Review

Animal models: an important tool in mycology

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Animal models of fungal infections are, and will remain, a key tool in the advancement of the medical mycology. Many different types of animal models of fungal infection have been developed, with murine models the most frequently used, for studies of pathogenesis, virulence, immunology, diagnosis, and therapy. The ability to control numerous variables in performing the model allows us to mimic human disease states and quantitatively monitor the course of the disease. However, no single model can answer all questions and different animal species or different routes of infection can show somewhat different results. Thus, the choice of which animal model to use must be made carefully, addressing issues of the type of human disease to mimic, the parameters to follow and collection of the appropriate data to answer those questions being asked. This review addresses a variety of uses for animal models in medical mycology. It focuses on the most clinically important diseases affecting humans and cites various examples of the different types of studies that have been performed. Overall, animal models of fungal infection will continue to be valuable tools in addressing questions concerning fungal infections and contribute to our deeper understanding of how these infections occur, progress and can be controlled and eliminated.

Keywords fungi, animal models, antifungal drugs, host-response, fungal virulence, fungal vaccines, pathogenesis, mice, rabbits

Introduction

The use of various animals in medical, biological and microbiological research has been ongoing since the era of Pasteur and before. Many of the advances made in medicine, infectious diseases and immunology stem directly from the use of one animal species or another as a model system. Although historical precedent exists for their use, there is strong opposition to the use of laboratory animals. As a scientist in today's society, one must be attuned to the ethics of the care and use of animals in experimental studies, follow governmental mandates and strive to determine whether or not the questions being raised can be answered by other means. Thus, scientists must answer the simple, yet elusive, question of 'Why are animal models needed?'.

As the field of Medical Mycology progresses and matures, those scientists investigating the areas of pathogenesis, therapeutics, and immune response must determine whether *in vivo* studies using animal models are necessary. Why are animal models of infection performed? This question has been answered in part above, but these models provide us the means to make significant progress in the direction of ultimately understanding fungal infections, allow investigations into the evolution and progression of disease (i.e., pathogenesis), studies of what makes a particular fungus virulent and able to cause disease, aspects of

innate and acquired immunity, how disease transmission might occur through fomites, contact or aerosols, and methods of prevention, and lastly, studies on therapeutics and diagnostics that might improve patient care and outcome.

The complexity of the host-parasite interaction cannot be mimicked by *in vitro* studies. Data obtained from animal experimentation aid us in demonstrating events or interactions that, up to the present time, we have no reliable alternative way to obtain. Although there are significant differences between the experimentally infected animals used in fungal models and naturally infected human beings, in numerous aspects the animal models mirror human infections remarkably well. This review will address a variety of issues including the philosophy of studying animal models, how the results derived from them are used and evaluated, the various types of models performed using different pathogenic fungi and what those studies are used for. We will document, from the work of others, but relying heavily on our own experiences, examples of engagement with these issues. Review articles are preferentially cited, as they may be used by the reader as a source for further examination of original articles, and to avoid repetition of points made in earlier reviews. Articles in English are preferentially used.

Strengths and benefits of animal models of fungal infection

Animal models have a number of strengths and benefits. These include mimicking clinical diseases, being predictive of clinical results, summation of *in vitro* data on drug activity, pharmacology, safety and drug interactions; affordability, the capacity to examine various questions rapidly and the capacity to control a multitude of variables. Animal models, particularly murine models, can be performed affordably using numbers sufficient to obtain valid statistical data, and mice (other than special congenitally or therapeutically immunocompromised mice) require less specialized animal care, support facilities and personnel.

The capacity to control different variables is the greatest benefit to performing animal models. Among the variables that can be controlled are the strains of organisms used, and increase or decrease of the inoculum size to control severity of infection. Furthermore, the choice of animal species is critical. The course of the disease in some species may mimic human disease better than others. Other animal model variables under the control of the investigator include the use of different immunosuppressive regimens to suppress particular host-cell types, choice of host and its genetics (e.g., inbred or outbred mice) and control over the route of infection to emulate different types of clinical disease. The control over the route of administration and duration of antifungal therapy when performing efficacy studies is also a benefit. Our capacity to use age and sex matched experimental animals also contributes to reproducibility. Animal models also are useful for the development of diagnostic assays. Thus, we are able to address issues *in vivo* that cannot be answered by *in vitro* tests.

Weaknesses of animal models of fungal infection

One also needs to be aware of the inherent weaknesses of an animal model. No single model of infection can be used to answer all questions. There can be substantial variability from one experiment to the next, which must be minimized if possible. Many murine models have the drawback of being too acute in the progression of disease, and some fungal models include the involvement of organs that are not prominent target tissues in patients. In addition, mice are difficult to use when repeated samplings of blood, for example, are desired from the same animal, if larger volumes of blood are needed. However, one may compensate for this in mice by adding extra animals to experimental groups and sacrificing cohorts at desired intervals during the experiment to obtain blood [1]. When performing therapeutic studies in animals there may be differences in drug penetration or metabolism from one species to the next and differences from that observed in humans. Lastly, as mentioned above, the model must be affordable to perform, and if the costs are too high whether in purchase price or personnel time and effort, the utility of the model is limited.

Parameters to follow

Because no single model can be relied on to answer the many questions one asks in experimental studies or mimic all the various diseases seen clinically, it is often necessary to use multiple models. The question in these studies becomes what parameters should be followed to obtain the best information. Two primary parameters of infection are followed, survival and fungal burden in the tissues. Survival studies result in clear data sets, but death as an end point is often not allowed by Animal Care and Use Committees, with ethical questions arising concerning the humane care and use of animals for survival studies. Many institutional committees now require euthanasia of the animals prior to death from disease. The criteria for euthanasia must be evaluated

carefully on an individual basis, since subjective judgments can skew the data (i.e., euthanizing animals too soon makes the infection appear more severe that it truly is). Thus, criteria for euthanasia have to be as quantitative as possible, applied objectively and reproducibly to ensure accuracy, and the judgements on individual animals made without knowledge of their study group, wherever possible. Statistical evaluation of these data are appropriately done by survival analyses using a logrank test or Gehan's Wilcoxon tests; where there are no censored data points a Wilcoxon rank sums test also can be used.

The determination of infectious burden in the target organs is another common, and sensitive, assay related to the severity of disease. For infections due to yeast (e.g., *Cryptococcus, Candida, etc.*) the quantification of CFU from the target organs by homogenization of the organ followed by serial dilution and plating is a useful and straightforward methodology. Infectious burden can be a sensitive parameter for studies of drug efficacy, comparative virulence or disease progression. Benefits of performing assays of infectious burden include shortened experimental durations and avoidance of survival studies. However, for hyphal organisms, like *Aspergillus,* the best method of infectious burden determination is controversial. Some investigators use a qPCR methodology or chitin assay, while others use CFU determinations [2–6]. Each method has benefits and drawbacks. The assay of chitin in the tissues [6] can be a tedious assay and does not indicate whether the organisms present were viable. A qPCR assay applied to the determination of *Aspergillus* burden [4] requires specialized equipment and reagents, and specialized sample preparation. These are drawbacks for some laboratories and commercial assay can be cost-prohibitive.

Statistical evaluation of the data obtained from determination of infectious burden is critical. A variety of statistical tests have been used by various investigators. These include parametric and nonparametric tests, including: ANOVA with or without tests used for multiple comparisons such as a Tukey's or Student Neuman-Kuels, Kruskal-Wallis followed by a Dunn's test and Mann-Whitney U or Wilcoxon rank sums tests. The advice of a professional statistician should be sought to determine the most appropriate statistical test for the types of experiments being done.

Alternative models

In the United States, The Office of Technologies Assessments (OTA) defined an alternative method as the protocol or technology that replaces the use of laboratory animals, reduces the number of animals required or refines existing procedures to minimize the level of stress produced to the animal [7].

Defining the term alternative model is difficult. Strictly, it can be defined as a model that replaces a living animal with a non-animal system. In these terms, *in vitro* systems (chemical or biological), plants, microorganisms or computer simulations should be the only alternative models used for medical research. Using a broader interpretation of the term, the use of non-vertebrates or cold-blooded animals also is considered an alternative model.

In vitro systems using chemical components are one of the most extensively used alternative models in medical mycology. *In vitro* tests for drug efficacy is a first step in pre-clinical trials and permits us to determine the concentrations of drug that are effective against an organism by the use of a well-defined culture media where microorganism growth is tested in the presence of differing concentrations of the drug. However, *in vitro* activity does not necessarily accurately reflect subsequent *in vivo* activity nor have the majority of antifungal drugs' *in vitro* activity been correlated with clinical outcome. Thus, it remains necessary to determine the efficacy of new antifungal drugs using one or more of the available animal models of fungal infection prior to testing in humans.

