Journal of the American Association for Laboratory Animal Science
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Vol 51, No 4
July 2012
Pages 401–406

Variation in Airway Responsiveness of Male C57BL/6 Mice from 5 Vendors

Herng-Yu Sucie Chang,[1] Wayne Mitzner,[1] and Julie Watson[2,*]

Mice are now the most commonly used animal model for the study of asthma. The mouse asthma model has many characteristics of the human pathology, including allergic sensitization and airway hyperresponsiveness. Inbred strains are commonly used to avoid variations due to genetic background, but variations due to rearing environment are not as well recognized. After a change in mouse vendors and a switch from C57BL/6J mice to C57BL/6N mice, we noted significant differences in airway responsiveness between the substrains. To further investigate the effect of vendor, we tested C57BL/6N mice from 3 other vendors and found significant differences between several of the substrains. To test whether this difference was due to genetic drift or rearing environment, we purchased new groups of mice from all 5 vendors, bred them in separate vendor-specific groups under uniform environmental conditions, and tested male first generation (F1) offspring at 8 to 10 wk of age. These F1 mice showed no significant differences in airway responsiveness, indicating that the rearing environment rather than genetic differences was responsible for the initial variation in pulmonary phenotype. The environmental factors that caused the phenotypic variation are unknown. However, differences between vendor in feed components, bedding type, or microbiome could have contributed. Whatever the basis, investigators using mouse models of asthma should be cautious in comparing data from mice obtained from different vendors.

Abbreviation: AHR, airway hyperresponsiveness.

In studies of the mouse lung involving measurement of pulmonary function, investigators commonly use inbred strains of mice to ensure a common genetic background. When mice are genetically identical the effects of specific environmental or genetic perturbations can be studied independent of background genotype. However, the need to similarly control for the source of the inbred mice is not always so apparent. In an effort to reduce costs in ongoing studies involving lung function measurements, we switched vendors from The Jackson Laboratory to the National Cancer Institute. Initial studies with the C57BL/6NCr mice unexpectedly showed substantially less responsiveness of the airways compared with the C57BL/6J mice. This preliminary observation called into question the validity of comparisons between studies of C57BL/6 mice of different substrains purchased from different vendors. If the differences were due to genetic drift rather than environmental factors, the effect of this variation could extend to genetically engineered mouse models generated by using different C57BL/6 substrains.

C57BL/6 substrains have a long history in the United States: they were so named because they originated as black offspring from female mouse number 57 and male mouse number 52 in a mating by Clarence Cook (CC) Little of Abbie Lathrop's stock in 1921. CC Little subsequently founded the Jackson Laboratory, and the substrain C57BL/6 was established at The Jackson Laboratory prior to 1937.[14] The sublines C57BL/6N and C57BL/6J were separated at NIH in 1951. Harlan and Charles River acquired their breeding colonies from NIH in 1974, Taconic in 1991, and the National Cancer Institute in 1996. These long passages of time would suggest that genetic mutations arising in different colonies could have resulted in genetically distinct substrains. However, several studies suggest only minimal differences exist.[29,32,44] Most recently, one study[44] evaluated 1449 single-nucleotide polymorphisms distributed over all 20 chromosomes in 10 C57BL/6 sources from Europe, Australia, and the United States. Of the 1449 single-nucleotide polymorphisms, only 12 were polymorphic between strains, and most could not be directly associated with a known gene. Although these single-nucleotide polymorphisms distinguished the B6/N substrains from the B6/J substrains, there were no differences within the 4 B6/N or the 3 primary B6/J sources, whereas a second group of 3 B6/J sources differed by 3 single-nucleotide polymorphisms from the primary B6/J sources.

