Urban particulate matter impairs airway-surface-liquid-mediated coronavirus inactivation

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Summary: Primary human airway surface liquid (ASL) inactivates SARS-CoV-2 and 229E-CoV in vitro. Air pollution particulate matter (PM) inhibits ASL viral inactivation, suggesting that PM may enhance coronavirus infection by impairing airway innate immunity.
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**Abstract:** Air pollution particulate matter (PM) is associated with SARS-CoV-2 infection and severity, although mechanistic studies are lacking. We tested whether airway surface liquid (ASL) from primary human airway epithelial cells is antiviral against SARS-CoV-2 and 229E-CoV (responsible for common colds), and whether PM (urban, indoor (IAP), volcanic ash) affected ASL antiviral activity. ASL inactivated SARS-CoV-2 and 229E-CoV. Independently, urban PM also decreased SARS-CoV-2 and 229E-CoV-2 infection, and IAP decreased 229E-CoV infection. However, in combination, urban PM impaired ASL’s antiviral activity against both viruses and the same effect occurred for IAP and ash against SARS-CoV-2, suggesting PM may enhance SARS-CoV-2 infection.

**Keywords:** Airway, viral infection, common cold, COVID-19, Innate immunity
**Background:**

Prior to the current COVID-19 pandemic, humans lacked virus-specific immunity against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Therefore, in immunologically naïve individuals, the airway’s innate immune response remains critical to combating SARS-CoV-2 and other infections. Airway surface liquid (ASL) is a thin layer of fluid lining airway epithelium. ASL contains antimicrobial peptides and proteins (AMPs) that provide a critical first line of defense against incoming pathogens [1]. Specific AMPs in the ASL may inhibit viral cell-binding, attachment, or alter subsequent viral life cycle stages to enhance viral clearance [2]. Additionally, AMPs within the ASL were recently reported to have antiviral activity against enveloped and non-enveloped viruses [3]. Upon viral challenge, other innate immune mechanisms are also activated (e.g. macrophages and neutrophils phagocytize infected cells and intracellular antiviral mechanisms interfere with viral replication, inactivating viruses).

Airway innate immunity is disrupted by air pollution. We have previously demonstrated that indoor and ambient air pollution particulate matter (PM) inhibits ASL-mediated bacterial killing, in part due to competition between cationic AMPs and negatively charged PM and bacteria [4]. Ambient PM is also associated with increased coronavirus infection incidence and mortality in epidemiological studies [5]. However, the mechanism(s) underlying this association are unknown.

Endogenous ASL with abundant and diverse AMPs, derived from primary human donor airway epithelial cell (hAEC) cultures at the air-liquid interface (ALI), has not been assessed for antiviral coronavirus activity. In this study, we hypothesize that ASL is antiviral, and PM impairs ASL antiviral activity, **Supplemental Figure 1.** If so, this effect likely contributes to the reported increased risk of SARS-CoV-2 infection related to urban air pollution exposures [6]. To test this, we assessed whether ASL from primary hAECs inactivates the causative agents responsible for COVID-19 (betacoronavirus
SARS-CoV-2), and a common cold (human alphacoronavirus 229E-CoV) then determined the effect of indoor and ambient PM on ASL antiviral activity in vitro.

Methods:
Following informed written consent, human donor lungs, confirmed PCR negative for SARS-CoV-2 infection at the time of tissue retrieval, were dissected to obtain bronchial and tracheal cells. Cells were seeded onto collagen coated filters at the ALI by the University of Iowa (UI) Cell Culture Core Repository, then washed to obtain endogenous ASL (n>4 donors/experiment), as previously described [4]. For donor demographics see Supplemental Table 1. This study is approved by the UI Institutional Review Board (IRB #199507432).

Washington [WA] strain human SARS-CoV-2 and 229E-CoV (ATCC® VR-740™) were quantified in Vero E6 cells (kindly provided by Dr. Wendy Maury, University of Iowa) and MRC-5 cells (ATCC® CCL171™) respectively, as previously described [7]. Both viruses are coronaviruses and share considerable virion and genome structure and organization but represent two different genera. Epithelial Vero E6 and MRC-5 cells were maintained at 37°C with 5% CO₂ prior to viral infection (MOI=1), after which they were maintained at 33°C with 5% CO₂. For cell viability and viral binding and entry methodology, see Supplemental Methods. SARS-CoV-2 work was performed in the UI Biosafety Level (BSL) 3 facility, while 229E-CoV studies were performed in UI BSL2 facilities.

