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Interacting genetic loci cause airway hyperresponsiveness

Kate G. Ackerman,[1,2] Hailu Huang,[1] Hartmut Grasemann,[3] Chris Puma,[3] Jonathan B. Singer,[4] Annie E. Hill,[5] Eric Lander,[4] Joseph H. Nadeau,[5] Gary A. Churchill,[6] Jeffrey M. Drazen,[3] and David R. Beier[1]

[1]*Division of Genetics, Brigham and Women's Hospital and Harvard Medical School;* [2]*Division of Emergency Medicine, Children's Hospital;* [3]*Division of Pulmonary and Critical Care Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston;* [4]*Whitehead Institute, Cambridge, Massachusetts;* [5]*Department of Genetics, Case Western Reserve University, Cleveland, Ohio; and* [6]*The Jackson Laboratory, Bar Harbor, Maine*

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Ackerman, Kate G., Hailu Huang, Hartmut Grasemann, Chris Puma, Jonathan B. Singer, Annie E. Hill, Eric Lander, Joseph H. Nadeau, Gary A. Churchill, Jeffrey M. Drazen, and David R. Beier. Interacting genetic loci cause airway hyperresponsiveness. *Physiol Genomics* 21: 105–111, 2005. First published January 18, 2005; doi;10.1152/physiolgenomics.00267.2004.—Airway hyperresponsiveness (AHR) is a key physiological component of asthma, and the genetic basis of this complex trait has remained elusive. We created recombinant congenic mice with increased naive AHR by serially backcrossing A/J mice (which have elevated naive AHR) with C57BL/6J mice and selecting for mice with an elevated naive AHR phenotype. The seventh backcross-generation hyperresponsive mice retained A/J loci in three regions. Quantitative trait linkage (QTL) analysis of 123 unselected N8 progeny demonstrated that the AHR phenotype was not associated with any single locus but was significantly associated with an interaction of loci on chromosomes 2 and 6. These findings were confirmed in an independent analysis of chromosome substitution strain mice. The identification of genomic regions containing loci causally associated with AHR and the demonstration that this trait requires their interaction have important implications for the dissection of the genetic etiology of asthma in humans.

complex trait; mouse model; asthma; quantitative trait linkage

UNDERSTANDING THE CAUSATION of asthma remains challenging; however, it is particularly compelling, as the disease is increasingly common. Multiple processes contribute to the disease state, including chronic and acute inflammation, allergy, bronchial hyperresponsiveness, and airway remodeling. Because each patient with asthma may have a variable constellation of the different components, and each component is a complex and multifactorial process, the study of the genetics of asthma in humans is a formidable task. Identification of candidate genes for asthma and other common complex diseases by genetic analysis in human populations has proven to be difficult, and most have failed to reveal significant linkages (2). In over 10 large human studies employing genome-wide analysis to detect asthma susceptibility loci, only two significant linkages have been detected (2, 13, 32, 33). Of these, only one has identified an association with a specific candidate gene (32). Many additional candidate genes have been suggested based on association analyses (1, 9, 11, 12, 16, 17, 25, 34).

Because there is substantially less genetic heterogeneity between any single pair of inbred mouse strains compared with the human population, genetic analysis of common disease phenotypes in this model system may prove to be more fruitful. A limitation is that we can only detect the small number of loci that are segregating between the selected strains. Inbred mice show strain-specific variation with respect to various traits related to asthma, such as bronchial hyperresponsiveness and inflammatory or allergic responses (3, 19). In particular, the A/J mouse shows markedly elevated naive airway hyperresponsiveness (AHR) relative to most inbred strains (18). In a quantitative trait linkage (QTL) analysis of A/J × C57BL/6J mice, loci for naive AHR have been reported on chromosomes 2, 15, and 17 (5), while a similar analysis of A/J and C3H/HeJ (hyporesponsive) mice identified loci on chromosomes 6 and 7 (6, 7). This could be due to the fact that there are different strains involved and different methods of delivery of bronchoconstricting agonists (different agonists and routes of delivery) as well as to issues of statistical power and the likelihood of detection of a QTL. To address this, we designed a new strategy to find loci for AHR utilizing an alternative phenotyping method and an entirely different study design. Specifically, we utilized a nonterminal technique for measuring AHR, which allowed us to select for mice retaining this trait in a serial backcross. These mice were then tested to identify which regions of the A/J genome were retained, which are likely to contain the loci that cause AHR.