In vitro systems that include biological material, i.e., cultured cells, have been used extensively in mycological research to study the interaction of the microorganism and a target tissue or specific cell-type or *vice versa*. Cell-cultures permit us to simplify an infectious event in a well-controlled environment. However, the *in vitro* system is a simple environment and does not reproduce the extreme complexity of an *in vivo* environment. The development of more complex cell cultures, where more than one type of cell is present can more accurately mimic the events *in vivo*. Another more sophisticated cell culturing system is based in three-dimensional (3-D) cultures, consisting in a rotating-wall vessel that generates low turbulence, allowing cells to associate, and form 3-D structures. It will be of interest to see how the use of these types of culture systems can be incorporated into mycological research.

Although extensively used in other disciplines, the use of non-mammalian models has been minimal in mycology. The fruit fly (*Drosophila melanogaster*), the helminth, *Caenorhabditis elegans*, the amoeba, *Acathamoeba castellanii*, the slime mold, *Dictyostelium discoideum* and the Lepidoptera, *Galleria mellonella* are the non-mammalian systems that have been used in mycology. Reviews on the use of these species in medical mycology studies have been published recently

[8,9]. The use of simple organisms is a helpful tool in the study of the host-parasite interactions by analogy to common processes conserved in mammalian cells. *D. melanogaster* has been used as model to study cellular defense mediated by macrophages against *C. albicans,* as well as virulence [10–12]. *A. castellanii* [13–18] and *D. discoideum* [9,19–22] have served as models to understand the phagocytic process, due the similarity of the process used by mammalian macrophages, but also may be relevant to interactions of the organisms in their natural ecological environments [16]. New genes involving pathogenesis of *C. neoformans* have been identified using the worm *Caenorhabditis* as screening method [23]. Similarly, *Drosophila* and *Galleria* models of aspergillosis have been described for use in pathogenesis, host-response and therapeutic screening [24–33].

The use of these types of alternative models in mycology is in its infancy and although their use may be highly desirable, the applicability of the results to human or animal infections is for the most part unknown. How the fungi differ in behavior in the environment of the *Drosophila* or other non-mammalian system where thermal environment, organ systems and immunological processes differ significantly from those of the mammalian or avian host remains to be determined. Thus, as with the results derived from conventional animal models of fungal infection, the results derived from alternative models should be interpreted cautiously.

Studies of pathogenesis

Mimicking clinical disease

The purpose in the development of animal models in clinical mycology is to mimic, as closely as possible, the progression and the clinical signs of the infection in human patients. This allows us to understand the mechanisms involving the infectious process, host-resistance to that pathogenic process and potentially, the cure for the infection. In this section we discuss the most common animal species used in mycology as models of infection.

Types of animals

A first step is to choose the correct animal species, which is one that will simulate the human infection as closely as possible, or if studying veterinary fungal diseases one may actually opt to use the same animal. As described in several review papers, a variety of animals have been used in mycological research and include: mice, rats, rabbits, guinea pigs, hamsters, dogs

and birds [34–43] and rarely other as toads, cats or bats [44–47].

The mouse (*Mus musculus*) is the species of choice in mycology due to its similarity to the human physiology, as well as the ease of availability, cost and other benefits discussed earlier. Both mice and humans have similarities in organ systems, biochemistries, pathologies, and similarities extend to their genomes as well. The mouse and human genome have approximately 30,000 genes encoding proteins and the proportion of genes with no homology between them is less that 1% [48]. In addition, the production of many genetically defined (i.e., inbred), genetically manipulated (e.g., gene knockouts) strains and the large numbers of immunological and genetic tools allow us to simulate and quantify what occurs during infection and be closer to human infection than models using other animal species. Mice have been used primarily as the model for systemic, pulmonary and central nervous system (CNS) infections by the most clinically important fungi.

Rabbits are the next most common species used in animal models of fungal infection. Rabbit models allow us to evaluate physical and physiological data unavailable or difficult to obtain with small mammals; for example, multiple large volumes of blood can be obtained from individual animals; serial bleeding is easier than in mice. Rabbits are also useful for the study of CNS infections because of the relative ease in obtaining high quality (i.e., without blood contamination) volumes of CSF (0.5 ml to 1 ml per sample time) from the same animal multiple times; this CSF is useful for determination of biochemical (e.g., glucose or protein) and cellular parameters (e.g., WBC counts). In contrast, the volume of CSF obtained from mice usually does not exceed 5 to 7 µl and is often a terminal procedure. The size of organs and internal structures makes possible more accurate and specific observation in rabbits than the mouse. For example, in our model of coccidioidal meningitis, the development and histological appearance of arteritis and vasculitis closely mimics that of humans [49]. Other models of CNS infection in rabbits include those using infection with *C. neoformans* [50] and *C. albicans* [51]. In addition, the use of larger animals, such as rabbits, allows for thorough clinical observations of signs and symptoms.

Rabbits have been used extensively as a model for mycoses affecting the eye due its size and similarity to the human anatomy and physiology. Experimental models of keratitis and endophthalmitis due to *A. fumigatus* [52]*, C. albicans* [53–56], *C. neoformans* [57], *Histoplasma* [58], *Fusarium solani* [59,60] or *P. boydii* [61] have been developed in rabbits.

Models of systemic fungal infection have also been described in rabbits and include *C. albicans* [62], *A. fumigatus* [63–66] and *S. prolificans* [67]. Other models including pulmonary aspergillosis [68,69], candidiasis [70,71] and cryptococcosis [72], sinusitis [73–75], endocarditis caused by *Aspergillus* [76] or *Candida* [77,78]. Rabbit models of *Aspergillus* have been reviewed in greater detail elsewhere [35,79–81].

Although rabbit models are quite useful for the study of fungal infections, and their treatment, they are more expensive to purchase, their husbandry requires special installations and careful monitoring, and greater efforts from support and animal care personnel, there are few immunological and biomolecular reagents available and there is a lack of genetically defined animals. Each of these factors needs to be considered and each can impose limits on the use of this species as an animal model for studies that includes these topics.

The next most commonly used species in medical mycology is the guinea pig. Guinea pigs are primarily used as a model for experimental cutaneous infections by *C. albicans* [82,83] *Trichophyton mentagrophytes* [84,85] and seborrheic dermatitis caused by *Malassezia* sp. [86]. In a model of tinea unguium in guinea pigs, inoculation with *T. mentagrophytes* between the toes resulted in nail collapse [87]. Guinea pigs have been used in therapeutic studies including systemic aspergillosis [88–92], cryptococcosis [93] and scedosporiosis [94]. Other models involving guinea pigs include a model of endocarditis caused by *A. fumigatus* [95], and a model of pulmonary histoplasmosis induced by aerosolized microconidia and hyphal fragments of *H. capsulatum* [96]. Guinea pigs are often used to test voriconazole (VCZ) efficacy [88,94,97–99] because, according to different authors, VCZ reaches therapeutic serum levels in this species comparable to those in humans [100–102] whereas, the drug is rapidly metabolized in mice and induces its metabolism. A drawback to the use of guinea pigs is that these animals have a complex social structure and are easily stressed under unfamiliar environments or housing conditions or the manipulations required during the course of the experimentation [103,104]. They are relatively expensive to maintain and board, and lack congenic strains.

The use of birds in models of aspergillosis deserves mention, since naturally acquired aspergillosis in the absence of immunosuppression is an important disease in avian medicine [35,105,106]. Both intravenous inoculation and pulmonary routes (intratracheal or aerosol) have been used to infect the animals, which include Japanese quail, turkeys, guinea fowl and chickens for studies of pathogenesis, therapy and vaccines [35,105]. The use of birds as a model for aspergillosis is beneficial in that therapeutics or vaccines against aspergillosis can be studied directly in the affected species rather than trying to translate results of therapy or vaccine work from rodents or other animals.

Routes of infection

When performing an animal model of fungal infection one must consider what route of infection to use. The various routes of infection include, pulmonary (intranasal or intratracheal), intraperitoneal, intravenous, intracranial, intrathecal or intracisternal, mucosal (vaginal or orogastric), dermal (skin or cornea) and subcutaneous. Each of these routes of infection has been used to study particular infections [34–36,38,41,107,108]. It is important to point out that animal hosts may differ in their susceptibility to challenge with the same pathogen by different routes. Our laboratory reported differences in mouse susceptibility to *Blastomyces dermatitidis* between intranasal and intraperitoneal infection. The same strain of *B. dermatitidis* at the same dose (30 CFU per animal) caused death of 50% of C3H/HeJ mice after intranasal infection vs. 17% when infection was made intraperitoneally. However, DBA/1J mice were more susceptible to intraperitoneal (83% mortality) than to intranasal infection (25% mortality). Thus, these data demonstrate that both route and mouse strain play a decisive role in modeling experimental fungal infection [109].

