reproducibly affect the margin between safety and efficacy (the therapeutic index) and the selection of safe doses for human testing.

Although there is growing pressure to use lower species and to limit the use of animals in drug development, NHPs cannot be easily replaced in numerous area of safety evaluation. Their phylogenetic closeness to humans has naturally identified them as a model of choice for the safety assessment of large molecules. The high homology of sequences of many proteins between humans and NHPs enables target receptor binding or epitope recognition by a human protein or mAb therapeutic more often in NHPs than in lower species. This sequence homology for many proteins also limits immunogenicity, often allowing treatment with a human or humanized protein over long periods of time [2]. The rationale and experimental means by which the appropriate species is selected differ for small and large molecules. Nevertheless, for some specific targets, similarities with human anatomy and physiology are crucial in evaluating the effect resulting from target occupancy and modulation. For example, aging primates are used as models for geriatric diseases and osteoporosis [3]. Primates also are a species of choice for many ocular indications such as macular degeneration, retinopathy, testing of retinal implants, treatments targeting the reproductive system, or bone remodeling in various pathophysiologic conditions. NHPs are especially useful in the risk assessment for immunotoxicology of biotherapeutic proteins [3].

Given ethical considerations, the number of NHPs used in a study or across a safety assessment program is limited when compared with the use of rodents and dogs. Nevertheless there is good knowledge of the background lesions and abnormalities that can be encountered, aiding in the interpretation of any findings [4–6].

Like rodents, NHPs are not a single entity. The most commonly used species are cynomolgus monkeys, rhesus monkeys, and marmosets. These three species have characteristics and physiological differences that may render them either attractive, inappropriate, or unsuitable for a given risk assessment (Table 19.1). The presence of the target protein and the pharmacological activity of the biopharmaceutical are required, but not always sufficient, to ensure an optimum safety assessment; other factors need to be considered. For example, reproductive and developmental functions cannot easily be evaluated in the rhesus monkey because of

TABLE 19.1 Comparison of NHP: Selected Species-Specific Differences

	Cynomolgus macaque (*Macaca fascicularis*)	**Rhesus macaque (*Macaca mulatta*)**	**Marmoset (*Callithrix jacchus*)**
Size (cm), range	44-53	47-53	18.5-18.8
Weight (kg), range	4.1-7.0	5.3-7.7	0.24-0.26
Blood volume (mL/kg)	65	55	70
Respiration rate (breaths/min), mean±SD	36±13	51±15	101±14
Tidal volume (mL)	46±6	39±11	0.98±0.1
Heart rate (beats/min)	123±30	174±27	400±45
PT (s)	12.1±1.1	14.5±0.9	6.7±0.5
Gamma GT (U/L; male)	70.5±16	76.5±16.7	5.1±4.3
ALT (U/L; male)	55.3±30.9	59±22.4	3±2.4
Reproductive physiology	Like human physiology	Like human seasonality	Different from human (males and females)
Sexual maturity	Females: 2.5-3 years Males: >4 years[a]	Females: 3 years Males: >4 years[a]	17-20 months
Menstrual cycle (days)	28±5	28±5 only 3-6 months per year	24-30 days (polyestrous, up to 4)
Gestation duration (days)	134-184	164	140-150
Litter size (*n*)	1	1	1-3 (usually twins)
Birth weight (g)	320	480-520	25-35
Interbirth interval (months)	15±5	14	6
Reproductive toxicology	Well established	Well established	Partially established
Developmental toxicology	Well established	Well established	Partially established
Palate fusion (gestational days)	45-50	45-50	75-80

[a]*Males may not be able to reproduce until 6-8 years of age.*
ALT, alkaline phosphatase; gamma GT, gamma glutamyl transferase; PT, prothrombin time.

seasonal variations. Some data can be generated from marmosets; however; their interpretation and their translatability to humans is questionable. One species may also be more predictive than another for a specific safety concern in humans. In addition, substrains of a given species from different geographical origins may also present genetic differences that may have a considerable impact on the outcome of biopharmaceutical evaluation.

Therefore selecting an NHP species for research or nonclinical development requires the careful evaluation of numerous ethical and scientific considerations. The possibility of using the clinical candidate is always an advantage. Confidence in the similarity of target distribution, density, downstream signaling, and physiology are important. Nevertheless, hazard

identification also has to be taken into consideration, and in this respect, NHPs are not always superior to other species, perhaps because of the limited number of individuals allocated to nonclinical studies. The purpose of this chapter is to help the reader understand how to best select the right NHP species or strain and to illustrate this with examples.

