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Chapter 10

Animal Models

No Model Is Perfect, but Many Are Useful

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Chapter Outline

1. INTRODUCTION

Full evaluation of the interactions between a virus and its host requires a living organism, because simpler systems cannot simulate either the host response or the evasion strategies of the invading virus. Advances in *in vitro* and *in silico* techniques now allow researchers to examine the intricacies of a virus, especially on a molecular level. Both primary cell cultures and immortalized cell lines derived from animal tissues are commonly used to study a virus' effect on the biology of a cell. However, an intact living organism is required to fully evaluate the interactions between a virus and its host. The innate and acquired immune responses play key roles in the course of a viral infection and can only be studied in an animal model (or a human host). Furthermore, animal models are required for testing therapeutics and evaluating vaccines.

For viral infections of humans, ethical considerations necessitate the use of animal models to address many aspects of the virus–host interaction. Even for infections of animals, it is not always feasible to use the natural host, and it may be necessary to use a different species as a model. The model animal should not be expected to identically mimic the disease seen in the subject being modeled, but rather to act as a surrogate in understanding the infection process and clinical disease produced. This information can then be compared to what is observed in the natural host. Because a single animal model may often not be able to duplicate every feature of a virus infection exactly as it would occur in the natural host, different species may be used to study different aspects of pathogenesis. For example, a rhesus macaque may be used to recapitulate the clinical disease seen with a virus that naturally infects humans, while a mouse may be used to study the immune response to virus infection at the cellular level.

As virus research has evolved over the last century, the use of animal models has increased dramatically (Figure 1). Many different species are used in virus research, from small rodents such as mice and rats to larger species such as guinea pigs, chickens, ferrets, and nonhuman primates. Prior to the 1970s, the rat, hamster, and the guinea pig were the most common animal models used in virus

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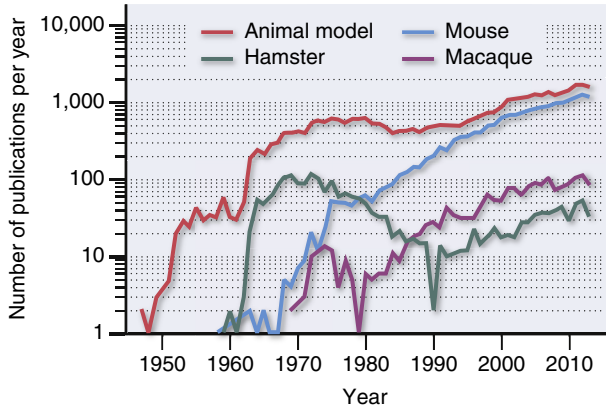

FIGURE 1 Trends in animal models used in virus research, 1940–2013. A Pubmed search was conducted using the search command "virus" + "animal model" +/− "species." Data are presented as the number of publications per year retrieved by each search.

studies. However, with advances in transgenic and knockout technology, mice have quickly become the dominant species used. In 2013, virology publications involving mice outnumbered publications for all other species combined. For many other animal species, full genome sequencing has recently increased the information available for analysis of the host response to virus infection. Also, the use of certain selected species has increased in use in recent years due to their fit as models for particular infections. For example, the ferret has proved susceptible to respiratory viruses, such as influenza and SARS coronavirus (SARS-CoV).

The choice of an animal model for a virus study requires careful consideration. Each species possesses advantages and disadvantages that can vary with the virus being studied. Understanding the basic biology and unique characteristics of different animals can help researchers select the ideal model for their virus and experimental goal. In this chapter, we will introduce the animals commonly used in virus research and point out the considerations required when developing and using an animal model, together with some of the potential pitfalls. Recent technical developments have made it possible to manipulate the genetic background of experimental animals, which has opened exciting new vistas for animal models. Other new methods have also markedly expanded the information obtained from animal experimentation. For instance, it is now possible to visualize viruses in the same living animal repeatedly over time and look deep into normal and infected tissues with three-dimensional imaging. Finally, we will describe some of the applications of animal models for the development of new therapies and vaccines.

2. COMMONLY USED SPECIES

The following brief descriptions provide some basic information about the more frequently used (and readily available) species of experimental animals (Table 1). More detailed information is available in several of the references provided.

2.1 Mouse

There is a rich history of biomedical research using mice (*Mus musculus*) as experimental models. French biologist Lucien Cuénot studied mouse coat color genetics in 1902, and demonstrated Mendelian inheritance in mammals for the first time. Mice used in research today were bred from "fancy mice" in the early part of the twentieth century. Clarence Cook Little developed the first inbred dilute brown agouti (DBA) mouse in 1918, and today there are hundreds of available inbred strains of mice, with varying amounts of interindividual genetic variation. Over the last century, the use of mice as an animal model has exploded, making these animals the most popular species used today in virus research.

Mice have many advantages as research animals and are arguably the most cost-efficient vertebrate in current use. Multiple animals can be housed in individual cages and occupy relatively little space. They are prolific breeders with a short life cycle, as little as 9 weeks between generations for certain strains. There are a wide variety of reagents and tools available for mice, providing researchers with the ability to study almost any aspect of the immune response. In addition, mouse and human species genomes share many similarities, such as the approximate number of protein-coding genes and regions of conserved orthologous sequences. Of particular relevance to studies of pathogenesis, the murine histocompatibility complex is well described, and many aspects of innate, cellular, and humoral immune responses were originally described in mice and subsequently identified in humans. Together, these characteristics have made the mouse the preferred animal for deciphering the host immune response to many virus infections.

Despite these advantages, host-range limitations make mice resistant to many human viruses. For example, dipeptidyl peptidase 4 (DPP4), the cellular receptor used by the newly emergent Middle East respiratory syndrome coronavirus (MERS-CoV), is absent in mice. Therefore, a virus native to another species must often be adapted to mice through multiple passages. This technique selects for viruses with mutations that increase virulence, but may also alter pathogenesis. In the case of SARS-CoV, 15 passages of the Urbani strain in young BALB/c mice resulted in a virus (MA15) that is lethal in mice. Aspects of disease seen in severe human cases of SARS were reproduced by MA15 as the result of six coding mutations associated with host adaptation. On the other hand, adaptation of yellow fever virus to mice altered the disease from hepatitis to encephalitis, and the mouse-adapted virus was encephalitogenic (not hepatotropic) in nonhuman primates.

