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Understanding asthma using animal models

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Asthma is a complex syndrome with many clinical phenotypes in children and adults. Despite the rapidly increasing prevalence, clinical investigation and epidemiological studies of asthma, the successful introduction of new drugs has been limited due to the different disease phenotypes and ethical issues. Mouse models of asthma replicate many of the features of human asthma, including airway hyperreactivity, and airway inflammation. Therefore, examination of disease mechanisms in mice has been used to elucidate asthma pathology and to identify and evaluate new therapeutic agents. In this article, we discuss the various animal models of asthma with a focus on mouse strains, allergens, protocols, and outcome measurements.

Key Words: animal model; asthma; mouse

INTRODUCTION

The prevalence of allergic airway disease is rapidly increasing world-wide in all age groups; in recent decades, the prevalence in children is reportedly over 30% in many countries and greater than 10% in some adult populations.[1,2] Although understanding of the pathogenesis of the disease has progressed enormously in the last several decades, the development and addition of new therapeutic agents in asthma has been very limited. The mainstay of therapy remains inhaled corticosteroids, which although impacting the disease in significant ways, does not address all issues and in all patients.[3]

Asthma is a complex syndrome with many clinical phenotypes. Common to all is chronic inflammation with reversible airway obstruction and airway hyperresponsiveness (AHR).[4] The most prevalent form of asthma is atopic asthma which is initiated by the exposure to (inhaled) allergens and resultant allergen-specific immune responses. Indeed, early sensitization to allergen, by 3 yr of age, may be an important predictor for persistent wheezing 10 yr later.[5,6] As we are all exposed to the same inhaled allergens, the genetic regulation of these responses is an important component in defining susceptible individuals. In addition to allergen exposure, other exposure risk factors such as viral infection, occupational exposure, air pollution, and environmental tobacco smoke are important contributors to the different phenotypes of asthma and asthma heterogeneity.[7-10] Similar to other diseases, clinical investigation and epidemiological studies are essential for the advancement of knowledge and disease management. However, the ability to comprehensively assess the different disease phenotypes and inherent ethical issues are limiting factors in conducting many of the required clinical studies. As a result, animal models have been developed to study the pathogenesis of the disease, including genetic factors, to define the pathogenetic pathways and suggest new therapeutic approaches.[11,12] That being said, it is clear that the results in animal studies are not easily translated to humans and therapeutic initiatives successful in animals have generally been of limited success in the clinic. This has prompted debate about the utility of animal models.[13]

ANIMALS

Animal models of asthma have been extensively used to examine mechanisms of disease, the activity of a variety of genes and cellular pathways, and to predict the safety of new drugs or chemicals before being used in clinical studies.[12] Advances in the understanding of the pathophysiology of asthma as an allergic airway disease would not have been possible without these models. Although asthma was associated with airway eosinophilia for more than a century, the contributors to this characteristic cellular inflammatory response followed on the descriptions of T helper cell functional heterogeneity and the

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distinct cytokine profiles described initially in mice. Indeed, most of the mechanisms of disease that are discussed today derive from the studies conducted in animal models.[14]

There is a wide variety of animal models of asthma in different species. Mice, rats, guinea pigs, ferrets, dogs, sheep, monkeys and horses have been employed to study the inflammatory processes and alterations in airway function.[15-19] Each animal possesses certain advantages and disadvantages as a model of allergic airway disease (Table 1).

Mouse models of allergic airway disease offer numerous advantages when compared to the use of other animals. IgE is the primary allergic antibody in mice, making this species appropriate for investigation of the role of humoral immune factors in the development of allergic airway disease. Further, mouse models offer the opportunity to explore detailed mechanisms of allergic reactions because of the availability of numerous immunological reagents such as antibodies against cytokines, growth factors, and cell surface markers. Numerous well-characterized inbred strains of mice are available, which allow direct transfer of cells between the same strain of animals for the assessment of function of specific factors and cells. In addition, the emerging technologies involving gene manipulation in animals is well-advanced in mice. The ease of breeding and short gestational period is an additional advantage. Accordingly, major advances in the understanding of the disease concept, "asthma as Th2-dominant disease", emerged from studies in mice.