USE OF NHPs IN RESEARCH AND DEVELOPMENT

Several NHP species have been used for research and drug development in many research areas because of their close evolutionary relationship to humans. Recent development of genomic resources also have boosted their value as model organisms. The Old World primates rhesus macaque (*Macaca mulatta*) and cynomolgus macaque (*Macaca fascicularis*) are by far the most commonly used species. The New World primate marmoset (*Callithrix jacchus*) is more evolutionary diverged from humans than Old World monkeys but is sometimes used when the two other species are not pharmacologically relevant for the biopharmaceutical or when specific models exist only in the marmoset. Each of these three species presents advantages and disadvantages for pharmacologic and toxicologic assessment of biopharmaceuticals, which are reviewed in this chapter.

Rhesus Macaques

Because of its relatively easy maintenance in captivity, wide availability, and anatomic and physiological proximity to humans, the rhesus macaque has been used extensively in medical and biological research on human and animal health-related topics and has given its name to the rhesus factor, one of the elements of a human blood group. The rhesus macaque is widely used as an NHP model to study infection and immunity because of the close genetic relationship with humans (93% average human-macaque sequence identity) and because of the homology between human and rhesus pathogen genomes. Rhesus macaques have been used to study fundamental aspects of immunology, including the development and maintenance of T-cell memory, immune dominance, and the aging immune system. There also have been many studies of immune responses in rhesus macaque models of human infections such as human immunodeficiency virus (HIV), influenza virus, tuberculosis, Epstein-Barr virus (EBV), cytomegalovirus, smallpox, measles, and severe acute respiratory syndrome [7]. Furthermore, rhesus macaques have been instrumental in designing and testing of vaccines against infections such as HIV and smallpox, in creating drugs to manage HIV/AIDS [7], and in understanding the female reproductive cycle and development of the embryo and the propagation of embryonic stem cells [8]. The genome of the rhesus macaque was cloned in 2007 [9]. Surprisingly, some normal gene sequences in healthy macaques cause profound disease in humans. For example, the normal sequence of phenylalanine hydroxylase in macaques is the mutated sequence responsible for phenylketonuria in humans. Some gene families are conserved or under evolutionary pressure and expansion in humans, macaques, and great apes such as chimpanzees (e.g., cholesterol pathways), whereas some are uniquely under expansion in humans, chimpanzees, or macaques (e.g., major histocompatibility genes are more abundant in macaques) [9]. The rhesus macaque was instrumental in understanding key features of reproduction. However, males and females are only fertile 4-5 months per

year; seasonality is influenced by the geographic origin and is still observed after several years of being housed in a research colony in Western countries [10].

Since the rhesus macaque is no longer available from India, it has started to be used less in research; there is often considerable lead time in sourcing these animals. In addition, it has been progressively replaced by the cynomolgus monkey in preclinical toxicology studies, in part because of its bigger size (requiring large amounts of a drug for dosing and making them more difficult to handle) and its seasonal reproduction, which makes its use in reproductive and developmental studies more difficult. This has resulted in less toxicology background data being available for interpretation of any findings.

Cynomolgus Monkeys

The cynomolgus monkey has become the most widely used primate species in preclinical toxicology studies. It is widely available, being purpose bred for laboratory use, is of a size that represents a good compromise with regard to the amount of drug required for dosing and the amount of blood/tissue that can be sampled since it is generally smaller than the rhesus macaque (Table 19.1). Cynomolgus macaques are especially useful for reproductive and developmental toxicology when lower species are not pharmacology relevant or given their close resemblance to human. They are also good models for the development of locally administered drugs, such as those for use in specific ocular diseases or prostate disorders, and for inhalation. Nevertheless, although specific information is easily obtained from numerous breeding colonies, the cynomolgus monkey has not been as extensively characterized as the rhesus macaque in terms of genomics; however, efforts are underway to comparatively screen rhesus, cynomolgus, and other macaque species genomes.

Both rhesus and cynomolgus macaque species exhibit substantial variations dependent on origin and genetic background. As an example, Chinese-derived populations of rhesus macaques have been shown to be more resistant to simian immunodeficiency virus than Indian-derived populations. Hematology and clinical chemistry differences also have been demonstrated in these two populations. In rhesus macaques, such dependence on genetic background is understood and used to guide research. Although cynomolgus monkeys are the most commonly used in the development of biopharmaceuticals such as mAbs and recombinant proteins, there is relatively little information on their genome in public domain databases. Nevertheless, cynomolgus monkeys originate from three different populations (e.g., Indochina, insular Asia and the Philippines, and Mauritius) with own genetic features. Animals from Mauritius and the Philippines exhibit potentially lower genetic diversity, even for genes involved in immune response [11]. Differences in the gene sequences of some target proteins have been encountered within cynomolgus monkeys sourced from different geographic origins (e.g., China, the Philippines, Mauritius), raising concerns that certain genetic variants might result in a loss or gain of pharmacologic responses in the cynomolgus monkey colony used for toxicology studies. Phenotypic differences in cynomolgus monkeys of diverse geographic origin, in particular for immune system functions, are well established, as illustrated by the diverse major histocompatibility complex (MHC) genetics of cynomolgus monkey from different geographic origin [12,13]. Collectively, these observations suggest that a prospective assessment of target gene variation may be warranted for cynomolgus monkey

(and other NHP species) pharmacology and toxicology studies. Moreover, identifying a sequence variation within a target gene requires complementary target expression/distribution, epitope/binding, and/or functional readouts.

