

Molecular mechanisms in allergy and clinical immunology

(Supported by a grant from Merck & Co, Inc, West Point, Pa)

Series editor: Lanny J. Rosenwasser, MD

Genetics of airway hyperresponsiveness

George T. De Sanctis, PhD,^a Massoud Daheshia, PhD,^b and Angelika Daser, PhD^c

Boston, Mass, and Berlin, Germany

Asthma is a disease characterized by intermittent airway obstruction, inflammatory cell infiltrates, increased mucus production, lung epithelial remodeling, and airway hyperreactivity. The genetics of asthma, as investigated in animal models, is poorly understood. Because no animal model of asthma mimics all of the pathologic and physiological features of asthma, genetic studies have focused on several phenotypes, including intrinsic or native airway hyperreactivity. It is generally accepted that both genetic and environmental factors determine the phenotypic expression of this complex disease. The genetics of airway hyperresponsiveness, as investigated in the mouse, are presented in this review. The inbred mouse currently represents the most valuable genetic resource for understanding the factors that control this complex phenotype. (J Allergy Clin Immunol 2001;108:11-20.)

Key words: Asthma, inbred mice, quantitative trait loci, genetics, methacholine, acetylcholine, airway hyperresponsiveness, airway reactivity

The genetic analysis of complex traits has been carried out in both inbred and outbred populations. Although outbred populations are more readily available than inbred populations, the latter are better suited for the study of "asthmalike" phenotypes. Specifically, inbred populations are genetically uniform, so genetic-environmental interactions are unconfounded. The main disadvantage of outbred populations is their genetic diversity. As a result of this heterogeneity, the impact of environmental influences on a particular phenotype can vary considerably within an outbred population. Although several studies have examined "asthmalike" phenotypes in outbred species such as the dog and rat, the most promising species to date has been the inbred mouse. It is important to note that the mouse and human genomes

Abbreviations used

AHR: Airway hyperresponsiveness
APTI: Airway pressure-time index
AR: Airway responsiveness
LOD: Logarithm of odds ratio
RL: Pulmonary resistance
QTL: Quantitative trait loci
Rrs: Respiratory resistance
SDP: Strain distribution pattern

share considerable homology and that mice develop many diseases similar to those observed in human beings. In addition, the availability, short gestation period, large litter sizes, and well-defined genetic map of numerous inbred mouse strains, as well as the ability to control the environment in which the animals are reared, all establish the inbred mouse as an ideal choice for these types of study.

Several recent studies have investigated "asthmalike" phenotypes such as airway hyperresponsiveness (AHR), a hallmark of asthma, in the inbred mouse. These studies have definitively shown that AHR is under genetic control in the inbred mouse. Two of these early studies measured AHR in a large number of inbred strains of mice and demonstrated that different inbred strains of mice display varying degrees of AHR in response to bronchoconstrictors.^{1,2} The results of these early studies quickly established the inbred mouse as an ideal species for dissecting out the genetic factors that contribute to this phenotype.

A comprehensive examination of the genetics of asthma, as studied in the inbred mouse, is presented in this article. In particular, our review focuses on the genetics of "intrinsic" AHR in the inbred mouse. Briefly, a summary of the airway responsiveness (AR) phenotypes used in these genetic studies is presented, along with a description of the strain distribution patterns (SDPs) of AHR generated from numerous strains of inbred mice. Subsequent sections review the strategies adopted in later studies to identify genes or chromosomal regions that contribute to this important "asthmalike" phenotype. Within these regions lie several candidate genes implicated in the pathobiology of asthma. In subsequent sections, data from other studies, in which knockout (KO) and transgenic (TG) mice were used to investigate the role of candidate genes on "intrinsic" AHR, are presented.

From ^athe Pulmonary and Critical Care Divisions, Department of Medicine, and ^bthe Department of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital and Harvard Medical School, Boston; and ^cthe Institute of Laboratory Medicine and Pathobiochemistry, Berlin.

Received for publication March 16, 2000; revised April 11, 2001; accepted for publication April 12, 2001.

Reprint requests: George T. De Sanctis, PhD, FCCP, Division of Pulmonary and Critical Care Medicine, Brigham and Women's Hospital and Harvard Medical School, 75 Francis Street, Boston, MA 02115.

Copyright © 2001 by Mosby, Inc.

0091-6749/2001 \$35.00 + 0 1/10/116429

doi:10.1067/mai.2001.116429

MEASUREMENT OF AIRWAY RESPONSIVENESS

AR, measured in the absence of allergen-induced airway inflammation, has been widely adopted as a quantitative "asthmalike" phenotype in the inbred mouse. To measure AR in this species, investigators have developed both noninvasive and invasive techniques. An extensive review of the various methods that have been used to quantitate lung function in mice has recently been reported by Drazen et al.³

Each of these techniques has inherent strengths and weaknesses. Invasive methods are used in situations in which the animal typically is studied at a discrete time point and then killed; accordingly, long-term studies cannot be performed through use of this method. These techniques require that the animal be anesthetized and mechanically ventilated and that the level of anesthesia be carefully controlled to prevent ventilatory efforts by the mouse, a factor that can complicate the interpretation of AHR. Anesthetic agents can also affect neural reflexes and hence airway caliber. Examples of these invasive techniques include measurement of pulmonary resistance (RL) and dynamic compliance, occlusion mechanics, and airway pressure measurements made during mechanical ventilation. The disadvantages of these techniques include the need to induce anesthesia and invasively instrument the airways.

Noninvasive measurements, on the other hand, can be repeatedly measured in the same animal over extended periods without anesthesia and manipulation of the animal. In addition, whereas noninvasive measurements involve both the lower and the upper respiratory system, invasive measurements involve tracheal intubation and thus assess only the lower respiratory apparatus. Representative techniques include barometric plethysmography, and airway resistance measured by means of dual-chamber plethysmography.

A brief review of specific techniques that have been used to study the genetics of asthma in the mouse is presented in the following section.

INVASIVE MEASUREMENTS OF AIRWAY RESPONSIVENESS

Respiratory resistance

In the respiratory resistance (Rrs) technique, the animal is anesthetized and intubated via the trachea. After insertion of the cannula, the animal is mechanically ventilated at constant volume. The tracheal cannula is connected to a flow transducer as well as to a tap for measurement of tracheal pressure. Rrs measurements are calculated from changes in recordings of airflow and transrespiratory pressure during occlusion (at the instant of end inspiration) and after release of the airway (when transrespiratory pressure reaches a plateau). Rrs represents the change in transrespiratory pressure (before and after occlusion) divided by the magnitude of the inspiratory airflow.⁴ Measurements of airway reactivity through use of Rrs have been shown to agree closely with the airway pressure-time index (APTI).⁴

Airway pressure-time index

The APTI method is an invasive method that measures total respiratory impedance. To accomplish these measurements, the animal is anesthetized and a tracheal cannula with a side tap for measuring pressure is inserted.¹ Thereafter, the animal is mechanically ventilated at constant frequency and tidal volume. Measurements of transrespiratory pressure swings are taken before (to establish a baseline) and after intravenous delivery of a bronchoconstrictor agent such as acetylcholine. The change in the pressure-time profile measured after the delivery of the bronchoconstrictor represents the APTI. The integrated change in the peak airway pressure over time is calculated to determine the degree of airway reactivity.