Using this approach, we were able to generate N7 B6.A recombinant congenic mice that have elevated AHR and retain A/J alleles at loci on chromosomes 2, 6, and 10. Genetic analysis of the retained regions demonstrates that AHR is not associated with any single locus, but is strongly correlated with the coinheritance of the loci on chromosomes 2 and 6, suggesting these interact. We used B6.A chromosome substitution strains (CSS) (24, 29) to independently confirm the effect of the interacting loci discovered in the congenic mice. In addition, we were able to rule out the effect of the chromosome 10 region on AHR.

Our genetic dissection of AHR in a mouse model has important implications not only for understanding human asthma, but also for the more general analysis of complex traits using association analysis. The observation that loci with a highly significant effect when analyzed in combination may show no association when analyzed individually illustrates the difficulty of discovering the etiology of complex genetic traits.

MATERIALS AND METHODS

Mice. All animal use was approved by the Harvard Medical Area Standing Committee on Animals (protocol no. 02863). A/J mice and C57BL/6J mice were obtained from Jackson Laboratory for breeding.

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Address for reprint requests and other correspondence: D. R. Beier, Genetics Division, Brigham and Women's Hospital, 75 Ave. Louis Pasteur, Boston, MA 02115 (E-mail: beier@receptor.med.harvard.edu).

Animals were housed in microisolation cages under viral antibody-free (VAF) conditions. CSSs were generated at Case Western Reserve University Medical School, as described previously (24). Combination CSSs were generated by crossing mice and analyzing three to five markers distributed across the consomic regions.

Phenotype analysis. Phenotype analysis was determined using a four-chamber noninvasive whole body plethysmograph developed by BUXCO. After calibration and regulation of chamber flow, animals were acclimatized to the chamber for 5 min before data collection. Baseline measurements were made before administration of 200 mg/ml methacholine (Sigma) in sterile PBS solution for 4.5 min, delivered by an ultrasonic nebulizer (DeVilbiss, Big Sandy, TX) into a central aerosolization chamber. Chamber flow was checked regularly to assure that all mice were exposed to the same dose of methacholine, and mice were rotated to different chambers on different days of testing. Each mouse was tested on 3 different days, with at least 1 day of rest between tests. This was determined to provide the most reproducible data for this dosing protocol in the inbred strains used. Average 1-min Penh readings were recorded after 3 min of aerosolization and continued for 12 min. This method of recording