Media: Vero E6 and MRC-5 cells were maintained in Dulbecco’s Modified Eagle Medium +5% fetal bovine serum (FBS), and Minimum Essential Media +10% FBS, +1% penicillin/streptomycin, respectively. Basolateral hAEC media (1:1 Dulbecco’s Modified Eagle Medium/F12, +2% Ultroser G, +1% penicillin/streptomycin, +0.1% gentamicin/fluconazole) was changed biweekly.

Particulate Matter: Urban PM SRM® 1648a, and NIST® Trace Elements in Indoor Dust SRM® 2584, hereafter IAP, was derived from the National Institute of Standards and Technology® (NIST®), and
volcanic ash from the Eyjafjallajökull eruption, see https://www.nist.gov/srm and [8]. Urban PM originated as atmospheric PM, containing polycyclic aromatic hydrocarbons (PAHs), nitro-substituted PAHs, polychlorinated biphenyl congeners, and chlorinated pesticides, common in urban air pollution. IAP was collected from residences. Lastly, we tested Icelandic volcanic ash as a relatively inert PM control condition. To measure the electrokinetic potential in PM colloidal dispersions, we assessed the zetapotential of PM and ASL in triplicate (Zetasizer nano ZS, Malvern Panalytical Ltd, Malvern WR14 1XZ, United Kingdom), per [9].

To assess the effect of ASL and PM on viral infection, PM was added to vehicle or ASL for 20 minutes, then virus was added and the mixture applied to permissive cells for 1h [10]. After 3-7 days, cells were washed and viruses were quantified [7, 11]. All experiments were performed in triplicate with vehicle and ASL controls.

Our PM dose was based on a healthy adult resting minute ventilation of 6 LPM (8.64 m³/24h) and the U.S. EPA PM_{2.5} standard (35 µg/m³), totaling 302.40 µg/24h of exposure. Accounting for the airway tree’s internal lung surface area (~2.471 cm², [12]) and the ASL lung volume (~1 µL/cm² in vivo, [13]), the airway tree contains ~2.47 mL ASL. Applying the 24h EPA standard PM_{2.5} exposure (302.40 µg/2.47 mL ASL), the normalized EPA standard PM_{2.5} concentration is 122.4 µg/mL. Therefore, respectively, 50 µg/mL and 500 µg/mL PM are approximately 40% and 400% the EPA standard normalized to lung surface area and ASL volume. Because urban PM is the most associated with COVID-19 infection and severity, we tested its effects at both doses. Exposure to PM varies widely worldwide [9], so we selected doses representative of different regions.
Results:

**ASL inactivates 229E-CoV and SARS-CoV-2.** We determined the effect of primary hAEC donor ASL on 229E-CoV or SARS-CoV-2 infectivity of epithelial cells by mixing virus with ASL or vehicle prior to their application onto MRC-5 or Vero E6 cells, then quantifying virus. ASL reduced 229E-CoV and SARS-CoV-2 infection by 28% and 41%, respectively Fig. 1A (p<0.001 and p=0.004).

**Effect of PM on 229E-CoV.** We then tested whether 50 and 500 µg/mL PM affected 229E-CoV infection of MRC-5 cells. 50 µg/mL of PM had no effect, while 500 µg/mL of IAP and urban PM resulted in less 229E-CoV infection (p=0.04 and 0.03, respectively) and ash had no effect (p=0.73). Fig. 1B. Because PM impaired viral replication, we tested whether it affected MRC-5 and Vero E6 cell viability (Supplemental Methods) and found no effects, Supplemental Figure 2A-B.

**Effect of PM on SARS-CoV-2.** Urban PM inactivated SARS-CoV-2 (50 and 500 µg/mL, p=0.02 and 0.06, respectively), Fig. 1C. Contrary to 229E-CoV, 500 µg/mL ash increased SARS-CoV-2 infection while IAP had no effect (p=0.03 and 0.54, respectively), Fig. 1C.