Pulmonary resistance

To measure RL, the animal is anesthetized and the trachea is cannulated. Next, an intravenous line is established for the delivery of a bronchoconstrictor agent such as methacholine or acetylcholine, and a bilateral thoracotomy is performed to make pleural surface pressure measurements. In this method, continuous measurements of RL are made on a breath-by-breath basis from the tidal volume, tidal airflow, and transpulmonary pressure recordings. Transpulmonary pressure represents the pressure differential between the tracheal cannula and the plethysmograph (pleural surface). Tidal airflow is measured from changes in plethysmographic pressure in a constant-mass-body plethysmograph. This method has been widely used to measure airway obstruction after delivery of bronchoconstrictors.⁵⁻⁸

NONINVASIVE MEASUREMENT OF AIRWAY RESPONSIVENESS

Whole-body barometric plethysmography

The whole-body barometric plethysmography technique is advantageous in comparison with the invasive techniques because the animal is unrestrained and not anesthetized. Numerous serial measurements can thus be accomplished in the same animal over extended periods of time. In this method, pressure fluctuations associated with the ventilatory cycle are measured in a whole-body barometric plethysmograph, and a detailed analysis of the expiratory period is performed to calculate the Penh (enhanced pause). Changes in Penh have been shown to reflect changes in RL during bronchoconstriction.^{7,9}

DISTRIBUTION OF AIRWAY RESPONSIVENESS AMONG INBRED STRAINS OF MICE

The first survey of AR among numerous strains of inbred mice was reported by Levitt and Mitzner¹ in 1988. It is important to note that all mice within an inbred strain carry the same allele at a given genetic locus. Outbred populations, on the other hand, demonstrate considerable heterogeneity at every locus. For this reason, phenotypic

variability within any given inbred strain reflects solely environmental influences. In this study, the distribution of AR phenotypes to acetylcholine and serotonin were determined in 9 common inbred strains of mice, the APTI being used as the measure of AR (Fig 1). These investigators observed varying degrees of intrinsic AHR among the different strains; the A/J strain was identified as the most hyperresponsive ("asthmatic") and the C3H/HeJ strain as the most hyporesponsive ("non-asthmatic").¹ Specifically, there was a 6-fold difference in AR between the C3H/HeJ and A/J strains. The C57BL/6J strain was relatively hyporesponsive, but in a range similar in magnitude to that of the C3H/HeJ strain. The major conclusion of these studies was that the AHR phenotype is a heritable trait.

Other investigators have confirmed this conclusion. Konno et al¹⁰ measured AR in 6 different strains of inbred mice after intravenous delivery of methacholine and reported that the A/J and DBA/2 strains displayed a hyperreactive phenotype whereas the C57BL/6 and C3H/He displayed a relatively hyperreactive phenotype. In a more recent study, De Sanctis et al⁶ examined the distribution of AR phenotypes in the A/J and C57BL/6J strains using RL as the measurable phenotype. RL was measured after injection of increasing doses of methacholine, and on the basis of these data the dose of agonist necessary to double RL was determined. In accord with what had previously been noted by Levitt et al,^{1,2,4} De Sanctis et al⁶ reported that the A/J mice were relatively hyperresponsive in comparison with the C57BL/6J mice. A subsequent study by De Sanctis et al⁸ demonstrated the A/J inbred strain to be significantly more reactive to methacholine challenge than the C3H/HeJ inbred strain. The relative difference in AR between these 2 strains confirmed the SDP reported with other bronchoconstrictors.^{1,2}

In other studies examining the distribution of AR phenotypes, Zhang et al¹¹ and Longphre and Kleeberger¹² reported that the A/J and AKR/J strains were relatively hyperresponsive in comparison with the C3H/HeJ and C57BL/6J inbred strains.

An investigation by Gavett and Wills-Karp¹³ into the mechanism(s) underlying the difference in AR between the hyporesponsive C3H/HeJ strain and the hyperresponsive A/J strain suggests that the A/J strain exhibits enhanced muscarinic receptor signal transduction, which in turn is due to increased agonist affinity for muscarinic receptors and upregulation of G-protein levels.

Although the aforementioned studies reported strain-dependent differences in reactivity to intravenous bronchoconstrictors, Chiba et al¹⁴ extended these findings in an important manner by demonstrating that the route of challenge was an important determinant of the rank order of AR. Specifically, whereas intravenous challenge with acetylcholine revealed that the DBA/2 strain was relatively hyperresponsive in comparison with the C57BL/6 strain, inhalation with the same bronchoconstrictor failed to produce a measurable difference in AR in the 2 strains.

Studies of serotonin-induced airway responses (assessed by the APTI) have been carried out in numer-

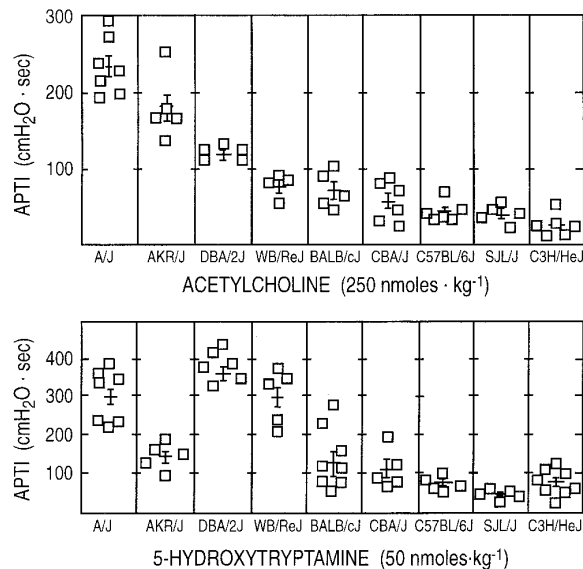


FIG 1. APTI response to either acetylcholine (250 nmol/kg) or 5-hydroxytryptamine (50 nmol/kg intravenously) in different strains of inbred mice. Each symbol represents the APTI response for an individual mouse. Reprinted with permission from the *Journal of Applied Physiology*.²

ous inbred strains of mice; they reveal that the rank order of AR among inbred strains of mice differ with different bronchoconstrictors. For example, the AKR/J strain was the least responsive inbred strain in a study by Levitt and Mitzner,¹ yet it was among the most reactive to acetylcholine. The dissimilarity in responses led the authors to hypothesize that different genes, inherited independently, were involved. These differential responses are strain-specific; Konno et al¹⁰ failed to observe a difference in airway responses to both acetylcholine and serotonin in C57BL/6J, WBB6F1-W/Wv, WBB6F1-+/+, DBA/2, C3H/HeN, and A/J mouse strains.