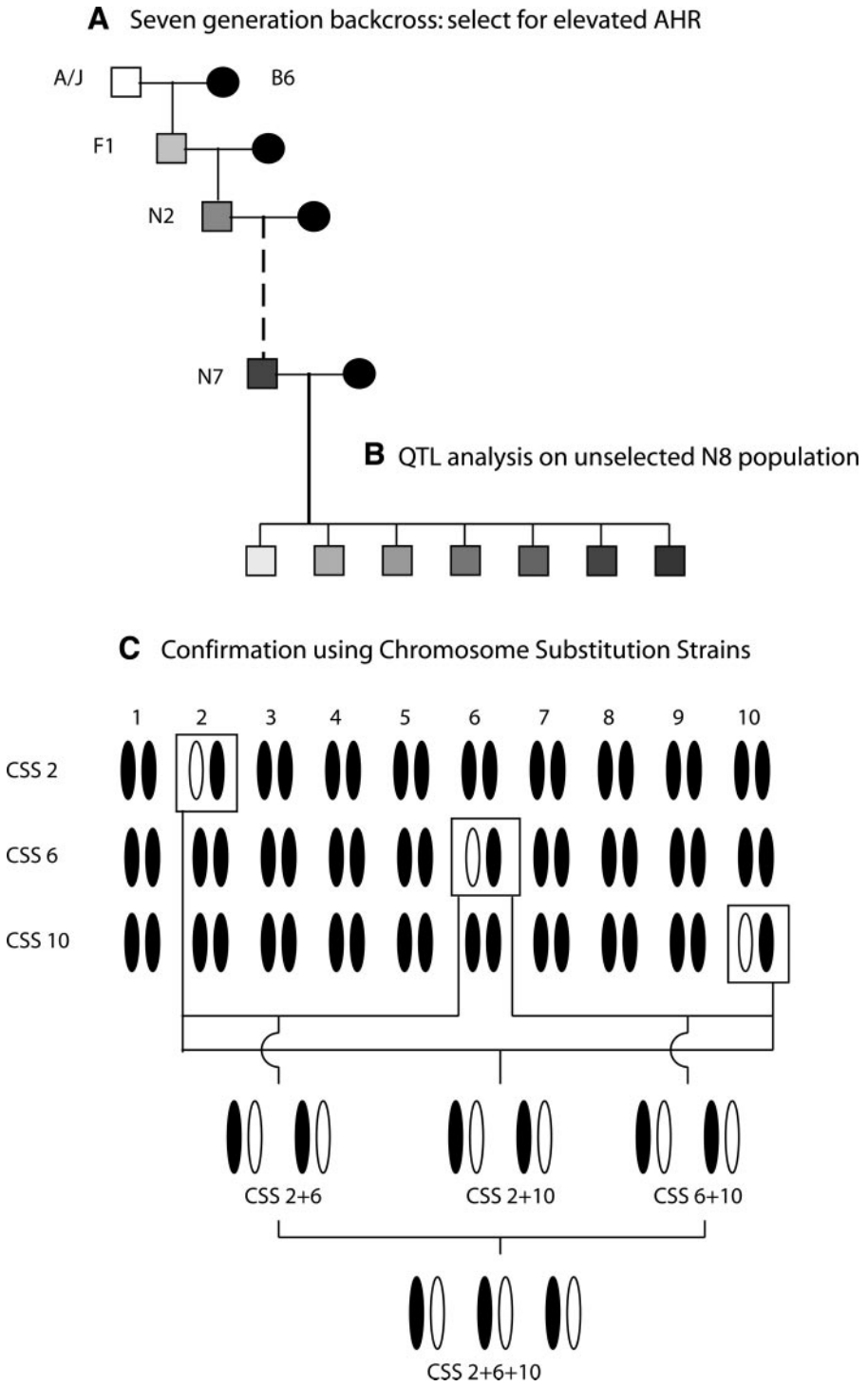

Fig. 1. Schematic diagram of the study design. *A*: a recombinant congenic line was created by serial backcrossing of mice with the airway hyperresponsiveness (AHR) phenotype to the hyporesponsive strain. B: at the 8th generation, a large population of male mice was evaluated for association between phenotype and genotype. QTL, quantitative train linkage. *C*: chromosome substitution strains (CSSs; Ref. 29) were obtained for chromosomes 2, 6, and 10. These mice were homozygous for A/J on chromosome 2, 6, or 10 (open chromosomes), whereas the other chromosomes were from the C57BL/6J strain (solid chromosomes). Mice heterozygous for each chromosome separately (first 3 rows) were created by crossing these CSSs to the background strain (C57BL/6J). Two-chromosome and three-chromosome combination strains (4th and 5th rows) were created by intercrossing the CSS strains and selecting for the appropriate genotype by analysis of polymorphic microsatellite markers.

assured capture of the peak response. Peak 1-min average Penh values were used for data analysis.

Pulmonary resistance after inhaled methacholine exposure was measured in anesthetized, ventilated mice with the Flexivent system (SCIREQ). Mice were anesthetized with pentobarbital sodium (80 mg/kg ip) diluted in sterile normal saline solution. Once adequate anesthesia was obtained, a tracheostomy tube (custom made from 20G tubing adaptors, BD Biosciences) was inserted and secured. Animals did not receive a paralytic agent but were studied in a deep anesthesia so that there were no spontaneous respiratory efforts. Standard ventilation was delivered at 10 ml/kg tidal volume, and a lung recruitment was performed immediately after placing the mice on the ventilator by occluding the expiratory loop circuit for three breath cycles. After an equilibration period, methacholine aerosol (5 mg/ml) was delivered by a nebulizer attached to the inspiratory ventilator circuit. Pulmonary resistance was measured immediately after methacholine delivery. Measurements were made every 4 s over a 2-min span, and peak pulmonary resistance was used for data analysis.

Genotype analysis. A genome-wide scan was conducted, using polymorphic microsatellite markers distributed at 10-cM intervals (list available on request). Beginning at the N2 generation, polymorphic microsatellite markers were used to genotype hyperresponsive recombinant congenic mice at 10-cM intervals across the genome. At subsequent generations, hyperresponsive males were genotyped for those regions that had been found to retain A/J alleles. DNA was extracted and PCR amplification was performed by use of standard techniques.