These minimal differences in genotype between B6 substrains suggested that environmental factors may have played the major role in the phenotypic differences we observed. Differences in phenotype attributable to environmental variation have previously been reported in several fields. For example, behavioral testing differences in inbred mice were attributed to different testing locations;[11] behavioral tests, tumor growth, and immunologic parameters were affected by veterinary treatments with fenbendazole,[15,17,25] and numerous research areas are affected by intercurrent infections.[9,14] In addition, differences in behavioral testing attributed to differences between B6/J and B6/N mice[5] may have resulted from differences in rearing environment rather than genetic differences. However, to our knowledge, there have been no reports of differences in airway responsiveness in B6 mice from different vendors. To further describe this finding and to evaluate the differing roles of genetics and environment, we tested airway responsiveness in 5 substrains of male B6 mice from 5 different vendors in the United States and then repeated the tests in the male offspring of mice of the same substrains purchased from the same vendors

Received: 06 Sep 2011. Revision requested: 11 Oct 2011. Accepted: 12 Dec 2011.
Departments of [1]Environmental Health Sciences and [2]Molecular and Comparative Pathobiology, Johns Hopkins University, Baltimore, Maryland.
[*]Corresponding author. Email: jwatso19@jhmi.edu

but bred and maintained under uniform environmental conditions at our institution.

Materials and Methods

Mice. Groups of 8 inbred male C57BL/6 mice were purchased at ages 7 to 9 wk from each of 5 United States vendors: C57BL/6J (The Jackson Laboratory, Bar Harbor, ME); C57BL/6NCrl (Charles River Laboratories, Wilmington, MA); C57BL/6NTac (Taconic Farms, Hudson, NY); C56BL/6NHsd (Harlan Laboratories, Indianapolis, IN); and C57BL/6NCr (National Cancer Institute, Frederick, MD). Mice were tested at 9 to 10 wk of age, approximately 1 wk after arrival. A second cohort of 2 male and 4 female mice of each of these substrains was purchased and subsequently bred by using 2 female and one male mouse to a cage. Offspring were weaned at 4 wk, and 5 to 8 male offspring per substrain were tested at 9 to 10 wk of age. Male mice were used for this experiment because we and others have observed a lower, and more variable, airway responsiveness in female mice, possibly related to estrus cycle variations.[7,8]

Husbandry. At our facility, mice were housed in same-sex groups of as many as 5 mice in individually ventilated cages (Allentown Caging Equipment, Allentown, NJ) on autoclaved corncob bedding (Harlan Teklad). They received filtered municipal water by means of an in-cage automated watering system (Edstrom Industries, Waterford, WI) and autoclaved diet (2018SX Teklad Global, Harlan Laboratories). Cages were changed on a 2-wk cycle by using chlorine dioxide-based disinfectant (MB10 tabs, 100-ppm solution, Quip Laboratories, Wilmington, DE) in filtered-air change stations (Lab Products, Seaford, DE), to minimize cross-contamination between cages. Sentinel testing indicated the colony was free of Sendai virus, pneumonia virus of mice, mouse hepatitis virus, mouse minute virus, mouse parvovirus 1 and 2, Theiler mouse encephalomyelitis virus, reovirus, epizootic diarrhea of infant mice, lymphocytic choriomeningitis virus, ectromelia virus, murine adenovirus, murine cytomegalovirus, *Mycoplasma pulmonis*, fur mites, and pinworms. Mice were not routinely tested for bacterial opportunists. The facility was accredited by AAALAC, and all procedures were in compliance with the *Guide for the Care and Use of Laboratory Animals*.[19] At vendors, mice were housed under varying conditions: information on husbandry at each of the vendors is summarized in Figure 1. Vendor health reports indicated that mice were free of the pathogens listed above that were excluded from our facility. Vendors also excluded several bacterial opportunists: all vendors excluded *Pasteurella pneumotropica* and *Bordetella bronchiseptica*, whereas Charles River, Harlan, The Jackson Laboratory, and the National Cancer Institute also excluded *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, and Charles River, Harlan, and the National Cancer Institute in addition excluded *Streptococcus pneumoniae.*

Pulmonary function testing. Mice are now the most commonly used animal model for the study of asthma, which is characterized by airway hyperresponsiveness (AHR). The essential measurement to assess AHR in mice is airway resistance, which can be approximated by lung resistance. In this study we measured changes in lung resistance in response to increasing doses of the cholinergic agonist methacholine.