Marmosets

C. jacchus (common marmoset) is one of the more primitive NHP species and is used widely in fundamental biology, pharmacology, and toxicology studies. Utilizing this species may provide the optimal compromise between ethical considerations, disease understanding, and drug development. Once the scientific rationale has been carefully considered and their use justified, there are several advantages to using the marmoset as a model in the nonclinical development of pharmaceutical products.

There are a number of unique physiologic differences between Old World primates such as rhesus and cynomolgus macaques and New World primates such as marmosets. For example, anatomic differences in placentation and frequent twinning lead to bone marrow chimerism of dizygotic twins. Limited diversity at both MHC class I and II loci has been identified in both tamarins and marmosets and may be responsible for differences in disease susceptibilities that are exploited in infectious disease studies. While the availability of research tools has been a limitation in the past, making marmosets less attractive than rhesus macaques as animal models, this is no longer the case in a number of key areas.

Experimental autoimmune encephalomyelitis (EAE) is a well-established animal model of multiple sclerosis (MS). The disease induced in common marmosets more closely resembles MS than that in rodent or other primate models in terms of clinical signs, lesions, and evolution. The unique particularities of marmosets in terms of full bone marrow chimerism of littermates have been key in the understanding of disease pathogenesis. The EAE model has been used in the preclinical evaluation of novel immunotherapies such as testing new anti-inflammatory agents, tolerance-based therapies, and costimulated target therapy [14]. Marmosets also have been used widely in neuroscience research programs in models of cerebral vascular disease, tardive dyskinesia, and neurodegenerative diseases and in studies of normal neurophysiology [15].

Common marmosets have been used extensively in infectious disease research in large part because of their unique sensitivity to a number of important human infectious agents, including viral, bacterial, and parasitic agents. In particular, marmosets are susceptible to a variety of herpes virus agents, including gammaherpes viruses, such as EBV and herpesvirus saimiri, herpes simplex virus, hepatitis A virus infection, Junin virus, malaria, measles, and GB virus B, a surrogate model of human hepatitis C virus. The reason marmosets are highly susceptible to infection with many of these agents is not understood, but limited diversity at MHC class I and II loci is hypothesized to play a role, as mentioned above. In particular, marmosets have been extensively used to investigate the molecular pathogenesis of viral-induced lymphoma and EBV and lymphocrypto virus (LCV) model of viral oncogenesis or viral persistence [15].

The marmoset also has been used as a nonrodent second species in drug safety assessment of new chemical entities (NCEs) and, more recently, of biopharmaceuticals based on side effects, findings of given drugs, and metabolizing enzymes or receptors found to be similar to humans [16–19]; because of the closer phylogenetic relationship to humans than other second species such as the dog, common marmosets may be more suitable for certain types of

pharmacokinetic and toxicological screening. When the test item is a biopharmaceutical and the mechanism of action is dependent on specific receptors or epitopes, relevant species may be restricted to NHPs. The small size of common marmosets may represent an additional advantage because it reduces the quantity of compound to be synthesized and may translate into shorter development times. However, their short stature, sensitivity, and activity level may also pose unique challenges [16,17]. Nevertheless, for some development programs, as for canakinumab, the marmoset may be the only suitable species [20]. In addition, marmosets are largely free of diseases harmful to humans.

As a result, this species is being used in studies ranging from behavioral studies to reproductive and developmental protocols. However, this species poses unique challenges because their sensitivity, small size, housing conditions, and high activity level.

EVALUATION OF REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Many drugs in development are intended for chronic administration to infants, women of child-bearing potential (WoCBP), fertile men, and infants, requiring the evaluation of hazard on fertility and/or early development of the offspring and juvenile toxicity. With biologics, often the only relevant species with which to assess these is the NHP.

If for NCEs the reproductive strategies benefit from standard and well-validated study designs in rodents and in rabbits, for which extensive historical databases are available, some specific considerations for biopharmaceutical proteins have to be taken into account. They do not freely diffuse across membranes, including placenta, and are therefore not expected to affect early stages of development, especially embryogenesis, because the embryo/fetus is not directly exposed during early life [21].