Statistical analysis. Genetic mapping analysis was carried out using the method of Sen and Churchill (28) with the pseudomarker software package (version 1.02; **http://www.jax.org/staff/churchill/labsite/software**). The genome scan was carried out at 2-cM intervals, using 64 imputations. The pairwise genome scan is described in detail elsewhere (28, 31). Briefly, we considered all possible pairs of loci on a 2-cM grid covering the targeted genomic regions. A 2-QTL model including an interaction term was fit to obtain the LOD scores. We repeated the scan 100 times using permuted data to establish multiple-test adjusted significance levels. A 2-QTL additive model (no interaction term) was fit to obtain a test for gene × gene interaction. ANOVA and *t*-tests for the strain comparisons were carried out with Minitab software (Minitab).

RESULTS

The study was carried out in three phases (Fig. 1). F1 (A/J × C57BL/6J) mice have been shown to retain the AHR phenotype by pulmonary resistance measurements (5). Penh measurements in F1 (A/J × C57BL/6J) mice also showed that the elevated naive AHR trait is dominantly inherited from the A/J strain: F1 mice retained 100% of the A/J AHR (Penh) phenotype (Fig. 2). This suggested that it would be possible to use a serial backcross to produce B6.AJ congenic mice that retain the elevated naive AHR trait. An F1 male mouse was backcrossed to female C57BL/6J mice, and 8-wk-old N2 male progeny were phenotyped for Penh, a measure of airway response (10). In each generation, at least 16 male mice were studied, and the mouse with the highest Penh was used for the next generation backcross (Table 1).

Table 1. *Phenotype (enhanced pause, Penh) and inherited loci of the male mouse with the highest AHR in each generation*

Generation	No. Phenotyped	Highest Male Phenotype, %A/J	Inherited Chromosomal Regions
N2	27	77%	1, 2, 5–7, 9–11, 13–17, 19
N3	21	100%	2, 6, 7, 9–11, 15–17
N4	33	85%	2, 6, 10, 11, 15
N5	48	85%	2, 6, 10
N6	16	96%	2, 6, 10
N7	28	80%	2, 6, 10

Penh is expressed as the % A/J response, the positive control strain. AHR, airway hyperresponsiveness.

Phenotype analysis for Penh was conducted with the use of a whole body noninvasive plethysmograph (BUXCO, Sharon, CT). The specific protocol utilized was designed to produce the best differentiation of Penh between the naive strains being tested. After an acclimation period, mice were challenged with a single dose of 200 mg/ml methacholine for 4.5 min, and Penh response was measured for 12 min. Peak 1-min average responses were used for analysis.

Mice at the N7 generation retained A/J genome in three regions. These were a 25-cM interval on distal chromosome 2 (*D2Mit259–D2Mit148*), a 36.5-cM interval on proximal chro-

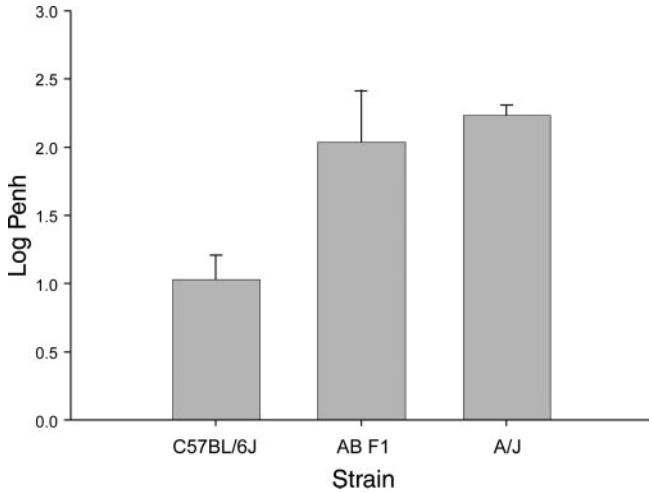

Fig. 2. AHR in C57BL/6J (hyporesponsive), A/J (hyperresponsive), and A/J × C57BL/6J (F1) mice measured by Penh. F1 mice retain the AHR phenotype, as the A/J alleles are acting in a dominant fashion.

