EFFECT OF SHORT TERM DIESEL EXHAUST EXPOSURE ON NASAL RESPONSES TO INFLUENZA IN ALLERGIC RHINITICS

Terry L. Noah, Haibo Zhou, Hongtao Zhang, Katie Horvath, Carole Robinette, Matthew Kesic, Megan Meyer, David Diaz-Sanchez, and Ilona Jaspers

ONLINE DATA SUPPLEMENT

DETAILED METHODS

Study design and subjects

This was a randomized, double blind, prospective study comparing the effects of DE (100 μg/m^3, 2 hr at rest) vs. clean air on subsequent nasal inflammatory responses to LAIV. At a screening visit, a medical history was obtained and subjects underwent spirometry, HIV testing, pregnancy test for females, and a panel of allergy skin tests. Subjects returned 2-4 weeks later on "day 0" to undergo baseline nasal lavage, followed by controlled chamber exposure to DE or clean air (randomized) for 2 hr, and a standard intranasal dose of LAIV (FluMist®) was given 2-3 hr after the end of the chamber exposure. Detailed description of the DE exposure has been previously described [1] and is in the online data supplement. Subjects then returned to the research facility on days 1, 2, 3, 4, and 9 for repeat nasal lavages. Nasal lavage was performed according to a method previously described by us [2]. Subjects were blinded as to exposure, as were all technicians performing assays on nasal lavage fluids (NLF). Serum was obtained at the screening visit and at 21 days post LAIV inoculation.

Both healthy, non-allergic young adult volunteers (NV), and allergic rhinitics (AR), age 18-40 years, were recruited. Allergic rhinitis was defined as a history of seasonal or perennial rhinitis plus at least one positive immediate hypersensitivity reaction to allergen among a standard panel (including *D. farinae*, *D. pteronyssius*, grass mix, weed mix, tree mix, dog, cat, *Alternaria*, and cockroach) administered at screening. Subjects were excluded for acute symptoms within 3 weeks prior to study participation. Other exclusion criteria were smoking, any cardiorespiratory disease including asthma, immune deficiency, receipt of an influenza vaccine or documented influenza illness in

past 12 months, egg allergy, and pregnancy. Subjects with no asthma history but with abnormal spirometry at screening (FEV_1 < 75% predicted) were also excluded. Subjects were prohibited from use of inhaled medications of any kind or medications expected to influence nasal inflammation (e.g., NSAIDs). Detailed description of sample estimate calculation is in the online data supplement.

We based study sample size on the magnitude and variability of the effects of LAIV on IL-6 (an important factor in post-viral inflammation) using preliminary NLF data from 20 healthy subjects inoculated with LAIV in a separate protocol. Using the formula $N \approx [2SD^2 \times (z_\alpha + z_\beta)^2] / \Delta^2$ [3], and assuming 2-sided $\alpha = 0.05$ and power = 80%, the number of subjects estimated to detect a DE-associated doubling of IL-6 in allergic rhinitics was 8-9 per exposure group (DE and air) or 16-18 total. The same number of normal, non-allergic volunteers was recruited.

The study was approved by the UNC Biomedical Institutional Review Board and by the U.S. Environmental Protection Agency. The study was registered with ClinicalTrials.gov (NCT00617110).

DE exposure protocol

Exposures were conducted using the EPA Human Studies Facility exposure chambers on the UNC campus as previously described [1]. Total exposure time was 2 hours. The exposure atmosphere was maintained at 40 $\pm$ 10% relative humidity and 22 $\pm$ 2^{o}C. DE was generated from a 6-cylinder, 205 hp, 5.9 L displacement diesel engine (Cummins, Columbus IN) mounted in a vehicle located outside of the building, and subsequently introduced into the exposure chamber after dilution with clean filtered and

humidified air by approximately 1/30th to give a chamber concentration of approximately 100 μg/m^3. This concentration of DE particulate matter was chosen as comparable to concentrations encountered at busy intersections in large urban areas [U.S. EPA. Health Assessment Document for Diesel Emissions. Office of Research and Development. Washington DC. Publication No. EPA/600/8-90/057C. 1998]. Preliminary testing showed the mean particle size to be approximately 0.14 μm. Diesel fuel used for the study was certified fuel (Chevron Phillips Chemical Co., Borger TX, 0.05 LS Certification Fuel, type II), and the same lot was utilized throughout the study.