Although mAbs and Fc fusion proteins are also large proteins, they can be actively transported from mother to fetus in mammals via the neonatal Fc receptor for immunoglobulin (Ig) G (FcRn), with variable efficacy as a function of the IgG subclass of antibodies from which they are derived, with $IgG_1 > IgG_4 > IgG_3 > IgG_2$ [21,22]. The long half-life of many therapeutic IgGs may result in prolonged pharmacological activity and effects on the developing fetus, including the immune system (developmental immunotoxicity). Transfer of antibodies occurs mainly during the second and third trimester in humans, when FcRn starts being expressed on the placenta. All antibodies or recombinant proteins with an intact IgG Fc portion can be transported across placenta, whereas antibody fragments (Fab, dAb, scFv, $F(ab)'_2$) without Fc are not [23]. Similar placental transfer of IgGs has been demonstrated in humans and NHPs [22,24,25].

Because IgG mAbs are not transferred to the fetus until later in pregnancy when organ development has ended, the evaluation of the effects of therapeutic IgG antibodies and Fc fusion proteins on embryo-fetal development is not systematically required prior the enrollment of WoCBP in phase II clinical trials (as for NCEs), according to the revised ICH M3 guideline [26]. This is because if a woman accidentally became pregnant while being administered an IgG mAb in a clinical trial, the risk of exposure to the developing offspring in the early weeks of pregnancy is very low. Potential adverse effects to the fetus in these early stages are more likely to be due to secondary effects of maternal toxicity rather than direct effects. However,

some cases of fetal abnormalities without maternal toxicity have been reported and considered secondary to altered placental function and/or reduced fetal growth, even if the direct inhibition of target signaling in the conceptus cannot be ruled out, as reported with anti-receptor for the insulin-like growth factor 1 (IGFR) [27]. Therefore the assessment should be designed based on the risks that may be related to target modulation in the mother or in the infant. A careful review of the distribution and the role of the target in different physiological conditions is important to appreciate risks and adapt the study design as a consequence. Standard study designs for use with rodents and rabbits allow all steps of reproduction and development to be evaluated (as described in ICHS5(R2)) and are generally predictive, provided the therapeutic mAb does not modulate a pathway that is physiologically different between rodents/rabbits and humans. Conversely, study designs for use with NHPs do not cover all aspects of reproduction (e.g., direct fertility assessment through mating or egg implantation—pregnancy cannot be confirmed until day 20 of gestation) and so may not allow a full evaluation of the specific risks in humans. However, the physiology of reproduction, pregnancy, and parturition, including hormonal control, are very close between human and NHPs, allowing better identification of hazards, provided an appropriate study design is applied.

Unlike rodents, the relevance of the NHP model for developmental toxicity is well established [8]. The spontaneous incidence of congenital defects is low [28,29]. Clinical relevance and superiority over rodents has been demonstrated for thalidomide, vitamin A, and valproic acid [28].

Choosing the NHP for assessing the effect of a therapeutic protein on reproductive and developmental functions may help identify hazards that would not be identified in other species and allows, in general, an evaluation of the clinical candidate. However, there are cases where no relevant NHP species is suitable for assessing reproductive toxicity of the clinical drug product. In those cases, mAb surrogates are used for reproductive toxicity studies. Well-known examples are infliximab (anti-tumor necrosis factor alpha (TNFα)), efalizumab (anti-CD11a), and eculizumab (anti-C5), respectively.

Among others, an important consideration is the choice of the NHP species or even substrain for reproductive and developmental testing (Table 19.1). The physiology of the menstrual cycle of Old World monkeys closely resembles that of humans. For this reason, monkey species belonging to this group are preferred as experimental models in studies of human reproduction, pharmacology, and toxicology. Especially for studies of sex hormones, synthetic steroidal compounds, or compounds that interfere with the hypothalamus-pituitary-ovarian (or testicular) axis, such monkey species are predictive [30].

Cynomolgus Monkeys

Because of its lack of reproductive seasonality, convenient body size, close resemblance to human physiology, and thanks to the availability of a large historical database, the cynomolgus monkey is the species of choice for reproductive toxicity studies. The average duration of the menstrual cycle in the female is 28 days, and the endocrinology of the ovarian cycle is similar to that in women, with one predominant follicle maturing to ovulation. Markers of the menstrual cycle are also similar to those in women, and the cycle can be monitored by daily vaginal smears [31].

There are also strong similarities between the male cynomolgus monkeys and men in terms of endocrine control of testicular function (follicle stimulating hormone (FSH) is especially

important for spermatogenesis and inhibin B is an important marker of testicular toxicity), prostate anatomy, and control of prostate function. Fertility rates are typically around 40-70% and preimplantation loss is evaluated at 25% [10].

