DIESEL EXHAUST EXPOSURE AND NASAL RESPONSE TO ATTENUATED INFLUENZA IN NORMAL AND ALLERGIC VOLUNTEERS

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DIESEL EXHAUST EXPOSURE AND NASAL RESPONSE TO ATTENUATED INFLUENZA IN NORMAL AND ALLERGIC VOLUNTEERS

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Running head: Diesel and influenza-induced nasal inflammation

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At a Glance Commentary: Diesel exhaust enhances allergic inflammation, and pollutants are associated with heightened susceptibility to viral respiratory infections. The effects of combined diesel and virus exposure in humans are unknown. This study demonstrates that prior exposure to diesel exhaust exacerbates influenza virus-induced nasal inflammation, in particular by promoting eosinophil activation in allergic rhinitics.

This article has an online data supplement, which is accessible from this issue’s table of content online at www.atsjournals.org
ABSTRACT

Rationale: Diesel exhaust enhances allergic inflammation, and pollutants are associated with heightened susceptibility to viral respiratory infections. The effects of combined diesel and virus exposure in humans are unknown.

Objective: Test whether acute exposure to diesel modifies inflammatory responses to influenza virus in normal and allergic humans.

Methods: We conducted a double-blind, randomized, placebo-controlled study of nasal responses to live attenuated influenza virus in normal volunteers and allergic rhinitics exposed to diesel (100 µg/m³) or clean air for 2 hr, followed by standard dose of virus and serial nasal lavages. Endpoints were inflammatory mediators (ELISA) and virus quantity (qRT-PCR). To test for exposure effect, we used multiple regression with exposure group (diesel vs. air) as the main explanatory variable and allergic status as an additional factor.

Measurements and main results: Baseline levels of mediators did not differ among groups. For most post-virus nasal cytokine responses, there was no significant diesel effect, and no significant interaction with allergy. However, diesel was associated with significantly increased interferon-γ responses (p=0.02), with no interaction with allergy in the regression model. Eotaxin-1 (p=0.01), eosinophil cationic protein (p<0.01), and influenza RNA sequences in nasal cells (p=0.03) were significantly increased with diesel exposure, linked to allergy.

Conclusions: Short term exposure to diesel exhaust leads to increased eosinophil activation and increased virus quantity after virus inoculation in allergic rhinitics. This is
consistent with previous literature suggesting a diesel “adjuvant” effect promoting allergic inflammation, and our data further suggest this change may be associated with reduced virus clearance.

Word count of abstract: 249

Key Words: Diesel, influenza, eosinophil cationic protein, eotaxin-1, interferon-gamma.
INTRODUCTION

Viral respiratory infections account for a large share of human morbidity and mortality worldwide, particularly during pandemic outbreaks of influenza virus (1). It is well established from epidemiologic data that exposure to air pollutants is associated with increased risk for severe influenza and other respiratory viral infections (2). While controlled human experiments to define the mechanisms for this are difficult to design, we recently reported reduced virus clearance and suppression of specific innate immune factors in nasal secretions of smokers compared to nonsmokers, after standard inoculation with live attenuated influenza vaccine (LAIV) (5). Influenza virus is also epidemiologically important in asthma (3, 4), as well as having obvious general public health importance in both its annual epidemic and pandemic forms. Epidemiological studies have demonstrated that exposure to air pollution is associated with increased asthma-related hospital admissions, medication use, and asthma exacerbation (6-8). Since respiratory viral infections are the leading cause of asthma exacerbations, and concurrent exposure to pollutants is likely to occur frequently, it is important to understand how air pollutants further aggravate asthmatic/allergic inflammation and how this impacts viral infection.

Previous studies have demonstrated that exposure to high levels of oxidant air pollutants increases the severity of virus-induced exacerbation of asthma (9). In experimental murine models, diesel exhaust exposure has been well documented to exacerbate allergic inflammation and Th2 immunity (10-14), and we have recently demonstrated that prior exposure to diesel exhaust particles increases influenza-
induced exacerbation of allergic inflammation (11). However, few studies have been
done to confirm these effects in humans.