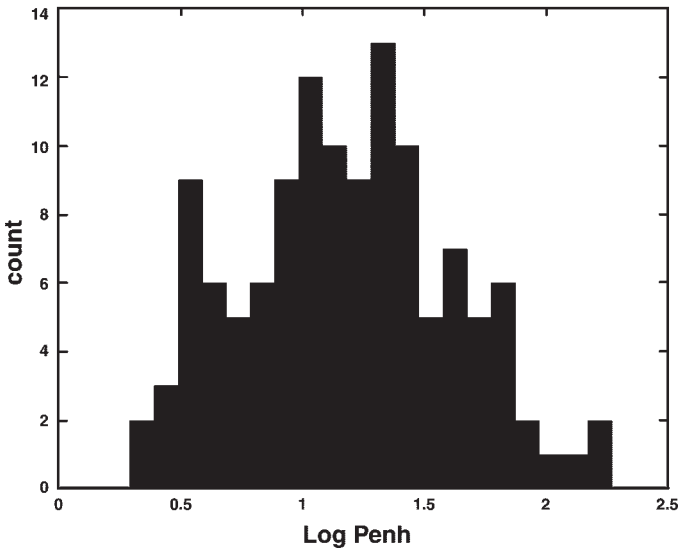

Fig. 3. Distribution of log-transformed AHR values (log Penh) among N8 progeny used for QTL analysis.

Table 2. *QTL analysis of N8 progeny*

Chromosome	Peak LOD Score	*P* Value
2	1.12	0.023
6	1.43	0.010
10	0.42	0.164
2+6	4.94	0.0001

QTL, quantitative trait linkage; LOD, xxx.

mosome 6 (*D6Mit86–D6Mit9*), and a 26-cM region on distal chromosome 10 (*D10Mit95–D10Mit103*).

Given the low probability of random retention in N7 mice of any single A/J locus, it is unlikely that the retained regions were inherited by chance. However, the A/J alleles may have been selected for reasons unrelated to an elevated AHR phenotype, such as increased viability or fertility. To assess this, we analyzed whether the retained A/J alleles were correlated with elevated AHR in an unselected population derived from an N7 recombinant congenic mouse. For this purpose, 123 N8 male progeny were analyzed for Penh at 8 wk of age. These mice were also tested for genotype at 13 markers distributed across the retained regions. The distribution of AHR values is shown in Fig. 3.

For the QTL analysis of the N8 population, the trait data were log transformed to reduce skewing and stabilize variances. Significance thresholds were determined, using permutation analysis to correct for multiple testing over three genomic regions (4). Only suggestive evidence was found for the retained regions on chromosomes 2 and 6. However, when a pairwise scan for interactions was done, a highly significant association was found for the retained chromosome 2 and 6 regions (Table 2, Fig. 4). A regression model is consistent with this result; in this analysis, 2 QTL + interaction LOD = 5.55. The total variance explained by these loci is 18.7%, and the fraction of the total variance attributable to the interaction is 11.3%. The chromosome 10 region was not found to be associated with AHR in either analysis.

To independently assess the contribution of the retained A/J regions to AHR, we analyzed B6.A CSS mice that were heterosomic for chromosomes 2, 6, or 10. This was feasible because the A/J alleles are genetically dominant, as A/J × C57BL/6J F1 mice retain 100% of the A/J phenotype (Fig. 2). In addition, combination heterosomic strains were created by breeding to test potential interactions between the loci retained by the congenic mice (chromosomes 2+6, 2+10, 6+10, and 2+6+10) (Fig. 1). Finally, CSS strains homozygous for each of the three single chromosomes were tested. Male mice were phenotyped at 8 wk of age with the same protocol used for the recombinant congenic mice. The trait data were log transformed, and one-way ANOVA was performed to evaluate the overall differences among strains. Pairwise comparisons between strains utilized a *t*-test with Tukey's correction for multiple comparisons. Data for CSS mice are shown in Fig. 5. Mice heterosomic for chromosome 2 showed some elevation above the B6 background ($P < 0.0001$). However, among heterosomic strains, the combination strain CSS 2+6 had the highest AHR phenotype (Fig. 5*A*). This is consistent with our finding in the recombinant congenic population and confirms the presence of interacting loci in the inherited regions. CSS 2+6+10 mice did not show any additional increase in the AHR phenotype, supporting the conclusion that the loci on chromosome 10 are not contributing to the phenotype. Interestingly, CSS mice homozygous for chromosome 6 or chromosome 2 had a high degree of AHR, even in the absence of an interchromosomal interaction ($P < 0.0001$ compared with B6; Fig. 5*B*).

