Ozone-Induced Acute Pulmonary Injury in Inbred Mouse Strains

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To determine if host factors influence the time course and extent of lung injury after acute inhalation of ozone (O_3), we evaluated the physiologic and biologic response of nine genetically diverse inbred strains of mice (C57BL/6J, 129/SvIm, BTBR, BALB/cJ, DBA/2J, A/J, FVB/NJ, CAST/Ei, and C3H/HeJ) exposed to O_3 (2.0 ppm $\times$ 3 h). Whole lung lavage determined that 129/SvIm, BTBR, DBA/2J, and FVB/NJ had a peak increase in polymorphonuclear cells (PMNs) at 6 h, whereas C57BL/6J and CAST/Ei had a peak increase at 24 h after exposure; airway PMNs were minimally elevated in A/J and C3H/HeJ; BALB/cJ had a predominant lymphocytic influx. Interleukin-6 concentration in the lavage fluid was associated with the influx of PMNs, whereas the total protein in the lavage fluid did not always correlate with lavage cellularity. Respiratory responses were monitored using whole body plethysmography and enhanced pause index. C57BL/6J, BALB/cJ, 129/SvIm, and BTBR were highly sensitive to O_3 and exhibited significant increases in enhanced pause to methacholine aerosol stimulation at 6 and 24 h after exposure to O_3. In contrast, DBA/2J, A/J, FVB/NJ, CAST/Ei, and C3H/HeJ strains had demonstrated increases in sensitivity to MCh at 6 h after exposure, but responses had returned to near baseline by 24 h after exposure to O_3. Epithelial cell proliferation as assessed by proliferating cell nuclear antigen staining was evident at 24 h after exposure to O_3. C57BL/6J and A/J showed 4% proliferating cell nuclear antigen–positive cells; 129/SvIm, DBA/2J, and FVB/NJ had 1–3%; and BTBR, BALB/cJ, CAST/Ei, and C3H/HeJ had $<$ 1%. Phenotypic measurements in six inbred strains were used for an *in silico* genome analysis based on the Roche mouse database. Consistent loci on chromosomes 1, 7, and 15 were among those identified to have a significant association with the phenotypes studied. In aggregate, our approach has identified O_3-resistant (C3H/HeJ and A/J) and -vulnerable (C57BL/6J and 129/SvIm) strains of mice, and determined novel genomic loci, suggesting a clear genetic basis for the lung response to inhaled O_3.

Ozone (O_3) is a ubiquitous environmental air pollutant. O_3 inhalation can induce toxic effects on the respiratory tract, including airway inflammation and hyperreactivity, epithelial cell lining injury, increased microvascular permeability, and impaired respiratory and immunologic function. Inflammatory cell accumulation in the lung has been reported in humans and several experimental animal models after O_3 inhalation. Interleukin-1β (IL-1β) and TNF-α, both proinflammatory cytokines produced by alveolar macrophages, contribute to O_3-induced inflammation (1) and also to O_3-induced airway hyperresponsiveness (2) in mice. Epidemiologic studies in humans have shown a relationship between O_3 and the prevalence of asthma (3, 4). Accordingly, the inflammatory response to O_3 in individuals with asthma is more intense than in healthy subjects, which may lead to aggravation of asthma symptoms.

Because O_3 does not penetrate cells, yet causes a number of airway and pulmonary responses, a cascade mechanism is proposed to account for its toxicity (5). It is hypothesized that O_3, as well as oxygen species generated during exposure, react with unsaturated fatty acids at the air–tissue barrier to form lipid ozonation products (aldehydes, hydroxyhydroperoxides, and Criegee ozonide), which in turn can activate membrane lipases and lead to the production and release of cell signal transduction molecules and proinflammatory mediators. Damage of sensory nerve fibers in the airway wall after O_3 can lead to release of tachykinins, which stimulate bronchoconstriction, mucus secretion, plasma exudation, immune cell responses (6), and enhance O_3-induced neutrophilic inflammation in mice (7).

Genetic factors are important in individual susceptibility to O_3; for example, although mean functional responses of men and women to O_3 exposure are similar (8), the difference in susceptibility among humans that received the same dose of O_3 suggests genetic control of the predisposition to the harmful effects of O_3. Mice also vary considerably in their response to ozone (9). Moreover, strain-related differences in O_3-induced responses indicate that susceptibility in mice may be genetically determined (10). Prows and coworkers tested mortality from acute lung injury after acute O_3 exposure (10 ppm) and found A/J mice sensitive and C57BL/6J resistant, with clear designation at 13 h after exposure (11). The authors concluded that several genes, including acute lung injury 1 (Ali1) on mouse chromosome 11 and Ali3 on chromosome 17, are involved in controlling susceptibility to death after oxidant-induced lung injury. A significant quantitative trait locus on chromosome 17 of mouse genome accounted for variance in an inflammatory cell phenotype (infiltrating PMNs) induced by exposure to 0.3 ppm O_3 has been reported (12). Recently, the same group reported another significant quantitative trait locus on chromosome 4, which controls O_3-induced hyperpermeability responsiveness in mice, and provide further evidence that *tlr4* is a potential positional candidate gene that affects O_3 responsiveness (13).

In considering the probability that genetics plays an important role in the development of O_3-induced lung injury, we believe that further refinement of the O_3 response lung phenotype in genetically diverse inbred strains of mice (14) is needed to inform and direct further efforts to identify O_3 response genes. Thus, the purpose of the present study was to investigate if genetic background modifies the severity and timing of lung response to acute ozone inhalation and to perform genetic linkage analysis to identify loci for O_3 sensitivity.

Materials and Methods

Overview

To determine whether host (genetic) differences influence the time course of lung response to acute O_3 exposure, nine genetically diverse inbred strains of mice were exposed to O_3 (2.0 ppm $\times$ 3 h). Concurrently age-matched controls were exposed to filtered air (FA). The mouse

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Abbreviations: filtered air, FA; interleukin, IL; megabases, Mb; methacholine, MCh; proliferating cell nuclear antigen, PCNA; enhanced pause, Penh; polymorphonuclear cells, PMN; parts per million, ppm; single nucleotide polymorphism, SNP; tumor necrosis factor-α, TNF-α.

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strains (C57BL/6J, 129/SvIm, BTBR, BALB/cJ, DBA/2J, A/J, FVB/NJ, CAST/Ei, and C3H/HeJ) represented various evolutionary backgrounds and therefore formed a genetically diverse group (14). Phenotyping was performed by physiologic, biologic, immunologic, and morphologic assessments before the exposure, and at the 6- and 24-h postexposure time points. *In silico* genome scan was performed to identify chromosomal regions that most likely contribute to observe O_3-induced phenotypes.