We hypothesized that in individuals with allergic rhinitis, exposure to diesel
exhaust increases allergic inflammation after subsequent viral infection, which in turn
modifies early innate immune responses and virus clearance. To test this, we
investigated the effects of short term, controlled diesel exhaust exposure on nasal
inflammatory responses to LAIV in human volunteers. Some of the results of this study
have been previously reported in abstract form (11b).

METHODS

Study design and subjects

This was a randomized, double blind, prospective study comparing the effects of
diesel exhaust (100 µg/m³, 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 diesel exhaust 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 diesel exposure
has been previously described (15) 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 (5).
Subjects were blinded as to exposure, as were all technicians performing assays on nasal lavage fluids. Serum was obtained at the screening visit and at 21 days post LAIV inoculation.

Both healthy, non-allergic young adult volunteers, and allergic rhinitics, 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.

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).

**LAIV and virus detection**
LAIV (FluMist®) was purchased from MedImmune, Inc. and used according to the manufacturer’s recommendations. Vaccine composition is described in the online data supplement. Virus quantification in NLF cells was done using quantitative RT-PCR for influenza type B hemagglutinin RNA as described before (5). Serologic response to LAIV was measured by hemagglutination-inhibition assay in sera obtained at screen visit and 21 days post LAIV (detailed methods in the online data supplement).

**Measurement of mediator levels in NLF**

Inflammatory cytokines (IL-1β, 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 nasal lavage fluids using a multiplex ELISA platform (MesoScale Discovery, Gaithersburg, MD) according to manufacturer’s instructions. Eosinophilic cationic protein (ECP) was measured in nasal lavage fluids using a specific commercial ELISA kit (Medical & Biological Laboratories Co., Nagoya, Japan) also according to manufacturer’s instructions.

**Statistical analysis.**

Raw nasal lavage fluid 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 (see online data supplement). To evaluate the effect of exposure and allergic status on the mediators, we reduced the longitudinal observations for each subject to a single point representing response to LAIV, namely
the area under curve (AUC), which was calculated for fold change over baseline. To formally test for the exposure effect, we employed a sequence of nested multiple regression models with exposure group (diesel/air) as the main explanatory variable and allergic status (normal/allergic rhinitis) as an additional factor. Since BMI is known to affect both influenza outcomes and vaccine responses in humans (16-18), we included it in all the models. The full model is a two-way ANCOVA model with interaction of exposure group and allergic status. Subsequent models tested were an additive two-way ANCOVA model and one-way ANCOVA model. Additional details of the statistical models are in the online data supplement.
RESULTS

Subject characteristics.

Demographic characteristics of the subjects completing the protocol are shown in Table 1. Subjects in the normal and allergic rhinitis groups were similar in age and BMI. Similarly, the normal and allergic rhinitis subgroups exposed to air and diesel also did not differ in age or BMI. In the normal group, there were more females than males but the distribution was similar between the diesel and air exposed subgroups. One subject in the allergic rhinitis/air group developed prominent allergic type symptoms (sneezing, conjunctivitis) felt to be unrelated to the study exposures. This subject was excluded from the final analysis, making the final N=7 for this group.

Chamber exposure data.

Mean measured average particle exposure for subjects exposed to diesel was 93.2 µg/m$^3$ (range 76.7-108.6 µg/m$^3$), and for air exposed subjects did not exceed 4.5 µg/m$^3$ in any case. Measured chamber concentration range for NO$_2$ was 0.00-0.01 ppm (air) and 0.86-1.32 ppm (diesel); NO concentration 0.00-0.03 ppm (air) and 3.26-4.67 ppm (diesel); SO$_2$ concentration 0.00-0.01 ppm (air) and 0.04-0.06 ppm (diesel); and CO concentration 0.01-0.08 (air) and 3.08-6.00 ppm (diesel). Subjects did not report significant symptoms with any exposure.

Nasal lavage fluid cytokines
At day 0, pre-LAIV baseline levels of nasal lavage fluid cytokines did not differ significantly between diesel vs. air exposure groups. For most cytokines, there was an increase in median levels in nasal lavage fluid from day 0 over the subsequent days 1-4 post LAIV, with a decline back toward baseline by day 9 (online data supplement Figures E1-E6). Statistical analysis of the diesel effect on post-LAIV cytokine responses based on AUC data for ratio to baseline (AUC\text{ratio}) for IL-1β, IL-6, IL-10, IL-12p70, and GM-CSF responses to LAIV, suggested no statistically significant effect of diesel (vs. air), and no significant interaction with allergy status. However, for IFNγ there was a significant diesel effect, not related to allergic status (p=0.02; Fig. 1). This was true whether AUC included days 1-9, or days 1-4 only.