TABLE 1 Select Animal Model Species and Their Commonly Studied Virus Families

	Species	Commonly Studied Virus Familes
	Mouse	Poxviridae, Herpesviridae, Adenoviridae, Hepadnaviridae, Parvoviridae, Retroviridae, Arenaviridae, Bunyaviridae, Orthomyxoviridae, Paramyxoviridae, Filoviridae, Rhabdoviridae, Reoviridae, Coronaviridae, Arteriviridae, Togaviridae, Flaviviridae, Caliciviridae, Picornaviridae
	Guinea Pig	Herpesviridae, Arenaviridae, Orthomyxoviridae, Paramyxoviridae, Filoviridae
	Hamster	Arenaviridae, Bunyaviridae, Filoviridae, Paramyxoviridae, Flaviviridae
	Woodchuck	Hepadnaviridae
	Prairie Dog	Poxviridae
	Cotton Rat	Adenoviridae, Orthomyxoviridae, Paramyxoviridae
	Ground Squirrel	Hepadnaviridae, Rhabdoviridae, Togaviridae
	Ferret	Orthomyxoviridae, Paramyxoviridae, Coronaviridae
	Chicken	Poxviridae, Retroviridae, Orthomyxoviridae
	Macaque	Poxviridae, Herpesviridae, Adenoviridae, Papillomaviridae, Polyomaviridae, Hepadnaviridae, Retroviridae, Arenaviridae, Bunyaviridae, Orthomyxoviridae, Paramyxoviridae, Filoviridae, Rhabdoviridae, Reoviridae, Coronaviridae, Arteriviridae, Togaviridae, Flaviviridae, Caliciviridae, Picornaviridae
	Pig	Poxviridae, Herpesviridae, Parvoviridae, Circoviridae, Orthomyxoviridae, Paramyxoviridae, Filoviridae, Rhabdoviridae, Reoviridae, Coronaviridae, Arteriviridae, Flaviviridae, Caliciviridae, Picornaviridae
	Ruminant	Poxviridae, Herpesviridae, Papillomaviridae, Retroviridae, Bunyaviridae, Paramyxoviridae, Reoviridae, Flaviviridae, Picornaviridae

Over the last few decades, numerous techniques to alter the mouse genome have been developed. With the sequencing and availability of more than 100 laboratory and wild-derived inbred strains of mice, researchers are now able to map genetic loci associated with disease susceptibility and identify quantitative trait loci underlying phenotypic variation. Methods to create knockout and transgenic mice have become increasingly accessible, and mice with spontaneous or engineered mutations are readily available for study. These important advances are described later in this chapter.

2.2 Other Commonly Used Species of Small Animals

2.2.1 Guinea Pig

The guinea pig (*Cavia porcellus*) is the only New World rodent commonly used in research. The Andean Incans in Peru originally domesticated the guinea pig to use as a food source and for sacrificial offerings. Dutch fanciers introduced them to Europe in the sixteenth century and bred them to create several colors and hair-coat varieties. They are more docile than smaller rodents, and their relatively low maintenance costs make them preferable to larger, more expensive nonrodent species. Although not as well characterized as the mouse, the guinea pig immune system shares many characteristics with that of humans. Despite this, few molecular and immunologic guinea pig reagents are commercially available, posing a hindrance to their use for viral pathogenesis studies.

Possibly the greatest advantage guinea pigs possess is the ability to recapitulate the gross and histologic pathology seen with many human viral diseases. They are most commonly used to study DNA viruses and negative-sense RNA viruses. Guinea pigs are highly susceptible to several arenaviruses and filoviruses and have been used in studying the pathogenesis of human hemorrhagic fevers including those caused by Ebola, Marburg, Junin, and Lassa viruses. These viruses generally replicate to high titers in immune-competent animals, and the clinical disease, hematologic profile, and pathology produced are similar to those observed in humans. Because of this, the guinea pig is a viable model for testing the efficacy of potential therapeutics. Guinea pigs are also popular models for several respiratory pathogens, including respiratory syncytial virus and influenza virus. These animals reproduce many of the characteristics of viral replication and pathology seen in humans and have been used to study aerosol infection and transmission efficiency between individuals.

2.2.2 Hamster

Hamsters were once one of the most commonly used animal models in virus research, but since 1973 their use has decreased in favor of the more genetically manipulable mouse. The Syrian or golden hamster (*Mesocricetus auratus*) is the most common hamster species used. Most of today's laboratory animals originated from a single litter whose progeny was imported into the United States in 1938, creating little individual genetic diversity. An advantage of hamsters is their extremely low rate of spontaneous disease compared to other animals, combined with their susceptibility to many viruses.

The Syrian hamster is often used for studying emerging RNA viruses, particularly hemorrhagic fever viruses. Infection of hamsters with nonadapted strains of Lassa fever virus, Rift Valley fever virus, or yellow fever virus produces pathological findings similar to those seen in humans. Hamsters infected with mouse-adapted Ebola virus are the only rodents that consistently develop the coagulopathies and vascular leakage seen in human infections. The hamster is currently the only animal model to reproduce many aspects of hantavirus pulmonary syndrome (caused by Sin Nombre virus), including the incubation period and disease pathology seen in humans.

In addition to reproducing clinical disease seen in humans, hamsters are particularly adept at amplifying many viruses, including lymphocytic choriomeningitis virus. In fact, federal regulations state that laboratory hamsters cannot be housed outdoors, lest they come in contact with wild rodents and become persistently infected; they are the only species with this stipulation. Because of their susceptibility, hamsters are commonly used for studying viral persistence and shedding.

The hamster immune system differs substantially from the human immune system, and there are only a limited number of immunologic reagents for hamsters; this has severely curtailed their use for studies of the immune response to infection. Due to the founder effect of the laboratory hamster population in the United States, very little alloantigenic variation exists.

2.2.3 Other Rodents

Other rodent species, including wild (nondomesticated) animals, are used for special situations. These species include the wood rat (*Neotoma* spp.), the deer mouse (*Peromyscus* spp.), the woodchuck (*Marmota monax*), the black-tailed prairie dog (*Cynomys ludovicianus*), the cotton rat (*Sigmodon hispidus*), and ground and rock squirrels (*Spermophilus* spp.).

2.3 Ferret

The ferret (*Mustula putorius furo*) is a member of the ancient and diverse *Mustelidae* family that includes weasels, minks, and martens. Although supposedly domesticated over 2000 years ago, the ferret was not identified as a potential model for biomedical research until the early twentieth century. They are an attractive "large animal" model for studying the pathogenesis of viruses in that they are a smaller and more cost-effective species than nonhuman primates. However, ferrets are large enough so that human neonatal equipment can be used to evaluate clinical parameters, such as temperature and blood pressure. In addition, the ferret respiratory system shares many anatomical and physiological features with humans, making the ferret a suitable model for the study of respiratory viruses.

Although ferrets were first used to study influenza in 1933, only recently has their use increased as their value has been recognized for the study of emerging respiratory pathogens such as henipaviruses, coronaviruses, and respiratory viruses. Oronasal challenge with henipaviruses produces both respiratory and neurological disease along with disseminated vasculitis, resembling the response of humans to these agents. When challenged with SARS-CoV, ferrets demonstrate severe alveolar damage and edema, though generally not to the extent seen in humans and nonhuman primates. The ferret's greatest impact on virus research lies in its contributions to the influenza field (Peng et al., 2014). It is an important model for understanding pathogenesis and evaluating potential vaccines. With the emergence of influenza strains with pandemic potential, including H1N1 and H5N1, the ferret has become an invaluable tool to predict transmission in humans (Figure 2), a major public health concern (see further discussion later in this chapter and Chapter 7, Patterns of infection).

2.4 Chicken

While rarely used as a model for studying human viruses, the chicken (*Gallus gallus*) holds an important place in history for its contributions to the field of tumor virology. In 1911, Peyton Rous first reported isolation of a "filterable agent" from a sarcoma in a Plymouth Rock hen that could be experimentally transmitted to other chickens. Further studies of this retrovirus have led to other pioneering discoveries in oncology, including *Src*, the first recognized oncogene.

Chickens have played a crucial role in unraveling the cellular basis of the adaptive immune system (Gitlin and Nussenzweig, 2015). Max Cooper in Robert Good's laboratory discovered that the progenitors of antibody-producing plasma cells were differentiated in the Bursa of Fabricius in the chicken, while the progenitors of cellular immunity were differentiated in the thymus. This discovery, which clearly distinguished B and T cells for the first time, was a key step in our current understanding of the immune system.