The majority of serious fungal infections are acquired by inhalation of infectious elements or by a breakdown of the natural body barriers i.e., skin and mucosa. Thus, in many instances a pulmonary route of infection would be optimal to initiate the model because it mimics the natural entry into the human body. For example, pulmonary inoculation with *C. neoformans* showed generalized dissemination of the fungus, with the brain and lungs heavily infected 42 days after challenge [110], mimicking human cryptococcosis. However, the development of infection and the resulting affected organs potentially can differ between animals and humans due to differential organ tropism of the infecting microorganism, the organism's greater ability to grow in a particular organ environment or host factors. An example is substantial renal involvement in various models of aspergillosis, which is less common in human infection [35]. In light of differences in pathogenesis and organ tropism, we have the option to mimic the infectious process, which is the goal of the animal modeling, by modifying the route of infection.

Several models of systemic or localized infection have been described. Systemic infections using intravenous

inoculation are used often in models of infection to reproduce in the animal a mimic of human dissemination, independent of the natural entry of the pathogen on the host. Systemic infections inoculating a suspension containing different fungal elements intravenously have been tested with the most common pathogenic fungi. Systemic infections of *Aspergillus* spp. [3], *Cryptococcus* spp. [111], *Candida* spp. [35,36,38,107], *Coccidioides immitis* [34,39], *Histoplasma capsulatum* [34,39], *Scedosporium* spp. [98,112,113], *Sporothrix schenkii* [114], *Paracoccidioides brasiliensis* [115], *Penicillium marneffei* [116], *Hansenula anomala* [117], *Fusarium* sp. [118] or *Trichosporon asahii* [97] have been described in the literature using different mammalian species. The intravenous route depends on anatomic characteristics of the animal species. In mice or rats systemic infection is performed most often by using the lateral tail veins. However, in other animals where tail veins are not easily accessible or do not exist, other routes are used. The lateral ear vein and the penile vein are the choose routes for infection in rabbits and guinea pigs, respectively.

Localized or site-specific infections have been developed over the years of animal modeling to simulate the pathology in the target organs associated with one site of infection in humans. With this purpose, pulmonary, brain, intraperitoneal, ocular, superficial, vaginal or subcutaneous models have been developed.

CNS is a common site of hematogenous dissemination of fungi such as *A. fumigatus, C. neoformans C. immitis, Scedosporium prolificans* and some phaeohyphomycetes in humans and infection there can result in high mortality. Mimicking the natural pulmonary establishment of a pathogen does not always result in dissemination to the CNS in animals. In the case of cerebral aspergillosis direct intracranial inoculation of conidia results in high tissue burdens in the brain, with the histopathological lesions and cellular host-response similar to the observations in human infections involving the CNS, although this is not the natural route by which humans acquire the infection [119].

Different techniques such as the intracerebral (or intracranial), intrathecal or intracisternal inoculation are available for the study of fungal infections associated with the CNS. Intracranial infection is performed by direct inoculation in the brain parenchyma through the midline of the cranium in mice [120]. However, as mice mature the sutures between the frontal and parietal plates fuse, making the bregma more difficult to locate. Thus, injecting through the fontanelle is useful only for mice up to about 8-weeks of age. Although infection spreads to other organs, CNS fungal burdens remain higher than in other organs [121,122]. In the example of aspergillosis, hyphal growth and development of abscesses and necrotic areas mimic the common complications observed in clinical cases of cerebral aspergillosis [119,123]. Intracerebral models of infection also have been used to test therapeutic options for treatment of aspergillosis, coccidioidomycosis, scedosporiosis, histoplasmosis and cryptococcosis [121,122,124–128]. Another route of infection to establish CNS infection in mice is intrathecal inoculation, which has been used in a model of coccidioidomycosis to simulate the meningitis and vasculitis associated with CNS complications caused by hematogenous dissemination of the fungus in human disease [129]. The technique requires a skin incision over the lumbar vertebrae and inoculation of an arthroconidial, spherule or endospore suspension into the subarachnoid space via lumbar puncture [129]. Via this route of infection, the fungus can be recovered from brain and meninges as early as three days after infection, and by 8 days postinfection, the histopathologic abnormalities found in the brain or spinal cord include acute meningitis, vasculitis and polymorphonuclear cells infiltrates [129]. Similar to the intracerebral route of infection, extrameningeal dissemination is observed; however, CNS involvement is greater than that reached in systemic or pulmonary infections [129]. Intracisternal inoculation for the establishment of infection in the CNS has been used as route of infection in rabbit models of meningitis by *C. neoformans* and *C. immitis* [49,50].

Pulmonary diseases can be initiated using different techniques. The most common technique is by intranasal instillation of a fungal suspension. However, the number of fungal elements arriving in lung tissue can be uncertain, depending on the anesthesia used and experience of the person doing the procedure. In early studies, our laboratory found that, using a standardized procedure, 1 h after intranasal inoculation with *B. dermatitidis* yeasts, 33.5% of the inoculum CFUs could be routinely recovered from lungs, and results in a consistent model of pulmonary blastomycosis [130]. This technique is easy to perform and several reproducible models of fungal infection have been established including pulmonary aspergillosis [3,35], cryptococcosis [131], histoplasmosis [34,39,46], blastomycosis [34,39], paracoccidioidomycosis [39] and zygomycosis [38,75,132] in different mammalian species. Some investigators prefer to use inhalation of dry fungal conidia as the method of pulmonary infection. A comparative study using titanium dioxide particles showed that inhalation results in a more homogenous distribution of the particles in lungs than does the instillation technique [133]. In another study, delivery

by inhalation was compared to intranasal instillation for the establishment of pulmonary aspergillosis in rats; higher fungal burdens with smaller standard deviation and more homogenous pneumonia was reported in animals infected by inhalation than by instillation [108]. The use of aerosol chambers reproduces, with the exception of higher numbers of fungal conidia inhaled, the natural entry of the microorganism in the respiratory tract. Separate studies have demonstrated the reproducibility of delivering *A. fumigatus* conidia to the lungs using inhalation [108,134,135]. The third method used for inoculation for pulmonary infection is the intratracheal route. Although better for the consistency of inoculum administration, it requires a minor surgical procedure. An incision needs to be made and organisms injected directly into the trachea. Various models using this method have been reported such as those using *Penicillium marneffei* [116], and *C. albicans* [70,71], as well as others [35,36,38,39].

Mucosal sites of infection include oral, gastrointestinal tract and vaginal and have been used primarily in rodents for studies of *C. albicans* [136,137]. Establishment of gastrointestinal infection is done by gavage with a suspension of the yeast, allowing the animals to drink from a suspension of yeast or by feeding the fungus in pellets. Immunocompetent, immunosuppressed, congenitally immunodeficient, germ-free and antibiotic-treated mice have all been used in this type of model [137]. In addition, adult and infant mice have been used [136,138]. The model can be manipulated to mimic different patient populations at risk for development of mucosal candidiasis by altering the immunosuppressive regimen or changing the strain of mouse used. Several studies have used neutropenic mice to examine *Candida* translocation from the gut as a source of disseminated disease [38,137,139–143], a syndrome that occurs in neutropenic and chemotherapy patients. In contrast, AIDS patient develop mucocutaneous candidiasis, but the yeast do not disseminate. Orogastrointestinal candidiasis in SCID mice shows a similar lack of dissemination from the gut and thus provides a good model for studies of therapy and host-interaction [144,145].

Studies on vaginal candidiasis are also done in rodents and primarily in mice, although other species have been used [38,146–148]. Infection of animals is easily done by lavaging a suspension of yeast into the vagina. To establish and maintain vaginitis in mice it is a requisite that estrogen be administered to induce estrus [38,149]. Interestingly, not all mouse strains are equally responsive to the effects of exogenous estrogen treatment, with CD-1 mice being particularly resistant to estrogenization [150]. This resistance to estrogeniza-

tion carries over to resistance to the establishment of experimental vaginal candidiasis [151,152]. Thus, choice of mouse strain can be critical to the success of the model. The use of the vaginal model has included pathogenesis, therapeutic and host-response studies [38,146,153–158].

Organ specificity

As mentioned before, when performing a model of fungal infection one must be aware that each fungus may preferentially grow in a particular organ system in the animal and these may not be the same as the primary tissues infected in humans. These tropisms may be apparent regardless of the route of infection or can also be determined by the route of infection as discussed above.

For example, in most murine models of candidiasis using outbred or inbred mice infected intravenously, the kidney is the primary organ infected, with lungs, brain, liver and spleen infected more transiently [159]. Although kidney infection is not so prominent in humans, renal infection in mice does mimic the course of sepsis in humans [1]. However, the use of a genetically deficient mouse strain, beige mice, shows a change in tissue tropism with the liver and spleens having progressive disease [160].