Inbred Mouse Strains

Mice were obtained from Jackson Laboratories (Bar Harbor, ME). Twenty-four males from each strain at 6–8 wk of age were used in this study. Animals were housed under pathogen-free conditions and were provided with water and food *ad libitum*. The study protocol was in accordance with guidelines set forth by the Duke University Animal Care and Use Committee.

Ozone Inhalation Challenge

Mice were removed from their cages and placed in individual compartments of two stainless-steel wire cage units. These units were set inside two separate 55-liter stainless-steel Hinners-style exposure chambers (one for O_3, the other for FA), equipped with a charcoal-filtered and high-efficiency particulate FA supply. Chamber air was renewed at the rate of $\sim$ 20 changes/h, with 50–60% relative humidity and a temperature of 20–22°C. O_3 was generated by directing a 100% oxygen gas source through an ultraviolet light ozone generator that was upstream from one of the exposure chambers. The O_3–oxygen mixture was metered into the inlet air stream and O_3 concentration was monitored continuously within the chamber with an O_3 ultraviolet light photometer (Dasibi model 1003AH; Dasibi Environmental Corp., Glendale, CA). The photometer was calibrated regularly against a standard source. Twelve mice from each strain were exposed to 2.0 ppm O_3 for 3 h and then put in room air for 6 or 24 h after recovery. Another twelve mice, assigned to corresponding control groups were exposed to FA in the inhalation chamber for the same duration.

The level and exposure duration for our protocol were based upon the popularity of this mouse model as a model of ozone/oxidant injury to the lower respiratory tract. This model has been used by Noviski and colleagues (15) to evaluate mast cell activation as a component of O_3-induced injury. Likewise, Longphre and colleagues (16) used this model to understand PAF modulation of O_3-induced pulmonary inflammation and epithelial proliferation. As inflammatory cell influx and epithelial proliferation were two of the several O_3-induced phenotypic responses in the mouse we were interested in, we chose this model, e.g., 3 h exposure to 2 ppm dose, to investigate strain dependent responses.

Barometric Plethysmography

A noninvasive method (whole body plethysmography) was used to estimate respiratory response to methacholine (MCh) aerosol challenge. Three assessments were performed: before the exposure, and at 6 and 24 h after exposure. Individual mice (unrestrained, unanesthetized) were placed in 3-in-diameter chambers (Buxco Electronics, Troy, NY) that were ventilated by bias airflow at 0.5 liter/min/chamber. Airflow obstruction in the mice was induced with an MCh (Sigma, St. Louis, MO) aerosol challenge. In each plethysmograph, a pressure signal is generated from the pressure difference of the main chamber containing the unrestrained mouse and a reference chamber, which cancels atmospheric disturbances. Signals were analyzed to derive whole body respiratory flow parameters (SFT3812, BioSystem XA version 2.0.2.48; Buxco), including respiratory rate, tidal volume, minute volume, inspiratory and expiratory times (Ti and Te, respectively), peak inspiratory and expiratory flows (PIF and PEF, respectively), and relaxation time (Tr). These parameters are used by the program software to calculate enhanced pause (Penh, unitless), using the following expression: $\text{Penh} = [(\text{Te-Tr})/\text{Tr}] \times (\text{PEF}/\text{PIF})$. Penh has been found to correlate with lung resistance (17) and is associated with changes in pulmonary resistance during bronchoconstriction. Mice were left to acclimatize inside the chambers for 15 min and then their respiratory responses were evaluated at baseline (saline-provoked) and after increasing doses of aerosolized MCh (0, 5, 10, and 20 mg/ml). The different concentrations of aerosolized MCh were administered for 1 min using an ultrasonic nebulizer and nebulizer control unit (Buxco Electronics). During MCh challenge, Penh changes have been demonstrated empirically to correlate with changes in pulmonary resistance and are therefore believed to represent airway obstruction; however, this remains controversial (18). Recording of breathing parameters began immediately after the end of MCh aerosolization and continued for 10 min. Average Penh values were determined over the first 3 min (early phase) of the respiratory response to the MCh aerosol.

Whole Lung Lavage

The degree of pulmonary inflammation was assessed by measuring the whole lung lavage fluid cellularity. The chest of each mouse was opened, the trachea was exposed and cannulated with polyethylene (PE-90) tubing, and lungs were lavaged with 6.0 ml of sterile saline, 1 ml at a time, at an infusion pressure of 25 cm H_2O. The return volume was recorded and was consistently $>$ 4.5 ml. Processing of the lavage fluid has been described previously (19). Briefly, immediately after collection the lavage fluid is cooled to 4°C and later centrifuged for 5 min at 600 $\times$ *g*. The supernatant is decanted and stored at 70°C for further use. The cell pellet is resuspended with Hanks' balanced salt solution (without Ca or Mg) and washed twice. A small aliquot of resuspended cells is used for counting the total number of cells using a Bright-Line hemocytometer (Hausser Scientific, Horsham, PA). Seventy-five to one hundred microliters of the cell suspension from each probe is spun onto a positively charged glass slide using a Shandon Cytospin centrifuge (Shandon, Southern Sewickley, PA). After cytospin, the cells are stained with HEMA-3 stain, air-dried, and covered with a coverslip with Cytoseal (Stephens Scientific, Kalamazoo, MI). Differential counts of lavage cells were performed at $\times$400 (dry objective) using an Olympus microscope (Olympus Corp., Lake Success, NY). Two hundred random cells were counted from each slide and were characterized as either macrophages, PMNs, eosinophils, lymphocytes, airway epithelial cells, or other. The total number of particular cell type per milliliter of lavage fluid was determined by multiplying the percent occurrence of the cell (i.e., the number of cells per 100) by the total number of cells per milliliter of lavage fluid.

Lung Lavage Total Protein and Cytokine Assays

The total protein concentration in the lavage supernatant was measured as an indicator of lung permeability. An assay kit (*DC* Protein Assay; Bio-Rad Laboratories, Hercules, CA) was employed according to the manufacturer's protocol. This colorimetric assay uses the method of Lowry (20) and is accurate from 5–2,000 μg/ml protein (bovine serum albumin standard, 750 nm). The levels of IL-6 in lung lavage fluid were quantitated by enzyme-linked immunosorbent assay using a mouse kit (Biosource International, Camarillo, CA).