On the other hand, because of considerable physiologic differences between mice and humans, extrapolation of findings to humans needs to be judicious. To a large extent these differences explain many of the failures to translate findings in the mouse to the clinic. Among these differences is the anatomy and differentiation and branching of the airways, the vasculature, and the somewhat limited airway musculature. Mice do not exhibit spontaneous AHR and smooth muscle hyperplasia is not easily demonstrated. The mice fail to develop airway constrictive responses to histamine and for studies of neurogenic inflammation, it is difficult to detect non-adrenergic/non-cholinergic (NANC) activated or inhibitory responses. A major limiting factor in mice and in virtually all animal models is the absence of a truly chronic model, a model where challenges are experienced over many months to years, akin to the human asthmatic.

Although a majority of studies of allergic airway disease are now carried out in the mouse, the guinea pig initially was utilized as an animal model of pulmonary hypersensitivity and AHR for many decades.[20] The guinea pig demonstrated airway constrictive responses, hyperreactivity towards cholinergic agonists, and production of hypersensitivity antibodies, including IgG1 and IgE.[21] The major benefit of the guinea pig was the ability to target the lung as the primary target organ of a hypersensitivity response. Further, both immediate- and late-phase airway responses following allergen challenge readily developed in this species, allowing mechanistic investigation of the distinct phases of altered airway function, as well as determining the interplay between the early- and late-phase responses. The associated pulmonary inflammatory response is consistent with asthma, composed of both eosinophils and neutrophils. The guinea pig model identified the importance of airway inflammation in the development of altered airway function.[21]

However, there are several disadvantages in using guinea pigs as a model of asthma. The shortage of inbred strains prevents

Table 1. Advantages and disadvantages of individual animal models of asthma

Animal	Advantage	Disadvantage
Mouse	IgE is the primary anaphylactic antibody Numerous immunological reagents Numerous inbred strains Easy breeding Short gestational period Small and relatively inexpensive	Do not exhibit spontaneous airway hyperresponsiveness Limited airway musculature Lung anatomical differences Do not respond to histamine Absence of a chronic model
Rat	IgE is the primary anaphylactic antibody Produce long-lasting airway response Show immediate and late phase airway responses	Requires injection of the allergen for sensitization Requires adjuvants for sensitization Species-specific immunological reagents are not abundant
Guinea pig	Lung is the primary target of anaphylaxis Response to cholinergic agonists Show immediate and late phase airway responses Pulmonary inflammation composed of eosinophils and neutrophils	IgG1 is the major anaphylactic antibody Shortage of inbred strains Few species-specific reagents
Rabbit	Show immediate and late phase airway responses Lung is the primary target of anaphylaxis IgE is the primary anaphylactic antibody	Neonatal immunization required for late phase airway response
Large animals	Horses naturally develop respiratory disorders when exposed to barn dust Monkeys develop natural sensitivity to Ascaris	Hard to handle Expensive

meaningful investigation of the genetic influences on susceptibility to sensitization and development of allergic airway disease. In addition, few species-specific reagents exist, making it difficult to identify and isolate particular cell types. Another major disadvantage to the guinea pig is the predominance of IgG1 rather than IgE as the major anaphylactic antibody.[21]

The rat, another small rodent, has been used by numerous investigators. The production of IgE as the major anaphylactic antibody enables the study of the control of the synthesis of allergen-specific antibodies, as well as the role of this antibody in the physiological and cellular responses to allergen. Another benefit is the ability to produce long-lasting airway hyperreactivity. Both immediate- and late-phase airway responses following allergen challenge have been produced in strains of rats, however, sensitization typically requires injection of the allergen, rather than administration via the inhalation route as is used with guinea pigs.[22,23] In addition, allergic sensitization, as in the mouse, requires use of adjuvants, specifically alum or *Bordetella pertussis*.[24] Species-specific immunological reagents are not as abundant as they are in mice. These are major limitations for the study of allergic airway responses.

The rabbit provides an animal model which resembles humans in that the lung is the target organ for anaphylactic responses. This species can demonstrate both immediate- and late-phase airway responses, has vigorous NANC responses and IgE is the primary anaphylactic antibody.