Subjects were monitored during chamber exposures by ECG telemetry, monitoring of CO and oxides of nitrogen, and by direct observation. Particle mass concentrations were determined by weighing filters obtained from Versatile Air Pollution Sampler impactors. Particle number and size distributions were monitored throughout the study and did not differ significantly between exposures.

Nasal lavage

This was performed according to a method we have previously described [2] by repetitive spraying of sterile normal saline irrigation solution (5 ml total) into the nostril, followed by voluntary expelling of fluid by the subject into a specimen collection cup. Both nostrils were lavaged in this way and the resulting NLF from both sides was combined. The NLF was centrifuged at 500g x 7 minutes to remove cells and debris, and the cell-free supernatant was stored in aliquots at -80 ^oC until used in mediator assays.

LAIV and virus detection

LAIV (FluMist®) was purchased from MedImmune, Inc. and used according to the manufacturer's recommendations. Vaccine composition in Year 1 of the study (2007-8) was based on influenza strains H1N1 A/Solomon Islands/3/2006, H3N2 A/Wisconsin/67/2005, and B/Malaysia/2506/2004. In year 2 of the study (2008-9) it was based on strains H1N1 A/Brisbane/59/2007, H3N2 A/Brisbane/10/2007, and B/Florida/4/2006. Virus quantification in NLF cells was done using quantitative RT-PCR for influenza type B hemagglutinin RNA as described before [2]. The rationale for use of this endpoint is that in the study cited [1], we found that the influenza B hemagglutinin sequence was more reliably detected and quantified in NLF cells than that of the A strains.

Serum hemagglutination inhibition assay

Antibodies to the LAIV strains used in the study were measured by hemagglutination inhibition using sera obtained at baseline screen visits, and day 21 post LAIV. Type O positive human blood was washed 3 times with PBS by mixing 5 ml of blood with 10 ml PBS and centrifugation at 4^0C, 1000 X g for 10 minutes. The human erythrocytes were diluted to a final suspension 0.5% with PBS. Hemagglutination-inhibition was tested as previously described (7). Briefly, the sera were treated with receptor destroying enzyme (RDE) (Denka Seiken, Tokyo, Japan) by diluting one part serum with three parts enzyme and incubated in a 37^0C water bath overnight. The RDE

was heat-inactivated by a 30 min incubation at 56^0C and hemadsorbed on human RBCs according to the sera preparation procedures of the CDC HI protocol for human influenza. The cleared sera were further diluted with PBS to a final 1:10 dilution. Two-fold serial dilutions in 25µl of PBS were performed in a 96-well U bottom microtiter plate. Next, 25µl of PBS containing 4 hemagglutination units (HAU) of the LAIV virus corresponding to the virus the subject was vaccinated with was added. The virus and sera mixture incubated at room temperature for 30 minutes after which 50µl of 0.5% human erythrocytes was added to all wells. Plates were incubated at room temperature and read after 1 hour. The serum titer result was expressed as the reciprocal of the highest dilution of serum where hemagglutination was inhibited. The back titer for both LAIV strains was run in duplicate.

Measurement of mediator levels in NLF

Inflammatory cytokines (IL-1β, IL-2, IL-6, IL-10, IL-12p70, GM-CSF, IFNγ) and chemokines (IL-8, IP-10, MCP-1, TARC, eotaxin-1, eotaxin-3, MDC, MCP-4) were measured in NLF using a multiplex ELISA platform (MesoScale Discovery, Gaithersburg, MD) according to manufacturer's instructions. Eosinophilic cationic protein (ECP) was measured in NLF using a specific commercial ELISA kit (Medical & Biological Laboratories Co., Nagoya, Japan) also according to manufacturer's instructions.