The most relevant period in which to evaluate adverse drug effects on organogenesis takes place mainly between gestation days 20 and 50, with palate fusion occurring around gestation days 45-50. Pregnancy lasts around 160 days, with a range of 134-184 days. The maturation of different organs and systems evolves similarly to that of humans, with some noticeable differences, including earlier bone ossification, respiratory and urinary systems being mature at birth, and more rapid maturation of the digestive system [32]. Twin pregnancies are extremely rare in macaques, with an overall twin live birth incidence around 0.1%.

The low fertility rates and high abortion rates (10-30%) in this species limit the detection of infrequent risks [10]. Nevertheless, its size, ease of housing, and extensive panel of reagents make it an attractive model. Several marketed products have been tested in this species (basiliximab, adalimumab, rituximab, natalizumab, ustekinumab, alefacept, and omalizumab) for a partial or complete evaluation of embryo fetal and postnatal development.

There is a much ethical pressure to limit the use of NHPs, especially in reproductive toxicology. However, there are examples where rodents are not helpful in hazard identification, as illustrated in the two cases below.

An IgG4 directed against a soluble target for inflammatory indications was developed in the cynomolgus monkey and general toxicity studies were uneventful. The potential effects on the fetus and developing infant were addressed in a single enhanced pre and postnatal development study in the cynomolgus monkey. The decision was based on normal fertility, reproduction, and development in target knockout mice, or in mice treated with a therapeutic antibody blocking upstream signaling of the receptor, an based on an uneventful embryo-fetal development (EFD) study in cynomolgus monkeys treated with a therapeutic antibody targeting the same signaling pathway from gestation days 20 to 50 and undergoing a cesarean delivery on gestation day 100. Therefore, pregnant female macaques were administered the IgG4 weekly from gestation day 20 to approximately gestation day 160 and were allowed to deliver their offspring naturally and raise their infants. Maternal animals tolerated the antibody treatment as in general toxicity studies, without changes compared with control animals, and delivered healthy infants that developed normally with a functional immune system. However, an increased incidence of treatment-related perinatal mortalities was observed in infants. Macroscopic examination of the decedents revealed skull bone luxation; subdural, cerebellar, or superficial brain hemorrhage, all suggesting difficult delivery; and an empty gastrointestinal tract, indicating the absence of suckling after birth. Unexpectedly, treated maternal animals experienced a longer pregnancy than controls (up to a mean of 10 days) and a higher incidence of retained placenta, and prenatal maternal mortality associated in some cases with genital hemorrhages was observed. Dystocia is one of the leading causes of late pregnancy loss in cynomolgus monkeys, rhesus monkeys, and baboons, often resulting in intracranial trauma/hemorrhages in the nonviable fetus/infant [33–35]. However, difficult delivery rarely results in maternal death and is not usually associated with important genital blood losses, even in the case of placental retention. A careful review of the literature in light of the findings of the study suggested that the soluble target may indeed play a role in parturition, as indicated by increased circulating concentrations of the target during the third trimester of pregnancy and increased messenger RNA and protein concentrations in the cervix,

myometrium, and choriodecidua during labor [36]. The target is thought to be involved in the local process of inflammation occurring at parturition, enabling recruitment of leukocytes in the cervix and uterine wall [37] to induce cervical ripening and dilation and probably resorption of the interface between the placenta and uterine wall [38,39]. It also promotes the expression of the prostaglandin synthase type-2 in the cervix, myometrium, choriodecidua, and placenta [40], enabling efficient contractions.

Although additional factors interfering with parturition cannot be ruled out, the inhibition of target signaling may explain the observations in maternal animals. Maternal mortality was considered to be a direct consequence of the long and difficult labor associated in some instances with hemorrhage caused by incomplete resorption of the placenta/uterine interface and with genitourinary infections resulting from the prolonged labor. Perinatal infant mortality was considered indirectly linked to the treatment, resulting from head injuries at parturition or lack of maternal care during the first critical hours after birth.

Such findings could not have been observed in mice, whose physiology of parturition is very different, or in monkey studies not specifically addressing parturition, but they highlight a risk for pregnant women still exposed to the mAb by the end of the pregnancy based on the similar physiology of pregnancy and parturition in NHPs and humans. This example illustrates the need to carefully evaluate target expression, the biology of the target, and its potential role in pregnancy, as well as the need to understand how close the test species and humans are for the pathway to be explored.

Another example in which the cynomolgus monkey was shown to be more sensitive than the rodent for detection of adverse effects on pregnancy is the case of the soluble receptor for interleukin-4 (IL-4R) [41]. The treatment of pregnant cynomolgus monkeys with human soluble IL-4R (0.2 and 2.0 mg/kg intravenous) during organogenesis (gestational days 20-51) resulted in increased incidence of spontaneous abortion, embryo/fetal death, and stillborn neonates. However, pregnant Crl:CD1 mice treated with an intravenously soluble mouse (surrogate) IL-4R during organogenesis presented no adverse effects up to the equivalent dose of 20 mg/kg. CD-1 mice may be Th1-biased (like C57BL/6), a possible reason why no reproductive effects were seen in this mouse study—i.e., they already have more Th1 bias, so tipping them further that way has little effect [42].