Pulmonary function testing was performed as previously described.[33] The mice from a given strain were not all delivered at the same time, and the order in which mice were tested was assigned randomly to eliminate bias due to time of testing. Testing was conducted over the course of several weeks. Briefly, each mouse was anesthetized with ketamine–xylazine (75 mg/kg, 15 mg/kg) administered by intraperitoneal injection under manual restraint. When the mice were deeply anesthetized, a tracheostomy was performed, and the lungs were connected to a Flexivent (SCIREQ, Montreal, Quebec, Canada) ventilator to measure lung resistance. Mice were ventilated at150 breaths per minute, and lung resistance was determined during a 2-s breath hold from a 2.5-Hz sinusoidal oscillation.

After 10 min of regular ventilation at a positive end-expiratory pressure of 3 cm H_2O, a standard lung volume history was established by delivering 2 deep sighs to a pressure limit of 30 cm H_2O. One minute later lung resistance was measured, and this measurement was followed by a 10-s inhalation of aerosolized methacholine from a nebulizer (Aerogen Galway, Ireland). Doses of methacholine were 0.1, 0.3, 1, 3, 10, 30, and 100 mg/mL. The dose–response curve was constructed in a cumulative fashion with measurements made 1 min after each dose and 2 min between doses. We continued increasing doses until lung resistance was more than double the baseline. From this dose–response curve, we extrapolated the dose at which resistance doubled (that is, at 200% of baseline), and this dose is defined as the PC200. The concept underlying this variable is similar to that used to define the PC20, which is routinely used to assess methacholine sensitivity in human subjects.[27] After the last dose, anesthetized mice were euthanized by occluding the tracheal cannula, allowing the oxygen to be absorbed, and allowing the heart to stop as monitored by electrocardiography. All procedures were approved by the Johns Hopkins IACUC.

Statistical analysis. PC200 data from the different groups were analyzed by using one-way ANOVA for the log values (Prism 4.0, GraphPad Software, La Jolla, CA). The log value was used because the range of PC200 can encompass several orders of magnitude. Significance levels between groups were assessed by using the Newman–Keuls Multiple Comparison Test, and significance was accepted at a P value of less than 0.05.

Results

Figure 2 shows the normalized mean (± SEM) dose–response curves from the different substrains of mice at 1 wk after arrival. Lung resistance was normalized to baseline resistance in each substrain, but there were no significant differences among strains in this baseline value, which averaged between 0.67 and 0.88 cm H_20/mL/s in all substrains. The 5 substrains showed substantial differences in response to methacholine, with the least responsive mice coming from the National Cancer Institute and the most responsive from The Jackson Laboratory. Figure 3 shows the dose–response curves from the various substrains of mice derived from the different vendors but bred and raised in the animal facilities at our institution. All of these F1 mice show similar airway responsiveness. Figure 4 shows the PC200 for the results in Figures 2 and 3. There were no significant differences in the mice bred locally (labeled F1), but there were significant differences between several of the different parental substrains (labeled F0) that were tested after purchase from the vendors. The C57BL/6NCr substrain was significantly different from all the others, and the C57BL/6J mice were significantly different from the C57BL/6NTac mice.

Husbandry information was obtained directly from vendors (Figure 1). Husbandry varied widely between vendors with the exception of the National Cancer Institute and Charles River Laboratories: C57BL/6NCr mice were raised under contract by Charles River, and both vendors used the same feed and bedding. All vendors provided autoclaved feed and bedding and treated water; however, the composition of feed and bedding and the method of water treatment differed between vendors.