Histopathology and Immunohistochemistry

Immediately after collection of the whole lung lavage fluid, the lungs were perfused with 0.9% saline through the pulmonary artery to remove blood from the vascular bed. Freshly prepared ice-cold 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) in 1 $\times$ phosphate-buffered saline (pH 7.4) was then instilled through the tracheal cannula at a constant pressure of 25 cm to inflation fix the lungs. Specimens (consisting of the whole single-lobed left lung) were taken out of the chest, immersed in 4% paraformaldehyde at 4°C overnight, and dehydrated in a graded series of ethanol solutions. Tissue was embedded in paraffin, and sections were cut at 5 μm thickness and mounted on Superfrost Plus slides (VWR Scientific, West Chester, PA) for evaluation. All histologic quantitation was performed in a blinded fashion using an established protocol (21). Proliferating cell nuclear antigen (PCNA), a nonhistone protein (MW 36 kD) that is found in the cell nucleus plays a role in the initiation of cell proliferation by mediating DNA polymerase. PCNA expression has a broad correlation with mitotic activity and can be used as a marker for cell proliferation. We evaluated PCNA expression with a staining kit containing biotinylated PCNA monoclonal antibody (clone PC10), streptavidin-peroxidase as signal generator, and 3,3′-diaminobenzidine (DAB) as the chromogen (Zymed Laboratories, Inc., South San Francisco, CA). Sections were also counterstained with hematoxylin. Images of cross-sections of bronchi were captured via an ECLIPSE E600 upright light microscope (Nikon Inc., Melville, NY) coupled to a Spot CCD camera system. At least 300 bronchial epithelial cells were counted in each section, and the percentage of epithelial cells exhibiting nuclear staining for PCNA (dark brown) was defined.

In Silico Genome Scan

The phenotypic results from six of the inbred strains (C57BL/6J, 129/SvIm, BALB/cJ, DBA/2J, A/J, and C3H/HeJ) were used in this analysis. To identify chromosome regions associated with the phenotypic response to inhaled O_3, we used a murine genome single nucleotide polymorphism (SNP) database that was created by investigators at Roche Pharmaceuticals (Palo Alto, CA) (22). Airspace inflammation, airway hyperreactivity, and total protein in lung lavage fluid at both time points (6 and 24 h after the exposure) were studied in this analysis. The average size of a predicted genomic region was 10 megabases (Mb). A total of 250 genomic intervals were analyzed for the phenotypes examined. Multiple pairwise comparisons across the genome of the available inbred mouse strains were performed to identify 10 Mb regions with correlated SNPs in strong linkage disequilibrium. Genome-wide threshold of 5% was used to separate the most highly correlated of 10 Mb chromosomal regions. Correlation value was derived using linear regression on the phenotypic and genotypic distances for each chromosomal interval and was expressed as SD above or below the average for all intervals analyzed.

Statistical Analyses

Data were analyzed for each mouse strain as the mean ± SEM of 24 animals before the exposure, and compared with the means of 6 animals for each postexposure group (at 6 and at 24 h) for FA- and for O_3-exposed animals, respectively. For comparison of the two group means (Penh, lavage cell counts, total protein, IL-6) and for statistical analysis, the *t* test and the Mann-Whitney *U* test were used (23). A P value of < 0.05 was considered to indicate a significant difference between the groups.

Results

Airway Physiology

Baseline respiratory function before the O_3 exposure revealed that all of the inbred mouse strains had similar values of Penh that ranged between 0.75 and 1.12 before MCh challenge (Figure 1A). Using Penh as a measure of respiratory response to MCh before the O_3 or FA exposure, hyperreactive (129/Svlm, BTBR, A/J), hyporeactive (C57BL/6J), or intermediate (BALB/cJ, DBA/2J, FVB/NJ, CAST/Ei, C3H/HeJ) phenotypes were observed (Figure 1B).

After exposure to O_3, but before MCh challenge, measures of Penh demonstrated differences between the strains, such that the strains were classified as either susceptible/vulnerable to O_3 (C57BL/6J, 129/SvIm, BTBR, and BALB/cJ) or as relatively resistant strains (DBA/2J, A/J, FVB/NJ, CAST/Ei, and C3H/HeJ) (Figure 1C). The susceptible strains showed increases in baseline Penh to saline aerosol even at the 24-h postexposure endpoint. Exposure to O_3 also caused a significant increase in the Penh response to aerosolized MCh, but the magnitude of change was different among the strains. The same strains that demonstrated an increase in their respiratory response to saline aerosol after the O_3 exposure were also the strains that were much more responsive to the MCh aerosol challenge. Similarly, the resistant strains were also observed to be less responsive to MCh after O_3 exposure (Figure 1D). Interestingly, using Penh as a measure of respiratory response the pre-O_3 values were not