Marmosets

Although the cynomolgus monkey is the established NHP model for reproductive and developmental toxicology, there are several advantages to conducting developmental toxicity studies using marmosets. Considerations include specific cross-reactivity—particularly with biologics—and some specific teratogenic risks that are more difficult to demonstrate in species with only one offspring.

From a reproductive point of view, marmosets are unique among NHPs and differ from humans in some key respects. Females have no menstrual bleeding, each cycle results in multiple ovulations, and postpartum conception is not uncommon. Duration of the menstrual cycle varies from 24 to 30 days, with a long luteal phase, a mid-phase FSH peak, and simultaneous maturation of one to four eggs [43]. Ovarian cyclicity can be controlled by monitoring progesterone after triggering luteolysis by administering prostaglandin-F2α. As a result, controlled EFD studies are feasible. Multiple, simultaneous ovulations result in litter

sizes ranging from one to four births; twins (30-70%) and triplets (20-25%) are most frequent. Interestingly, marmoset have a naturally high abortion rate to reduce the number of fetuses in utero, as they generally can nurse only two offspring. Compared with Old World monkeys, the father has to stay with the mother for the entire duration of the pregnancy to prevent abortion. Gestation lasts between 140 and 150 days. The interbirth interval is approximately 6 months, but conception can occur 10-50 days postpartum. In contrast to humans, twins' placental disks are supplied by blood vessels of both fetuses. Shared anastomoses in the chorion commonly occur, exhibiting a hematopoietic XX/XY chimerism in the case of twins of opposite sex. In addition, most marmosets are chimera from at least two fertilized eggs or early embryos fused together. The difference in phenotype may be subtle (hitchhiker's thumb and straight thumb, different hair growth on opposite sides of the body, etc.) or completely undetectable [43]. In marmosets, as in most New World monkey species, chorionic gonadotropin hormone plays the role of luteinizing hormone (LH). Key differences are present in males too. The control of Leydig cell function and testosterone production by LH is entirely different from that of humans. Regulating genes on the Y chromosome are different from those of male Old World monkeys and humans, and some key fertility genes (e.g., *DAZ*) are absent. As a consequence, even if the organization of the testicular germinal epithelium is similar to that of man, the correspondence with the functional and hormonal spermatogenesis in human remains unknown. The reproductive cycle is considerably shorter than in macaques, and males attain fertility at 17-20 months of ages (for a review, see refs. 10,16,44).

This species is valid and relevant for EFD studies (segment II) in the event that cynomolgus monkeys cannot be used (e.g., a biopharmaceutical that is not pharmacologically active in cynomolgus or rhesus monkeys). Effects of canakinumab on embryo-fetal development were assessed in marmosets using the drug product. The only finding associated with canakinumab treatment was a slight reduction in the number of fetuses at the high dose, which correlated with a slight trend toward decreased placental weight. There were no treatment-related effects on fetal development. Despite the confirmation of placental transfer of IgG at the time of cesarean delivery (gestational days 112-114), given that transfer of IgG across the placenta starts late during gestation, it is unlikely that the fetus was exposed to canakinumab during organogenesis [22]. There are no other published examples of the use of marmosets in reproductive and developmental toxicology. The feasibility of the marmoset model for a full range of developmental studies, including segment III pre/postnatal studies, must be more fully evaluated [10,16,44].

EVALUATION OF IMMUNE FUNCTIONS

The high species specificity of the sequence/structure of most immune receptors targeted by mAbs makes the selection of an appropriate animal model challenging. This is probably less true for small-molecule drugs. However, the NHP is the most commonly selected animal model for safety assessment of immunomodulators, given its genetic similarity to humans and the homology of its immune system to that of humans. For reasons of practicability/availability, the cynomolgus monkey is the preferred NHP species, although occasionally marmosets and rhesus macaques are used. Nevertheless, there are safety-relevant functional differences between primate and human immune systems, despite a close evolutionary relationship. These include,

for example, differences in MHC genes, distribution and function of Fc receptors and Toll-like receptors, and sensitivity to complement activation, which all need to be considered while assessing the safety of immunomodulatory compounds and, more specifically, agonists [45].