Larger animals such as monkeys, sheep, and horses have been used in asthma models. However, they are hard to handle and too expensive to use on a regular basis. Horses are the only animals which naturally develop respiratory disorders characterized by acute airway obstruction when exposed to barn dust.[25] Monkeys may develop natural airway sensitivity to Ascaris.[26]

In light of the predominant use of mice as surrogates, this review article focuses on mouse models. In considering the use of mice, several distinct components need to be considered, the genetic background of the mice, the allergen, the experimental approach, and the outcome measures. The interplay between these four components is exceptionally complex and understanding the biological implications is essential to the extrapolation to human disease.

STRAINS OF MICE

The availability of various inbred mouse strains is an advantage, but problems can arise if an inappropriate mouse strain is selected. The ability to induce parameters of allergic airway inflammation and AHR varies greatly among the different strains with both responders and non-responders. A/J and AKR/J mice display high levels of allergen-induced AHR and reactivity to methacholine.[27] In contrast, C3H/HeJ and DBA/2 mice are comparatively resistant to the development of allergen-induced AHR.[27,28] Among the strains, BALB/c and C57BL/6 mice are the most widely used due to their well-characterized immunological responses. BALB/c mice typically mount Th2-dominated immune responses, and the induction of parameters of allergic responses such as allergen-specific IgE, AHR, and eosinophilic airway inflammation are robust. Conversely, C57BL/6 mice exhibit Th1-dominated immune responses, and have limitations in the development of allergic airway responses compared with BALB/c mice especially in the development of allergen-specific IgE responses and airway responsiveness to inhaled methacholine. Surprisingly, in response to allergen challenge, for example to ovalbumin (OVA), they do develop a robust BAL eosinophilic response,[29] and in the tissue tend to accumulate more eosinophils in the parenchyma than around the airways, in contrast to BALB/c mice where eosinophils accumulate around the airways.[30] However, C57BL/6 mice are widely used as strains of most gene-manipulated mice are on this background.

The availability of genetically-manipulated animals enables the investigation of the genetic influence on susceptibility to sensitization and development of allergic airway inflammation. Targeting of specific genes has served to identify pathways uniquely involved in disease development and progression. Thus, the contribution of a given molecule in disease development has been identified through the use of gene-deletion or

Table 2. Advantages and disadvantages of non-invasive and invasive airway function measurements

Methods	Advantage	Disadvantage
Non-invasive	Fast and easy	Interference from upper airways
	Use aerosolized stimulants	Prone to artifacts (movement)
	Repeated and long term measurements in the same animal	Breathing patterns only but not physiological values
	Normal breathing pattern with no need for anesthesia or tracheal instrumentation	No direct assessment of pulmonary mechanics
Invasive	Reproducible and precise	Need for tracheostomy
	Avoid changes in the upper airways	Need for anesthesia
	Opportunity for bronchoalveolar lavage fluid collection	Need for mechanical ventilation
	Based on physiological principles	No repeated measurements
	Intact anatomical relationship in the lung	Expertise

knockout mice, which are genetically-manipulated to produce a defective or non-functional version of a gene-related product or to overexpress a gene product. However, such studies require careful scrutiny as the effects are developmental in origin, and genetic manipulation circumvents naturally occurring regulatory or control mechanisms or may be compensated by unknown pathways. To circumvent some of these limitations, technologies using conditional knockout approaches have been developed in which the developmental concerns are bypassed and where the target gene can be directly manipulated at defined times. Transgenic mice have also been used to examine the impact of overexpression of a gene of interest on the course of disease development. The transgenic genes can disrupt the expression of other genes which can be induced by the random insertion of target genes. Therefore, once animals expressing the transgene have been made, it is important to confirm that insertion did not disrupt the expression of other genes.