Because our phenotyping method used for these experiments is a surrogate measure of true AHR, we measured pulmonary resistance in CSS 2+6+10 and control mice after exposure to aerosolized methacholine. Peak pulmonary resistance after

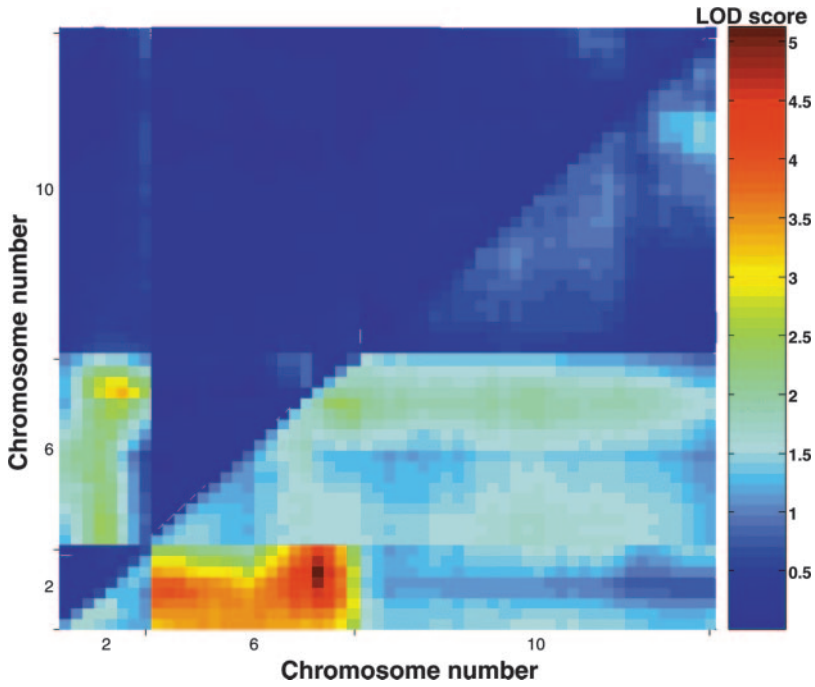

Fig. 4. QTL analysis: pairwise genome scan. LOD scores obtained from a simultaneous scan of all locus pairs are shown using a color scale; *x*- and *y*-axes are genomic positions, and the image is pixilated in 2-cM intervals. *Bottom right* triangle displays the full LOD score, accounting for two main effects and an interaction term. The peak (LOD = 4.79) occurs at the intersection of chromosome 2 at 8 cM (*y*-axis) and chromosome 6 at 28 cM (*x*-axis). *Top left* triangle displays the component of the LOD score that is attributable to the interaction alone; the peak value ($LOD_{int} = 3.07$) occurs at the same position and is highly significant ($P = 0.0002$, unadjusted).