Chickens also have made valuable contributions to vaccine development. In the 1930s before the era of cell

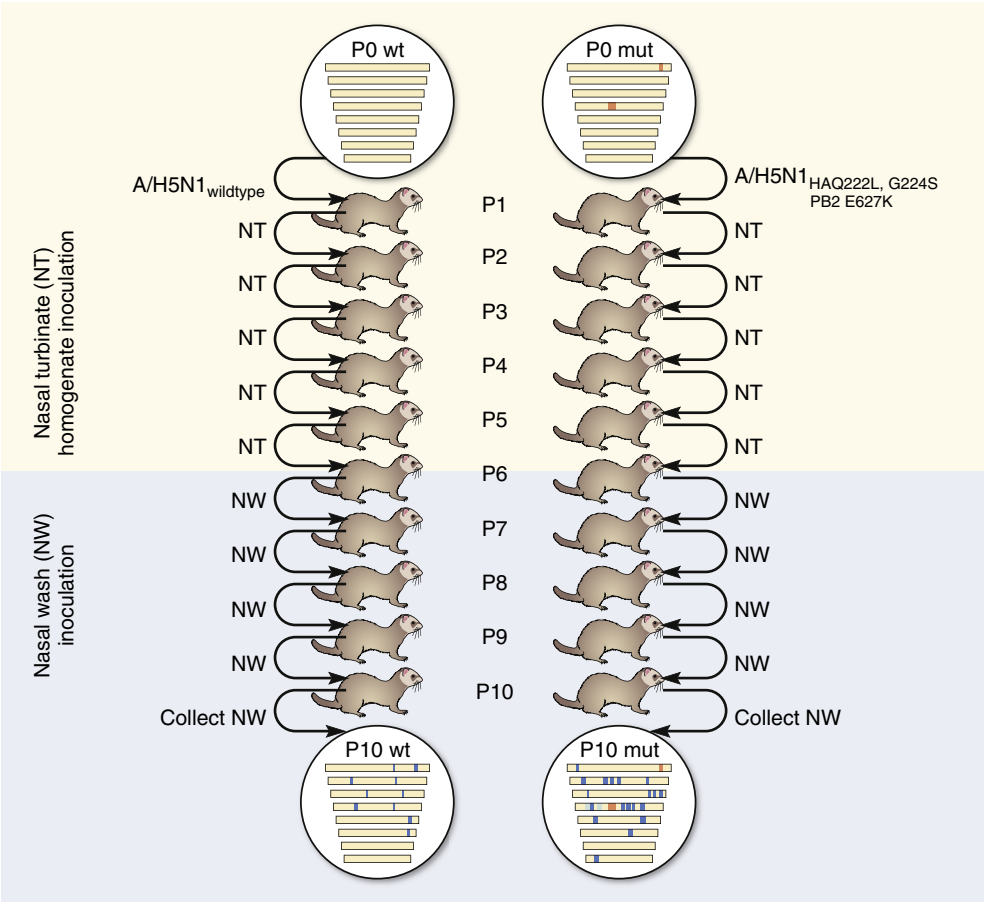

FIGURE 2 Unique animal models can be critical for pathogenesis studies of specific viruses. Ferrets are an important experimental host for pathogenesis studies of influenza, in part because they respond to influenza type A virus infection in a manner similar to humans. In addition, ferrets differ from mice and many other experimental animals because they have the same distribution of sialic acid receptors as do humans, with α-2,6-linked receptors in the upper respiratory tract and α-2,3-linked receptors in the lower respiratory tract. In the experiment illustrated above, a human isolate of influenza type A H5N1 viruses was genetically modified to introduce mutations that would change its receptor preference from α-2,3 to α-2,6 sialic acid receptors. Both wild-type and genetically modified strains were passaged in ferrets 10 times, to determine if they could be adapted to spread by aerosol from animal to animal. The modified (but not the wild-type strain) was aerosol-transmissible after passaging in ferrets. *Redrawn after Herfst et al. (2012).*

culture, Alice Woodruff and Ernest Goodpasture reported the successful propagation of fowlpox virus in the chorioallantoic membrane of chick embryos. Their technique allowed for the cultivation of uncontaminated virus for the first time, revolutionizing the field of virology. Subsequent work with embryonated chicken eggs led to vaccine development for several viral diseases including yellow fever and smallpox. Even though cell culture has largely replaced embryonated chicken eggs for virus production, the vast majority of influenza vaccines are still produced in embryonated chicken eggs.

2.5 Nonhuman Primates

Nonhuman primates share many anatomical, physiological, and immunological characteristics with people and tend to be more susceptible than other animals to infection with human viruses. Old World primates include baboons, many species of African monkeys, Asian macaques, and great apes such as chimpanzees, while New World primates include marmosets, tamarins, spider, and squirrel monkeys. Because of their closer phylogenetic relationship to humans, the Old World primates, particularly macaques and chimpanzees, are more commonly used to study virus infections.

Asian-origin macaques, specifically rhesus, cynomolgus, and pigtail macaques, are commonly used in virological studies. Macaques have made contributions to countless studies involving viruses, particularly in developing vaccines, where a higher order mammal is often required for preclinical trials. They have been used to study global diseases such as AIDS (discussed in more detail later), childhood diseases such as poliomyelitis and measles, tropical diseases such as yellow fever and dengue fever, and potential bioterrorism agents including smallpox and Ebola virus. Due to their close relationship to humans, findings from

these studies often also benefit nonhuman primates that are naturally susceptible to many of these diseases.

The chimpanzee requires special mention for its historical contributions to viral pathogenesis and vaccine research. With over 99% shared genetic identity, they are our closest animal relatives. Research using these animals has resulted in vaccines for hepatitis A and B and has increased our understanding of HIV, respiratory syncytial virus, cytomegalovirus, and hepatitis C virus infections. Chimpanzees are the only great apes used in biomedical research, but federal legislation has significantly diminished their role. In 1995, the NIH enacted a moratorium on breeding chimpanzees in captivity, and in 2013 all but 50 of the NIH-owned chimpanzees were retired from research. Certain high-impact, noninvasive studies are still permitted.

2.6 Large Domesticated Animals

Large domesticated animals, such as cows, sheep, horses, camels, and pigs, are used for specialized research problems. Their description is beyond the scope of this chapter but may be found in reference books (see Further reading).

3. CONSIDERATIONS WHEN USING ANIMAL MODELS

3.1 Genetics

The genetic background of the host animal plays an important role in the phenotype produced by a virus infection. In outbred populations, individual animals commonly differ in their responses to virus infection. For example, in pigtail and rhesus macaques, some major histocompatibility complex alleles can present an immunodominant epitope of the SIV Gag protein, leading to lower viral loads than that seen in animals with other alleles.

Likewise, the immune response to pathogens and outcomes after infection can differ greatly among inbred mouse strains. For example, mice that have functional Mx1 proteins (e.g., wild *Mus spretus* mice) are less susceptible to influenza virus infection than those with nonfunctional proteins (e.g., C57BL/6 and BALB/c). Different mouse strains can also have biased immune responses that can affect susceptibility to a virus infection. For example, C57BL/6 mice tend to skew toward a Th1 response whereas other strains, such as BALB/c and DBA/2 mice, tend to have a predominant Th2 response. Therefore, it is essential to use the same strain of mouse in experiments where consistency of a phenotype is important, such as for "knockout" and "knockin" comparisons. Because many substrains exist within a strain, it is also important to use mice from the same vendor for a set of experiments.