Substrain	Caging system	Watering system	Water treatment	Diet	Bedding	Cage-change frequency
C57BL/6J	Polycarbonate cages with filter bonnets or individually ventilated cages	Bottles	Filtered and acidified	Purina 5K67 pelleted	Aspen shaving–chip mix	Weekly or biweekly
C56BL/6NHsd	Polycarbonate cages with wire-bar lids	Automatic inside cage	Hyperchlorinated	Harlan-Teklad 2018S pelleted	Aspen shredded	Weekly
C57BL/6NTac	Polycarbonate cages with wire-bar lids	Automatic outside cage	Hyperchlorinated	NIH 31M pelleted	Hardwood chip	Weekly
C57BL/6NCr	Polycarbonate cages with wire-bar lids	Automatic inside cage	Filtered and acidified	Purina 5L79 pelleted	Hardwood chip	Weekly
C57BL/6NCrl	Polycarbonate cages with wire-bar lids	Automatic outside cage	Filtered, UV- and ozone-sterilized, and chlorinated	Purina 5L79 pelleted	Hardwood chip	Weekly

For all vendors, feed and bedding was autoclaved, and caging was autoclaved prior to entering the barrier.

Figure 1. Husbandry characteristics at vendor facilities of evaluated substrains.

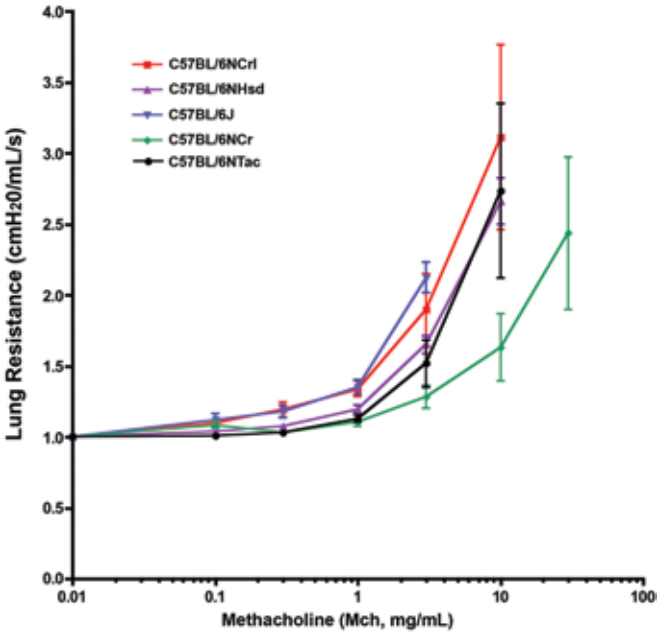

Figure 2. Dose–response curves plotting normalized mean (± SEM) lung resistance compared with methacholine dose for each of the C57BL/6 substrains tested 1 wk after arrival from the vendor. The wide variation in these curves is visually apparent.

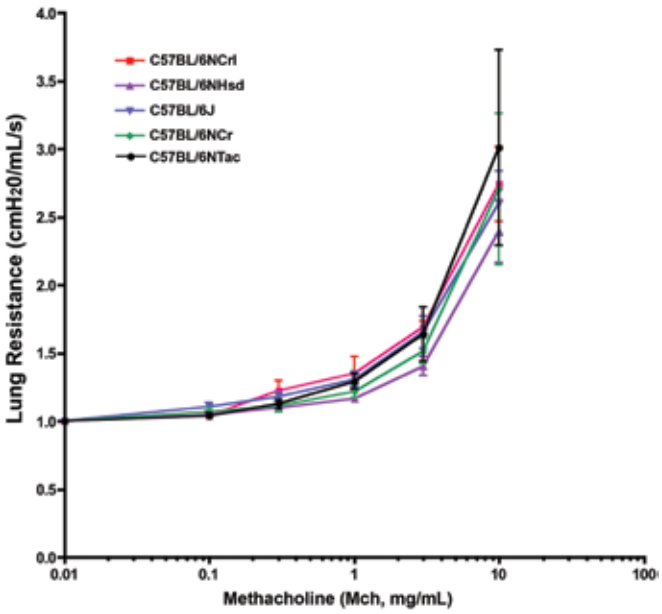

Figure 3. Dose–response curves plotting normalized mean (± SEM) lung resistance compared with methacholine dose for mice from each of the C57BL/6 substrains bred and raised inhouse. The curves for all groups overlap.