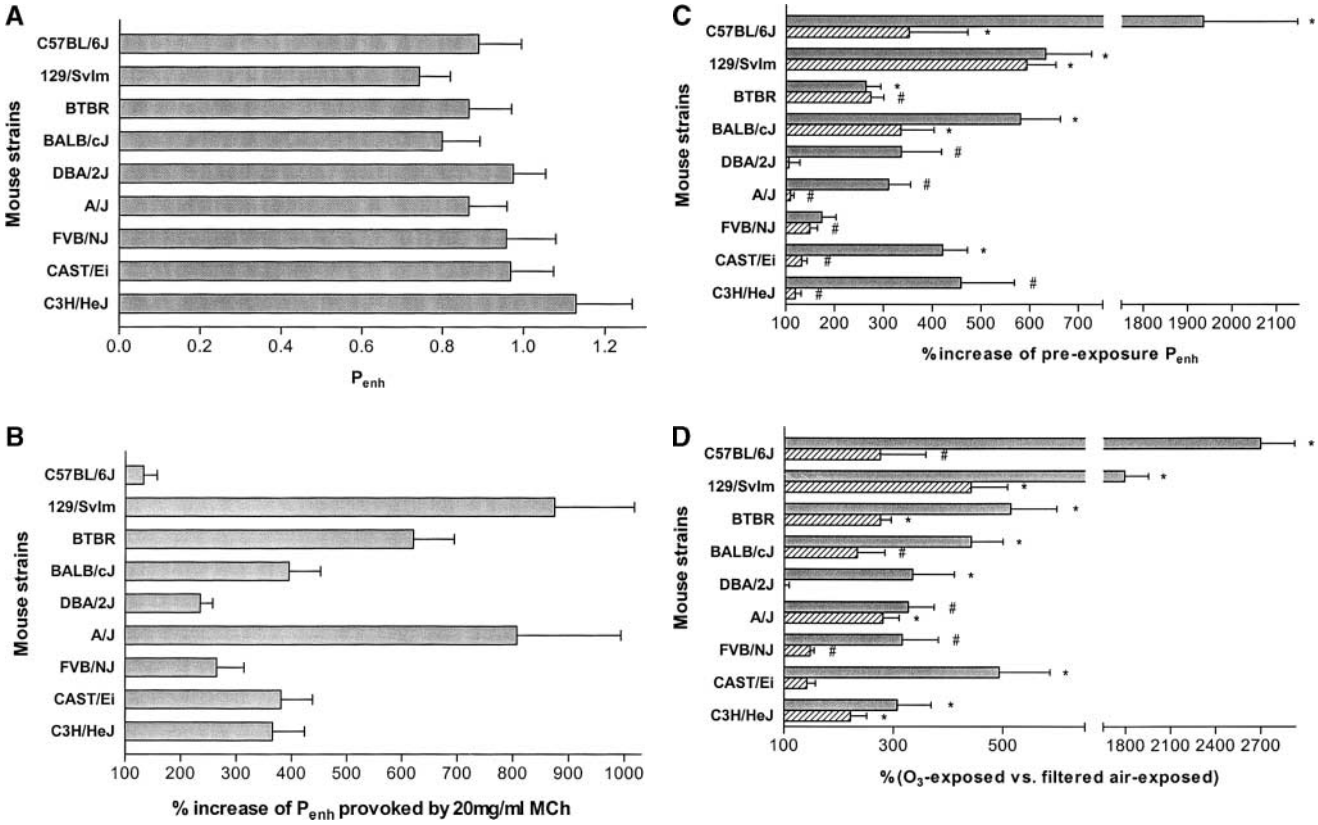

Figure 1. Barometric plethysmography measures. (*A*) Pre-exposure saline provoked (baseline) respiratory breathing response (expressed as Penh) measured with whole body plethysmography. Values are given as the mean ± SEM for 24 mice of each strain. (*B*) Pre-exposure mouse strain differences in respiratory Penh response to inhaled MCh. Percent increase of Penh ± SEM after inhalation of aerosolized 20 mg/ml MCh. Relatively hyperreactive (129/Svlm, BTBR, A/J), hyporeactive (C57BL/6J), or intermediate (BALB/cJ, DBA/2J, FVB/NJ, CAST/Ei, C3H/HeJ) phenotypes could be distinguished. (*C*) O_3 effect on the baseline (saline-induced) Penh at 6 h (*shaded bars*) and 24 h (*hatched bars*) after exposure. (*D*) Increase in Penh sensitivity to inhaled MCh aerosol after O_3 exposure after 6h (*shaded bars*) and 24 h (*hatched bars*). Data are expressed as a percent over the FA-exposed groups, challenged with the same MCh dose (5 mg/ml). $^*P < 0.005$, $^\#P < 0.05$ versus pre-exposure.

predictive of the post-O_3 measures. For instance, the A/J strain was very responsive to MCh before O_3 exposure, and surprisingly after O_3 exposure, the respiratory response to MCh actually decreased. In contrast, the C57BL/6J strain had a relatively low respiratory response to MCh aerosol before exposure, but had a pronounced increase in Penh after the O_3 challenge. At 24 h after exposure, the Penh responsiveness to the lower doses of MCh (5–10 mg/ml) was fairly uniform throughout all the strains (Penh values ranged 1.5–3 times greater than the air-exposed controls); however, several of the strains (C57BL/6J, C3H/HeJ, and A/J) still demonstrated an enhanced respiratory response to the highest dose of MCh aerosol (data not shown).

Inflammation in the Lower Respiratory Tract

All nine strains of mice had an inflammatory response to the acute 3 h O_3 inhalation challenge by increased total lung lavage cellularity. At 6 h after the end of the 3-h exposure to O_3, all of the inbred strains showed significantly greater total number of lavage fluid cells compared with corresponding air-exposed controls. In particular for C57BL/6J, BTBR, FVB/NJ, CAST/Ei, and C3H/HeJ, this response was highly significant ($P < 0.005$). Moreover, 129/SvIm, BTBR, DBA/2J, and FVB/NJ strains were found to have a similar pattern of inflammatory cell recruitment with significantly more ($P < 0.005$) PMNs at both 6 and 24 h after exposure as compared with the air-exposed animals (Figure 2). The total absolute number of PMNs per ml of lavage fluid at 24 h was 40–80% lower than the level observed at 6 h after exposure, and demonstrates that the influx of PMNs is greater during the early hours after the O_3 challenge. Of note, the PMN response by C3H/HeJ mice was substantially diminished when compared with the other strains, and BALB/cJ mice recruited a high concentration of leukocytes but the predominant cell type among the BALB/cJ strain was lymphocytes. Although the concentration of PMNs in the lavage fluid was highest at 24 h after exposure for the C57BL/6J and CAST/Ei strains, the A/J strain had similar concentrations of lavage PMNs at 6 and 24 h after the challenge with O_3.

Concentration of Lung Lavage Total Protein and IL-6

The total protein content in the lavage fluid was analyzed to determine whether there were interstrain differences in this index of airspace injury, because after acute O_3 inhalation, increases in protein may be caused by transudation of proteins from the blood into the alveolar space, lysis of injured cells, and/or enhanced protein secretion by cells of the respiratory tract. Two strains, C57BL/6J and A/J showed that total protein concentrations in the lavage fluid were not significantly affected by acute O_3 exposure when compared with FA-exposed controls (Figure 3A). 129/SvIm strain demonstrated significant ($P < 0.005$) increases in lung lavage total protein amounts only at 6 h after O_3 inhalation. In contrast, BALB/cJ, DBA/2J, and C3H/HeJ achieved significant levels of lavage fluid protein only at 24 h after O_3 inhalation. BTBR, FVB/NJ, and CAST/Ei strains had elevated protein in lavage supernatant at both time points after the completion of the O_3 exposure, compared with the air-exposed controls.

All nine inbred mouse strains have significantly elevated levels of IL-6 in the lavage fluid at 6 h after O_3 exposure (Figure 3B). This difference in O_3-exposed as compared with FA-exposed mice was pronounced ($P < 0.005$) in C57BL/6J, BTBR, and CAST/Ei inbred strains, whereas the rest of the strains had only modest elevations in IL-6 after O_3 inhalation ($P < 0.05$). Measurement of the IL-6 levels at 24 h after O_3 exposure showed that concentration decreased in all the strains but was still significantly ($P < 0.05$) elevated in C57BL/6J, 129/SvIm, BTBR, DBA/2J, and CAST/Ei.