GENETIC STRATEGIES USED TO IDENTIFY AIRWAY HYPERRESPONSIVENESS SUSCEPTIBILITY GENES

Two general techniques have been used to analyze the genetics of AHR in the mouse: quantitative trait loci (QTL) analysis and candidate gene studies. Both techniques represent powerful tools for dissecting the genetics of AHR. QTL analysis is typically used for constructing primary genetic linkage maps of experimental and natural populations.¹⁵ Although QTL analysis allows one to determine the genomic regions where AHR susceptibility genes might be found, it does not allow the determination of the genetic contribution of specific candidate genes of biological relevance lying close to or within these intervals. One advantage to this approach, however, is that no prior knowledge of the gene(s) involved in the disease process is required for the identification of QTL. Through use of this approach, a genetic association between the phenotype—in this case, AR—and a locus (a

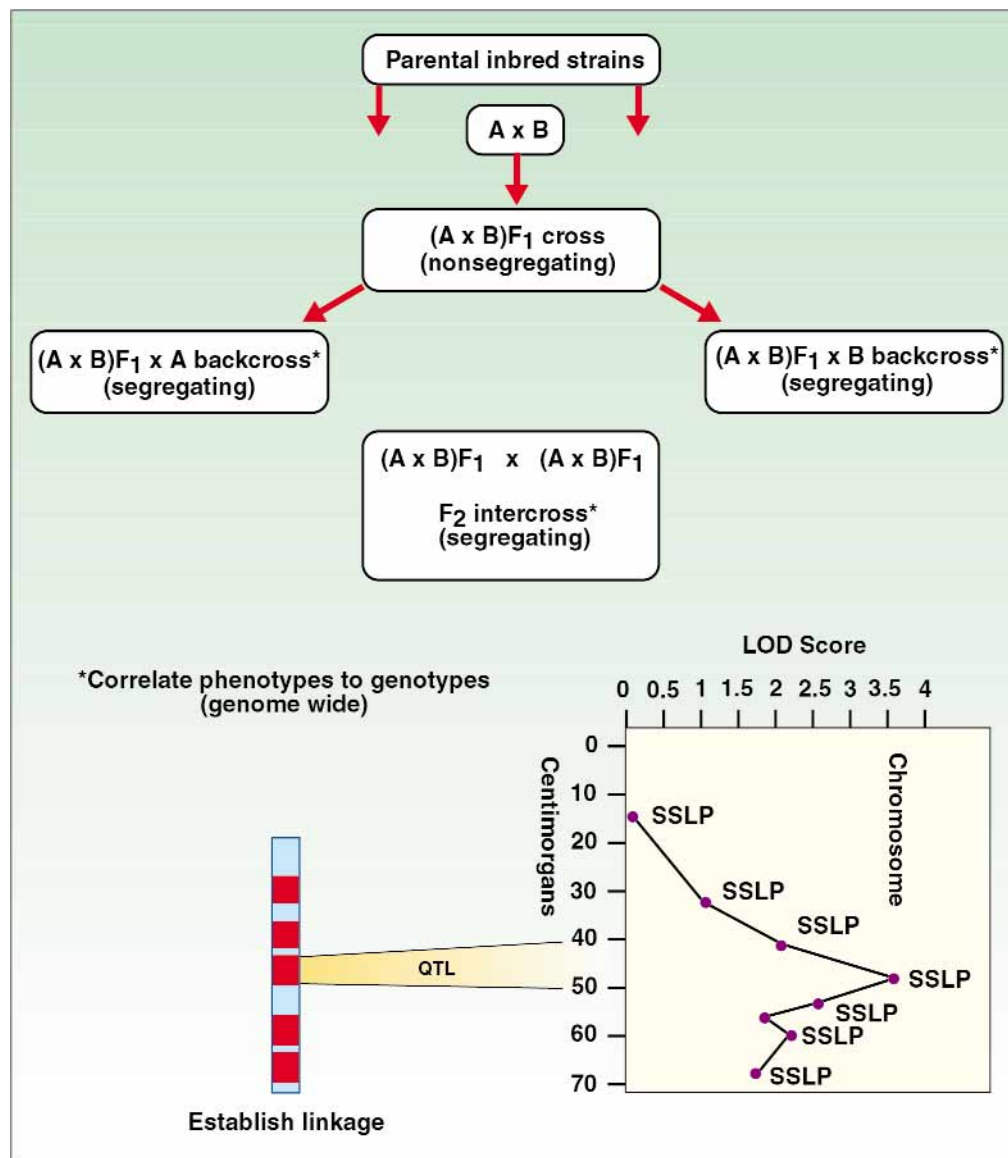


FIG 2. Schematic presentation of the genetic approach used to identify QTL. Parental strains that differ substantially in the trait of interest are mated together to produce the F₁ generation (nonsegregating). Subsequent simple crosses yield the backcross and intercross progeny (F₂; segregating) that are phenotyped and genotyped across the whole genome to identify by QTL analysis chromosomal regions harboring disease susceptibility gene(s).

genome fragment) can be established by means of the strategy about to be described.

The initial step involves breeding a hyperresponsive inbred strain with a hyporesponsive inbred strain (which differs substantially in this phenotype) to yield the first filial (F₁) generation (nonsegregating) and then backcrossing the F₁ progeny back to the appropriate parent (depending on the F₁ phenotype) to produce segregating offspring. Alternatively, the F₁ progeny can be bred together (F₂ intercross) to generate segregating offspring. The segregating progeny in either the second filial (F₂) intercross or the backcross can be used for QTL analysis. If the overall phenotype represents a summation of major and minor QTL, it is advantageous to pick parental strains that differ

substantially in the AHR phenotype. For example, if 2 parental strains differ by several SDs, considerably fewer segregating progeny would be required to phenotype and genotype (genomewide at intervals of approximately 10 centimorgans [cM]) to uncover potential regions harboring QTL.¹⁵ Once an initial screen is completed on the phenotypically extreme progeny (which provide the most informative genetic data), all segregating progeny are analyzed for the regions of interest. A schematic representation of this approach is presented in Fig 2.