3.2 Age and Sex

Host susceptibility to a virus can be markedly affected by age and sex. For many viruses, young animals have a higher mortality than older animals (Figure 3). For other viruses, older animals may exhibit increased susceptibility, such as with human coronaviruses (see Chapter 16, Emerging virus

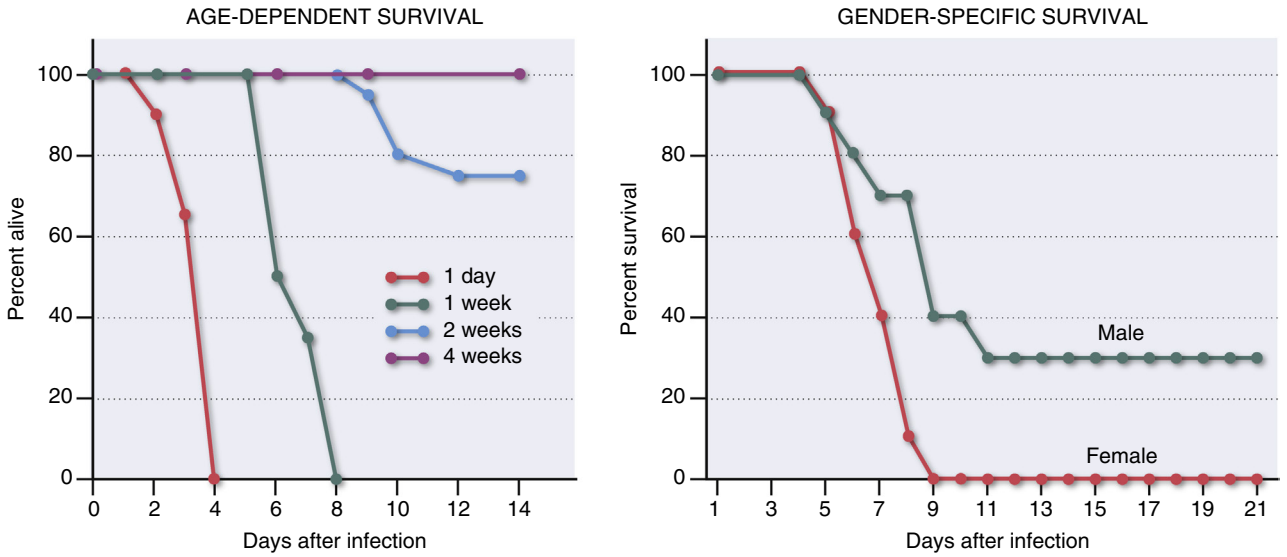

FIGURE 3 Age and sex influence susceptibility to virus infections. Left panel. Age-dependent survival in mice infected with Sindbis virus. Mice infected with Sindbis virus at a younger age have a higher mortality than mice at an older age of infection. Mice infected at 1 day of age (red line) and at 1 week of age (blue line) show 100% mortality, while mice infected at 4 weeks show no mortality. Mice infected at 2 weeks of age (purple line) have an intermediate rate of mortality Griffin (1976). Right panel. Survival curves for male and female mice infected with 10^3 TCID50 of an H1N1 strain of type A influenza virus. The LD50 was 11-fold higher for male than female mice in this experiment. *After Lorenzo et al. (2011).*

diseases). The sex of the animal often affects the outcome of virus infection. Male mice and humans are more susceptible to myocarditis caused by Coxsackie virus infection, and females have higher morbidity and mortality when infected with influenza A virus. These confounders should be identified, studied, and controlled whenever possible.

3.3 Virus Strain and Route of Infection

Different strains of the same virus can have different courses of pathogenesis, tissue tropisms, and varying disease severity (this variable is discussed in detail in Chapter 3, Basic concepts, and Chapter 7, Patterns of infection). These differences can often be exploited to identify viral determinants of virulence or attenuation. The route of infection, such as intraperitoneal versus intranasal, intracerebral or subcutaneous, can also affect the course of infection and nature of disease (Belser et al., 2013).

3.4 Coinfections

As more natural pathogens are discovered in laboratory animal species, the possibility of confounding infections affecting a virus phenotype increases. Dramatized in Richard Preston's *The Hot Zone*, a quarantined shipment of cynomolgus macaques in Reston, Virginia, created significant concern when it was discovered that the monkeys were infected with a previously unidentified strain of Ebola virus. However, the monkeys were also infected with simian hemorrhagic fever virus, an arterivirus, which exacerbated the disease severity and contributed to their deaths.

Coinfection has the potential to markedly affect the immune response to an experimental virus infection. Immunosuppressive viruses, such as simian retrovirus in macaques, can result in an altered immune response to experimental infection and disease from opportunistic secondary infections. Subclinical infection can alter the immune response of immune-competent animals and have an impact on virus studies. A number of natural infections can spread silently in colonies of laboratory mice and affect experimental outcomes. Examples include viruses such as lymphocytic choriomeningitis virus, lactate dehydrogenase virus, mouse hepatitis virus, Theiler's murine encephalomyelitis virus, murine norovirus, and mouse parvovirus, bacteria such as *Helicobacter*, *Pasteurella*, and *Staphylococcus* species, and parasites such as pinworms and fur mites. Many commercial vendors sell and animal facilities maintain "specific-pathogen free" animals, and sentinel programs monitor resident colonies for subclinical infections and infestations.

3.5 Practical Considerations

Cost can influence the selection of animal models. Smaller rodent species are less expensive than larger animals. From a research standpoint, the availability of immunologic reagents and tools needs to be taken into account. As noted, the extensive arsenal of antibodies and molecular reagents available for mice, in addition to their genetic manipulability, make them very attractive models. However, as full genome sequences become available for more species, reagents and technologies available for those animals will expand.

3.6 Ethical Considerations

Ethical considerations are important when planning the use of an animal model. The "3 R's" govern the humane use of animals in research: Replacement, Reduction, and Refinement. Whenever possible, animal experiments should be replaced with nonanimal techniques. This includes using animal cells in tissue culture (relative replacement) and in vitro techniques or computer simulations in place of animals (absolute replacement). The number of animals used for an experiment should be reduced to the minimum needed to obtain significant results. Refinement refers to improvements that minimize pain and distress for animals and allow them to participate in natural behaviors. Whenever possible, social species, which include mice, rats, dogs, and most nonhuman primates, should be housed together in socially compatible groups. When appropriate, animals should also be provided enrichment, such as nesting material for rodents, balls and other toys for dogs, or swings for nonhuman primates.

In the United States, there are Federal regulations regarding the care and use of laboratory animals. These regulations are implemented at the institutional level by Institutional Animal Care and Use Committees (IACUC). The IACUCs evaluate animal protocols prior to the initiation of a study to ensure that the use of laboratory animals conforms to government standards. Distress and pain can be powerful confounders of experiments, and optimal welfare is more likely to produce reliable, consistent results. Laboratory and husbandry staff should be properly overseen and trained in the care of laboratory animals. Compassionate care is both ethical and supportive of the highest quality of animal research.