Infection of mice with *C. neoformans* provides an excellent example of a fungal model that demonstrates the same organ involvement, CNS, as is found in humans. Although the reasons are not entirely elucidated, *C. neoformans* requires exogenous substrates to synthesize melanin, and some suggest than the tropism of *C. neoformans* for the brain could be due to its capacity to make melanin from catecholamines. Observations showing that areas of the brain rich in catecholamines are frequently infected by *C. neoformans* support this suggestion. Recently, we have reported differences in organ involvement between *C. gattii* and *C. neoformans* var. *grubii* in a systemic model of cryptococcosis in BALB/c mice [161]. Infection with *C. grubii* showed a temporal increase of CFU in brain and liver but no yeast were recovered from the brain of animals infected with *C. gattii*. Interestingly, and reported for first time, long-term infection with *C. gattii* resulted in unusual skin and intestinal mucosal lesions in hydrocortisone-suppressed and in immunocompetent BALB/c mice, whereas these types of lesions were not found in *C. grubii* infected mice [161].

Murine models of blastomycosis provide examples for tissue involvement based on routes of infection. In humans, primary pulmonary disease with dissemination occurs; cutaneous lesions are common sites of

disseminated disease. In mice infected intranasally with *B. dermatitidis*, pulmonary disease is quite severe and lethal [39,41,130]. Although the organism does not disseminate from the lungs to other tissues in the acute infection produced in the mouse, cutaneous blastomycosis is mimicked by using subcutaneous inoculation as the route of infection [39,41].

Virulence studies

Historically, the comparative virulence of strains of fungi (e.g., a mutant and the parent, or multiple isolates of the same species) has been studied in these models and comparisons made based on lethality or fungal burden in the target tissues. Animal modeling is a useful method to determine the severity of the experimental infection as a correlate of the virulence of the infecting fungus. Severity is primarily evaluated by mortality and quantitative recovery of the fungus in affected organs, while histopathology of affected tissues and the host response give us a picture of the interaction between host and pathogen *in vivo*. Understanding the mechanisms regulating fungal virulence and the interaction with the host is necessary for a better understanding of the infectious process. The ability of the fungus to survive, grow, and evade the immune system in mammalian tissues seems to be a strain attribute that depends on the expression and regulation of various genes (e.g., virulence factors).

Classically, virulence factors have been considered to be those traits that are present in the microorganism that are needed to cause disease and when absent, the microorganism loses its ability to produce disease, but the loss of the virulence factors does not alter the ability of the organism to grow under other conditions. In bacteria, adhesion, colonization, invasion, immune-response evasion, capsule formation and toxin release are strongly related to virulence. Although capsule formation by *Cryptococcus* spp. is clearly a virulence factor [162], other factors, responsible for virulence in fungi, are not as well defined.

Because most fungi have a free living saprobic phase, not requiring a host to complete its biological cycle, it would seem that infection is merely an accidental encounter between the fungus and the host. As a consequence, we can postulate that fungal physiology has developed as a result of a natural selection outside of human tissues, and the mechanisms that give the fungus the ability to cause infection may be cross-linked to the necessity of survival, in the environment.

For example, melanin is a hydrophobic pigment present in several species of pathogenic fungi [163,164]. Melanin seems to play an important role as a virulence factor and contributes to the severity of the disease. Nonpigmented strains of *C. neoformans,* generated by UV spontaneous mutation, were shown less virulent than pigmented strains in animal models, but the mutation did not result in avirulence [165]. Similarly, melanin-deficient *Wangiella dermatitidis* [166,167] and *A. fumigatus* [168,169] showed loss of virulence in comparison to their respective parental wild-types in murine models. Important roles have been attributed to the melanin as a virulence factor, contributing protecting the organism from oxidative stress and macrophage digestion in mammalian tissues and give higher resistance to antifungal agents [170,171].

Strains of fungi

Studies of comparative virulence among isolates of a fungal species have shown, for many organisms, that when inoculated into an animal in equal numbers of organisms, some isolates are more pathogenic than are others. Differences among these may be large or relatively small depending on the organism. Thus, it is crucial to determine the relative virulence of the fungal strain under study prior to embarking on additional studies looking for virulence factors or pathogenic mechanisms. Studies on virulence and host-resistance most often are done using one strain of fungus and several strains of mice or several strains of fungus and one strain of mice. An example of how this approach can be troublesome comes from our experience with studies on the pathogenicity of *Saccharomyces cerevisiae*. Using a murine model studied in CD-1 mice we surveyed 28 clinical and nonclinical isolates of *S. cerevisiae* for their capacity to proliferate and persist in the brain [172]. A continuum of virulence was found ranging from those able to proliferate by about 5-fold to those that were rapidly cleared from the tissues [172]. In a subsequent set of studies we examined these same isolates in a different strain of mouse, DBA/2, and found that some of the isolates were lethal (increase in virulence), but that the rank-order of virulence was not the same in DBA/2 mice as it was in CD-1 mice [173]. These differences indicate the potential for multiple strategies of virulence [173]. Thus, one should not assume that because an organism appears virulent or nonvirulent in one model that it will appear the same way when used in a different model of infection.

Molecular tools and fungal gene knockouts

With the advent of molecular biological techniques, studies of virulence factors have routinely used a 'gene knockout' approach, where one or more genes are removed or inactivated or replaced and the virulence of

this strain is compared with the wild-type parental strain [174]. Numerous studies have been done to identify virulence factors and mechanisms that mediate the pathogenesis of the fungi and have been presented in detail elsewhere [3,9,35,41,75,115,163,164,175–186].

Examples of these types of studies follow. Filamentous growth of *C. albicans* is important in establishing mucosal infection [187]. Disruption of genes involved in filamentation resulted in decreased virulence in animal models. Mutation of *int1*, a gene involved in filamentation and adherence in *C. albicans* [188] showed reduced mortality in a systemic murine model, but fungal burden demonstrated higher persistence in kidneys than did the wild-type strain [189]. *Cph1* and *efg1* are transcriptional regulators in controlling filamentous growth in *C. albicans* that activate different pathways involving filamentation. *Efg1* seems to drive the major pathway for filamentation and *cph1* has a lesser role. However single mutants for each of these regulators and double mutants still showed capacity to form filaments as a function of the medium used, suggesting that additional genes are involved in filamentation [190]. Double *cph1* and *efg1* mutants were found to be avirulent in a mouse model [191].

Several genes and proteins of *A. fumigatus* have been also been linked to pathogenesis. Genes involved with cell wall integrity, evasion of the immune response, toxins and extracellular enzymes related to direct attack of the host tissues have been recently and extensively reviewed [192]. *Aspergillus* strains defective in particular genes involved may demonstrate a reduction of virulence, but can still retain pathogenic capability. For instance, double chitin synthase mutants (*chs*C-/*chs*G-) showed reduced chitin synthase activity (required for cell wall assembly), as well as anomalous growth in *A. fumigatus*. However, the double mutant, despite showing reduced pathogenicity in comparison to the wild-type strain, still caused pulmonary disease in a neutropenic mouse [193]. Similar results were found in *chs*E mutants [194]. Deletions of *rhbA*, *fos-1* and *pksP* genes also show reduction of virulence in models of murine aspergillosis [195–197]. Histidine kinases [198,199], and calcineurin [200–202] serve as additional examples of genes linked to virulence by studies done in animal models.

As should be evident, using the approach of studying single genes and their impact on virulence is a slow and tedious process, requiring many animals. More recent advances in the study of virulence factors have taken other approaches. One method to assess virulence is that of *in vivo* gene expression and there are two related methodologies for gene-profiling of fungi during infection that use reporter genes. These are *in vivo* expression technology (IVET) or constructing fusion proteins tagged with green fluorescent protein (reviewed in [203,204]). IVET has been successfully used for *C. albicans* [205] and *Histoplasma capsulatum* [206]. In *Candida albicans* IVET demonstrated that *SAP2* was expressed during systemic, but not mucosal murine infection. For *A. fumigatus*, Langfelder *et al*. [207] formed a green fluorescent protein (GFP) fusion with polyketide synthase (*pksP-egfp*) and followed the *in vivo* expression of this GFP-fusion protein during pulmonary infection in corticosteroid-suppressed mice. They showed expression of *pksP* in the hyphae of germinating conidia in the lungs of infected animals.

A second method of assessing virulence is that of inoculating animals with a pool of mutants and letting the mouse eliminate those that are not virulent. In studies on *S. cerevisiae*, pools of isogenic strains were used to infect mice to determine genes important to survival *in vivo* by determining which of the isogenic strains was recovered from the animals [208]. Signature Tagged Mutagenesis (SMT) has been applied successfully to the study of *in vivo* gene expression of *Cryptococcus* [209] and *A. fumigatus* [210] in murine models. This methodology uses a transposon mutagenesis system in which each transposon mutant is tagged with a unique DNA sequence that is to be followed using PCR and Southern blotting. To examine *in vivo* expression, a pool of individually tagged mutants is prepared and inoculated into mice and organisms recovered at various times postinfection. In the *Aspergillus* studies pools consisted of 84 or 96 unique mutants, which increases the number of mutants that can be screened in one experiment [210]. The use of this methodology for *A. fumigatus* resulted in the identification of PABA synthase as an essential gene for virulence [210]. Similarly, the *Cryptococcus* work resulted in the identification of 5 strains with reduced virulence and one strain with increased virulence [209].