Bronchiolar Epithelial Cell Proliferation

Expression of nuclear PCNA was not present at 6 h (data not shown) but clearly identified in the bronchial epithelia of O_3-exposed animals at 24 h after exposure (Figure 4A). Two of the strains (C57BL/6J and A/J) have increased levels of expression in bronchial epithelial cells with 4% of cell counted positive for PCNA; whereas three strains (129/SvIm, DBA/2J, and FVB/NJ) had between 1 and 3% of the epithelial cells counted express PCNA (Figure 4B). Four strains (BTBR, BALB/cJ, CAST/Ei and C3H/HeJ) had < 1% of the epithelial cells counted positive for PCNA.

In Silico Genome Scan

Our results identified 10 Mb chromosomal regions associated with O_3-induced airway hyperreactivity, inflammation, and air-

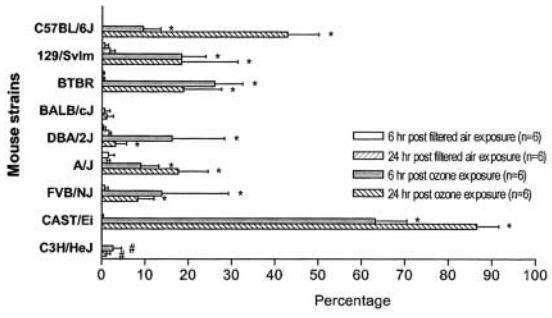

Figure 2. Percentage of PMNs in BAL fluid after a 3-h O_3 versus filtered air inhalation challenge. *Open bars* represent 6-h time point; *hatched bars*, 24-h time point. $^*P < 0.005$, $^\#P < 0.05$ versus corresponding air-exposed.

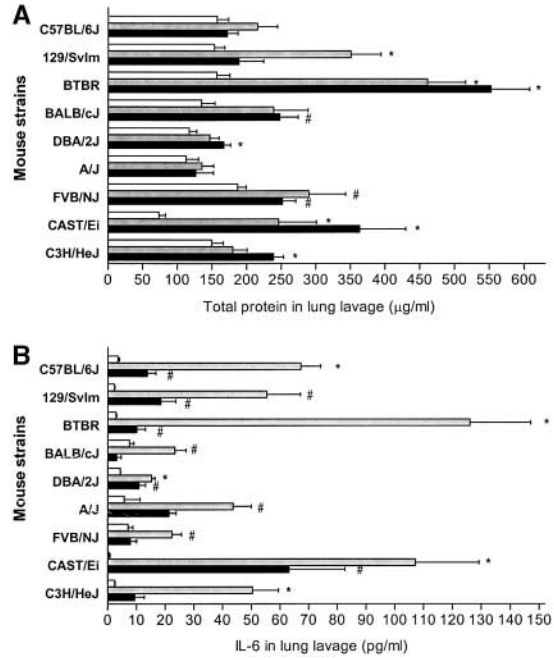

Figure 3. (*A*) Total protein in the lavage fluid recovered from the nine inbred strains at 6 h (*shaded bars*) and 24 h (*solid bars*) after O_3 exposure. Air-exposed controls (at 6 and 24 h after exposure) are presented as a total group ($n = 12$; *open bars*) because of the similar numbers. (*B*) Comparison of IL-6 levels in the lavage returns at 6 and 24 h after O_3 as detected by ELISA. *Open bars* represent levels in total of 12 individual air-exposed controls for each strain that were assessed at 6 ($n = 6$) and 24 h ($n = 6$) after FA exposure. $^*P < 0.005$, $^\#P < 0.05$ versus air-exposed.

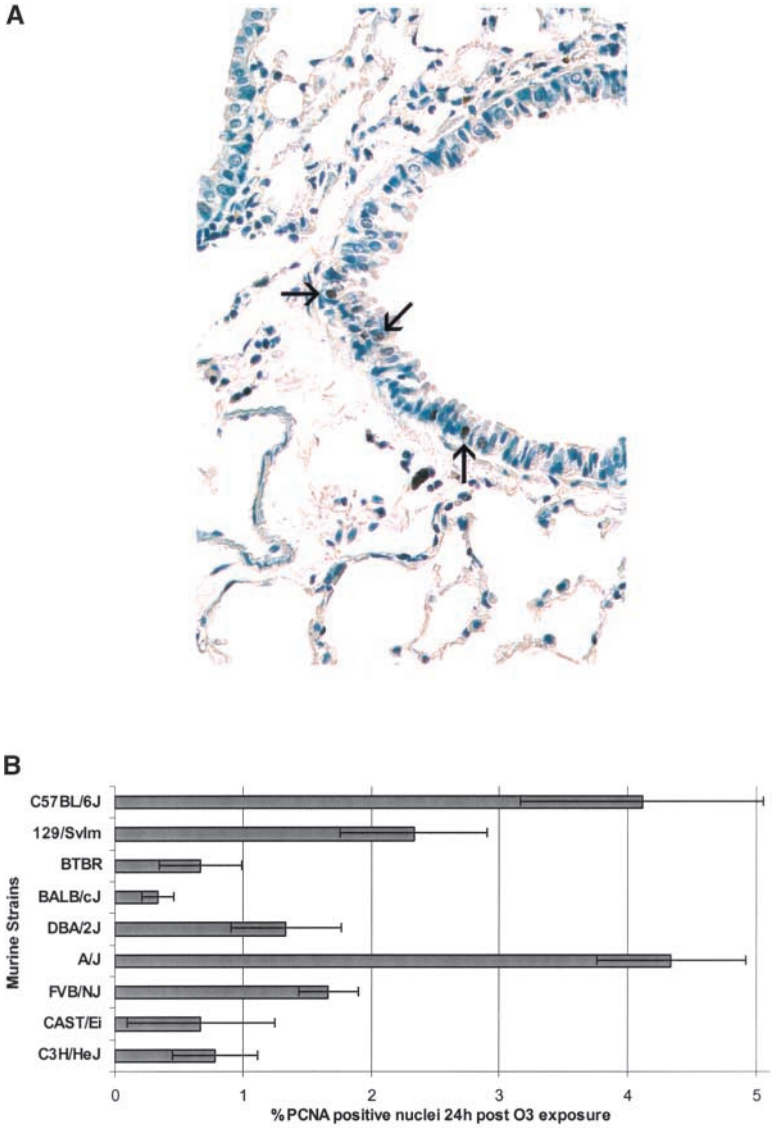

Figure 4. (*A*) A representative bronchial profile immunostained for PCNA. Positive nuclei are visualized by a brown color (3,3′-diaminobenzidine reaction product). *Arrows* point to the bronchial epithelium labeling. *Original magnification:* ×200. (*B*) PCNA index expressed as percentage PCNA positive bronchial epithelial cells at 24 h after a 3-h (2.0-ppm) O_3 exposure.