In contrast to the QTL strategy, the candidate gene approach targets known genes of biological relevance. Typically, the suspected gene is manipulated and the contribution of the specific gene on the disease model

assessed. These methods include targeted deletion of a specific gene (commonly referred to as a KO) and overexpression of the protein(s) of interest (TG). The advantage of this technique is that the methods are well established for generating KO or TG mice and provide more detailed genetic information than does QTL analysis. The disadvantage of the technique is that only a limited number of genes can be manipulated.

MENDELIAN AND COMPLEX MODELS OF AIRWAY RESPONSIVENESS

Mendelian traits result from the contribution of a single mutant gene and occur when the transmission of the trait follows simple patterns of inheritance. Complex traits, on the other hand, result from the contribution of multiple genes that interact to produce a complex pattern of inheritance. The groundwork that set the stage for the first formal segregation analysis of mediator-induced AR came from earlier work by Levitt and Mitzner,^{1,2} who generated SDPs of acetylcholine and serotonin-induced airway responses, as measured by the APTI. From these data, they identified hyporeactive and hyperreactive inbred strains that differed substantially in the AR phenotype and set up simple genetic crosses to follow the transmission of the phenotype from generation to generation. Through use of this strategy, a hyperreactive, or “asthmatic,” strain is mated with a hyporeactive, or “non-asthmatic,” strain to produce the F1 generation. The variance of the F1 offspring (nonsegregating) represents environmental effects, whereas that of the segregating offspring represents both genetic and environmental effects. The transmission of the phenotypic trait is then followed in subsequent matings in the F2 intercross (F1 × F1, segregating) progeny and in the backcross [(F1 × Parent), B1] offspring (segregating).

The parental strains chosen in the first segregation analysis of acetylcholine-induced AR were the hyperreactive A/J and hyporeactive C3H/HeJ inbred strains; there was a 6-fold difference in AR between the 2 strains.¹ Analysis of the APTI phenotypes revealed that the F1 progeny and backcross progeny were uniformly hyporeactive to acetylcholine challenge and that the F2 intercross progeny segregated out with an observed 3:1 ratio of hyporeactive to hyperreactive progeny (Fig 3). The ratio of affected to unaffected progeny (in the segregating progeny) led the authors to conclude that the distribution of AR phenotypes was best described by an autosomal recessive pattern of inheritance. It is important to note that Mendelian traits of autosomal origin are encoded by genes on chromosomes other than sex chromosomes and are considered recessive if they are not expressed in the heterozygous animals.

Using serotonin as a mediator, Levitt and Mitzner² carried out another study 1 year later with the hyperresponsive DBA/2J and hyporesponsive C57BL/6J inbred strains. As in their previous study, the authors concluded that the distribution of AR phenotypes to this mediator was best described by an autosomal recessive pattern of inher-

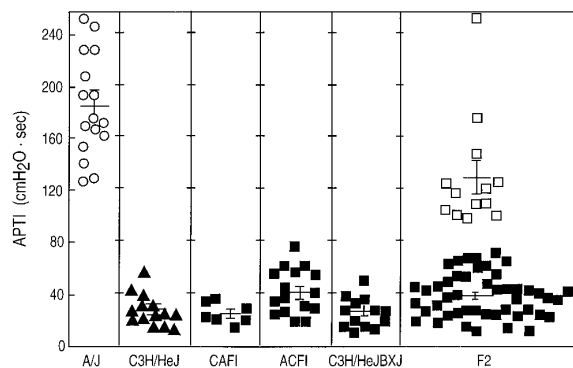


FIG 3. Airway responsiveness to 25 μ g acetylcholine (intravenously) in C3H/HeJ and A/J and their progeny. Means $\pm$ SEMs are presented. The relationship of the APTIs from individual animals of each cross is illustrated. CAF1, C3H/HeJ female $\times$ A/J male; ACF1, A/J female $\times$ C3H/HeJ male; C3H/HeJ BXJ, C3H/HeJ female $\times$ CAF1 male; F2, ACF1 $\times$ CAF1. Reprinted with permission from *The FASEB Journal*.¹

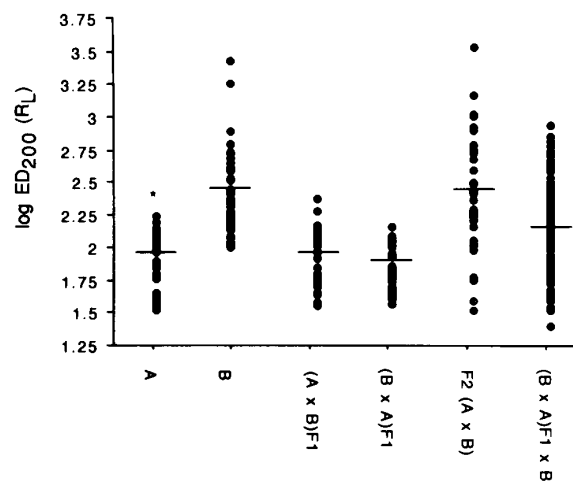


FIG 4. Scatterplot of log ED₂₀₀ RL from individual animals in parental, F1, F2, and backcross mice. Asterisk indicates significant difference from C57BL/6J mice ($P < .05$). A, A/J; B, C57BL/6J. Reprinted with permission from *Nature Genetics*.⁶

itance and that the responsiveness genes to acetylcholine and serotonin were not closely linked and are inherited independently.² On the basis of their studies, Levitt and Mitzner² concluded that AR to serotonin and AR to acetylcholine were each controlled by a single gene.

More recent genetic studies of AHR by De Sanctis et al⁶ and Ewart et al¹⁶ suggest that the pattern of inheritance is more complicated than previously reported. In the first study conducted by De Sanctis et al,⁶ the “asthmatic” A/J and “non-asthmatic” C57BL/6J parental strains were studied along with simple crosses derived from the parental strains. RL was measured after injections of increasing doses of methacholine chloride, a congener of acetylcholine. The results confirmed previously published data¹: the A/J mice had a hyperresponsive phenotype and the C57BL/6J mice a hyporesponsive phenotype.⁶ An analysis of the distribution of phenotypes in the F1 offspring from both reciprocal crosses demon-