Lastly, *in vivo* gene profiling is a desirable method to use for the detection of genes potentially relevant to virulence. Comparisons of *A. fumigatus* transcription *in vivo*, during pulmonary infection of mice, to *in vitro* transcription, demonstrated higher transcription of *rhbA*, a RAS-related protein and *fos-1*, a gene encoding histidine kinase protein *in vivo* than *in vitro*. Interestingly, expression of *pksP* related to conidial pigmentation was only detected in pulmonary infection, but was not detected *in vitro* [211]. *In vivo* gene expression has also been examined by serial analysis of gene expression (SAGE) technology in elegant studies done on *C. neoformans* in a rabbit model of meningitis [212]. Generation of sequence tags (ca. 14 bp) for each cryptococcal gene allowed for RNA expression of the

organism to be followed. The results of the study indicated that *C. neoformans in vivo* showed over 300 highly expressed sequence tags (i.e., genes), involved in a variety of cellular functions [212] and will allow more focused efforts on particular pathways and gene sets.

Studies of host response

Infection can be considered as an imbalance between the host-defenses and the infectious agent, with the host unable to control the proliferation of the infectious agent. How the host is able to fend off fungal infectious or why the host is susceptible to fungal infections is an area of intense study, for which animal models of infection have proven invaluable.

Historically the use of animal models, and particularly murine models, has been crucial to our understanding of host-response to fungal infection. The greater susceptibility of immunosuppressed animals to the mycoses, the increased refractoriness of immuno-suppressed animals to antifungal therapy, and, in aspergillosis, the requirement for immunosuppression in order for progressive invasive pulmonary disease to develop, corroborate findings in clinical medicine. Various methodologies and strains of mice have been used to correlate the importance of various cell types (e.g., PMNs, macrophages, different lymphocyte populations, antibodies, etc.) [39,213,214]. Good examples of these studies are the use of spontaneously occurring mutations in mouse strains such as athymic nude mice, depletion of particular cell populations or cytokines by antibody treatment (e.g., anti-PMN, anti-mu, or anti-interferon-γ) or other means of cell depletion like silica or carageenan treatment or immunosuppressive cyto-toxic regimens [39,213]. Much progress has been made with the advent of site-directed mutagenesis for generation of cytokine or receptor deficient strains of mice, cloning and availability of specific cytokines for use *in vitro* or *in vivo* and in the methods that can be used to detect and follow the global genesis of the host's response to infection. These methodologies continue to reveal to us the complicated nature of the host-parasite interaction. Given the vast number of articles on these subjects, we will only present a brief overview addressing more recent studies and use examples of how animal models of fungal infection have been used to move the field forward.

How does the host respond during a fungal infection and which aspects of the immunological response are most important? This question is multifaceted and quite complex, and as yet not fully answered as the host-response can be tissue specific and organism specific. Animal models provide us the means with which to address different issues.

Laboratory manipulations

As the reader will remember from the introductory remarks, no single model can be used to answer all questions. Therefore, the investigator may need to perform a manipulation of the animals to address a particular immunological question. These manipulations include, but are not limited to administration of an immunosuppressive regimen (e.g., cytotoxic chemotherapy or glucocorticoids) given alone or in combination with antibiotic regimens to prevent secondary bacterial infections, antibodies to interfere with or inactivate a cell population (e.g., anti-PMN) or particular cytokine (e.g., anti-IL-12), administration of a chemical or drug to induce a metabolic change (e.g., streptozocin to induce type II diabetes or estrogen to induce estrus), surgical alteration of animals (e.g., thymectomy, orchidectomy, or ovariectomy), gamma-irradiation, and changes in diet (e.g., amino acid deficiency). Each of these manipulations affects the host-response to infection and those effects should be defined. For example, in recent studies on orogastrointestinal candidiasis we found that administration of 5-fluorouracil resulted in dissemination of *C. albicans* from the gut tissues to visceral organs, nicely emulating a clinical situation of the cancer chemotherapy patient that develops systemic candidiasis [139]. However, during our preliminary studies we noted that mice were succumbing to secondary bacterial infection rather than fungal disease and found it necessary to include a combination regimen of oral and parenteral antibacterials to allow for the development of the candidal infection [139].

Genetic manipulations

Most useful for studies of host response to fungal infections are animals, particularly mice, with defined genetic defects in their immune systems. Animals of this type include T-cell deficient nude mice, beige mice with phagocytic cell degranulation defects, chronic granulo-matous disease mice with defects in generating reactive oxygen species by phagocytes, and SCID mice with T and B cell defects. Each of these types of animals has been used in studies of fungal infection examining host-response [35,36,38,39,107,215,216]. However, a drawback to these types of animals is that often the mutation responsible for the defect has pleiotropic effects, which may or may not influence the results. Although nude mice have no mature T cells, their macrophages have been described as being at a higher

basal state of activation and they have increased numbers of NK cells [217] and have, for example, shown paradoxical resistance early during infection with *C. albicans* [218].

More desirable and now possible with the advent of modern molecular techniques is the generation of gene knockout (KO) mice, which have one or more specific genes inactivated or deleted. The commercial availability of these animals, although quite expensive to purchase, makes a variety of studies possible. Several of these strains of animals have been mentioned already, but include KO for a specific cytokine, enzyme, cell receptor, etc. These types of mice have become invaluable for determining the role played by a particular molecule in host response [177,214,219]. Possibly confounding effects can occur because of redundancy in the immune system (more than one gene may control a defense pathway), or compensatory hyperactivity (up-regulation of gene expression to compensate for the gene knocked out).

Genetic susceptibility to fungal infection in humans is a growing field of study [220], and it benefits from observations of genetic susceptibility in mice [150,173,221–224]. In addition, the continued accumulation of haplotype polymorphisms into databases for mouse strains allows investigators to further refine their searches for genes or gene families involved in susceptibility [220]. Furthermore, the advent of molecular manipulation has allowed for the insertion of human genes into mice resulting in a transgenic humanized mouse. For example CD13 insertion has been studied in relationship to the pathogenicity of human coronavirus in mice [225]. These types of studies further refine and extend the potential of murine models for the study of disease, since they permit study of human-specific molecules and/or cells in an *in vivo* environment. Future studies using transgenic mice humanized by the insertion of a single human gene (e.g., cytokine or chemokine receptor molecules, Toll-like receptors, etc) will likely be applied to fungal diseases and will undoubtedly provide extremely interesting results.

Cytokine expression

As we have come to learn over the last 30 years, cytokines are the primary cellular signals responsible for the activation and modulation of the primary innate cellular host-response, as well as the genesis and development of the adaptive response to infection. The expression of particular cytokines signal whether the host-response will be protective or nonprotective as they drive it towards a Th1 or Th2 response. Studies on

the genesis of host response can be greatly aided by examination of the cytokine response *in vivo* [214,219,226–229]. Animal models provide the possibility of examining specific tissues or organs for cytokine expression and the availability of immunological reagents for mice and variety of strains of mice make them ideal for these studies. Methodologies include ELISA assays for cytokine proteins, cytokine protein macroarrays, RT-PCR assays for individual cytokines and mouse DNA microarray for use in examining the global host-response to infection. The use of other animals as models for studies of host-response is more limited due to the severely limited availability of immunological reagents and genetically defined animals.

Examples of studies examining cytokine expression include infection with *Aspergillus, Candida, Cryptococcus, Coccidioides, Histoplasma, Paracoccidioides,* and *Pneumocystis* [230–244]. Each of these used a mouse model. In contrast, examination of cytokine expression in rabbit models of fungal infection is extremely limited due to a lack of reagents. Using a rabbit model of meningeal coccidioidomycosis our group has examined the temporal expression of mRNA for several cytokines in the basilar artery. We accomplished this by developing our own primers for sequenced rabbit cytokines and used PCR methodology to demonstrate up-regulation of mRNA encoding for different proinflammatory interleukins, as well as MCP-1, iNOS and MMP-9 in the brain basilar artery [245]. The rabbit will become an even more useful animal for modeling as additional immunological reagents become available and research is done on the immune response of the rabbit.

Innate and adaptive immunity: genesis and regulation of the immune response

Innate immunity is phylogenetically the oldest mechanism of defense and is present in all multicellular organisms [246]. Soluble proteins made by the host play an important role in innate immunity to fungal infection. The activation of complement promotes inflammation via chemotactic subunits as well as opsonization of fungi [247–249]; several fungi activate complement through the alternate pathway [247–249]. Studies in murine models have shown the importance of complement controlling infection by *C. albicans.* DBA/2 mice, which are defective in C5, are extremely sensitive to systemic candidiasis and aspergillosis [221,222]. Infusion of C5-sufficient serum into DBA/2 mice infected systemically with *C. albicans* increased survival of the animals, reduced tissue burdens of fungus and reduced the severity of lesions in the

kidney, but not in brain [250]; transfer of C5-sufficient serum to normal and neutrophil-depleted DBA/2 mice suggested that the complement system plays an important role controlling the proliferation of yeast during initial stages of the infection [250,251]. However, based on murine studies of comparative susceptibility, complement may not play a significant role in host-resistance in other fungal infections such as histoplasmosis, coccidioidomycosis or paracoccidioidomycosis [39,213,247,248].