**Effect of PM on ASL inactivation of 229E-CoV.** We tested whether PM affected ASL anti-229E-CoV activity by applying 50 and 500 µg/mL PM or vehicle to ASL for 20 minutes then incubating the mixture with virus and quantifying infectious virus by plaque assay. Compared to baseline ASL viral inactivation, 500 µg/mL of urban PM resulted in significantly more 229E-CoV plaques (p<0.0001), while IAP and ash had no significant effect, Fig. 2A. We then assessed whether PM physically interfered with the virus’s ability to infect cells by quantifying 229E-CoV binding and entry in MRC-5 cells (Supplemental Methods). Including PM in media did not lead to significant differences in viral binding or entry compared to vehicle control (Supplemental Figure 3, 4A-C).
**Effect of PM on ASL inactivation of SARS-CoV-2.** Urban PM affected both viral viability and impaired ASL 229E-CoV inactivation. We therefore probed whether it affected ASL inactivation of SARS-CoV-2 at both doses, and tested IAP and ash at 500 μg/mL. In the presence of PM and ASL, there was increased SARS-CoV-2 infection compared to either alone; however, only ash (500 μg/mL) significantly increased SARS-CoV-2 infection, Fig. 2B.

Finally, we tested whether PM’s electrostatic state affected its affinity to AMPs within the ASL and found that the zeta potential (mV) of each PM was significantly more negatively charged than the ASL (p<0.001; -17, -15, -13 mV, respectively). Nevertheless, there were no significant differences between particle type, Supplemental Fig. 5, thus, charge alone is unlikely to explain the antiviral effect of PM, or its inhibitory effect on ASL.

**Discussion:**

It is established that ASL can be antimicrobial, and this is often mediated by AMPs [1, 2, 4]. Our findings are the first to demonstrate that primary human airway ASL significantly inactivates 229E-CoV and SARS-CoV-2 *in vitro* (Fig. 1A). Therefore, ASL likely contributes to innate immune protection against coronavirus infection.

Epidemiological studies demonstrate an association between urban PM air pollution and viral respiratory infection incidence (influenza and influenza-like illness), hospitalizations (respiratory syncytial virus, pneumonia), and mortality (SARS), including increased risk of COVID-19 incidence and severity [5, 14]. After establishing that ASL was antiviral against both coronaviruses, we asked two distinct research questions: 1) in isolation, does PM affect 229E-CoV and SARS-CoV-2 infection of epithelial cells and 2) does PM impair ASL-mediated viral inactivation of 229E-CoV and SARS-CoV-2?

Urban PM independently reduced 229E-CoV and SARS-CoV-2 infection of epithelial cells. 500 μg/mL IAP also decreased 229E-CoV, but not SARS-CoV-2, infection. Distinctly, volcanic ash had no effect on
229E-CoV epithelial cell infection, but increased SARS-CoV-2 infectivity (500 µg/mL), Fig. 1B-C, potentially due to “viral hitchhiking” on ash PM. These findings demonstrate that different sources of PM differentially affect viral infection.

The addition of urban PM to ASL led to significantly more 229E-CoV (500 µg/mL) and SARS-CoV-2 infection (50 µg/mL) relative to untreated ASL, Fig. 2A-B. Adding IAP and ash to ASL did not significantly affect 229E-CoV infection, but each impaired anti-SARS-CoV-2 ASL activity (500 µg/mL, Fig. 2A-B). These data demonstrate that PM decreases the efficacy of ASL against coronaviruses. They also reveal that while both PM and ASL are antiviral, effects are not additive. Delineating the percent of viral inactivation contributed by each component is not feasible as it would require quantification of all ASL proteins and free/bound PM for any given assay.

We expected PM charge state to affect viral viability. While we did not find differences between charge state in the PM tested (Supplemental Fig. 5), viral stability and adsorption are affected by many factors including pH, humidity, temperature, and surface conditions, and the role of each of these factors in the context of ambient PM is underexplored in SARS-CoV-2 [15]. The complexity of PM, including metal content, charge, size, and conformation may explain the differences we observed.