**Nasal lavage fluid chemokines and ECP**

At day 0, pre-LAIV baseline levels of nasal lavage fluid chemokines did not differ significantly between diesel vs. air exposure groups, except for TARC levels which were higher in the diesel exposed normal subgroup than the air exposed normal subgroup (p=0.01). Similarly to the cytokine responses to LAIV, most of the measured chemokines increased during days 1-4 after LAIV inoculation, then declined back toward baseline by day 9 (online data supplement Figures E7-E12). For the CXC chemokines IP-10 and IL-8, there was no significant diesel effect in the regression model, although if AUC excluded day 9, a significant increase with diesel was noted for IL-8, in allergic rhinitics Among the CC chemokines, eotaxin-1 (CCL11) showed a statistically significant diesel-associated increase in the regression model, an effect
interacting with allergic status (p=0.01; Fig. 2). This was true whether AUC included days 1-9, or days 1-4 only.

At day 0, pre-LAIV baseline levels of nasal lavage fluid ECP did not differ significantly between diesel vs. air exposure groups. In diesel-exposed allergic rhinitis subjects, ECP levels were elevated compared to air exposed controls after LAIV, and persistently elevated at day 9 (Fig. 3A). ECP response expressed as area under curve was significantly increased with diesel exposure in the regression model, an effect linked with allergy status (p<0.01; Fig. 3B). This was true whether AUC included days 1-9, or days 1-4 only.

**Virus quantity and antiviral antibody responses**

While within-group data for influenza B hemagglutinin RNA sequences in nasal lavage fluid cells were quite variable, median virus quantity on day 1-4 post LAIV inoculation tended to be 1-2 log_{10} higher in diesel-exposed than in air-exposed groups (Fig. 4A). Since there was no virus in nasal lavage fluids at day 0 (pre-LAIV) baselines, area under curve analysis for influenza B hemagglutinin RNA sequences in nasal lavage fluid cells used only “raw” virus quantity (AUC_{raw}) to assess diesel effects. Levels of viral RNA sequences in NLF cells were significantly increased with diesel exposure, an effect modified by allergy status (p=0.03; Fig. 4B).

Pre-LAIV hemagglutination-inhibition titers in serum did not differ at baseline among the subject groups. Paired pre-LAIV and 21 days post-LAIV sera were available for testing in the majority of the subjects, and all four groups had significantly increased reciprocal titers after LAIV. Mean (SD) fold change increase in reciprocal titers post-
LAIV were similar among groups: normal/air = 2.5 (1.0), N=4; normal/diesel = 2.9 (2.5), N=7; allergic rhinitis/air = 3.0 (2.8), N=5; and allergic rhinitis/diesel = 1.7 (0.5), N=7.

DISCUSSION

In a randomized, controlled sequential-exposure study in young adults with or without allergic rhinitis, we found that short-term diesel exhaust exposure in a chamber setting had significant effects on subsequent post-LAIV nasal responses, specifically an increase in IFNγ levels and in virus quantity. Subjects with allergic rhinitis had particularly elevated quantities of virus, and additionally had statistically significant and prolonged increases in post-LAIV eotaxin-1 and ECP responses after diesel compared to air controls. Thus, allergic individuals may be especially susceptible to diesel-induced eosinophil recruitment and activation, either as a direct result of diesel exhaust particle interactions with nasal cell types relevant to allergic inflammation, or secondary to diesel-induced reduction in virus clearance. While we have previously reported that young adult smokers also have reduced clearance of LAIV and altered nasal inflammation (5), we believe the current study is the first to measure the impact of an inhaled pollutant on viral respiratory infection in a controlled-exposure setting in human volunteers.