A range of potential liabilities associated with immune agonism may be explored in NHPs [46]. The cynomolgus monkey has been used as a relevant toxicology species for immunostimulatory agents such as Toll-like receptor agonists; however, while special recognition regarding the clinical risk of systemic cytokine release for certain targets is warranted, the NHP is not always predictive of this potential toxicity in humans. An important difference between humans and other NHPs such as cynomolgus macaques is the stronger human T-cell proliferative responses upon activation via the T-cell receptor. This is the consequence of human-specific loss of T-cell Siglec expression during the later stages of evolution. CD33-related Siglecs, which are inhibitory signaling molecules expressed on most immune cells, downregulate cellular activation pathways via cytosolic immunoreceptor tyrosine-based inhibitory motifs. Concordant with this species-related difference in Siglec expression is the observation that several common human T-cell-mediated diseases, such as bronchial asthma, rheumatoid arthritis, and Type 1 diabetes, have not been reported in chimpanzees or other great apes. The lack of Siglec expression in humans may partially explain why the cytokine release syndrome (CRS) in humans cannot be accurately predicted by NHPs or rodents. The life-threatening cytokine storm effects induced by TGN1412 were a result of the unexpected TGN1412-mediated activation (proliferation and cytokine release) of CD28-expressing $CD4^+CD45RO^+$ effector memory T cells predominantly residing in tissue [47]. These effects were not observed in cynomolgus monkeys, which do not express CD28 on effector memory T cells, nor in mice, in which the rapid T regulatory cell expansion quenched the proinflammatory effects of the relatively small effector T-cell population in these animals compared with a mature human immune system [48,49]. These human-specific differences in T-cell activation need to be carefully considered during safety assessment of immunostimulatory mAbs against T-cell targets.

Immune genes are expanded in macaques. The macaque genome has 33 major histocompatibility genes, three times that of humans. This has clinical significance because the macaque is used as an experimental model of the human immune system. By contrast, marmosets exhibit limited diversity at MHC class I and II loci [50,51].

Fc receptors are plasma membrane glycoproteins that bind to the Fc region of antibodies. Crosslinking of Fc receptors by Ab-opsonized antigen complexes initiate cellular immune responses, including phagocytosis, Ab-dependent cell-mediated cytotoxicity, respiratory burst, release of cytokines and inflammatory mediators, and antigen presentation. Binding human IgG to cynomolgus monkey FcγRs is broadly similar to that observed with human FcγR [52]; however, there are some important differences. In humans, FcγRIIIA (CD16A) is expressed on monocytes, macrophages, and NK cells, whereas the FcγRIIIB (CD16B) isoform is expressed on neutrophils, eosinophils, and other cells. In NHPs there is only one CD16 gene, homologous to the human CD16A, which is restricted to NK cells and monocytes [53]. This difference necessitated the testing of an antihuman CD16 (FcγRIIIA and FcγRIIIB)-specific mAb in human CD16 transgenic mice to assess the impact on neutrophils [54]. Even greater differences in Fc receptor expression, distribution, and internalization profiles between humans and mice also are observed [55]. Anatomic differences in human and animal immune systems and their responses to immunomodulatory drugs have been reviewed [56].

The importance of ensuring that human-relevant Fc binding and effector function activity of IgG is occurring in the toxicology species is further illustrated by the case of hu5c8, an anti-CD40L IgG1. Development was stopped in clinical phase II after it induced thromboembolism in patients [57]. No thromboembolisms were detected in preclinical studies conducted using cynomolgus monkeys. Further investigations showed that treatment in rhesus monkeys, but not in cynomolgus monkeys, resulted in thrombi formation at various locations and vasculopathy characterized by intimal hyperplasia. The full-length IgG1 induced platelet aggregation and serotonin release once bound to its target in humans and in rhesus monkeys. Other constructs based on Fab fragments or full-length antibody with aglycosyl Fc did not cause platelet aggregation or activation in vitro, nor thromboembolisms or intimal vascular hyperplasia in vivo, while binding to the target with comparable affinity. The mechanism is not fully understood, but one hypothesis is that CD40 may play a role in hemostasis and platelet biology by functioning as a primary signaling receptor for CD40L and by serving as a docking molecule for CD40L immune complexes. CD40 may drive translocation of FcγRIIa (CD32a) to the platelet surface, enabling the Fc part of antibodies bound to their target to be captured and inducing strong platelet activation [58]. Based on these observations, the nonclinical development of a new anti-CD40L with a different format preventing Fc interaction was therefore conducted in the rhesus monkey [59]. This case study also demonstrates that the rhesus monkey may for some toxicities be the most relevant NHP species.

IgG1 and IgG3 antibodies are capable of mediating complement activation (binding to C1q). By contrast, IgG2 shows little complement activation and IgG4 shows none at all. Therefore, for immunomodulatory mAbs that bind to membrane-based targets, to avoid cell depletion, the IgG2 or IgG4 format or silent IgG1 (with reduced Fc effector function) is often used because of the absence of Fc effector function. In addition to complement-dependent cytotoxicity, which is an intended pharmacological effect of certain mAbs (e.g., direct killing of tumor cells), complement activation may lead to a number of safety-relevant consequences, including cytokine release, hematological effects, and/or potentially life-threatening hemodynamic disturbances. Despites large concentrations of complement components in cynomolgus monkey serum compared with human serum, the hemolytic activity is comparable. However, mechanistic differences in terms of (cross-)species-specific IgG/Fc-mediated complement activation, particularly related to IgG aggregation status, cannot be excluded and deserve further investigation [60].