Ingestion of microbes into lysosomes and phagolysosomes first requires recognition of antigens, which is mediated by several cell surface receptors. The receptors and the signaling pathways involved have become an area of intense study. Pattern recognition proteins play a significant role and include various molecules such as dectin-1, TLR, and collectins [26,27,214,252–255]. The following paragraphs provide examples of how animal models have been used to elucidate the role played by these molecules in fungal immunity.

Mannan-binding lectin (MBL) is a triple helix of peptides that recognizes terminal mannose residues. MBL binds to the pathogen and activates MBL-associate serine protease-2 (MASP-2), which is involved in activation of C4 and C2 fragments via C3b through the lectin pathway of complement, stimulating the innate response against a spectrum of infectious diseases [256,257]. MBL recognition of pathogens stimulates the immune system through the activation of complement, promoting the killing of pathogens either directly through complement activation or by opsonization, as well as effects on the pathogen by targeting the ligands [258–261]. MBL deficiency has been associated with higher risk for infection by virus, bacteria and fungi [258,262–266]. MBL appears important in resistance to vaginal infection due to *C. albicans* and deficiency is associated with recurrent episodes of disease [260]. Experimentally, administration of recombinant human MBL to MBL knockout mice significantly reduced the number of CFU of *C. albicans* in vaginal mucosa and potentiated the effect of itraconazole therapy [267].

The soluble collectin surfactant proteins, SP-A and SP-D, are important in innate immunity to pulmonary infection, binding to organisms, in some cases killing, and contributing to regulation of inflammatory response in the lung [268,269]. Several studies have examined the role of these collectins in innate immunity and their interactions with fungi [224,270–273]. The generation of SP-A and SP-D deficient mice allowed for *ex vivo* studies with *Blastomyces* indicating the SP-D is bound by the organism and thus blunts the host reaction and production of TNF-α [272].

The cellular receptor TLR family of proteins recognizes microbe-derived molecules and stimulates the innate immune response by inducing the production of proinflammatory cytokines. The inflammatory response is induced after binding of a TLR with its ligand, which subsequently induces, such as through the MYD88-dependent pathway, transcriptional regulation of TNF-α through the activation of the NF-κB pathway in macrophages and dendritic cells [26,214,219]. Since this family of proteins was first described in *Drosophila* sp., 10 different TLR have been identified in mammals based on sequence homology to the *Drosophila* Toll protein [274]. TLR-4 and TLR-2 contribute strongly in signaling responses to *A. fumigatus* and *C. albicans,* as studies relating deficiencies in TLRs to severity of disease have shown [26,275–277].

Experimentally, peritoneal and alveolar macrophages from TLR-2$^{-/-}$ mice showed a significant decrease in TNF-α production compared to naïve cells following *A. fumigatus* infection [24]. Experimental aspergillosis in TLR2 knockout mice resulted also in low production of TNF-α and IL-12, as well as reduced survival and higher fungal burdens in the tissues than competent mice [24]. Blease *et al.* reported that TLRs could be regulated by circulating interleukins, when IL-18 defective mice infected with pulmonary *A. fumigatus* showed a down-regulation of TLR-2 mRNA in comparison to that from IL-18 competent animals [278]. Regulatory processes acting on TLRs play an important role in orchestrating an adequate innate response against specific pathogens, introducing a new concept about 'specificity' of the innate response [279,280].

Natural killer (NK) cells are a population of large granular lymphocytes present in non-immune animals and are well known for their capacity to kill tumor cells by cytotoxic mechanisms. NK have also been proposed to play a role in innate immunity against virus, bacteria, parasites and fungi [281]. Cell wall components from *C. neoformans* and *C. albicans* increase NK activation [282–284]. Murphy *et al.* demonstrated than NK recognize cell wall components of *C. neoformans* and made contact with the microorganism by microvilli, demonstrating differences in the binding mechanisms used by NK for *C. neoformans* and tumor targets [284]. Inhibition of NK by anti-NK antiserum increased the CFU of *C. neoformans* in lungs of mice after systemic infection in comparison to the control animals but not in liver, kidneys or brain. However, no differences were reported when the intrathecal route of infection was used [285]. From later murine experiments it appears that NK cells potentiate macrophage anticryptococcal activity by production of IFN-γ rather than by direct killing [286,287].

Lymphocyte T Helper (Th) cells are subsets of the $CD4^+$ T cell lineage that modulate the immune response by cytokine release. From the $CD4^+$ subsets, Th1 and Th2, differentiation of Th1 cells is the predominant response to infections by invasive fungi and Th2 in allergic responses to inhaled fungi on mucosal surfaces [288]. Although these patterns of response are constant in mammals, there are significant differences between human and, for example, mouse immunology that must be taken into account. Differences in TLR expression, cytokine secretion, Th1/Th2 differentiation, and antigen-presenting functions, among others, have been extensively reported and should be taken into account to interpret data obtained from animal modeling [214].

Classically, cytokine release by Th1 cells has been known as pro-inflammatory cytokines, because of their roles in enhancing macrophage activation and promoting inflammation. Activated macrophages and dendritic cells induce Th1 cell development through a STAT4-dependent pathway by major release of IL-12. The major effector function of Th1 cells is to promote activation of macrophages by IFN-γ. The use of IL-12 knockout mice has shown them to be highly sensitive to *C. albicans*, *H. capsulatum*, *C. neoformans* and *C. immitis* infection, demonstrating the importance of this cytokine to resistance to these organisms [289–293]. However, the up-regulation of this cytokine can be also responsible for an uncontrolled inflammatory process that affects the host tissues. It has been reported that administration of exogenous IL-12 induces a less severe infection by *P. brasiliensis* in mice, but increases the severity of inflammation [294].

In contrast to IL-12, IL-10, secreted by Th2 cells, plays a role in inhibiting the inflammatory response mediated by Th1 cells and also promotes the Th2 response [295,296]. Down-regulation of Th1 response results in diminution of resistance to various fungal pathogens. Data from animal models support this inflammatory interaction, since IL-10 KO mice have been shown more resistant than control wild-type mice to infection by clinically important fungi [234,297–299]. It has been reported that neutralization of IL-10 up-regulates production of nitric oxide, contributing to an effective fungicidal effect against systemic candidiasis [300]. However, a reported pivotal function of IL-10 shows the flexibility of the immune response and its regulation. For example, physiological levels of IL-10 were required for Th1 development in IL-12 KO mice indicating the relevance of a finely regulated balance in cytokine expression [301].

Although Th1 cells are viewed as necessary in defending the host against acute fungal infections, Th2 cytokines principally down-regulate the inflammatory response. In systemic aspergillosis in mice, administration of exogenous administration of IL-12 failed to induce resistance, whereas IL-4 cured 70% of the mice and protected them from a second lethal challenge [235]. Stimulation of IgE production and eosinophil activation by Th2 cytokines are responsible for the prolonged immune response and the hyper-reactivity reported in allergic aspergillosis [302,303]. Recent studies suggest members of the IL-17 cytokine family as regulators of the Th2 response instead of Th1 in pulmonary aspergillosis [304].

The function of T cells is clear against fungal infections, but the role of B cells in protection from most fungal infections is less certain. Recent studies showed the importance of B cells in regulating the severity of systemic cryptococcosis. B cell defective mice showed greater susceptibility to systemic and pulmonary infection than did C57BL/6J mice suggesting a protective B response. However, transferring IgG to B-cell defective animals did not improve the course of the infection, but did in C57BL/6J. In addition, higher levels of IFN-γ, MCP1 and MIP-1α and lower levels of IL-12 were found in B cell defective mice than in C57BL/6J animals after infection [305]. These observations may be due to an over-exuberant Th1 response in response to the infection and suggest immunomodulatory effects of B cells and antibodies [305,306]. Similarly, Magee *et al*. demonstrated by using microarray technology, up-regulation of B-cell related genes in mice immunized with formalin-killed spherules, and that immunization of B-cell deficient MuMT mice, resulted in no protection against coccidioidomycosis, which supports the protective role of B cells [307].

Although the innate immune response is a primary host-defense mechanism, long-lasting resistance to re-infection due to acquired immunity is also very important [214,219,226]. Adaptive immunity is the result of long-lived memory CD4 and CD8 T cells, which upon restimulation with a specific antigen initiate the inflammatory immune response, phagocytic cell activation and enhancement of cellular mechanisms of killing. There is strong evidence that infection and recovery from infection with various systemic fungi, *Histoplasma, Coccidioides, Blastomyces* and *Paracocci-dioides,* corroborated in animal models, lead to life-long immunity [214,219,226]. In contrast, infection and recovery from disease due to an organism such as *Candida* does not appear to lead to life-long immunity and re-infection can occur (e.g., recurrent vaginitis).