TABLE I. Summary of quantitative trait loci studies of native AHR in mice

Year	Investigators	Strain		Agonist	LOD score	Chromosome	Candidate genes
		Hyperreactive	Hyporeactive				
1995	De Sanctis et al ⁶	A/J	C57BL/6J	MCh	3.0	2	<i>Il1B</i>
					3.7	15	<i>Il2rb</i> , <i>Il3rb1</i> , <i>Il3rb2</i> , <i>Pdgfb</i>
					2.85	17	<i>Tnfa</i> , <i>Mcpt6</i> , <i>Mcpt7</i>
1996	Ewart et al ¹⁶	A/J	C3H/HeJ	ACh	3.1	6	<i>Il5ra</i> , <i>Gnb-3</i> , <i>Ly-36</i> , <i>Ly-49</i>
					2.07	6	
1999	De Sanctis et al ⁸	A/J	C3H/HeJ	MCh	3.32	6	<i>Il5ra</i> , <i>Gnb-3</i> , <i>Ly-36</i> , <i>Ly-49</i>
					3.8	7	<i>Klk</i>
					1.7	17	<i>Tnfa</i> , <i>Mcpt6</i> , <i>Mcpt7</i>

strated, however, that the progeny were uniformly hyperresponsive, similar to the A/J parental strain. These results suggested that the AHR phenotype was inherited as a dominant rather than a recessive trait.⁶ Moreover, the distribution of AR phenotypes in the backcross and F2 intercross progeny failed to segregate out in a manner consistent with a single gene trait; they were unimodally and normally distributed (Fig 4). Indeed, the distribution of AR phenotypes in the backcross, the F2 intercross, and different recombinant inbred strains (derived from the A/J and C57BL/6J strains) exhibited a continuous distribution consistent with the hypothesis that the pattern of inheritance was complex and not Mendelian. Interestingly, the broad and unimodal distribution of AR phenotypes in the backcross progeny is similar to the distribution of AR phenotypes observed in human populations by Pattemore et al.¹⁷

De Sanctis et al⁶ extended the results of previous genetic studies of AHR^{1,2} in an important manner by considering AR as a quantitative trait and genotyping the phenotypically extreme offspring in the backcross progeny to identify specific genomic regions linked to the expression of the AHR phenotype. The phenotype, log ED₂₀₀ R_L, was used as the quantitative measure of AR. Numerically lower values of log ED₂₀₀ R_L are consistent with a higher degree of AR; conversely, numerically lower values of log ED₂₀₀ R_L are consistent with a lower level of AR. Briefly, a comprehensive set of unique genetic markers that are polymorphic for the 2 parental strains (A/J and C57BL/6J) were used with the AHR phenotype data in an initial cohort of the segregating backcross progeny. De Sanctis et al⁶ used 157 simple sequence length polymorphic markers spaced approximately 9 cM apart across the entire genome in the initial screen. The polymorphic markers used in the initial screen of the phenotypic extremes were evenly distributed across the entire mouse genome. Using the QTL MAPMAKER program,¹⁵ they determined whether a correlation existed between the quantitative data and the genotypic data—ie, linkage. This genome-wide search strategy uses interval mapping in which both genotypic data and phenotypic data are used to estimate the probable genotype and the most likely QTL effect across the

entire mouse genome.¹⁵ In this manner, De Sanctis et al⁶ identified 3 potential regions of linkage on chromosomes 2, 15, and 17. Through use of a denser map of markers (average spacing, approximately 1 cM) around the 3 regions of initial linkage, all of the backcross progeny were genotyped and significant linkage was found at 2 of the 3 QTL (designated *Bhr1*, *Bhr2*, and *Bhr3*). The third QTL (*Bhr3*) fell slightly below the threshold of significance (logarithm of odds ratio [LOD] = 2.83, chromosome 17; Table I).

When De Sanctis et al⁶ subsequently tested the 3 QTL for interactions using an ANOVA with cross terms for 2- and 3-way interactions, they found evidence of significant interactions between *Bhr1* and *Bhr3* and between *Bhr2* and *Bhr3*.⁶ Finally, several candidate genes of biological relevance to asthma are located in the vicinity of these QTL. These include IL-1* (*Il1B*), IL-2b (*Il2rb*) B, IL-3b1 (*Il3rb1*), IL-3b2 (*Il3rb2*), platelet-derived growth factor (B chain; *Pdgfb*), tumor necrosis factor α (*Tnfa*), and mast cell protease (tryptaselike) genes 6 (*Mcpt6*) and 7 (*Mcpt7*).

Using an approach similar to that of De Sanctis et al,⁶ Ewart et al¹⁶ subsequently examined the genetics of acetylcholine-mediated AHR in the A/J and C3H/HeJ inbred strains. In their study, a single dose of acetylcholine was delivered intravenously and AR was assessed by 2 different outcome indicators: the APTI and Rrs. Interestingly, when the distribution of AR phenotypes among the segregating progeny was analyzed in their study, the ratios of observed and expected hyperreactive and hyporeactive phenotypes did not reveal a Mendelian mode of inheritance; instead, it showed a more complex mode of inheritance.¹⁶ These results are at variance with previously reported results in the same genetic background and involving the use of the same phenotype (the APTI).¹

When Ewart et al¹⁶ genotyped and phenotyped the segregating progeny, a single significant QTL was obtained with the APTI of AR but not with the Rrs phenotype.¹ The QTL for AHR was detected on chromosome 6 with a maximum LOD of 3.11 for the APTI phenotype. Several genes of interest were found around this QTL, including IL-5 R (*Il5ra*), G protein B3 subunit (*Gnb-3*), and several T cell antigens that are important in

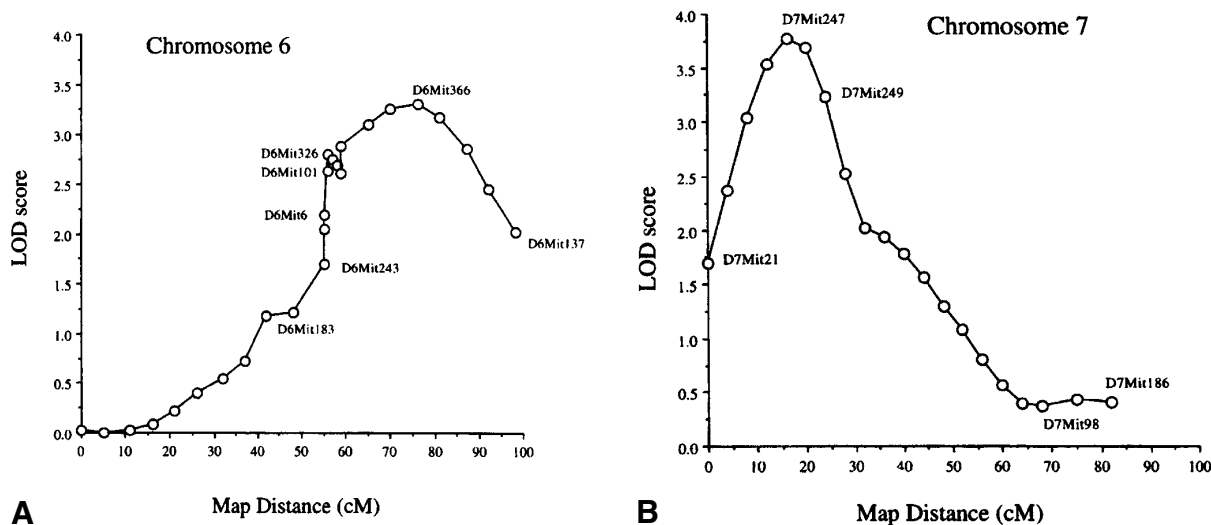


FIG 5. A, LOD score from genotypes of murine simple sequence length polymorphic markers for 128-361 informative backcross progeny on chromosome 6. **B**, LOD scores from genotypes of murine simple sequence length polymorphic markers for 137-224 informative progeny on chromosome 7. Reprinted with permission from the *American Journal of Physiology*.⁸

T-cell activation (*Ly-36*, *Ly-49*). When the segregation data were modeled through use of multiple inheritance models, the investigators concluded that the data were best described with a mixed general model in which the inheritance pattern was determined by one major locus along with a polygenic component.