4. EXAMPLES TO ILLUSTRATE THE SELECTION OF ANIMAL MODELS

4.1 Influenza

Disease resulting from influenza A virus infection in humans can range from a mild, self-limiting febrile illness to a fulminating lethal disease with severe acute respiratory syndrome. Vaccines produced each year for seasonal influenza reduce morbidity and mortality, but are far from optimal. With the emergence of new strains with pandemic potential,

such as H1N1, H5N1, and H7N9, animal models are needed to investigate pathogenesis and immune responses and to develop new vaccines.

Waterfowl are the natural reservoir for influenza A virus, and certain avian strains can infect several mammalian species, including pigs, dogs, horses, and ferrets. Of increasing public health concern is the transmission of highly pathogenic influenza strains from birds to humans. Host restriction is highly dependent on the linkage of the sialic acid (SA) receptors found on respiratory cells. Like humans, ferrets and nonhuman primates have both α2,6- and α2,3-linked sialic acid receptors in the lower respiratory tract, but only α2,6-linked SA receptors in the upper respiratory tract. In contrast, birds and mice predominantly have α2,3-linked sialic acid receptors throughout the respiratory tract. Avian influenza isolates preferentially use α2,3-linked receptors, whereas mammalian isolates use α2,6-linked receptors. If there is a mutation in the receptor-binding site of an avian isolate, it could potentially jump species and infect mammals. If such a strain of influenza virus to which humans have no preexisting immunity crosses the species barrier, there is the potential for a pandemic, such as occurred in 1918.

When designing influenza virus studies, there are several choices of animals, including mice, ferrets, and nonhuman primates. In practice, each of these species is used for certain studies.

Mice are the most frequently used animal model for influenza studies, even though they are not a natural host of this virus. Many mouse strains, including C57BL/6 and BALB/c, have mutations or deletions in the antiviral *Mx1* gene, rendering the protein nonfunctional and the animal more susceptible to influenza virus infection. However, for productive infection, influenza viruses usually must be passaged to select for isolates that replicate efficiently in mice. Although upper respiratory signs such as nasal discharge and coughing are not seen in mice, animals do develop weight loss, lethargy, and dyspnea when infected with highly pathogenic strains of influenza virus. Histopathology tends to mirror that seen in humans.

Ferrets have become the gold standard model for influenza research because they have the same distribution of influenza virus receptors that occur in humans (see above). Ferrets develop rhinitis, fever, coughing, sneezing, appetite loss, and weight loss, similar to that seen in humans. Infection with highly pathogenic influenza virus can result in severe disease. Ferrets can transmit influenza to other ferrets and are used in transmission studies of seasonal isolates and emerging strains such as H5N1. Also, they are used to study the secondary bacterial infections that can complicate influenza.

Macaques are also used in influenza research, especially for vaccine studies. Cynomolgus macaques replicate seasonal influenza viruses in their lungs and upper respiratory tracts without developing severe clinical signs. However, when infected with virulent 1918 H1N1 or H5N1 influenza type A viruses, they tend to develop the more severe clinical signs and pathology seen in humans. Although expensive and cumbersome, macaques are selected for preclinical studies of either vaccines or antiviral drugs, as the best predictor of efficacy in humans. The investment is justified as a screen prior to initiating very expensive and time-consuming human trials.

4.2 SIV

HIV causes a disease with a very complex pathogenesis, much of which can only be studied in an animal model. Also, animal models are essential for the development of therapies, functional cures, or the ever-elusive HIV vaccine. Despite species-specific virus-host restrictions, animal models have been developed and used extensively in HIV research, through modification of the virus or studies with homologous animal lentiviruses. The following account is very brief, and a more comprehensive exposition can be found in Chapter 9, HIV/AIDS.

The SIV macaque model has become the premier model for HIV research. It was found by happenstance that strains of SIVsmm cause an AIDS syndrome when transmitted to Asian macaques, although SIVsmm is not pathogenic in sooty mangabeys, its natural host. The natural routes of HIV transmission (sexual contact, mother-to-child, and so forth) can be used to infect macaques with SIV. However, there are differences between this SIV model and HIV in humans. SIV-infected macaques progress to end-stage disease faster than HIV-infected humans, and the species of macaque and specific isolates of SIV influence the course of infection.

HIV and SIV are only about 50% identical at the nucleotide level, and neutralizing antibodies have limited cross-reactivity, indicating that SIV-infected macaques are not appropriate models for HIV-1-based vaccine testing. This problem has been partially circumvented through the development of chimeric simian-human immunodeficiency viruses (SHIVs), which can be used in preclinical vaccine and therapeutic trials.

The lack of an effective small animal model has seriously impaired HIV research. Rodents are not permissive to HIV and do not possess an analogous lentivirus of their own. Therefore, much effort has been expended to develop "humanized" mice with human immune system components. The first engineered humanized mice, such as hu-PBL-SCID and SCID-hu thy/lv mice, did not support long-term productive HIV-1 infection, despite reproducing certain aspects of viral replication and pathogenesis. Recent improvements in humanized mice using NOD/SCID/γ_c knockout (NSG), NSG-BLT, and Rag2/γ_c knockout mice have resulted in models that can support a productive HIV-1

infection and induce an HIV-1-specific immune response, allowing for potential candidate vaccine and therapeutic testing. It is unknown whether the responses seen in humanized mice will predict the responses of humans in clinical trials.

5. GENETIC INTERVENTIONS IN THE MOUSE: THE FUTURE OF ANIMAL MODELS

A great strength of the mouse as an animal model lies in the ability to manipulate its genome. As part of the Human Genome Project in 1990, the *Mus musculus* genome was sequenced as one of the first model organisms. The full genome of the C57BL/6 mouse was published in 2005, and today the genomes of over 20 other inbred strains and outbred stocks have been sequenced. This achievement has helped identify many genes for researchers to target, greatly aiding in the production of knockout and transgenic mice.

5.1 Inbred Mouse Strains

An inbred line is a mouse strain that has undergone at least 20 consecutive generations of brother/sister mating making the animals genetically identical at virtually every locus except for the sex chromosomes. This is beneficial for biomedical researchers in that genetic homozygosity decreases variability, leading to the need for fewer animals to obtain statistically significant results. Furthermore, inbred mice accept grafts from homozygous donors, a condition that has been critical for a vast number of immunobiological studies, some of Nobel Prize quality.

The Jackson Laboratory in Maine, one of the largest suppliers of mice in the United States, currently has over 200 inbred mouse strains available. Most strains also have multiple substrains due to the distribution of parent strains to different researchers and vendors. They in turn established their own colonies, eventually resulting in genetic divergence from the progenitor strain. Although this may not seem like a major problem to many researchers, the genetic discrepancies between substrains can result in phenotype differences, and so the same substrain should be used for a set of experiments.

Trait differences can be further analyzed by using established inbred lines to create recombinant inbred lines. To do this, inbred strains are crossed to make F1 and F2 generations, and those progeny are then intercrossed and inbred for 20 generations. This allows phenotypes of different traits to be mapped to a chromosome, and the larger the family of recombinant inbred strains, the greater the power and resolution of the mapping. To expand the variety (and genetic diversity) of inbred mouse strains, the Collaborative Cross Consortium (2012) is cross-breeding eight founder lines of inbred mice, as described in Chapter 13, Host genetics. To mimic the genetic diversity of a human population, outbred stocks are also available from commercial vendors.