Our results are consistent with data from experimental models suggesting that diesel exhaust exacerbates allergic inflammation. In ovalbumin (OVA)-sensitized BALB/c mice, exposure to diesel exhaust particles at 2000 µg/m³ for 4 hours/day for 4 days resulted in increased eosinophils, neutrophils, lymphocytes, and IL-6 in
bronchoalveolar lavage fluids following OVA challenge, while non-sensitized controls responded to diesel with increased neutrophils only (14). Data from a similar model suggest that diesel exhaust particles may function as an adjuvant in the induction of Th2 immune responses by OVA (13). Two recent studies (10, 12) have reported that repeated exposure to low-dose diesel exhaust after allergen challenge induced increased expression of MDC and the Th2 cytokines IL-4, IL-5 and IL-13 in murine lung tissue. Oxidant stress appears to be involved in diesel-associated increases in allergic inflammation and airway hyperresponsiveness as evidenced by experiments in Nrf2 knockout mice (19) and exposure of dendritic cells (DC) to diesel exhaust particle extracts (20, 21). Ambient and diesel particulates instilled into the oropharynx have been shown to activate pulmonary DC and promote eosinophilia and Th2-type responses in mice (22). In a study comparing responses to several chemically distinct preparations of diesel exhaust particles, potentiation of murine allergic immune responses appeared to correlate with diesel exhaust content of polycyclic aromatic hydrocarbons (23). Additionally, nasal challenge studies in humans have shown that diesel exhaust exposure predisposes to heightened allergic responses after allergen stimulation, particularly in GSTM1 null individuals (24, 25). However, conclusions regarding the clinical or mechanistic significance of our data must be made with caution given the fundamental differences between LAIV and wild type viruses (see caveats below).

Several recent reports have described experiments using human tissue to investigate mechanisms for diesel effects. In experimental in vitro studies using human bronchial epithelial cells in co-culture with myeloid dendritic cells (mDC), it was reported
that diesel exhaust particles induced oxidative stress which upregulated epithelial production of thymic stromal lymphopoietin (TSLP), driving mDC to Th2 polarization (26). Incubation of nonatopic human peripheral blood mononuclear cells with diesel exhaust particle derived PAH resulted in increased IL-13 and decreased IFNγ production and increased chemotaxis of Th2 lymphocytes (27). *In vitro* stimulation of human DC with diesel exhaust particles induced production of several cytokines including IFNγ, and direction of a Th2-like cytokine production pattern by CD4+ lymphocytes in co-culture (28). Thus, limited research using human tissue seems consistent with data from murine studies.

The effects of diesel exhaust exposure on susceptibility to infection have also been investigated in BALB/c mice. Exposure at varying levels and durations increased inflammatory mediators, including IFNγ, but suppressed expression of surfactant proteins A and D, which function as important host defense factors against both bacterial and viral pathogens in the lung (29). In murine experiments analogous to the design for our current study, we found that OVA-sensitized C57BL/6 mice instilled intratracheally with diesel exhaust particles, then infected with influenza A, had increased lung levels of eosinophils and Th2 cytokines compared to mice exposed to either diesel or virus alone (11). Furthermore, the Th2-like cytokine responses to influenza noted in neonatal mice (compared to adults) are associated with delayed migration of T cells to the lung, and increased susceptibility to influenza virus infection (30). As noted above, no previous studies have tested the effects of sequential exposures to diesel exhaust and virus in human volunteers.
Epidemiologic evidence exists for an impact of diesel exhaust or "near roadway" effects on asthma in humans, particularly children (31). Behndig et al. (32) recently reported a study of normal volunteers and subjects with mild to moderate asthma who were exposed to a similar controlled diesel exhaust exposure as in our study (100 µg/m³, idling truck source, 2 hr with intermittent exercise), followed by lung function testing and bronchoscopy 18 hours later. While the normal volunteers had mild increases in neutrophils in the airways, asthmatics had no change in inflammation or in bronchial hyperreactivity, and the authors concluded that "the increased sensitivity of asthmatics to traffic-related air pollution is not necessarily associated with classical acute inflammation or aggravation of standard cellular indicators of allergic asthmatic inflammation." It is possible that the lower airways are relatively protected from real-world DE effects compared to the nasal airway, or that the diesel fuel or engine characteristics used by Behndig et al. were significantly different from ours. Furthermore, a study at our center in which diesel exhaust particles were applied directly to the nasal mucosa in asthmatic volunteers showed no inflammatory effect (33). However, a reasonable hypothesis, consistent with our current results and with our data in mice (11), is that lower-level exposures may have minimal impact alone, but can predispose to exacerbation of allergic or asthmatic inflammation by increasing virus-induced eosinophilic inflammation and reduced early virus clearance. This might occur via effects of diesel exhaust on airway cells driving Th2 immune responses, by diesel-induced reduction of virus clearance through non-immune mechanisms, or both. While such a sequence of events is complex and cannot be proven in an observational human study such as ours, it could conceivably be common in allergic individuals in urban
settings. A very similar sequence was suggested in a previous report in which severity of virus-induced exacerbation was increased in asthmatic children with recent exposure to elevated levels of ambient NO\textsubscript{2} (9).