Four of the vendors used feed that contained multiple natural ingredients (variable formula), whereas one vendor used a diet with limited ingredients (constant formula).

Discussion

Our results clearly demonstrated substantial differences in airway responsiveness between C57BL/6 mice from different vendors. Moreover, those differences resulted from environmental factors rather than genetic variation, because vendor-associated differences in airway responsiveness were not present when mice were raised in a uniform environment.

Although the absolute variation we demonstrated between substrains when mice were obtained from vendors (not raised inhouse) is not large compared with the variation reported among different labs in the literature, under our testing protocol the variation is significant. This effect is partially a result of the relatively small variation demonstrated within substrains. In addition, the variation in our testing protocol is influenced by our use of the PC200 to assess airway responsiveness. For example, if one mouse had an extremely large response at a low methacholine dose, this outcome would result in a very large SD at that dose. However the PC200 for that one mouse could never be less than the previous dose (even if the airway resistance response was infinite), so this 'outlier' would have a smaller effect on the variation in PC200. For comparison, we note that the coefficient of variation of PC200 within a strain in our lab averages 0.22, whereas the variability between vendor-reared strains averaged more than double this, at 0.48.

Given the multiple possible environmental factors that could have contributed to the phenotypic variation documented in this study, it is not possible to confirm a specific explanation. However, we can speculate on several reasonable possibilities that may have played a role. Husbandry differed between vendors