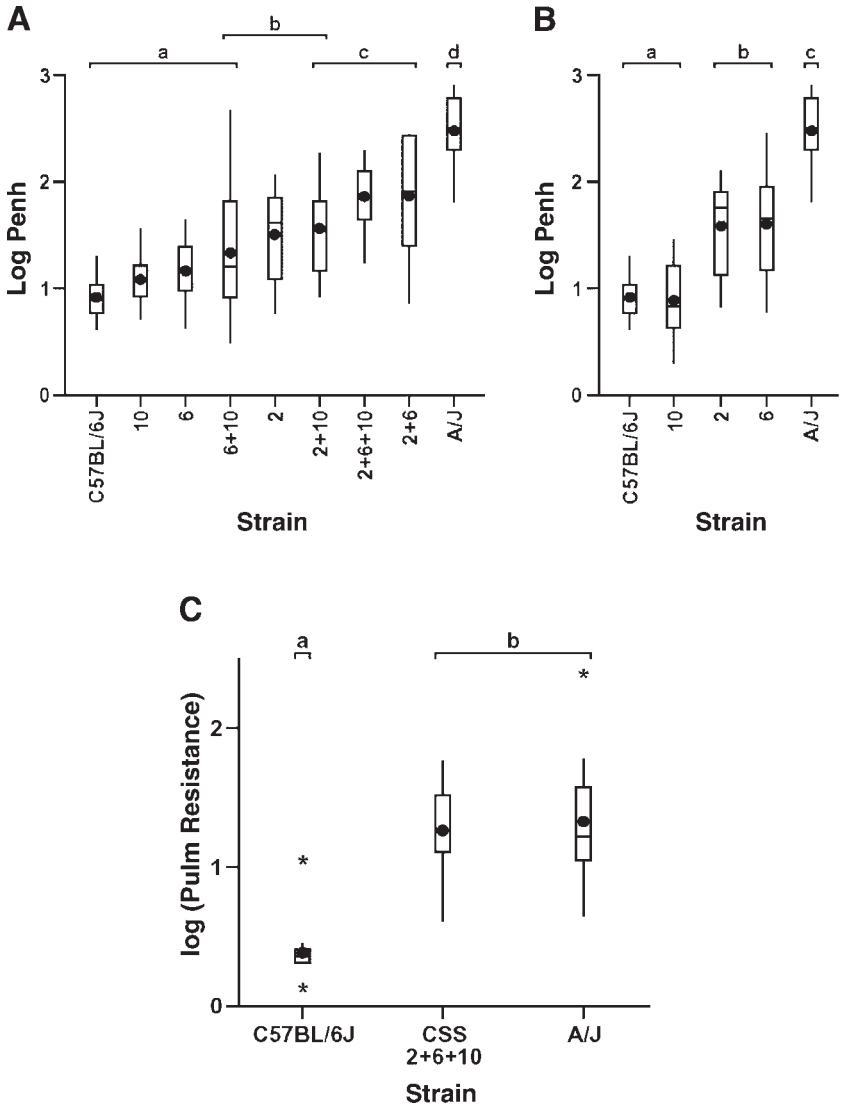

Fig. 5. Analysis of AHR (*A* and *B*: log Penh; *C*: pulmonary resistance) in CSSs. Box shows the median and interquartile range of the data, whiskers show the range, and the mean is indicated by ●. Sets of strains that cannot be distinguished by a *t*-test using Tukey's adjustment for multiple comparisons are grouped as indicated by horizontal bars *a*, *b*, *c*, and *d*. Strains not in the same grouping are statistically distinct. *A*: mice heterozygous for A/J on chromosomes 2 plus 6 had higher AHR than mice with heterozygous loci on chromosome 6 alone. The addition of loci on chromosome 10 did not increase the AHR phenotype in any of the strain comparisons. *B*: in homozygous single CSSs, chromosome 2 and chromosome 6 loci are associated with an elevated AHR that is significantly higher than the B6 strain but not as high as for A/J. *C*: pulmonary resistance was measured after exposure to aerosolized methacholine. CSS 2+6+10 mice had significantly elevated pulmonary resistance compared with the C57BL/6J hyporesponsive strain and are comparable to A/J mice.

methacholine exposure was significantly higher in CSS 2+6+10 mice than in hyporesponsive C57BL/6J control mice ($P < 0.0001$). The elevated pulmonary resistance observed was similar to that measured in the hyperresponsive A/J control strain (Fig. 5*C*).

DISCUSSION

We have used selection for a disease phenotype in a serial backcross to definitively identify loci associated with a genetically complex trait. In this strategy, genomic regions that are causally associated with the trait are retained, while unassociated regions are highly likely to be lost as breeding proceeds. Although there is no apparent association between inheritance of any single locus with elevated naive AHR in the phenotypically derived recombinant congenic line, we found a highly significant association with the trait when loci on both chromosomes 2 and 6 were inherited together. The evidence that interactions can play an important role in complex traits suggests that combinatorial analysis should be a feature of the association studies that are being facilitated by characterization of human haplotypes. The theoretical possibility that this type of interaction could confound human genetic association studies has been raised (14). However, a result with this magnitude of effect has not been previously shown using contemporary methods of linkage and association analysis.

The loci retained on chromosomes 2 and 6 have been reported in previous QTL experiments; however, an interaction was not previously shown. On chromosome 2, the previously reported QTL for the A/J × C57BL/6J cross reported a peak at *D2Mit409*(5). This marker maps to 74.3 Mb, and this region is retained in the recombinant congenic mice. Loci on chromosome 6 had been reported in previous A/J × C3H/FeJ crosses (6, 7). On the basis of current physical mapping of the previously reported markers, the QTL on chromosome 6 localizes to the 72- to 135-Mb region. This region has a small overlap with the region we report on chromosome 6, which extends from 0 to 88 Mb. The significance of this overlap is qualified by the fact that, in both previous studies, the analysis of chromosome 6 revealed a large region of recombination suppression, and recent studies reveal the C3H strains used were likely to have contained a cytogenetic inversion (**http://jaxmice.jax.org/library/notes/491p.html**). One cannot readily assess how this

large region of recombination suppression may have affected the QTL localization in those experiments.

We found A/J loci retained on chromosome 10 but could not show an association with the AHR phenotype in either population of mice. Because it is improbable that this region was inherited only by chance, it is likely that it was selected for reasons unrelated to AHR and may contribute to survival, fertility, or another unknown factor. This demonstrates the importance of testing whether the retained regions in congenic mice are associated with the desired phenotype.