The second phenotype, *Rrs*, also mapped to chromosome 6 within the interval spanning the QTL; however, the maximum LOD achieved with *Rrs* through this interval was 2.07, which was below the accepted threshold of significance.

In a subsequent study, De Sanctis et al⁸ performed a similar QTL analysis in the A/J and C3H/HeJ inbred strains in which intrinsic or native AR was assessed through use of a phenotype different from that used by Ewart et al.¹⁶ In their study, they reported 2 QTL on chromosomes 6 and 7 (Fig 5); the QTL on chromosome 6 confirmed a previous QTL by Ewart et al.¹⁶ The replication of the QTL on chromosome 6 is important because it demonstrates a common linkage with 2 different methods of phenotyping—ie, the APTI and RL. Thus the linkage on chromosome 6, designated as *bhr5*, is in close proximity to that reported previously by Ewart et al¹⁶ and is likely to represent the same locus. The significant QTL on chromosome 7 in their study was unexpected, inasmuch as no evidence of linkage had been demonstrated by Ewart et al in this region. The QTL on chromosome 7, named *bhr4*, maps close to an interesting gene, kallikrein (*Klk*), which has been implicated in the pathobiology of human asthma. An ANOVA with cross terms for 2- and 3-way interactions failed to find significant interactions between the QTL on chromosomes 6 and 7. This is in contrast to findings in the previous linkage study by De Sanctis et al,⁶ who reported significant interactions

between QTL in the A/J and C57BL/6J backgrounds.

The absence of linkage to chromosome 7 with the APTI might be due to any of several factors, including (1) differences in environmental factors during housing and (2) the greater number of segregating progeny that were both phenotyped and genotyped in the study by De Sanctis et al.⁸

Finally, there was evidence of a previously linkage, albeit at the suggestive level, on chromosome 17. This QTL was in the region of the *Bhr3* linkage reported in the A/J and C57BL/6J cross.⁶ Although the evidence for linkage is only suggestive, the QTL mapped in 2 different crosses, suggesting that the presence of A/J alleles in this region can affect the AHR phenotype in multiple genetic backgrounds.

In addition to the 3 previously mentioned linkage studies of intrinsic AHR in the mouse,^{6,8,16} Nicolaides et al¹⁸ tested for evidence of linkage between the AR phenotype and genetic markers in the mouse chromosomal regions that are syntenic to human chromosome 5q31-q33. The rationale for this approach was based on the observation that human studies had localized a major gene for asthma to the chromosomal region 5q31-q33 and that the IL-9 locus was suggested as a likely candidate on the basis of linkage disequilibrium between log serum total IgE level and a marker within this gene. In their study, 4 intervals on 3 mouse chromosomes (11, 13, and 18) were considered. Using bronchial responsiveness as a quantitative trait, the authors demonstrated linkage to chromosome 13 in a region harboring the IL-9 gene. When the gene was subsequently investigated as a gene candidate, the authors found that the expression of IL-9 was significantly reduced in the hyporesponsive mice, suggesting a role for IL-9 in the pathogenesis of AHR.

CANDIDATE GENE APPROACH: GENE DELETION AND OVEREXPRESSION STUDIES

The candidate gene approach has been successfully used as a tool for determining the biological contribution of candidate genes in various disease models. Indeed, several candidate genes have been implicated in the genesis of native or intrinsic AHR. Candidate genes can be selected for several reasons, including their proximity to QTL, subtractive hybridization, and RNA chip study findings. The interpretation of candidate gene studies can be complicated by the possibility that compensatory or redundant pathways might develop in the genetically manipulated mouse.

KNOCKOUT MICE STUDIES

The traditional manner of generating a KO mouse takes advantage of coat color to identify the deleted allele in the offspring; this is accomplished by using chimeric mice. The deletion is commonly done in the embryonic stem cells of mice on a 129-strain background. Once selected, the embryonic stem cells are subsequently inserted in another strain of mouse, traditionally C57BL/6. The chimeric mice are then backcrossed to the C57BL/6 strain to generate the heterozygous mice, and the heterozygous mice are in turn intercrossed to generate the F1 homozygote KO. Although the method is technically simple, one of the important issues in obtaining a KO mouse is the genetic purity of the KO. For genetic heterozygosity to be bred out, 9 or 10 generations of backcross breeding are required. Because this is costly and time-consuming, many studies report data on KO mice that have not been fully backcrossed and retain some degree of genetic heterogeneity from the 2 background strains, a possible confounding factor.

IL-8 receptor homolog KO

There is growing evidence to suggest that IL-8 is implicated in the pathobiology of asthma. IL-8 is a potent eosinophil chemoattractant,¹⁹ and several clinical studies have demonstrated the presence of IL-8 in the bronchoalveolar lavage fluid of patients with asthma.²⁰ Furthermore, there is an upregulation of free and complexed IL-8 in the blood and bronchial mucosa in people with asthma.²¹ Using a murine asthma model, De Sanctis et al²² measured methacholine-induced AR in the IL-8r homolog KO mice and found that their baseline responsiveness, in the absence of antigen challenge, was reduced in comparison with similarly treated wild type (WT) controls.

PDE4D KO

Hansen et al²³ reported an essential role for muscarinic signaling in the control of airway function and in the development of cholinergic-induced AHR. Specifically, mice deficient in a cAMP-specific phosphodiesterase (PDE4D^{-/-}) were no longer responsive to cholinergic stimulation, and baseline AHR present in the background strain was abolished in comparison with the

WT controls. Because serotonin-induced AR was not altered in the KO mice, the investigators concluded that the reduction in cholinergic AR was not due to anatomic changes in the airways that might affected the contractile properties of the smooth muscle.²³

TRANSGENIC MICE STUDIES

TG mice, like KO mice, have been invaluable for determining the functional role of specific genes on the genesis of native or intrinsic AHR. Using this approach, one can overexpress a protein in multiple organs in the body (ubiquitous expression) or in a defined tissue or cell type (tissue/cell-specific). The method is based on engineering different regulatory sequences linked to the gene or cDNA of the protein of interest. The ubiquitous promoters, such as cytomegalovirus and β -actin, do not always lead to an "all-body" expression, mainly because of the chromosomal site of integration or postinsertional modification of the transgene. The copy number of the transgene integrated is a major concern because the amount of protein expression is affected by this parameter. As the TG mouse is typically on a pure genetic background, there is no need for backcrossing.