Modulation of host response

There are a number of different methods used to modulate the host response. The inflammatory response can be down-regulated with glucocorticoids, inhibitors of TNF-α such as pentoxifylline [308], antibodies to specific cytokines or cytotoxic chemotherapies. Up-regulation or stimulation of the immune response can be done by administration of specific cytokines (e.g., TNF-α, IFN-γ, IL-12, CSF's), specific antigen administration, and nonspecific stimulators such as alum, BCG, muramyl dipeptide, CpG DNAs, monophosphoryl lipids and saponins. The latter are used most often as adjuvants in vaccine preparations [309].

A primary focus of immuno-modulation of fungal infections has been in therapy using particular cytokines as adjunct to conventional therapy [107,227,228,310–314]. Administration of exogenous IFN-γ to mice has proven effective in cryptococcosis, histoplasmosis, paracoccidioidomycosis, candidiasis, and aspergillosis [115,315–324]. Similarly, IL-12 and IL-18 stimulate mice to be more resistant to cryptococcosis and histoplasmosis [287,293,325–333].

Colony stimulating factors (CSFs) have also been used as adjunctive stimulators of the host response [107,213,227,228,310,311]. For example, efforts to reverse neutropenia or enhance PMN production in normal mice by exogenous administration of G-CSF have failed in different models of systemic fungal infection. G-CSF in combination with antifungal therapy showed no advantage in comparison to the antifungal treatment alone in murine models of scedosporiosis [334], mucosal and intra-abdominal candidiasis [144,335] or meningeal cryptococcosis [336]. Similar results have been observed with GM-CSF and M-CSF. However, successful enhancement of antifungal therapy against candidiasis [310,337,338], histoplasmosis [339] and aspergillosis [340,341] in mice by G-CSF has been reported.

Vaccines

A long-term goal in the field of medical mycology has been the development of preventative vaccines. Approaches using strains defective in virulence or killed organisms in different forms, i.e., conidia, hyphae or yeast, have shown the capacity to induce at least partial protection of animals from many common fungal pathogens [342–347].

Early studies showed that sub-lethal doses of *A. fumigatus* or viable avirulent and acapsular strains of *C. neoformans* followed by lethal challenge of *A. fumigatus* or *C. neoformans*, respectively, protected animals from a lethal challenge [348–350]. In the 1960s Levine *et al.* demonstrated that injections of formalin killed spherules (FKS) of *C. immitis* protected mice from pulmonary infection [351–353]. However, clinical trials done in 2,867 subjects showed no significant protection in subjects that received three intramuscular injections (1.75 mg of FKS each) over the placebo group [354]. Unfortunately, the high incidence of severe reaction at the site of injection precluded future studies using doses equivalent to those used in the animal studies.

Today, the focus of vaccine research is on specific antigens and several have shown protective effects in laboratory animals against different fungal infections. The efforts to develop an effective vaccine against coccidioidomycosis continue, with several different specific proteins and preparations having shown promise in murine studies [355,356]. A respiratory protein, identified as β-1,3-glucanosyltransferase (Gel1), confers protection against pulmonary coccidioidomycosis by *C. posadasii* in mice; there was an increase of IL-12 and IFN-γ as well as IgG in mice receiving rGel1, demonstrating the importance of the Th1 response in protection against this dimorphic fungus [357,358]. Proline-rich antigen (PRA), also called Antigen 2 (Ag2), and a glycoprotein of the lipid-rich outer wall (SOWgp) layer from *C. immitis* have also demonstrated protective properties by enhancing Th1 response in animal models [359,360].

The use of Th1 stimulators has demonstrated an increase in the protective effect of vaccines in animal models. For example, unmethylated CpG oligonucleotides in combination with *A. fumigatus* allergens [361], or antigens from *C. immitis* [362] stimulated Th1 response increasing resistance. However, the use of CpG increased the susceptibility of mice to challenge with *C. albicans* [363]. These contradictory effects on the use of CpG tell us about the complexity of general immunity.

Other evidence about the activation of acquired responses against a fungus and production of specific antibodies and the importance they may play in coordinating immunity exists [364]. Detection of specific antibodies, used for diagnostic and epidemiologic purposes [365–368], supports this role. Mannans and glucuronoxylomannan that stimulate protective and nonprotective IgM production have been associated with protection against *C. albicans* and *C. neoformans*, respectively [369,370], suggesting that individual epitopes differ in their ability to elicit protective antibody. Immunizations with liposomal encapsulated adhesin from *C. albicans* protected BALB/c mice against systemic candidiasis and polyclonal anti-serum from the immunized mice protected naïve BALB/c and SCID

mice [369]. Because SCID mice are defective in B and T cells, these cells appear not to be involved in the protection. The authors suggest that protection could be transferred by antibody that agglutinates *C. albicans* cells [369]. Similarly, mice immunized with capsular glucuronoxylomannan covalently bound to tetanus toxoid conferred protection against *C. neoformans,* and antibody titer during the early phase of infection correlated with clearance of the organisms [371]. The true potential of antibodies may have been recently demonstrated by studies showing that a laminarin-diphtheria toxoid conjugate prolonged survival of mice infected with *C. albicans* or *A. fumigatus* and that transfer of whole immunogenic serum to naïve animals resulted in immunoprotective properties ascribed to IgM and IgG1 [372]. One may hope that this type of study could lead to the goal of having a Universal Vaccine against clinically important fungi.

Interestingly, acquired immunity may be tissue specific and not translate into other organs. In a murine model of vaginal candidiasis it was observed that once animals cleared the infection a second challenge resulted in an enhanced delayed hypersensitivity, reduced numbers of *Candida* in the vagina and less hyphal penetration of the mucosa [156]. Furthermore, the protective effects of the primary infection did not protect mice from gastrointestinal or systemic candidiasis [156]. These studies present the interesting possibility that vaccines should be site directed.

A desirable aspect for using a vaccine would be one that would remain effective in immunocompromised patients or could be used in immunocompromised patients [373]. In murine studies of aspergillosis, Ito et al. [374] demonstrated vaccine efficacy in corticosteroid suppressed animals. Although CD4$^+$ cells are a primary cell involved in protective immunity resulting from vaccines, more recent studies have demonstrated that CD8$^+$ cells also have a role to play. Long-lived memory CD8$^+$ cells could induce and mediate resistance to *H. capsulatum* and *B. dermatitidis* in absence of CD4$^+$ subsets [375], as well as *Coccidioides* [376]. These findings are especially encouraging since potentiation of CD8$^+$ cells could be designed to protect even immunosuppressed patients.

Antifungal therapy

The ever increasing numbers of fungal infections, whether primary or opportunistic, especially over the last two decades, has proven again and again the limitations of the antifungal armamentarium. Although the drugs have demonstrated valuable activity against many mycoses, therapy is not always effective. Clinical experience tells us that additional studies are needed in order to find more effective drugs, new routes of administration or new regimens, and animal models play an important role in refining monotherapy with antifungal drugs. Each class of drugs has limitations in spectrum, cidal activity and utility as a result of pharmacodynamics. In addition, the increasing occurrence of unusual fungi as opportunistic pathogens, such as the Zygomycetes or other filamentous fungi such as *Fusarium*, which are innately resistant to almost all antifungal drugs, places the development of new and effective therapies as a prime pursuit [377,378].

In therapeutics, the gold standard is the (double-blind) randomized clinical trial. However, when performing clinical trials one has to know the right question to address before embarking on a big adventure. Moreover, such clinical trials usually require many patients, are expensive, sometimes must compete with other studies for the same patients, usually require coordination of multiple centers, take a long time, and are confounded by variations in treatment groups (different stages of disease when therapy starts, co-morbidities not randomly distributed, different ages, sex, genetic backgrounds).

When a randomized clinical trial is not possible, the alternatives are clinical case series and open trials, and the deficiencies are that the plural of anecdote is not data. Animal model studies can tell you *which* clinical trials you should try to do, can be fashioned to mimic a variety of human diseases, are relatively inexpensive, provide sufficient subjects for appropriate statistical analysis, can be relatively easily repeated (at which time study conditions can be intentionally varied), and give prompt answers (enabling decisions about future directions). All the subjects can be the same age and sex, be genetically identical, and have the same co-morbidity (or none) and same stage of disease at entry. Different regimens can be studied concurrently to avoid unknown external factors (e.g., referral patterns, treatments for co-morbidities) influencing outcome that are changing over time, as occurs in the clinical setting. The outcome endpoints can be devoid of subjectivity (not 'the patient is improved/stable/worse'). While death can be an objective endpoint in clinical trials or animal models, in models the amount of infection after therapy can be quantified, even in survivors. One can do pharmacologic and toxicologic studies not possible in patients, including sampling parts of or whole organs for histopathology and/or drug concentration, and study uncommon conditions that would be difficult or impossible to study in a randomized clinical trial, because of insufficient numbers of subjects available in a reasonable time frame.