Statistical analysis. Revise per Haibo additional comments onnested model?

Raw NLF data are presented in descriptive fashion for each mediator endpoint, and levels of most mediators tended to rise then fall back to baseline levels over the 9 days post LAIV inoculation. To evaluate the effect of exposure and allergic status on the mediators, we reduced the longitudinal observations for a subject to a single point representing response to LAIV, namely the area under curve (AUC). To reduce within-group variability in responses, we adjusted the baseline variability in absolute mediator concentrations in NLF by calculating each day's data as a ratio to the Day 0 (the baseline day). The adjusted AUC, denoted by $AUC_{ratio,}$ were calculated based on baseline-adjusted ratio from day 1-9 for each subject. Additionally, since data collection did not occur on days 5-8, all comparisons were repeated using AUC data for days 1-4 only. For endpoints which had baseline (Day 0) results of 0 for more than 20% of subjects, statistical analysis was applied to "raw" AUC data (AUC_{raw}) rather than to ratio to baseline AUC data. This was the case for IFNγ and for influenza virus sequence quantity. To formally test for the exposure effect, we employed a sequence of nested multiple regression models with exposure group (DE/Air) as the main explanatory variable and allergic status (NV/AR) as an additional factor. We log transformed the AUC value to achieve normality in responses and used the backward selection procedure to arrive at the final model for each response. Since BMI is known to affect both influenza outcomes and vaccine responses in humans [4-6], we included it in all the models. The full model is a two-way ANCOVA model with interaction of exposure group and allergic status ($\gamma_i = \beta_0 + \beta_1 * I\ (group_i = DE) + \beta_2 * I\ (allergic_i = AR) + \beta_3 * I\ (group_i = DE)*I(allergic_i = AR) + \beta_4\ BMI$). Subsequent models tested were an additive two-way ANCOVA model ($\gamma_i = \beta_0 + \beta_1 * I\ (group_i = DE) + \beta_2 * I\ (allergic_i = AR) + \beta_3\ BMI$)

and the one-way ANCOVA model ($\gamma_i = \beta_0 + \beta_1 * I\ (group_i = DE) + \beta_2\ BMI$). Hypothesis testing was performed at the 0.05 significance level. All statistical analysis was conducted using SAS 9.2.

RESULTS

For Figures E1-E12, data are shown as medians at each time point. Break in x-axis reflects that data were not collected on days 5-8. NV/air = normal volunteers exposed to air; NV/DE = normal volunteers exposed to diesel exhaust; AR/air = allergic rhinitics exposed to air; AR/DE = allergic rhinitics exposed to diesel exhaust.

ONLINE DATA SUPPLEMENT REFERENCES

1. Sawyer, K., et al., *Responses measured in the exhaled breath of human volunteers acutely exposed to ozone and diesel exhaust.* Journal of Breath Research, 2008. **2**(3): p. -.

2. Noah, T.L., et al., *Tobacco Smoke Exposure and Altered Nasal Responses to Live Attenuated Influenza Virus.* Environ Health Perspect, 2010.

3. Motulsky, H., *Intuitive biostatistics*. 1995, New York: Oxford University Press. xx, 386 p.

4. Hagau, N., et al., *Clinical aspects and cytokine response in severe H1N1 influenza A virus infection.* Crit Care, 2010. **14**(6): p. R203.

5. Karlsson, E.A., P.A. Sheridan, and M.A. Beck, *Diet-induced obesity impairs the T cell memory response to influenza virus infection.* J Immunol, 2010. **184**(6): p. 3127-33.

6. Louie, J.K., et al., *A novel risk factor for a novel virus: obesity and 2009 pandemic influenza A (H1N1).* Clin Infect Dis, 2011. **52**(3): p. 301-12.