It is important to note that the random chromosomal insertion of the transgene can affect the expression of some other endogenous gene(s), which in turn can conceivably alter the phenotype of interest. To control for this, the observed phenotype should be studied in at least 2 different TG lines. If the desired effect of the gene is absent, this might be due to insufficient expression of the gene.

In studies of the functional contribution of a candidate gene on airway function, a lung-specific promoter has been used to have pulmonary-specific expression. Because most epithelial cells in the trachea, bronchi, and bronchioles of mice are Clara cells,²⁴ which produce a 10-kDa protein referred to as *CC10*, the regulatory sequences of this protein have been used for lung-specific expression.

IL-4 transgenic mice

The contribution of IL-4 on baseline and methacholine-induced AHR has been investigated in IL-4 TG mice. This cytokine is produced mainly by T_H2 and mast cells, both of which are major players in the pathobiology of asthma. IL-4 plays an important role in IgE production by directly affecting the isotype switching of the B cell and has been shown to cause an increase in pulmonary infiltrates²⁵ and degree of eosinophil activation.²⁶ Rankin et al²⁷ generated TG mice expressing murine IL-4 in the lung using the lung-specific *CC10* promoter. Examination of lungs from these TG mice revealed hypertrophic changes in the airway epithelium and increased numbers of eosinophils and macrophages. Similarly, the total number of cells in the bronchoalveolar lavage fluid from TG mice was increased in comparison with WT animals.

With regard to an effect of IL-4 on AR, measurement of baseline airway resistance revealed a significantly greater airway tone in the TG mice than in the WT con-

trols. Methacholine-induced AR, however, was not significantly different between the TG and WT mice.²⁷

IL-5 transgenic mice

In another study examining the role of a cytokine on airway function, Lee et al²⁸ overexpressed the T_H2 cytokine IL-5 in the lung. In their study, the peribronchial spaces were infiltrated with leukocytes and there was an expansion of bronchus-associated lymphoid tissue. Histologic examination revealed an increase in the number, distribution, and staining of goblet cells in the TG mice and deposition of collagen in the bronchial epithelium. There was a 4-fold increase in the number of infiltrating cells in the TG mice relative to the Wt; the bronchoalveolar lavage fluid contained approximately 50% eosinophils. When AR was measured after methacholine challenge (whole-body plethysmography), the TG animals displayed greater AHR than the WT controls.

IL-6 transgenic mice

DiCosmo et al²⁹ generated TG mice overexpressing human IL-6 in the lung. Histologic evaluation of the lungs in the TG IL-6 mice revealed normal lung epithelium in the TG mice but an overwhelming infiltration of mononuclear cells in areas adjacent to large and mid-sized airways. Most of the infiltrating cells consisted of B and T lymphocytes; eosinophils were not present in the cell infiltrates. When AR was evaluated in the WT and TG mice, there was no difference in baseline RL despite a significant degree of airway inflammation in the IL-6 TG mice. Interestingly, methacholine challenge revealed a lower degree of AHR in the IL-6 TG mice as a result of unknown mechanisms. The findings of this study are particularly important because pulmonary inflammation, per se, did not lead to an enhancement of baseline responsiveness. The results of the IL-6 TG gene on AHR studies has recently been confirmed by Wang et al.³⁰

IL-11 transgenic mice

Using a human IL-11 cDNA, Tang et al³¹ generated TG mice that expressed IL-11 solely in the lung. Histologic examination of the lungs revealed airway wall thickening and increased numbers of B and T lymphocytes adjacent to bronchi and bronchioles. Increased deposition of collagen caused subepithelial fibrosis in the TG animals. Baseline AR was approximately 3-fold greater in the IL-11 TG mice than in the WT controls. Methacholine-induced AR was similarly greater in the IL-11 TG mice than in the WT controls. Although there was an increase in AR in the IL-11 TG mice, eosinophils were not present in the cell infiltrates. It is of interest that the composition of the cell infiltrates in the IL-11 TG mice was similar to that observed in the IL-6 TG mice.

IL-13 transgenic mice

IL-13 is another T_H2 cytokine suspected as a major player in the expression of AHR. IL-13 TG mice were generated through use of an IL-13 cDNA and the CC10

promoter in a C57BL/6 background. As mentioned earlier, C57BL/6 mice are characteristically hyporesponsive. However, the TG mice (on a C57BL/6 background) exhibited a significant increase in methacholine-induced AHR.³² There was evidence of predominantly mononuclear cell infiltration around the airways with increased numbers of macrophages and eosinophils. Eotaxin was detected in the lungs of TG animals, which can account in part, for the eosinophil infiltration. Airway epithelial cell hypertrophy, mucus production, and goblet cell hyperplasia were also evident.

Platelet-activating factor receptor transgenic mice

Platelet-activating factor (PAF) has been reported to be involved in the manifestation of AHR by different investigators.^{33,34} To establish a role for PAF on airway reactivity, Ishii et al³⁵ overexpressed PAFr using the guinea pig PAFr cDNA coupled to a β -actin promoter and cytomegalovirus enhancer. PAFr expression was detected in the lung as well as in some other tissues in the TG mice. Although there was no difference in baseline airway resistance, the TG mice exhibited a degree of methacholine-induced AR greater than that seen in WT controls. It should be noted that the enhanced response to methacholine was in large part reversed by the prior intravenous administration of a PAF antagonist.

CONCLUSION

These findings demonstrate that AHR, a hallmark of the asthmatic response, is a complex trait in the inbred mouse. Several QTL that confer AHR have been mapped to regions of the mouse chromosome that harbor candidate genes believed to be involved in the pathobiology of asthma. Given the high degree of homology between the mouse and human genomes, further work is required to evaluate the contribution of these gene candidates in asthma.