Another ongoing approach to gene mapping is the introduction of mutations in individual genes, which can be linked to phenotypes. While previously limited to yeast and other nonvertebrate models, the accessibility of forward genetic technology in mice has dramatically increased over the last few years. Whole exome sequencing can be applied to the progeny of mice exposed to N-ethyl-N-nitrosourea (ENU) or other mutagens to identify all mutations produced and help elucidate the genetic basis of both Mendelian and complex traits (Moresco and Beutler, 2013). Further discussions of the use of genomic technology to study viral pathogenesis are presented in Chapters 12 and 13, The virus-host interactome, and Host genetics.

5.2 Transgenic Technology

While most inbred strains had been developed by the mid-twentieth century, the gene knockout and transgenic technology that has set mice apart from other models has emerged more recently. Rudolf Jaenisch and Beatrice Mintz published the first account of a genetically modified mouse in 1974, where they injected simian virus-40 viral DNA into a mouse blastocyst and showed that it was present in every cell of the resulting animal. In late 1981, four different groups reported that plasmid DNA injected into the pronuclei of fertilized mouse eggs had integrated into the host genome with stable germline transmission, thus producing transgenic mice (Figure 4). The first mouse lacking a gene through targeted mutation ("knockout") was generated in the late 1980s (Figure 5), a feat for which the 2007 Nobel Prize for Physiology or Medicine was awarded to Mario Capecchi, Martin Evans, and Oliver Smithies. Through this technology, thousands of strains of genetically modified mice have now been produced.

Virologists have used genetically modified mice to overcome some host-range limitations. To confer permissiveness to virus infection, mice that lack immune response or other host defense genes are often used. "Knocked out" genes commonly code for antiviral proteins or cytokines, such as interferons or proteins involved in immune signaling, or for genes involved in the development or maturation of immune cells, such as recombination activating gene-1 (*Rag1*). By removing antiviral proteins or populations of immune cells, researchers can identify the immune components necessary for virus control and clearance. This method presents a powerful approach to elucidating the intricacies of virus pathogenesis and the host immune response.

Another advance is the conditional knockout mouse (Figure 6). This technology, which is based on the Cre-Lox recombination and tetracycline-controlled transcriptional regulation systems, allows for targeted gene elimination or activation in either a specific cell or tissue or at a certain

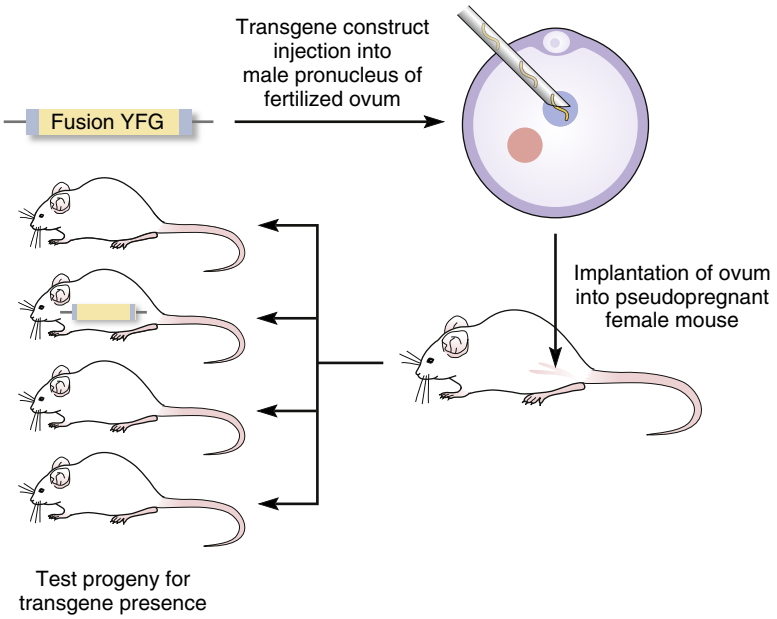

FIGURE 4 Pronuclear injection for the generation of transgenic mice. A transgenic construct is injected into the male pronucleus of a fertilized mouse ovum. The ovum is then implanted into a pseudopregnant female mouse. Once the resulting offspring are born, they can be genotyped for the presence of the desired transgene and further propagated.

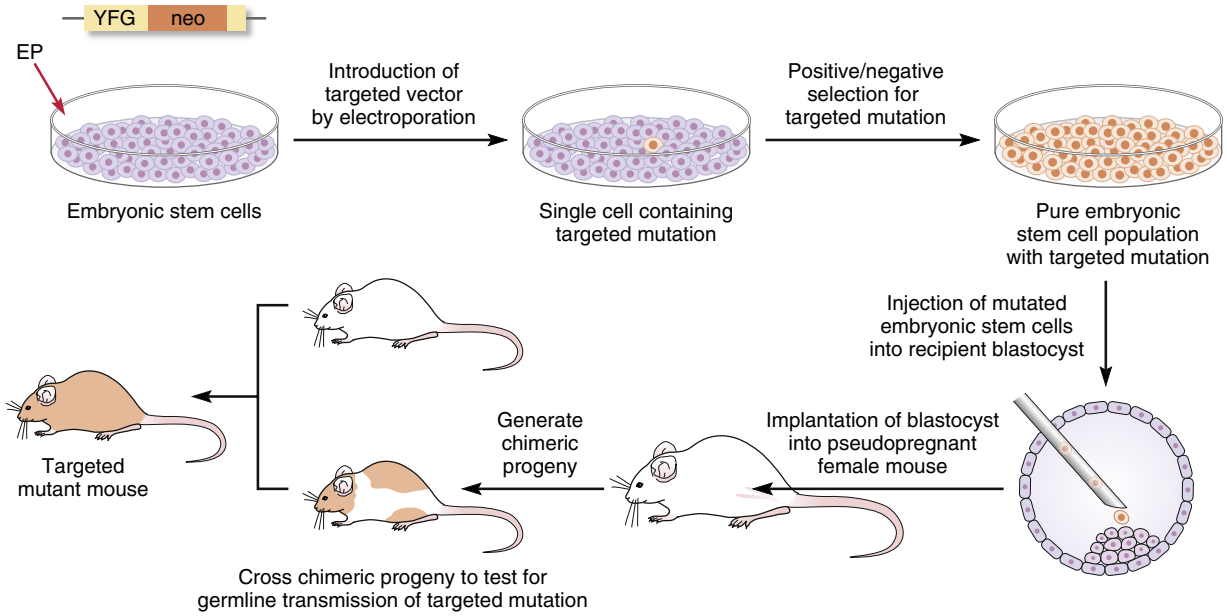

FIGURE 5 Production of "knockout" mice. A DNA vector containing a genetically disrupted gene, usually with a selectable marker, is introduced into stem cells from the blastocyst of a mouse by electroporation. The new DNA sequence is incorporated into the chromosomes of some of the stem cells in place of the original gene by homologous recombination. The stem cells containing the altered gene are then selected through a combination of positive and negative selection, and those cells are propagated until a uniform population is obtained. The mutated stem cells are injected into the blastocyst of a mouse of a different coat color than that of the mouse from which the stem cells originated. The blastocyst is implanted in a pseudopregnant mouse, and resulting chimeric progeny (as indicated by a parti-colored coat) are crossed with a mouse with the same coat color as that of the recipient blastocyst. If the germ cells of the chimeric mouse contain the targeted mutation, the resulting progeny's fur will be the same color as the coat of the mouse strain from which the stem cells originated. Genotyping is used to confirm the presence of the cassette disrupting the desired gene.