Several caveats are appropriate for our study. First, the relatively small number of subjects we studied may limit definitive conclusions. Baseline levels of most factors in NLF were quite variable, but our main method of data reduction for statistical analysis (area under curve for ratios to baseline) was chosen to minimize the impact of baseline variability. Second, the temperature-sensitive LAIV is replication-limited and therefore does not fully mimic a natural influenza infection, though it clearly is immunogenic and simulates many important features of natural mucosal host defense (34). For practical reasons and based on our prior published experience (3), we limited our data collection to the first several days after LAIV inoculation plus a later time point (day 9), leaving open the possibility that significant effects could have been missed in the day 5-8 interval. However, it seems unlikely that such changes could have been sufficient to negate the significant results we observed in our analysis, which were similar whether area under curve data for days 1-4 only or including day 9 were used.

Diesel exhaust particles are a complex mixture of organic carbons adsorbed to elemental carbons and small amounts of sulfates, nitrates and minerals (35). Our study, designed to approximate real-world exposures by employing an actual truck engine and levels of particulates reported in common urban situations (36), used a fuel and exposure setup identical to that reported by Sobus et al. (37), but different engines and running conditions can alter the chemical composition of diesel exhaust, which in turn may have a significant impact on inflammatory outcomes (23). Our study design
did not include control groups for LAIV itself, raising the possibility that any mediator effects we observed were related to the diesel or air exposures alone rather than to LAIV. However, the time course and pattern of mediator responses we observed in the current study were very similar to those in our previous LAIV study which did not involve diesel exposure (3), and a previous study at our center showed no effect of diesel exhaust particles alone on nasal inflammation (29). It thus seems very unlikely that the cytokine elevations observed in the current study were independent of LAIV. A final caveat is that nasal mucosal responses may also not entirely mimic those in the lower airways, though there is evidence from whole-genome studies that nasal epithelial responses resemble bronchial epithelial responses to toxins (38).

In summary, we have observed evidence for reduced nasal virus clearance and increased and prolonged virus-induced eosinophil activation in allergic rhinitics randomized to exposure to low-level diesel exhaust compared to air prior to LAIV inoculation. These results are consistent with data from our previous study conducted in OVA-sensitized mice (11), supporting the relevance of these models, and add to the evidence that diesel exposure at commonly-encountered levels may influence the course of infection in humans, particularly in allergic individuals. While the clinical importance of this kind of interaction cannot be defined from our data, additional studies regarding the effect of novel preventive or treatment strategies such as antioxidant upregulation seem warranted. Additionally, our data suggest that detailed studies of mucosal responses to respiratory viruses may be safely carried out in humans using nasal exposure models, as we have previously shown for LAIV (5) and as recently shown for RSV by DeVincenzo et al. (39). The general experimental approach of
sequential controlled exposures to environmental pollutants and attenuated or well-tolerated wild type virus strains may allow further advances in our understanding of the complex interactions between environmental agents and infectious diseases in humans.

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REFERENCES


Inflammatory Responses on Instillation of Engineered, Environmental Diesel Emission Source or Ambient Air Pollutant Particles in vivo. *J Innate Immun*


Table 1. Demographic characteristics of normal (NV) and allergic rhinitic (AR) volunteers exposed to air or diesel exhaust (DE). Data are shown as mean (SD).