REFERENCES

1. Levitt RC, Mitzner W. Expression of airway hyperreactivity to acetylcholine as a simple autosomal recessive trait in mice. *Faseb J* 1988;2:2605-8.
2. Levitt RC, Mitzner W. Autosomal recessive inheritance of airway hyperreactivity to 5-hydroxytryptamine. *J Appl Physiol* 1989;67:1125-32.
3. Drazen JM, Finn PW, De Sanctis GT. Mouse models of airway responsiveness: physiological basis of observed outcomes and analysis of selected examples using these outcome indicators. *Ann Rev Physiol* 1999;61:593-625.
4. Ewart S, Levitt R, Mitzner W. Respiratory system mechanics in mice measured by end-inflation occlusion. *J Appl Physiol* 1995;79:560-6.
5. Martin TR, Gerard NP, Galli SJ, Drazen JM. Pulmonary responses to bronchoconstrictor agonists in the mouse. *J Appl Physiol* 1988;64:2318-23.
6. De Sanctis GT, Merchant M, Beier DR, et al. Quantitative locus analysis of airway hyperresponsiveness in A/J and C57BL/6J mice. *Nat Genet* 1995;11:150-4.
7. De Sanctis GT, MacLean JA, Hamada K, et al. Contribution of nitric oxide synthases 1, 2, and 3 to airway hyperresponsiveness and inflammation in a murine model of asthma. *J Exp Med* 1999;189:1621-30.
8. De Sanctis GT, Singer JB, Jiao A, et al. Quantitative trait locus mapping of airway responsiveness to chromosomes 6 and 7 in inbred mice. *Am J Physiol* 1999;277(6 Pt 1):L1118-L1123.
9. Hamelmann E, Schwarze J, Takeda K, et al. Noninvasive measurement of

- airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* 1997;156(3 Pt 1):766-75.
10. Konno S, Adachi M, Matsuura T, et al. Bronchial reactivity to methacholine and serotonin in six inbred mouse strains [in Japanese]. *Arerugi* 1993;42:42-7.
 11. Zhang LY, Levitt RC, Kleeberger SR. Differential susceptibility to ozone-induced airways hyperreactivity in inbred strains of mice. *Exp Lung Res* 1995;21:503-18.
 12. Longphre M, Kleeberger SR. Susceptibility to platelet-activating factor-induced airway hyperreactivity and hyperpermeability: interstrain variation and genetic control. *Am J Respir Cell Mol Biol* 1995;13:586-94.
 13. Gavett SH, Wills-Karp M. Elevated lung G protein levels and muscarinic receptor affinity in a mouse model of airway hyperreactivity. *Am J Physiol* 1993;265(5 Pt 1):L493-L500.
 14. Chiba Y, Yanagisawa R, Sagai M. Strain and route differences in airway responsiveness to acetylcholine in mice. *Res Commun Mol Pathol Pharmacol* 1995;90:169-72.
 15. Lander ES, Green P, Abrahamson J, et al. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* 1987;1:174-81.
 16. Ewart SL, Mitzner W, DiSilvestre DA, Meyers DA, Levitt RC. Airway hyperresponsiveness to acetylcholine: segregation analysis and evidence for linkage to murine chromosome 6. *Am J Respir Cell Mol Biol* 1996;14:487-95.
 17. Pattemore PK, Lampe FC, Smith S, Clough JB, Holgate ST, Johnston SL. Asthma survey items as predictors of respiratory problems in children 2 years later: a longitudinal study. *Eur Respir J* 1999;14:650-8.
 18. Nicolaides NC, Holroyd KJ, Ewart SL, et al. Interleukin 9: a candidate gene for asthma. *Proc Natl Acad Sci U S A* 1997;94:13175-80.
 19. Shute J. Interleukin-8 is a potent eosinophil chemo-attractant. *Clin Exp Allergy* 1994;24:203-6.
 20. Chanez P, Enander I, Jones I, Godard P, Bousquet J. Interleukin 8 in bronchoalveolar lavage of asthmatic and chronic bronchitis patients. *Int Arch Allergy Immunol* 1996;111:83-8.
 21. Shute JK, Vrugt B, Lindley IJ, et al. Free and complexed interleukin-8 in blood and bronchial mucosa in asthma. *Am J Respir Crit Care Med* 1997;155:1877-83.
 22. De Sanctis GT, MacLean JA, Qin S, et al. Interleukin-8 receptor modulates IgE production and B-cell expansion and trafficking in allergen-induced pulmonary inflammation. *J Clin Invest* 1999;103:507-15.
 23. Hansen G, Jin S, Umetsu DT, Conti M. Absence of muscarinic cholinergic airway responses in mice deficient in the cyclic nucleotide phosphodiesterase PDE4D. *Proc Natl Acad Sci U S A* 2000;97:6751-6.
 24. Plopper CG. Comparative morphologic features of bronchiolar epithelial cells. The Clara cell. *Am Rev Respir Dis* 1983;128(2 Pt 2):S37-S41.
 25. Gavett SH, Chen X, Finkelman F, Wills KM. Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *Am J Respir Cell Mol Biol* 1994;10:587-93.
 26. Favre C, Saeland S, Caux C, Duvert V, De Vries JE. Interleukin-4 has basophilic and eosinophilic cell growth-promoting activity on cord blood cells. *Blood* 1990;75:67-73.
 27. Rankin JA, Picarella DE, Geba GP, et al. Phenotypic and physiologic characterization of transgenic mice expressing interleukin 4 in the lung: lymphocytic and eosinophilic inflammation without airway hyperreactivity. *Proc Natl Acad Sci USA* 1996;93:7821-5.
 28. Lee JJ, McGarry MP, Farmer SC, et al. Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *J Exp Med* 1997;185:2143-56.
 29. DiCosmo BF, Geba GP, Picarella D, et al. Airway epithelial cell expression of interleukin-6 in transgenic mice. Uncoupling of airway inflammation and bronchial hyperreactivity. *J Clin Invest* 1994;94:2028-35.
 30. Wang J, Homer RJ, Chen Q, Elias JA. Endogenous and exogenous IL-6 inhibit aeroallergen-induced Th2 inflammation. *J Immunol* 2000;165:4051-61.
 31. Tang W, Geba GP, Zheng T, et al. Targeted expression of IL-11 in the murine airway causes lymphocytic inflammation, bronchial remodeling, and airways obstruction. *J Clin Invest* 1996;98:2845-53.
 32. Webb DC, McKenzie AN, Koskinen AM, Yang M, Mattes J, Foster PS. Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. *J Immunol* 2000;165:108-13.
 33. Miwa M, Miyake T, Yamanaka T, et al. Characterization of serum platelet-activating factor (PAF) acetylhydrolase. Correlation between deficiency of serum PAF acetylhydrolase and respiratory symptoms in asthmatic children. *J Clin Invest* 1988;82:1983-91.
 34. Ishida K, Thomson RJ, Beattie LL, Wiggs B, Schellenberg RR. Inhibition of antigen-induced airway hyperresponsiveness, but not acute hypoxia nor airway eosinophilia, by an antagonist of platelet-activating factor. *J Immunol* 1990;144:3907-11.
 35. Ishii S, Nagase T, Tashiro F, et al. Bronchial hyperreactivity, increased endotoxin lethality and melanocytic tumorigenesis in transgenic mice overexpressing platelet-activating factor receptor. *EMBO J* 1997;16:133-42.