Immunophenotyping of peripheral blood cells in NHPs is readily available for a variety of populations of lymphocytes and is usually included in the safety assessment of immunomodulators. A standard panel can include total T lymphocytes, helper T lymphocytes, cytotoxic T lymphocytes, B lymphocytes, and NK lymphocytes. Populations that typically are not evaluated unless warranted to measure a specific pharmacodynamic response may include regulatory T helper lymphocytes, activated T helper lymphocytes, activated cytotoxic T lymphocytes, effector memory T helper lymphocytes, effector memory cytotoxic T lymphocytes, central memory T helper lymphocytes, central memory cytotoxic T lymphocytes, naïve T helper lymphocytes, and naïve cytotoxic T lymphocytes. The selection of such assessments should be driven by the biology of the drug target and should be considered as part of the safety assessment on a case-by-case basis [60]. Some qualitative and quantitative differences exist with respect to surface markers on lymphocytes relevant for studies of immunotoxicity. For example, the marmoset lacks natural killer cells with typical markers

found in humans ($CD2^{-}CD56^{+}$); conversely, cytotoxic killer effector $CD4^{+}$ cells ($CD4^{+}CD56^{+}$) found in marmosets are not found in humans [17].

The T-cell-dependent antigen response (TDAR) assay encompasses multiple aspects of the immune response (i.e., antigen capture and presentation, T-cell help, and antibody formation [including class switching]) or the ability to mount an immune response in one end point: antigen-specific antibody titers [61]. In toxicology regulatory guidance documents, the TDAR is considered to be one of the functional assays of choice to evaluate potential immunotoxicity of investigational new drugs. However, the interanimal variability limits the sensitivity of the assay in studies powered for general toxicology evaluations, despite the similar behavior of sexes or animals from different countries of origin. Inhibition of the TDAR can be clearly evidenced with immunosuppressive agents in NHPs, but demonstrating a lack of activity in the assay has been problematic and the definition of biologically irrelevant variations is not established. Keyhole limpet hemocyanin, (KLH), sheep red blood cells (SRBC), and tetanus toxoid (TT) are the most commonly used antigens for challenge; KLH and TT show similar interanimal variability and a trend to less variability than SRBC. Power analysis of numerous studies has demonstrated that animal group sizes of 8-12, combining males and females, could detect ≤3.1-fold differences in anti-KLH IgG responses. Assay optimization and harmonization of the TDAR in NHPs remains a priority [60]. A functional *ex vivo* assay for NK cell-mediated lysis is available for NHPs (using the K562 human myelogenous leukemia target cell line), but the development of a functional assay for CTL activity is needed and is the subject of significant effort.

If there is generally a good concordance of pharmacodynamic effects in humans and NHPs, there is often limited concordance for adverse effects when considering mAbs or fusion proteins for cell surface targets or soluble targets [62,63]. The rare but serious adverse events that are of greater concern for patients are usually not predicted in studies conducted in normal healthy animals. Part of the reason why the animal studies fail to detect these effects is that NHP studies are not powered to detect rare events. Also, serendipitous infectious challenge may not occur in a controlled laboratory environment, and animals are generally prescreened for certain infectious agents before inclusion in studies; only those animals that test negative are included in the study. Laboratory animals do not receive comedications and are healthy when enrolled in toxicology studies. Regarding cell surface targets, downstream signaling may also be different because the antibody or fusion protein tested does not bind to the same epitope.

NHPs are often the only relevant species for the development of biopharmaceuticals; however, a comprehensive assessment of immunotoxicity remains a challenge because of the interanimal variability associated with certain parameters. The stage of development of certain immune assays, the incomplete knowledge of target distribution or effector function compared with humans, and a greater understanding of the background incidence of certain pathogens in NHPs are needed for proper interpretation of infectious agent-related findings that may be misinterpreted as treatment-related findings as a result of immunomodulation.

CONCLUSION

NHPs are used for research and development and are often the only relevant species for the development of biopharmaceuticals for many different indications. Many similarities with humans generally result in a good concordance of pharmacodynamics effects in humans and

NHPs. Several examples have demonstrated the need to use NHPs, especially to identify hazards in the reproductive and developmental assessment of biopharmaceuticals. Nevertheless, some important challenges that may impact safety and risk assessment for humans remain. These include limited numbers of animals and interanimal variability associated with some parameters, the stage of development of certain assays, the incomplete knowledge of target distribution or effector function compared with humans, and differences inherent to the species used or the provenance.

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