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FIGURE LEGENDS

Fig. 1. (A) Time course of IFNγ in nasal lavage fluid after Day 0 inoculation with LAIV in subjects exposed just prior to LAIV inoculation to clean air or to 100 µg/m³ diesel exhaust particles for 2 hr at rest. Data are shown as medians at each time point. (B) Individual area under curve (AUC) data for IFNγ in nasal lavage fluid. Horizontal lines represent medians for each exposure group. 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.

Fig. 2. (A) Time course of eotaxin-1 in nasal lavage fluid after Day 0 inoculation with LAIV in subjects exposed just prior to LAIV inoculation to clean air or to 100 µg/m³ diesel exhaust particles for 2 hr at rest. Data are shown as medians at each time point. (B) Individual area under curve (AUC) data for eotaxin-1 in nasal lavage fluid. Horizontal lines represent medians for each exposure group. 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.

Fig. 3. (A) Time course of eosinophil cationic protein (ECP-1) in nasal lavage fluid after Day 0 inoculation with LAIV in subjects exposed just prior to LAIV inoculation to clean air or to 100 µg/m³ diesel exhaust particles for 2 hr at rest. Data are shown as medians at each time point. (B) Individual area under curve (AUC) data for ECP in nasal lavage fluid. Horizontal lines represent medians for each exposure group. 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.

Fig. 4. (A) Time course of influenza virus RNA in nasal lavage fluid after Day 0 inoculation with LAIV in subjects exposed just prior to LAIV inoculation to clean air or to 100 µg/m³ diesel exhaust particles for 2 hr at rest. Data are shown as medians at each time point. (B) Individual area under curve (AUC) data for influenza virus RNA in nasal lavage fluid. Horizontal lines represent medians for each exposure group. 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.
Figure 1

A

IFN\(\gamma\) (pg/ml NLF) vs Study day

- AR/DE
- AR/air
- NV/DE
- NV/air

B

IFN\(\gamma\) AUC_{raw} vs NV/air, NV/DE, AR/air, AR/DE

Log scale on the y-axis.
Figure 2

A

Eotaxin-1 (pg/ml NLF)

Study day

B

Eotaxin-1 AUC_{ratio}

NV/air

NV/DE

AR/air

AR/DE
Figure 3

A

ECP (ng/ml NLF)

Study day

B

ECP AUC ratio

NV/air  NV/DE  AR/air  AR/DE
Figure 4

A

Influenza RNA

Study day

B

Influenza AUCraw

NV/air  NV/DE  AR/air  AR/DE
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 (FEV₁ < 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
\[ N \approx \left[2SD^2 \times (z_\alpha + z_\beta)^2\right] / \Delta^2 \]
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 ± 10% relative humidity and 22
± 2°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/m³. 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 °C 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°C, 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°C water bath overnight. The RDE
was heat-inactivated by a 30 min incubation at 56°C 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 on nested 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 ($\gamma_i = \beta_0 + \beta_1 \ast I(group_i = DE) + \beta_2 \ast I(allergic_i = AR) + \beta_3 \ast I(group_i = DE)\ast I(allergic_i = AR) + \beta_4 BMI$). Subsequent models tested were an additive two-way ANCOVA model ($\gamma_i = \beta_0 + \beta_1 \ast I(group_i = DE) + \beta_2 \ast I(allergic_i = AR) + \beta_3 BMI$)
and the one-way ANCOVA model ($y_i = \beta_0 + \beta_1 \times I(group_i = DE) + \beta_2 BMI$). 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


Fig. E1. Time course of IL-1β in nasal lavage fluids.

Fig. E2. Time course of IL-6 in nasal lavage fluids.

Fig. E3. Time course of IL-10 in nasal lavage fluids.
Fig. E4. Time course of IL-12p70 in nasal lavage fluids.

Fig. E5. Time course of GM-CSF in nasal lavage fluids.

Fig. E6. Time course of IP-10 in nasal lavage fluids.
Fig. E7. Time course of IL-8 in nasal lavage fluids.

Fig. E8. Time course of TARC in nasal lavage fluids.

Fig. E9. Time course of Eotaxin-3 in nasal lavage fluids.
Fig. E10. Time course of MDC in nasal lavage fluids.

Fig. E11. Time course of MCP-1 in nasal lavage fluids.

Fig. E12. Time course of MCP-4 in nasal lavage fluids.