space total protein. Because 5% of mouse genome was selected (as cutoff) and assumed as having predicted chromosomal regions for O_3 phenotypes, twelve 10-Mb intervals were observed as peaks with the highest correlation value in each analysis. At both time points, O_3-induced airway hyperreactivity was associated with genomic intervals having a correlation value between 2 and 5.35 SD above the average (Figure 5) with strongest significant evidence for linkage at 6 h after exposure on chromosome 1 at 170–180 Mb (SD = 5.06), and at 24 h after exposure on chromosome 17 at 30–40 Mb (SD = 5.35). In silico mapping also identified regions associated with O_3-induced airspace inflammation at 6 h and at 24 h after exposure (Figure 6). For the earlier time point, the strongest correlation was observed on chromosome 7 at 30–40 Mb (SD = 5.58), whereas for the later point, it was chromosome 1 at 170–180 Mb (SD = 4.68). This latter region was also one of the intervals correlated with the O_3-induced airway hyperreactivity. Airspace total protein content also demonstrated a strong association at 6 h after exposure with a peak on chromosome 7 at 30–40 Mb (SD = 5.14) (Figure 7). This very same region was the strongest association for airway neutrophilia.

Discussion

Our results demonstrate that the physiologic and biologic response to inhaled O_3 differs between inbred strains of mice. Importantly, several strains could be grouped in sensitive and resistant categories. However, the physiologic response did not always correlate with the biologic response, and the response to O_3 was clearly time-dependent. We conclude that the response to inhaled O_3 is, in part, genetically determined and that the identification of sensitive and resistant strains is limited to specific physiologic and biologic phenotypes. An *in silico* genome scan demonstrated that novel genomic loci are associated with the physiologic and biologic phenotypes and may contain critical genes that regulate the response to inhaled O_3.

The molecular mechanisms of O_3-induced lung injury are not fully understood. Aldehyde production, secondary radicals (lipid ozonation products) generated after O_3 reaction with cell membrane lipids, are considered to be involved in the early stage of the inflammatory response (24, 25). Other studies suggest that IL-1β or TNF-α (1), or even reactive oxygen species (26), may play a role in this process. A number of soluble by-products arising from epithelial lining fluid, and cellular reactions with O_3, may also upregulate transcription factors and proinflammatory

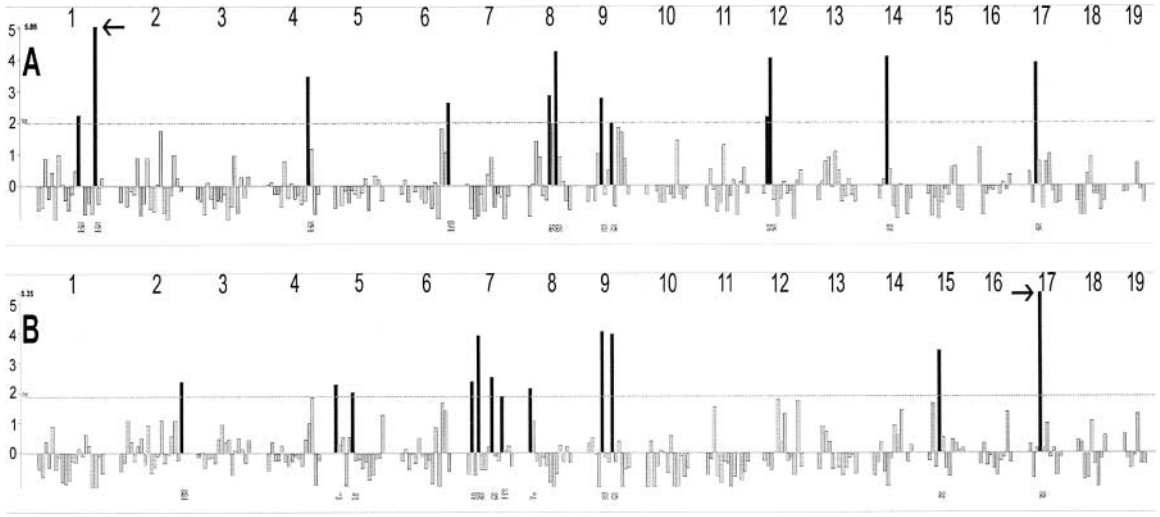

Figure 5. Graphic presentation of the chromosomal loci involved in determining airway hyperreactivity. Each *bar* represents a 10-Mb interval. The *dotted line* represents the cutoff for this analysis; the most highly correlated 5% of the loci are above this line and are presented as *black bars*. *Arrows* point to the regions (*bars*) with the best correlation value. *y*-Axis is SD of the correlation value above the average for all loci analyzed. (*A*) Six hours after O_3 exposure. (*B*) Twenty-four hours after O_3 exposure.

genes (27, 28). Our data suggest that the susceptibility to acute O_3 exposure is a complex trait with strain variation in expression, combination, and distribution of several main phenotypic features (e.g., lung inflammation, increased lavageable protein levels, and airway epithelial cell injury/proliferation). The pathogenesis of these features and their association or casual relationship is also not completely elucidated. However, focusing on understanding the genetics that are responsible for the between-strain differences may provide insight into the pathogenesis of this injury.

In a series of reports, Kleeberger and colleagues (29–32) have studied the genetic control of susceptibility to O_3-induced lung inflammation. They have shown that inbred strains of mice have a maximum increase in lavage PMN concentration at 6 h after acute O_3 exposure, and have demonstrated significant interstrain differences in the magnitude of PMN infiltration. Moreover, they have been able to map this phenotype to at least two loci—on chromosome 17 and on chromosome 11 (12). In the present study we distinguished three patterns of PMN influx, whereby the lavage numbers of PMNs: (*i*) waned after a high level at 6 h after exposure (129/Svlm, BTBR, DBA/2J, FVB/NJ); (*ii*) waxed over time in two of the strains (C57BL/6J, CAST/Ei); or (*iii*) were without extreme change (A/J, BALB/cJ, C3H/HeJ) within the time frame studied. These findings differ from the previous data in the literature. Our results predicted several additional genomic loci that are potentially involved in the development of O_3-induced airspace neutrophilia. In aggregate, these findings suggest that the strain-related differences in O_3 responsiveness are, in part, genetically determined.