ALLERGENS

A number of allergens have been used in animal models of asthma. In most studies, OVA has been used to sensitize and challenge host animals. OVA is relatively inexpensive, can be highly purified, the immunodominant epitopes have been well-characterized, and recombinant peptides have been generated. Purified OVA can be prepared without protease or endotoxin which is abundant in other allergens.[31] In most strains, repeated inhalation of OVA may induce the development of tolerance, rather than sustained allergic airway responses. For sensitization, OVA is usually combined with adjuvant and injected to prime for allergic airway inflammation.[32] Following sensitization, a series of inhaled or intranasal challenges are administered to elicit responses. OVA-induced allergic airway models may not represent the same conditions experienced by asthmatics where allergen exposure may be more frequent and for much longer periods of time. Although allergens represent an important component of allergy induction, other factors such as lipopolysaccharides (LPS, endotoxin) may contribute as they are ubiquitous in the environment. Small amounts of LPS usually contaminate preparations of OVA and are likely essential for sensitization in mice. In one study sensitization to OVA via the intranasal route was shown to be dependent on LPS in the preparation but this requirement was bypassed when sensitization systemically was combined with adjuvant.[33] LPS-free OVA may induce tolerance and prevent development of allergic airway inflammation or AHR.[34] Extracts or purified proteins derived from potent human allergens including house dust mite (HDM), cockroach, ragweed, or fungi have been increasingly used as allergens in mice and other species.[35-41] Repeated administration of a combination of three allergens may avoid the development of tolerance.[42] These allergens induce asthma attacks in humans and some studies have demonstrated their potency to induce chronic airway inflammation and tissue remodeling in mice. It is also apparent that even following the same protocols, different allergens in different strains elicit quite different responses.[43]

EXPERIMENTAL PROTOCOLS

At the present time there is no standardized experimental protocol; most laboratories have developed their own protocols either with major or minor modifications. Simple reliance on a single model poses many problems when data are analyzed. Similarly, because of the number of models used, it is very difficult to compare results from different studies. As the experimental approach is so important to the outcomes, it is an area ripe for consolidation and standardization. Our approach has been to investigate several different models in order to fully explore the complexity of the outcomes under different conditions.

Primary allergen challenge model

Mice do not spontaneously develop AHR or allergic airway inflammation. Therefore, to investigate the development of lung inflammatory responses, an artificial asthma-like reaction has to be induced in the airways. Primary allergen challenge models have been widely used to elucidate the mechanisms underlying the allergen-induced immunologic and inflammatory responses in the airways. Although many different sensitization and challenge protocols exist, the basic model is shared, with a sensitization phase and a challenge phase. Single or multiple systemic injections of allergen with or without adjuvant are used to induce sensitization. Adjuvants such as aluminum hydroxide are known to promote the development of Th2-type responses. Adjuvant-free protocols are also effective, but these usually require a greater number of exposures to achieve the proper sensitization, and even then, elicit less robust responses on allergen challenge.[44] In the challenge phase, generally 2-4 weeks after completion of sensitization, allergens are administered in a nebulized form or administered by deposition through the intratracheal or intranasal route. The primary allergen challenge models are relatively short-term and show high reproducibility. The responses elicited include elevated levels of allergen-specific IgE, eosinophilic airway inflammation, and AHR. However, due to the short-term nature of primary allergen challenge models, one of the key features observed in asthmatics, airway remodeling, is minimal and many of the other parameters (AHR, eosinophilic inflammation) are transient. Airway inflammation and AHR have been shown to resolve within a few weeks after the final allergen inhalation.[45] It is this model which has been most used in pre-clinical studies, as a prelude to a clinical trial. This model has several limitations and results are often overinterpreted to represent asthma. As a

result, it is not surprising that most extensions to asthma have failed in the clinic. This model is not a model of asthma, the human disease.

Secondary allergen challenge models

The responses to primary allergen challenges, including AHR, eosinophilic inflammation, and goblet cell metaplasia can be induced by the first exposures to allergen in sensitized mice. Often described as an acute exposure model, this contrasts with asthma where allergen exposure is repeated and chronic. Therefore, secondary allergen challenge models were developed to more closely mimic the conditions of allergic asthma with allergen re-challenge after animals received sensitization and primary allergen challenge. Animals are exposed to secondary allergen challenge 2-6 weeks after primary allergen challenge when airway eosinophilia and AHR have resolved and returned to baseline levels. A single provocative or secondary allergen challenge elicits airway eosinophilia and neutrophilia, lymphocyte accumulation, and altered airway function. The advantage of this protocol is the ability to monitor the kinetics of inflammatory parameters induced by a single allergen challenge and the effect of treatment. This approach revealed that the requirements for some of the responses to secondary challenge differed from those suggested to be critical in the response to primary allergen challenge.[46,47]