Study strengths include use of primary human donor hAEC-derived ASL to examine airway immune function with a reductionist approach, use of common air pollutants (urban/indoor PM), and similarity between ASL findings despite having used two discrete human coronaviruses propagated in distinct epithelial cell lines (lung and kidney) from two organisms (human and monkey). Study limitations include the narrow mechanism tested (infection), and the limited PM sources. For example, we cannot speculate on PM composition from rural environments, which may influence COVID-19 severity. Although we were not able to measure outcomes such as infection severity, other studies have reported increased viral circulation, infection, length of hospital stay, illness severity and mortality for influenza, H1N1, SARS and rhinovirus, due to PM and its associated air
pollution exposures [14]. Further, we strictly examined ASL viral inactivation and the effect of PM without examining the role of macrophages or neutrophils, or the effect of PM on intracellular antiviral pathways. Nevertheless, our in vitro findings provide rationale to further examine the effects of air pollution PM on viral replication in vivo. Future directions include translational research to validate in vitro results using an in vivo model, and identification of specific physicochemical characteristics in PM responsible for ASL antiviral impairment. Our in vitro data provide new insight into how PM exposures may influence respiratory viral infections and thus may inform future PM exposure policy discussions.

Our data suggest PM influences coronavirus infectivity via two distinct mechanisms, at minimum. PM decreases viral viability and impairs ASL viral inactivation. Urban PM consistently inactivated both coronaviruses, thereby decreasing ambient viral titers prior to inhalation. Once urban PM was introduced to ASL, it consistently inhibited the antiviral effect of ASL, while IAP and ash had differential effects depending on the virus studied. These results support epidemiological observations concerning urban air pollution and viral infection. Additionally, IAP and ash results indicate air pollution from other sources may affect the risk of viral infection. To our knowledge, this is the first report of viral inactivation by primary human ASL, and the first to report its inhibition by PM in vitro.
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Figure legends.

**Figure 1. A.** Percent viral 229E-CoV plaques (left) or TCID$_{50}$ of SARS-CoV2 (right) with ASL compared to viruses with vehicle, dots represent experiments performed on 1 cell passage in triplicate using freshly obtained ASL from >4 donors; significance determined by Wilcoxon matched-pairs signed rank test on untransformed data. **B.** 229E-CoV plaque formation with PM compared to 229E-CoV plaques with vehicle, significance determined by comparing triplicate data from four experiments per condition using a one sample t-test with theoretical mean=100. **C.** SARS-CoV-2 TCID$_{50}$ with PM compared to SARS-CoV-2 TCID$_{50}$ with vehicle, significance determined by comparing triplicate data from four experiments per condition using a one sample t-test with theoretical mean=100. Error bars represent standard error of the mean and *p<0.05, **p<0.01, ***p<0.001.

**Figure 2. A.** Percent viral 229E-CoV plaque formation with ASL combined with 50 or 500 µg/mL PM, normalized to PM control; significance determined by comparing triplicate data (per condition from three experiments) to ASL control using one-way ANOVA. **B.** Percent viral SARS-CoV-2 TCID$_{50}$ with ASL combined with 50 or 500 µg/mL PM, normalized to PM control; significance determined by comparing triplicate data (per condition from three experiments) to ASL control using one-way ANOVA when two doses were tested, or by unpaired t-test for single-doses (IAP, ash). Error bars represent standard error of the mean and *p<0.05, **p<0.01, ***p<0.0001.
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Figure 1

A  

% Virus relative to ctrl (ASL vs vehicle) 

229E  SARS-CoV-2

B  

% 229E plaques (vs vehicle ctrl) 

PM (µg/mL)

0 100 200 300 400 500

Ash  IAP  Urban

C  

% SARS-CoV-2 TCID50 (vs vehicle ctrl) 

PM (µg/mL)

0 100 200 300 400 500

Ash  IAP  Urban
Figure 2

A

\[ \% \text{229E plaques (vs PM ctrl)} \]

- Ash
- IAP
- Urban

\[ \text{PM alone} \]

\[ \text{ASL alone} \]

\[ \text{PM (µg/mL)} \]

0 100 200 300 400 500

B

\[ \% \text{SARS-CoV-2 TCID}_{50} \text{ (vs PM ctrl)} \]

- Ash
- IAP
- Urban

\[ \text{PM alone} \]

\[ \text{ASL alone} \]

\[ \text{PM (µg/mL)} \]

0 100 200 300 400 500