The region of the human genome that has conserved synteny with the retained A/J region of chromosomes 2 and 6 is large, which makes the prediction of candidate genes for causation of AHR impractical. However, it is notable that the metalloprotease ADAM33 is located within the retained region on chromosome 2. Human studies have associated single-nucleotide polymorphisms (SNPs) in the ADAM33 gene with asthma (15, 32), although this has not been replicated in all populations (20, 26). Recent data in the mouse suggest that differences in *Adam33* activity are not likely to account for the strain-specific AHR phenotypes, as a multistrain comparison of 198 potential SNPs in *Adam33* genomic sequence (including extensive upstream and downstream regions) found that none differed between the A/J and C57BL/6J strains (**http://mousesnp.roche.com/**). Examination of an additional 26 nonoverlapping SNPs in the Celera proprietary database also revealed no differences. Because the A/J and C57BL/6J strains appear to retain the same ancestral haplotype at *Adam33*, and because the association between human asthma and polymorphisms in ADAM33 is uncertain (27), we suggest that it may be a closely linked gene that is contributing to the phenotype in both humans and mice.

Recently, the dipeptidyl peptidase DPP10 was found to be associated with asthma in humans and was reported to be in a region of conserved synteny with mouse loci on chromosome 2 linked to elevated AHR (1). However, the mouse ortholog of DPP10 (Genbank accession no. AK046842) resides on chromosome 1 and is thus not a candidate for causing elevated naive AHR in A/J mice.

Both the recombinant congenic and heterosomic CSS 2+6 mice have a lesser degree of AHR than the parental A/J strain. This suggests that the maximally severe phenotype depends on inheritance of more than the two interacting loci we have identified. Given that the number of mice that could be practically generated and analyzed at each generation of a phenotype-driven selection is constrained, it is not unexpected that we would not capture all of the contributing loci. In this regard, it is notable that in our previous analysis of A/J × C57BL/6J mice, loci on chromosomes 15 and 17 were found associated with AHR with approximately the same significance as the region of chromosome 2 that was confirmed in this study (LOD values of 3.7 and 2.95, respectively) (5). One could potentially reintroduce these regions using the appropriate CSS mice to assess their possible contribution to the AHR trait.

We used unrestrained plethysmography to assess the AHR phenotype in this study. This noninvasive method was crucial to our phenotypic selection strategy, because the tested animals could be utilized for breeding future generations. In addition, measurement of invasive pulmonary resistance requires anesthesia and surgical intervention, both of which may alter an animal's bronchial hyperresponsiveness. Penh, or enhanced pause, does not directly measure airway resistance and is a calculated variable based on the characteristics of an animal's breathing pattern. The respiratory pattern waveform in a mouse with bronchoconstriction changes in a characteristic manner, and these changes are incorporated into the Penh calculation, as bronchoconstriction causes greater expiratory respiratory effort and results in a longer expiratory time. Others have shown that Penh correlates well with the "gold standard" measurement of invasive pulmonary resistance (10), and we have found good correlation in our lab between the two methods (8). However, it has been suggested that a correlation between pulmonary resistance and Penh does not require that these phenotyping methods measure the same thing, and the likelihood that Penh is purely a measure of airway resistance has been disputed (21–23). Because Penh is an empiric value, we compare our study animals to hyper- and normoresponsive control strains in every experiment. We developed the specific protocol used based on extensive trials designed to provide the best differentiation of methacholine-induced breathing pattern between the two parental strains, and we feel that the phenotype measured is a true surrogate of bronchial hyperresponsiveness. In addition, we have confirmed our AHR phenotype by showing that heterosomic 2+6+10 CSS mice have elevated pulmonary resistance when measured with standard techniques.

As we have noted, our study has important implications for the genetic analysis of common diseases in a human population. Our results also have specific utility for the analysis of human asthma. While model systems indisputably cannot capture all of the causal factors for common diseases in humans due to their genetic homogeneity, controlled environmental exposures, and fundamental differences in biology, there is evidence for striking concordance between loci of major effects found in human and animal studies (30, 31). In this report, the regions of chromosomes 2 and 6 that are retained correspond to 153 Mb of DNA that have conserved synteny distributed among seven human chromosomes. A comprehensive analysis of these regions using association analysis may prove fruitful for uncovering additional loci that contribute to the human disease.

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Present addresses: E. Lander, Broad Institute, 320 Charles St., Cambridge, MA 02141-2023; J. B. Singer, Novartis Institutes for BioMedical Research Inc., 250 Massachusetts Ave., Cambridge, MA 02139.

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