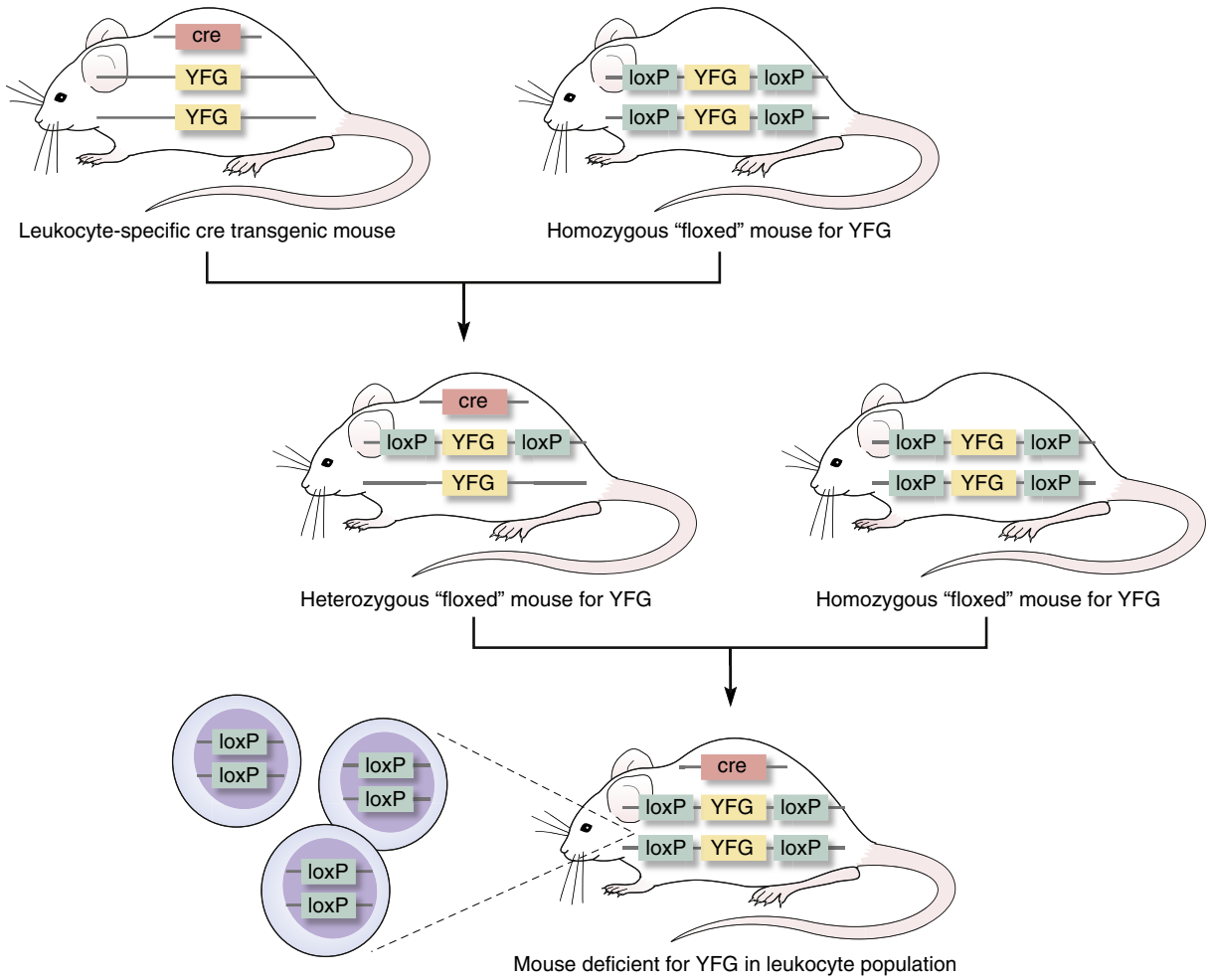

FIGURE 6 The cre/lox recombination system to develop conditional knockout mice. A mouse expressing a transgene for cre recombinase under the control of a tissue-specific promotor, such as CD4 for CD4+ T cells, is crossed to a mouse homozygous for a gene of interest flanked with loxP sites ("floxed" strain). Resulting progeny is automatically heterozygous for the floxed gene, and genotyping is performed to determine cre transgene status. A cre-positive mouse from the original cross is then crossed to another mouse homozygous for the floxed gene. Approximately, 25% of the progeny will be homozygous for the floxed gene and hemizygous/heterozygous for the cre transgene. These mice are incapable of transcribing the floxed gene in the tissue lineage containing the cre transgene and thus are the conditional knockouts.

time in development. As removal of many genes is lethal in mice during the embryonic or fetal stages of development, deleting a gene after that stage allows for the study of such knockout animals. Removal of a gene from a certain cell line can also help elucidate the role of that gene during a virus infection. However, this method can be time-consuming, labor intensive, and expensive, as interbreeding between multiple different strains is often required to obtain mice with the desired genotype.

Mice often lack the cellular receptor for a human virus (such as DPP4 used by MERS-CoV), or the mouse homologue is sufficiently different to prevent virus entry into mouse cells. In these cases, the human gene for the cellular receptor can be introduced into the mouse genome, and the subsequent human protein is then translated in the mouse, allowing for virus binding and entry into the cell. Transgenic mice can also be used to study the specific host adaptive immune response to a human virus. For instance, T cell receptors, specific for a peptide epitope from the virus of interest can be introduced into the mouse genome. When mice are challenged with the virus, it is possible to characterize the focused immune response resulting from activation of cells with that receptor.

The development and use of CRISPR/Cas9 technology (Harrison et al., 2014) to simultaneously induce targeted mutations in multiple genes has greatly facilitated the analysis of gene function at a structural level, as well as the

interactions between different genes (described in Chapter 13, Host genetics). Other recently developed approaches for inducing targeted mutations include hit and run, double replacement, and PiggyBac recombinase systems, and the use of engineered nuclease pairs such as ZFNs or TALENs. These advances allow virologists to apply techniques, previously limited to virus manipulation, to animal models to better elucidate viral pathogenesis and the host immune response.

Although advances in the genetic manipulation of mice far exceeds that available for other species, the process can be very time and labor intensive. Several attempts to knock out genes or insert transgenes must generally be made before a genetically manipulated animal is successfully produced. To create mice congenic for a mutation on the desired background strain, multiple generations of backcrossing are required. Using traditional techniques, the process can take 2–3 years if no problems are encountered, but more commonly takes up to 5 years. This timeline has been significantly decreased with the advent of more advanced genetic screening technology. While ten generations of backcrossing are traditionally required to create a congenic mouse, the use of speed congenics has reduced this timeline to as little as five generations. This process uses microsatellite markers or single nucleotide polymorphisms to select for progeny that not only possess the desired genetic manipulation, but also the greatest amount of genetic information from the desired background strain. Advances in DNA sequencing technology and computational analysis have further decreased the amount of time and labor required to produce genetically manipulated mice. New developments in mouse genetics are constantly being published, and as these technologies are fully validated and optimized, the availability of genetically manipulated mice to better study viral pathogenesis will further improve.

Researchers should be cautious when interpreting data using genetically modified mice, because these artificial models are far removed from infection in the natural host. In those cases where mice prove to be insufficient for the study at hand, another animal model should be considered. Genetic modification is starting to become more commonplace in other species such as rats, pigs, ferrets, and macaques, but the process is more difficult and less well understood in these species.