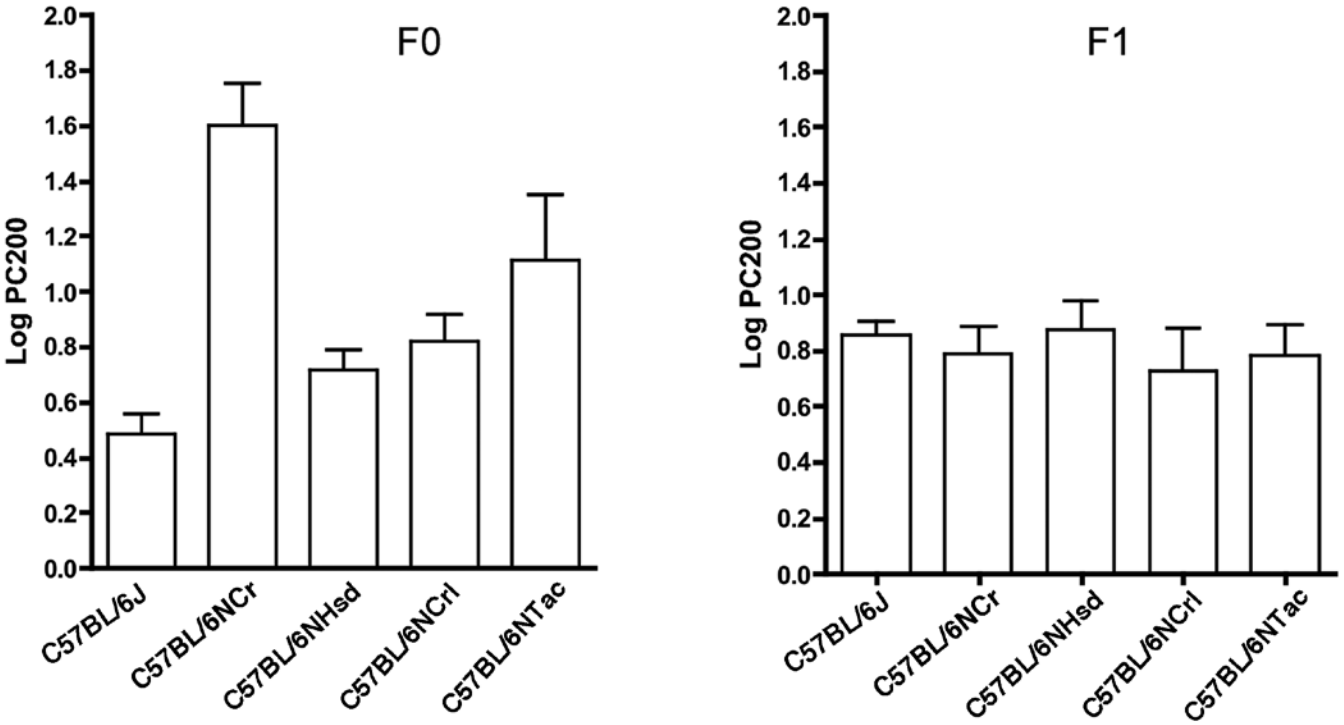

Figure 4. Log of the PC200 from each of the parental and locally bred C57BL/6 substrains. Among the F0 vendor-supplied substrains, C57BL/6J mice were significantly different from C57BL/6NCr and C57BL/6NTac mice. In addition, C57BL/6NCr mice were significantly different from C56BL/6NHsd, C57BL/6NCrl, and C57BL/6NTac mice ($P < 0.05$). PC200 did not differ among locally bred substrains.

with respect to caging system, methods for water delivery and treatment, feed components, and bedding type. Caging system can affect in-cage air quality: ammonia generated by bacteria from nitrogenous wastes can cause respiratory irritation and corneal injury.[2] The maximum 8-h time-weighted average ammonia exposure for humans is 25 ppm,[2] whereas concentrations in closed mouse cages changed weekly can reach 500 to 710 ppm.[10,36] However, none of the vendors in this study used closed cages, and the open cages (4 vendors) and ventilated caging (1 vendor) that were used are not typically associated with high in-cage ammonia levels.[10,20,34]

Although mice were conditioned for at least 1 wk at our facility before testing, disruptions in circadian rhythm and physiologic parameters due to shipping cannot be discounted as a cause for variation,[30] particularly as mice from the different vendors traveled over distances varying from 46 (the National Cancer Institute, Harlan) to 659 (The Jackson Laboratory) miles, The period required for these parameters to recover has not been established, but in one study,[6] rats transported for 5 h required only 3 d for body weight, heart rate, and activity to return to preshipping levels. Further, even fairly stressful procedures do not have long-lasting effects on AHR. For example, residual effects on AHR after anesthesia for bronchoalveolar lavage with 1 mL of fluid, were gone in 3 d.[41] We also know that repeated measurement of airway resistance in individual mice measured with nonlethal intubation does not vary over the course of several weeks.[28] Whether riding in a truck is more stressful than either of these procedures is not known, but 7 d would seem to be sufficient time for recovery. Finally, even if shipping was associated with neurologic activation, the effect of the vagus nerve on airway resistance in the mouse lung is relatively minor.[26]

Feed components such as vitamin A,[35] α tocopherol (vitamin E),[31] and prebiotic oligosaccharides[40] can affect mouse models of AHR. Vitamin A increases AHR, and vitamin E decreases it. Vitamins A and E are hypersupplemented in sterilizable diets to compensate for losses during autoclaving. Because the actual reduction in vitamin content during autoclaving may vary,[38] vitamins are supplemented sufficient to ensure that minimum requirements are met. Therefore some mice may have been fed higher-than-normal amounts of these vitamins, with potentially variable effects on AHR. Supplementation with oligosaccharides similar to those found in breast milk has been shown to decrease parameters of allergic asthma in mice by increasing Th1 relative to Th2 responses.[40] Vendors in the current study used different diets; therefore, oligosaccharide components likely varied between vendor—and with the exception of the constant formula diet used at one vendor—between batches of the same diet. Although contaminants such as heavy metals, pesticides, mycotoxins, phytoestrogens, and nitrosamines have been found in low concentrations in some laboratory rodent diets,[38] they are not reported to affect airway responsiveness. However, steam-sterilized feed may contain small quantities of the additive 2-diethylaminoethanol, which is used to reduce corrosion in steam sterilization systems. 2-diethylaminoethanol is a respiratory irritant and has been associated with development of asthma in people after accidental exposure during a steam leak.[16]