Animal models have been used extensively to test experimental antifungal therapy against localized or systemic infections by clinical important fungi, and past experience demonstrates that efficacy in one or more animal models is predictive of efficacy clinically. Perhaps no other use of animal models can be so directly and promptly linked to benefits in the care of patients. Studies in animals are directed to test the efficacy and safety of new agents, as well as new indications for licensed molecules. After *in vitro* tests have shown efficacy against a fungus, animal modeling is a mandatory step prior to clinical trials and final governmental approval. Once a model of infection has been established, demonstrating reproducibility and mimicking the clinical disease, determination of the route and regimen of administration, pharmacokinetic/pharmacodynamics, efficacy and toxicity of the drug may be tested. Varying the time of onset of therapy in animal models can give valuable clues as to the importance of timing of initiation of therapy in clinical diseases. The types of studies performed are those of monotherapy, combination therapy and pharmacokinetic or pharmacodynamics studies. We, and others, have reviewed these types of studies extensively and will thus present only examples of the types of studies done [35,36,38,41,42,107,108,312,379–382].

Monotherapy studies

Determination of drug efficacy in an animal model is assessed by many investigators using the parameters of survival and fungal burdens in the tissues, by colony-forming units, after a defined period of dosing. Several aspects of performing these types of studies need to be considered. Many of these already have been discussed in the initial sections of this review. Based on *in vitro* susceptibility studies it is critical to know which organisms within a genus appear to be susceptible and which are resistant to the antifungal being tested, as well as how the organisms behave during the experimental infection. Similarly, one must be aware of differences between the animal species being used for the models, as the efficacy of antifungal drug can be also species-dependent. Because of physiological differences between animal species, the pharmacokinetic and pharmacodynamics of the new drug must be considered before drug testing *in vivo*. The example of VCZ in guinea pigs versus mice has been discussed in a previous section of this review. However, administration of grapefruit juice in the drinking water of mice, which inhibits gut P450 enzymes, has been shown to increase the serum concentration of the drug to therapeutic levels [383,384] and allows the use of murine models to test VCZ efficacy

against different experimental infections [98,101,385]. Variability in drug efficacy can also depend on the animal model used and for this reason data should be carefully interpreted, as treatment efficacy in one model may not occur in a different one. For example, we have previously reported differences in efficacy for several antifungal drugs between murine models of systemic and pulmonary aspergillosis [386,387].

A true benefit of using animal models for the determination of drug efficacy is that doses and regimens can be easily monitored, and modified in animal models, in order to establish an optimal effect [36,37,388]. Higher dosages of an antifungal are not always more curative, and the use of animal models has clearly demonstrated this for the lipid-carried amphotericin B preparations and the echinocandins in murine models of CNS aspergillosis [2,5,385,387,389].

As noted previously, animal models are used in lieu of clinical trials where those trials may be difficult to perform or put patients at serious risk. A more recent example of the utility of a model is that of a rabbit model of indwelling catheter colonization by a biofilm of *C. albicans*, which mimics the very serious clinical problem of candidal line infections and sepsis. These studies demonstrated the effectiveness of using liposomal amphotericin B to clear the biofilm from the catheter [390] and also demonstrated ethanol inhibition of biofilm formation by *C. albicans in vivo*, which may suggest novel methods of clearing the catheter [391]. Studies such as this directly address important clinical problems and can be indicative of better methods of treatment.

Combination therapy studies

Another strategy in face of serious fungal disease is the use of combinations of drugs. Combination therapy can enhance efficacy over monotherapy, avoid toxic effects of one of both drugs by reducing the dosage and increase the therapeutic benefits against infections caused by multi-resistant species. When performing antifungal combination studies it is critical to know the efficacy of the monotherapies being considered for use in combination. Significant enhancement of efficacy by the combination cannot be demonstrated if one or both of the compounds is already curative at the dosage being tested. Thus, if one or both drugs alone show good efficacy, the combination of the two is assessed by using them at suboptimal dosages [36,37]. The combined efficacy over the monotherapy must be seen in animal models by comparing the combination and demonstrating a significant enhancement in efficacy over that of either drug tested alone at the same dosing

concentration. Combination can be done by administering both drugs simultaneously or sequentially.

Recently published examples of combination therapy studies demonstrated that lipid-carried AMB in combination with VCZ given concurrently or sequentially with AMB for three days followed by VCZ showed greater efficacy against CNS aspergillosis than did the respective monotherapies [385,387,389]. In another study, liposomal AMB followed by administration of VCZ was superior to VCZ followed by L-AMB [91].

A variety of drugs have been studied in combination particularly in murine models of candidiasis and aspergillosis. The combinations include amphotericin B preparations with azoles or echinocandins and azoles and echinocandins. Of note and discussed earlier in this review are the use of immunostimulatory cytokines in combination with a conventional antifungal agent such as amphotericin B or fluconazole. IL-12, and IFN-γ particularly, have been shown to be useful adjuncts to conventional therapy in a number of murine models including cryptococcosis and histoplasmosis [107,213,228,229,311,312,324,392].

Pharmacokinetic and pharmacodynamic studies

Determination of *in vivo* pharmacological parameters, such as C_{max}, $t_{1/2}$, AUC_{0-24}, volume of distribution and elimination and toxicity, is necessary for antifungal drugs as well. Numerous studies of this type have used mice and rabbits and are usually performed in uninfected animals [102,393–395]. Less frequent and sorely needed are pharmacodynamic studies where the same pharmacokinetic parameters are determined in infected animals [102,394,395]. Important for the future is the application of physiologically based pharmacokinetics to antifungal agents. These types of studies are done modeling pharmacokinetic parameters for two or more animal species in order to predict the parameters in humans. Studies of this type are very limited with antifungal drugs and one example is a study with terbinafine done in rats showing the modeling from rat data correlated well with published human data [396]. Additional studies on other antifungal drugs are much needed.

A subdivision of pharmacologic studies is toxicologic studies. As mentioned in regard to other pharmacologic studies, these are usually performed in uninfected animals, often the same species in which an efficacy trial and/or a pharmacologic study are conducted. Early studies of polyene toxicity to nephrons, and the localization of the effect, necessarily relied on animal models. As examples of more recent studies, we have utilized animal models to illuminate endocrinological effects of azoles [397], drug-drug interactions resulting in toxicity [398] and to study the neurotoxicity of amphotericin preparations [399], information that would not have been possible to obtain from humans. Toxicological studies in infected animals are also sorely needed, largely because of the possibility that disease toxicity and drug toxicity may summate, such as that reported in studies of murine blastomycosis [400].

Developing diagnostic tools

Surrogate markers of infection and alternatives to survival or infectious burden assays have included radiographic imaging techniques applicable to pulmonary models of aspergillosis in rabbits [3,35,65,81], metabolites [401] and other clinical parameters such as body weight and temperature. In addition to the PCR methods, assays for antigenemia (galactomannan and glucan) have been used to study disease progression, as well as for diagnostic purposes [401–406]. Rabbit studies with aspergillosis have shown that galactomannan assays are useful for diagnostics and gave results similar to those from humans [3,402,404,407,408]. The course of cryptococcal polysaccharide concentrations in animal serum in the presence or absence of therapy corroborates correlations of the concentrations, fungal load, and disease severity in humans [111,409,410]. In each example mentioned, the clinical utility of a particular assay is based on the corroboration of animal data in later clinical experience.

Summary and conclusions

A primary aim of this review has been to demonstrate to the reader that animal models in mycology are a useful and necessary tool for studies of pathogenesis, host-response, clinical infection and therapeutics. We have discussed various aspects of the benefits and weaknesses, types of data generated and evaluation of these data. From the numerous fungal animal models discussed it should be apparent that each is unique and requires careful performance and evaluation before reasonable conclusions concerning the results can be drawn. One cannot make overall generalizations that all fungal infections progress or behave in the same manner nor does the host respond in the same way to all fungi. Thus, before embarking on studies utilizing animals a series of questions must be addressed and answered by the investigator. What is the infection being modeled and is the model well defined and reproducible? What is the immune status of the host? Is the fungal infection being modeled an acute or chronic disease? When therapeutic studies are the goal, does the investigator

know what the efficacies of monotherapy regimens are, what are the optimal doses, duration, frequency and routes of therapy? Regardless of the model and purpose of using the model, what parameters of infection should be followed? Are these parameters survival and CFU or is a surrogate marker of infection applicable? And finally, how are the data best evaluated, with which statistical analyses? Answering these questions first will lead to better experimental results, sound conclusions and allow us to better understand medically important fungi and their interactions with the host.

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