Lavage fluid originates from the airway lining fluid, the vascular space, and lysis of cells. Importantly, we did not observe a consistent correlation between influx of inflammatory cells and the concentration of total protein in the lavage fluid, suggesting that inflammatory cells recruitment may be an active integrin-mediated process and is not simply a function of leaky vessels. This observation is consistent with the literature (29), where differences in levels of total protein and PMNs in the lavage

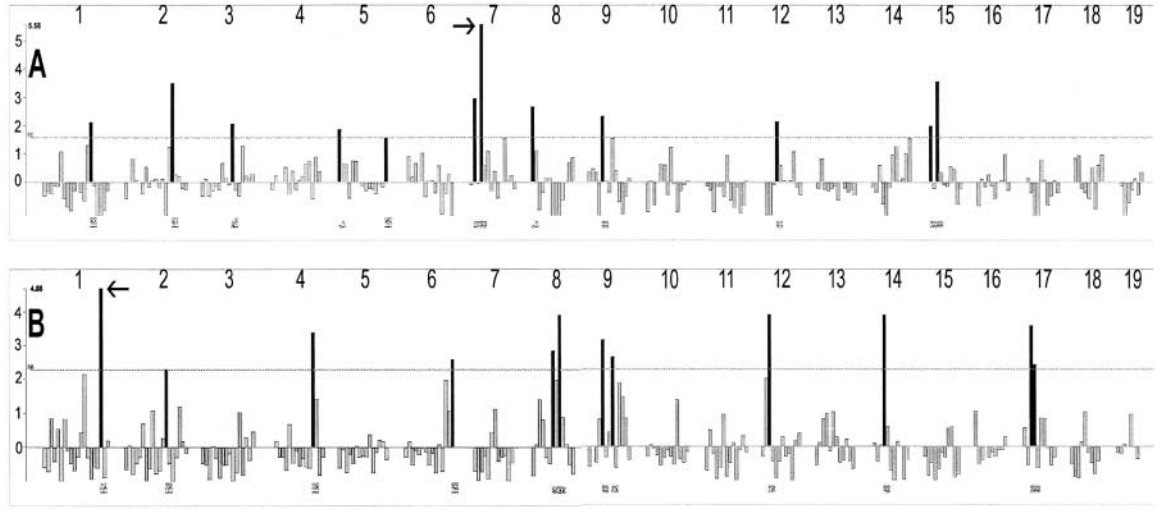

Figure 6. Computational prediction of the chromosomal loci involved in determining airway neutrophilia. All the labels are the same as in Figure 5. (*A*) Six hours after O_3 exposure. (*B*) Twenty-four hours after O_3 exposure.

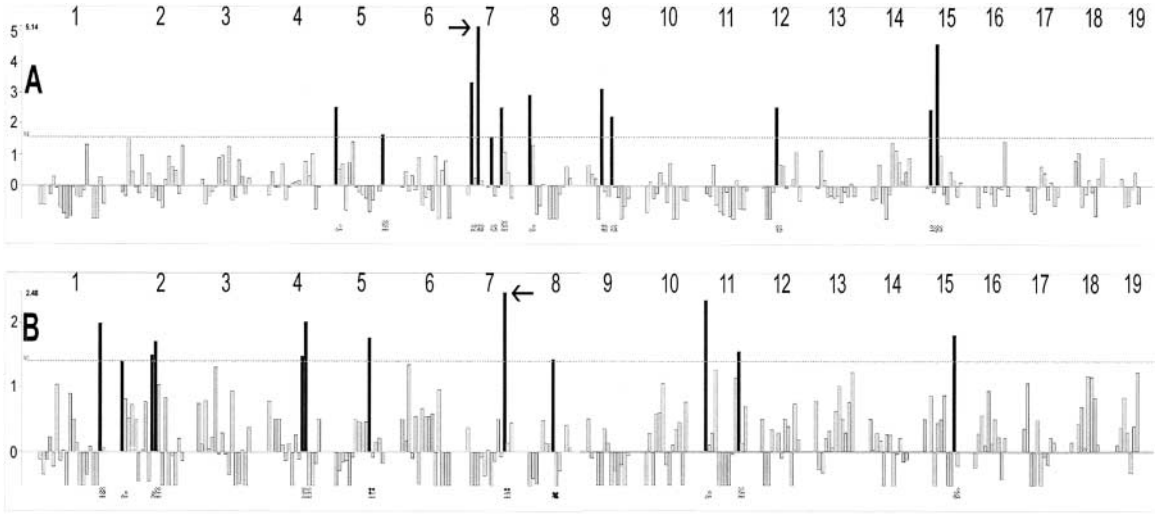

Figure 7. Computational prediction of the chromosomal loci involved in determining total protein content in the lavage fluid. All the labels are the same as in Figure 5. (*A*) Six hours after O_3 exposure. (*B*) Twenty-four hours after O_3 exposure.

after acute O_3 exposure have been described. In fact, in humans, protein leak into the airspace has been shown to be independent of inflammatory cell recruitment (33, 34). In aggregate, these data suggest that different pathophysiologic mechanisms are involved and these independent phenotypes may be controlled by different genes. Nevertheless, our *in silico* results suggest that a region on chromosome 7 (30–40 Mb) may contain a gene (or genes) that regulates the inflammatory response (neutrophilic and protein) to O_3.

Our observation in the present study, that increases in IL-6 in lung lavage fluid were correlated with the concentration of PMNs in the lavage returns, is a novel finding of O_3 response in the mouse. Time-dependent correlations between IL-6 and PMN increases after O_3 have been noted for human lavage (35) and for human epithelial cell lines (BEAS 2B) that secrete IL-6 following exposure to O_3 (36). To our knowledge, a relationship between this inflammatory cytokine (IL-6) and PMN influx has not been reported previously in O_3-exposed mice. However, there was some dissociation in the pattern of the relationship: for example, all the strains showed a decrease of IL-6 after a peak level at 6 h after O_3 exposure, whereas the PMN influx continued to increase at 24 h in a couple of strains. IL-6 is considered a pleiotropic cytokine that could be produced by a variety of cells. Some recent studies have shown that IL-6 has potent anti-inflammatory and protective properties (37), and that this cytokine could be involved in regulating proliferation and repair of injured bronchoalveolar epithelium (38).