Ambient particulate matter can affect the immune system parameters that impact AHR. Even relatively inert particulate matter (such as carbon black) has been show to activate pulmonary dendritic cells and promote a Th2-type cytokine response from naive $CD4^+$ T cells.[4] Airway dendritic cells also are known to play important roles in initiating the allergic innate immune response in the lung in humans and murine models of asthma.[13,24,39] This dendritic cell activation is consistent with studies of airway responsiveness in mice in vivo, where cytokines from both Th1 and Th2 T cells were shown to be increased after exposure to particulate matter collected from inner-city air.[42] The same study also showed upregulation of

several genes associated with innate immune responses, chemotaxis, and complement system pathways. However, how this relates to the potential effect of bedding particulates is uncertain, because the particulate matter inside the cage is a complex mixture of particles with varying properties. Nevertheless, at least one study has found that there was an increase in large (greater than 5 μM) particles in air from mouse cages compared with room air.[34] In addition, there are other factors in bedding that could affect airway responsiveness, including endotoxins from gram-negative bacteria and (1→3)-β-D-glucan, a cell wall component of various plants, molds, and bacteria.[12] Exposure to endotoxins is a risk factor for asthma in humans,[37] and endotoxins and glucans in bedding were implicated as causes of pulmonary inflammation in rats.[12] Levels of endotoxin in bedding vary widely: in one study, minimal levels were reported for processed paper products (maximum, 100 EU/g) compared with corncob (2749 ± 260 EU/g) and hardwood chip (4925 ± 1443 EU/g).[43] A second study reported much higher levels of endotoxin (81,000 EU/g) in aspen chip bedding,[23] although the results for the 2 studies may not be comparable because bedding was autoclaved in the former study but not the latter. It should be noted though that endotoxins are not destroyed at temperatures below 180 °C.[18] Two of the vendors used aspen bedding, whereas the other 3 vendors used hardwood chip composed of maple, beech, birch, or poplar.

We cannot completely discount differences in bacterial infections as a cause of variation in vendor-reared animals, but we think this unlikely. All the vendors excluded primary rodent pathogens and most also excluded most bacterial opportunists, with minor variations. Bacterial opportunists are not excluded from colonies at our facility. Because we did not test the mice for opportunists, minor variations in bacterial status among mice received from vendors could have equalized in mice bred at Johns Hopkins, particularly if the cage barrier system was compromised. However, immune-competent mice rarely support active infections with bacterial opportunists. In contrast, recent reports indicate that gastrointestinal infection with filamentous bacteria can affect IL17 production in organs other than the gastrointestinal tract.[21,22] Although these cited studies did not assess the lungs, they suggest the possibility that the lungs also might experience upregulation of IL17 activity when filamentous bacteria are present in the gastrointestinal tract. This effect may be important, given that IL17 has been shown to have direct and indirect effects on airway responsiveness in animal models and humans.[1,3] Although the gastrointestinal colonization in inhouse-bred mice should have remained isolated within the mice from different vendors, it is possible that the gastrointestinal bacterial environment among the different mice became similar during the 8- to 10-wk breeding periods.

C57BL/6 mice have become an increasingly common asthma model because of their widespread use as a background strain for genetic manipulation. We do not know whether these results would be applicable to other strains commonly used for asthma studies; however, we have no reason to expect that our findings are peculiar to the B6 strain.

In summary, we observed significant variations in airway responsiveness between substrains of male C57BL/6 mice from different commercial vendors. These differences do not reflect underlying genetic factors, because F1 mice bred in the same environment at our facility showed no differences in responsiveness among substrains. The reason for the observed differences are unclear, but regardless, investigators using mice as models of asthma should be cautious when comparing results from different substrains of mice obtained from different vendors.

Acknowledgments

This study was supported in part by NIH grant HL10342 and by Research Animal Resources at Johns Hopkins University.

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