6. OTHER NEW METHODOLOGIES

A host of new methods have been developed that enhance the utility of animal models, providing information that was previously unavailable. Two of these will be noted briefly: systems biology and imaging.

The host response consists of a series of antiviral and immune programs. These orchestrated molecular events, captured through transcriptomics, proteomics, and metabolomics, can provide heat maps that capture large groups of data to elucidate the host response to infection. Influenza provides an example; a comparative cross-species transcriptomic analysis found that the 2009 pandemic H1N1 influenza virus elicits differential expression of proinflammatory genes in the lung following infection of nonhuman primates, mice, and pigs (Go et al., 2012). When these inflammatory responses in the lung are overly exuberant, they may endanger the host, as shown in a comparison of highly pathogenic avian influenza virus infections (H5N1 and 1918 strains) with influenza viruses of lower virulence (Tisoncik et al., 2012; Peng et al., 2014).

Bioimaging is undergoing a renaissance through a panoply of new methods. For instance, it is possible to introduce a fluorescent genetic label into a virus that permits its visualization during infection of a living animal (Golding and Zaitseva, 2014). Repeated imaging of a single animal during the course of infection provides a dynamic view of virus replication and dissemination. Counterpart studies of T lymphocytes, dendritic cells, and macrophages capture the movements and interaction of cells that participate in the innate and adaptive immune responses (Germain et al., 2012). Furthermore, new methods for visualizing living or fixed tissues can be used to construct three-dimensional views of tissues whose architecture is preserved.

7. ANIMAL MODELS FOR EMERGING VIRUSES

Developing an animal model that accurately mimics the disease presentation of a new virus infection is an important first step in studying an emerging virus. The first animal usually evaluated is the macaque, because nonhuman primates are closely related to humans and often exhibit a similar disease outcome. However, different species sometimes more appropriately model the observed disease, such as common marmosets for the coronavirus-induced Middle East respiratory syndrome (MERS), or Syrian hamsters for hantavirus pulmonary syndrome induced by Sin Nombre virus. Because many emerging viruses must be studied at high containment levels, such as BSL-3 or BSL-4, it is often desirable to use smaller animal species that are easier to house and handle.

More often than not, emerging viruses are zoonotic, with one or more animal species acting as a reservoir. Examples include passerine birds for West Nile virus, field mice or rats for the hantaviruses, pteropid fruit bats (flying foxes) for the henipaviruses, and camels for MERS-CoV. These animals act as long-term carriers and transmitters of the virus and generally do not develop disease. However, it is informative to understand how the virus establishes persistence

or latency in the reservoir species while facilitating virus shedding or transmission.

8. VACCINE DEVELOPMENT

The development of vaccines requires the use of animal models (see also Chapter 19, Viral vaccines). The United States Food and Drug Administration (FDA) requires that all new investigational drugs be tested in "relevant species" to ensure safety before beginning clinical trials in humans. Animals are used at several stages of vaccine development, including the host immune response to vaccination, efficacy against viral challenge, and selection of optimal vaccine formulations and delivery routes.

Animals have been involved in vaccine development for several hundred years. Edward Jenner observed that milkmaids, who often were naturally exposed to cowpox from their cows, were resistant to smallpox, leading to the development of vaccination in the late-eighteenth century. In 1885, Louis Pasteur reported the successful creation of an attenuated vaccine for postexposure prophylaxis against rabies by desiccating infected spinal cords from rabbits that had been inoculated with serially passaged rabies virus. The poliomyelitis vaccine was developed as a result of 40 years of research using nonhuman primates. Where species-specificity prevented use of an animal model for vaccine development, such as human papillomavirus, related animal viruses were used as surrogates. In this case, virus-like particle vaccines containing papillomavirus L1 capsid protein were tested in rabbits, cattle, and dogs infected with species-specific papillomaviruses before starting clinical trials in humans.

In choosing an animal model for the development of a vaccine, the species should reflect the course of natural virus infection in humans as closely as possible. The same organs should be affected, the pathology should be comparable, and the immune response should be similar. Additionally, disease should occur in a high proportion of animals to decrease sample size, and the route of virus infection should be the same as it is in humans. Although mice are often considered the ideal animal model for immunogenicity studies, other animal species may better fit these criteria and be a more appropriate choice for vaccine studies.

Animals are required for immunogenicity testing of candidate vaccines. This includes characterizing the specificity, level, durability, class and subclass of antibodies produced, and the cellular immune response. For most viral vaccines, the ability to induce neutralizing antibodies has proved a good predictor of efficacy. Once a candidate meets some criteria of immunogenicity, the protective efficacy of the vaccine should be evaluated by viral challenge. Challenge dose should be as low as possible to cause infection and should be administered by the same route as in natural infection. Although historically commonplace, using death as an endpoint has fallen out of favor, and if possible, morbidity and clinical signs should be used to evaluate vaccine efficacy.

In addition to evaluating immunogenicity, the safety of a vaccine must be tested in an animal model prior to clinical trials. The viral antigen itself and adjuvants, both alone and in combination with the antigen, may cause adverse effects. Unfortunately, a vaccine that meets desired criteria in an animal model may not be adequately immunogenic or safe in humans.

There are cases where human efficacy studies for a vaccine may not be feasible because of the high mortality rate or infrequency of infection. In these situations, the FDA may permit two relevant species to be used in place of humans to evaluate the effectiveness of a vaccine for licensure. This process is known as the "Animal Rule" and further points to the importance of animal models as contributors to vaccine research.

9. REPRISE

Pathogenesis studies require animal models for several reasons: (1) for human virus diseases, another host is required for experimentation; (2) in vitro or in silico systems cannot simulate the complex response of a living host, with its innate and adaptive immune response; and (3) only an in vivo system can reproduce the sometimes unpredictable response of a natural host. Pathogenesis studies in animal models are also of great importance to applied science, since they constitute an essential foundation for the development, assessment, and production of drugs and vaccines to treat and prevent important human and animal viral diseases.

Choosing an animal model is a complex decision, involving scientific and practical considerations. In many instances, it may be best to use several animal models to address different aspects of pathogenesis or to test candidate treatments or preventive interventions. The mouse has become the default animal for many virus infections because there is a vast scientific database and a large set of reagents, and because it is the least expensive animal model. However, there are many situations where other animal models are required to best address experimental questions.

The use of animals in research is required for the production of new drugs and vaccines and plays a critical role at several stages in the development process. Small animals may be used to screen large numbers of candidate drugs or immunogens for potential efficacy and also for unwanted toxicity. Larger species, particularly nonhuman primates, may provide models that better simulate human disease, where promising products can be tested to select those qualified for human trials. In special instances, the Animal Rule may be applied, where data from two animal species substitute for human trials.

Animal-based research is entering a new and exciting phase because of a variety of evolving methods to select mice with specific and variable genetic backgrounds or to manipulate the mouse genome. It is now possible to delete or inactivate specific genes ("knockout"), to insert new genes ("knockin"), or to introduce specific mutations, using an armamentarium of techniques. These methods enable the assessment of individual host genes in the response to viral infection. Recent developments in "omics" and systems biology have made it possible to record a vast number of discrete host responses during a single infection, enormously enhancing the dissection of a very complex process. Another technical advance is the new set of imaging methods to visualize both viral invader and host responses in the living animal or to capture three-dimensional images of intact functioning tissues. Together these new technologies are opening an expanded vista of virus-host interactions, which will take viral pathogenesis studies to an increasing level of sophistication.

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