The present study also demonstrated a good correlation between O_3-induced changes in the baseline breathing response (Penh) to saline aerosol and increases in sensitivity to MCh aerosol after exposure. Those two parameters allowed us to classify the inbred strains as susceptible (C57BL/6J, 129/SvIm, BTBR, and BALB/cJ) and resistant to acute O_3 (DBA/2J, A/J, FVB/NJ, CAST/Ei, and C3H/HeJ). However, none of the post-O_3 changes of these physiologic parameters were a function of pre-exposure airway physiology measurements and therefore it was our conclusion that respiratory P_{enh} response to MCh prior to exposure is not predictive for the susceptibility to acute ozone. Whether inflammatory cell recruitment (in various lung compartments) is essential to the development of MCh sensitivity is not clear. Noviski et al. (15) suggested that multiple cell types may participate in the pathogenesis of airway obstruction after O_3 exposure. Mast cells and PMN influx were not required for its development; perhaps no single inflammatory cell type is causal to the development of O_3-induced bronchospasm. Recent studies suggest that TNF-α contributes to O_3-induced airway hyperresponsiveness (2). Making mice neutropenic attenuated PMN infiltration, but did not affect acute O_3-induced hyperreactivity (9). Our data suggest that there was not a consistent relationship between level of inflammation and respiratory Penh sensitivity to MCh. For example, two strains (C57BL/6J and CAST/Ei) demonstrated marked increase in lavage PMNs with reduction in sensitivity to MCh at the 24-h time point. Several other mechanisms are possibly involved in the development of airway hyperresponsiveness: for example, O_3 can stimulate C-fibers in the lungs, which can lead to a variety of physiologic responses, including increased

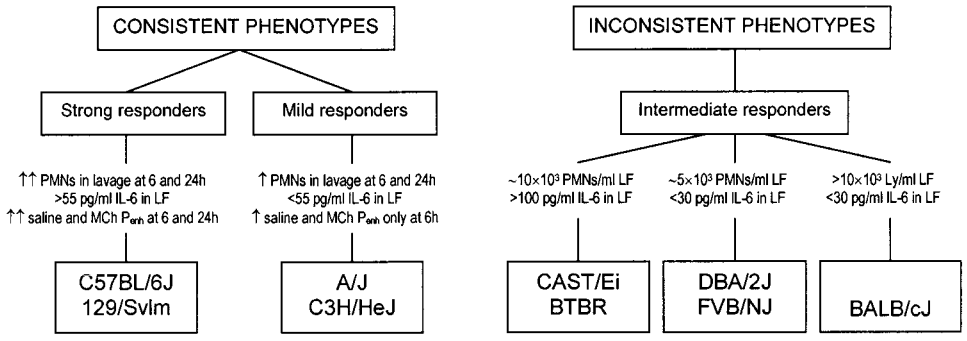

Figure 8. Summary of the different phenotypes observed in the nine inbred strains of mice that were exposed to 2.0 ppm O_3 for 3 h.

mucous secretion, bronchoconstriction, airway microvascular leak, increased blood flow to the bronchial mucosa, and alterations of inflammation (39, 40). Edema within the wall of conducting airways might also develop as a reaction to acute O_3 exposure (41). This could compromise airway patency and, together with the surfactant dysfunction that is seen with O_3 exposure (42), contribute to increases in airway obstruction. Interestingly, the *in silico* genome scan determined some similarity and overlap between the loci linked with O_3-induced airway hyperresponsiveness and those linked with airway neutrophilia. The 10-Mb intervals that were most highly correlated with airway hyperresponsiveness at the early time point were very similar with the regions that were linked with the late time point airway neutrophilia (most convincing on chromosome 1). These data suggest genetic regulation in the presentation of O_3 response phenotypes being expressed at different time points in the post-exposure period.

There are probably multiple mechanisms by which O_3 can damage epithelial cells within the airway. Results from previous studies have been demonstrated that O_3 exposure could induce apoptosis of lung cells in murine models (43). Epithelial injury or loss of epithelial layer (barrier) contributes to increased airway responsiveness. Hypothetically, nonspecific bronchoconstrictors (like MCh aerosol) delivered to airway surfaces would be able to penetrate the mucosal layer more easily and diffuse to airway bronchial musculature (44, 45). Repair of the damaged epithelium in the stage of recovery from an acute lung injury is important for reestablishing normal tissue architecture (bronchiolar and alveolar). We found that significant bronchial epithelial cell regeneration did not occur within 6 h after the end of the acute 3-h, 2.0-ppm O_3 inhalation exposure. However, PCNA (used as mitotic marker in this study) was expressed 24 h after O_3 exposure, suggesting that epithelial proliferation and repair is induced by O_3. The mechanism of bronchial epithelial cell proliferation after O_3 inhalation exposure is not completely clear. It is disputable whether O_3 directly stimulates cell division or the proliferation seen as a part of the reparation process following cell injury and lost. Data in the literature suggest that in rats after O_3 exposure, PCNA expression in bronchial epithelial cells was maximal between 24 and 48 h after exposure, was dose-dependent, could be inhibited by apocynin and dexamethasone, and appeared to involve PMNs (46). Because our results indicate that O_3-induced PCNA index is not related to PMN recruitment, non–PMN-dependent mechanisms appear to play a role in this process. In this connection Gohil and coauthors (47) have recently identified, using differential microarray analysis of lung tissue from air- and O_3-exposed mice, increases in at least 15 mRNAs that are linked with the progression of the cell cycle.

In summary, we were able to classify genetically diverse mouse strains as either O_3-sensitive or -resistant using our physiologic and biologic phenotypes (Figure 8). C57BL/6J and 129/Svlm strains were highly sensitive, with increased PMNs and IL-6 in the lavage fluid, increased respiratory Penh, and over 2% positive for PCNA epithelial cells. At the other extreme, A/J and C3H/HeJ strains were resistant to inhaled O_3, having only modest increases in lavage PMNs and IL-6, modest elevations in sensitivity to MCh, and divergent PCNA epithelial indices (A/J $\sim$ 4% and C3H/HeJ $<$ 1%). Our data, along with those of previous studies, support the premise that genetically diverse inbred mouse strains are useful for identifying genetic factors that modulate susceptibility to acute O_3-induced lung injury. The data from the *in silico* scan demonstrate that several novel chromosomal regions may contain genes that are important in the physiologic and biologic response to O_3.

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