Chronic allergen challenge models

Asthma is defined as a chronic inflammatory disorder of the airways with structural changes in the airways including subepithelial fibrosis, goblet cell metaplasia, smooth muscle thickening, and increased vascularity.[48,49] Although primary and secondary allergen challenge animal models elucidate many aspects of human asthma, there are limitations to these models when compared to asthmatics. Several laboratories have investigated chronic allergen challenge models in mice to more closely mimic the human disease. Various models have been developed by increasing the number of allergen exposures over many weeks. OVA as well as other allergens such as HDM extract and/or grass pollen have been used.[50-53] In many of these studies long-term challenge leads to the development of tolerance and progressive decreases in AHR and airway eosinophilia.[54] The induction of tolerance may be dependent on the strain of mouse used and the nature and route of allergen administration.[45,55] Chronic models have been shown to reproduce some of the hallmarks of asthma such as goblet cell metaplasia, epithelial hypertrophy, subepithelial fibrosis and limited smooth muscle hyperplasia. Regrettably, chronic allergen challenge models have had limited use in the preclinical evaluation of novel therapeutic agents where compounds can be tested on a background of established allergic airway disease. Certain atypical features are observed in these chronic allergen challenge models that are not observed in humans, for example, inflammation is not restricted to the conducting airways in the mouse, whereas it is restricted in humans. In addition, there are few mast cells in the airway walls or epithelium of mice.[56,57] Despite these concerns, development of a standardized chronic model in mice is important as many of the clinical studies were initiated based on outcomes in primary allergen challenge models.

Exclusive airway exposure to allergen and passive sensitization to assess lung allergic responses

These were some of the first models which assessed airway function in mice.[58] Briefly, sensitization and induction of AHR were achieved by airway allergen challenge exclusively for 10 days or following passive sensitization with allergen-specific IgE followed by two days of airway allergen challenge.[59] In both cases there was no systemic sensitization. This is similar to the natural mode of sensitization to airborne allergens in asthmatics. Since no adjuvants are used, IgE production and airway inflammatory responses are somewhat lower than observed following systemic sensitization.[60] Importantly, these models, unlike those incorporating systemic sensitization, are both IgE and mast cell-dependent.[61]

Dendritic cell–dependent models

In mice, the sensitization phase can be bypassed using adoptive transfer of allergen-primed dendritic cells (DCs) which are efficient antigen-presenting cells (APC).[62] Bone marrow-derived DCs (BMDCs) can be differentiated and expanded from precursors in vitro in the presence of GM-CSF (with or without IL-4). The bone marrow-derived DCs are primed to allergen and initiate naive T-cell differentiation in vivo when they are adoptively transferred into mice that are then exposed to allergen via the airways on three consecutive days.[63] When allergen-primed DCs are administered directly into the airways of mice followed by exposure to allergen, an asthma-like phenotype is induced.[63,64] Such experiments have highlighted the importance of DCs in the initiation of allergic airway disease and identify DCs as therapeutic targets.[65] BMDCs are different than the naturally occurring lung DCs, the myeloid and plasmacytoid subsets, that differentially regulate lung allergic responses.[66]

MONITORING OUTCOMES IN DIFFERENT MODELS

The physiologic hallmark of asthma is reversible airway obstruction, accompanied by airway hyperreactivity to bronchoconstrictors and non-specific stimuli. In humans, allergen challenge can induce immediate- and/or late-phase responses. Although assessments of early allergic responses are feasible in mouse models,[67] most studies assess changes in airway responsiveness to non-specific agonists such as methacholine (inhaled or administered intravenously) or acetylcholine (administered intravenously) after allergen challenge(s). Allergen

exposure in sensitized mice results in significant increases in airway responsiveness to cholinergic agonists when compared to the non-exposed controls. Several different methods are used to measure airway responsiveness in mice following allergen exposure (Table 2).

Non–invasive airway function measurements

Non-invasive approaches have been used to assess airway function. Spontaneously breathing, non-anesthetized mice are placed in the main chamber of a whole body plethysmograph. The response of the airways to inhaled methacholine is measured and compared to the response to a control inhalant (aerosolized saline). Airway function is expressed as the calculated parameter "enhanced pause" (Penh). Briefly, Penh is an empirical parameter that reflects changes in the box flow waveform between inspiration and expiration and combines these results with the early and late expiratory box flow (Pause). Non-invasive measurements such as this have several potential advantages compared to invasive methods as they are technically less demanding and can be used to monitor AHR over time in the same animal. When carried out in experienced hands, the results correlate with those of the invasive methods.[68] Although faster and somewhat easier, this approach requires careful calibration, may be better suited to responsive strains such as BALB/c mice, and the results can be affected by any impingement on nasal airflow. The validity of this method has been questioned and in fact has engendered a great deal of debate because it is difficult to exclude the influence of the upper airways, and Penh is based on breathing patterns but not physiological values.[69] Investigators have used this technique for screening large numbers of compounds and defining genetic control of airway responsiveness, often in concert with invasive methods to confirm the results.[70]

Invasive airway function measurements

Invasive methods more directly measure pulmonary mechanics in mice and represent the current gold standard in airway physiology. AHR can be assessed more directly by measuring changes in respiratory (lung) resistance (R_L) and dynamic compliance (C_{dyn}) in response to increasing concentrations of inhaled or intravenously administered methacholine or acetylcholine. R_L is the sum of airway and tissue resistance, which is fairly comparable at normal breathing rates.[71] C_{dyn} is calculated by relating the volume changes to the concomitant elastic recoil pressure changes between end inspiration and end expiration. The advantages of the invasive measurements are the precise assessments of transient changes in pulmonary mechanics. The insertion of a tracheal tube avoids changes secondary to the upper airways and provides the opportunity for extracting bronchoalveolar lavage fluid after lung function measurements are performed. The disadvantages include the need for surgical tracheostomy, thus precluding repeated measurements, the needs for anesthesia, mechanical ventilation, and expertise in handling.

Electrical field stimulation (EFS)

Although the in vivo response to inhaled methacholine is the most widely used method of assessing AHR in mice, it was initially limited in the mouse due to the difficulty of delivering an aerosol to the airways. To determine the effects of EFS, tracheal smooth muscle segments (TSM) are isolated after allergen challenge and placed in baths with supporting longitudinally stainless steel wire. Repetitive currents are applied at increasing frequencies to define the frequency that elicits a 50% maximal contraction (ES_{50}). Following allergen challenge, the reduction in ES50 was shown to reflect M2 receptor dysfunction and the increase in acetylcholine release.[72] These alterations may be induced by eosinophilic major basic protein.[73] EFS has been used to investigate mast cell-dependent increases in airway responsiveness and neural airway control.[74]

SUMMARY

For the last several decades, various animal models have been studied and have contributed to the further understanding of the mechanisms underlying allergic airway disease. There are very few pathologic features not shared in mouse models and human asthma. The advances in technology, availability of new equipment, introduction of potent reagents and genetically-manipulated mice continue to provide unique opportunities to explore the pathogenesis of allergen-induced airway inflammation and airway dysfunction in ways not available to clinical research in patients. What is lacking is development of guidelines to establish more uniformity in approach and outcomes so that different studies are more easily compared.

Nonetheless, the mouse like other species has several limitations including the facts that they do not have spontaneous symptoms or long-lasting bronchoconstriction as seen in asthmatics, their lungs are more fully developed at birth so environmental influences have different effects, the structure of mouse lungs is very different than in humans, mice are obligate nasal breathers, and chronic asthma models and evidence for remodeling, e.g., smooth muscle hypertrophy is limited. Although no mouse model fully mimics the full range of clinical manifestations of asthma, many do reproduce a collection of the features that characterize its most common forms and they yield a basic core of phenotypic consequences.

In summary, mouse models cannot be considered a surrogate for human asthma or a panacea for difficulties encountered in human study design and outcomes, but rather should be seen as an important opportunity to generate and test hypotheses in simple, controlled systems. The clinical relevance of the findings in mice or any other species can only be determined in human studies.

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