Online data supplement

GENE EXPRESSION PROFILES DURING IN VIVO HUMAN RHINOVIRUS INFECTION: INSIGHTS INTO THE HOST RESPONSE

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Experimental Rhinovirus Infection: This was a randomized, parallel group study conducted in healthy volunteers with no neutralizing antibody to HRV-16 (titer <2) in a non-sequestered setting. The protocol was approved by the Human Investigations Committee of the University of Virginia and all volunteers gave written, informed consent before participating in the study. A schematic of the study protocol is included in Figure E1. Subjects were eligible to participate in the study if they were between 18 and 60 years of age, could read, understand and sign the informed consent form, and were healthy based on medical history. Subjects also had to be willing to refrain from taking any allergy or cold medications (including dietary supplements and homepathic preparations indicated for allergy or colds) starting on day –14 and through day 5 of the study. In addition, intranasal medications or treatments were not permitted from day –14 to day 5 and all other medications (except contraceptive preparations or topical acne medications) from day –2 to day 5. Subjects were excluded if they had abnormal nasal anatomy, had experienced cold or allergy symptoms in the previous 2 weeks, had a history of nosebleeds, had used any investigational medication in the past 30 days or had used any tobacco products in the prior 6 months.

One group of subjects was inoculated with a total dose of 1000 50% tissue culture-infective dose ($TCID_{50}$) units of an inoculum of HRV-16 that was safety-tested for human use as described (E1), while a control group were sham inoculated with vehicle. Nasal epithelial scrapings were collected from the anterior portion of the inferior turbinate under direct visualization as described (E2). For each subject, scrapings were obtained from surface of the turbinate 5 times with a disposable cytology collection curette (Rhinoprobe). This procedure was then repeated with a second curette for a total of 10 scrapes. Scrapings were performed on day -14 (prior to virus/control inoculation), on day 0 at 8 hours after inoculation, and on day 2.

For each subject, scrapings were obtained from alternating nostrils. In other words, if day-14 scrapings were from the left nostril, the 8 h scrapings were from the right nostril, and the 2 day sample from the left nostril. This meant that 16 days passed to permit full epithelial regeneration between consecutive scrapings from the same nostril. For all subjects, the nostril (left or right) from which the initial nasal scraping sample was collected was randomized, stratifying on sex. Both curettes were placed into an RNase-free screw-capped cryovial containing Trizol Reagent (Invitrogen, Carlsbad, CA) to preserve RNA. The cryovial was vortex-mixed to remove the cellular material from the curettes and then stored frozen at -70 °C until extraction of RNA.

On day 0, subjects underwent nasal lavage immediately prior to inoculation, and just prior to nasal scraping. To rule out the possibilility of intercurrent viral infection, molecular tecniques were used to screen both this lavage, and the lavage from day –14, for the presence or absence of respiratory syncytial virus, adenovirus, coronavirus, parainfluenza, influenza and rhinoviruses. Detection of any of these viruses excluded subjects from further participation in the study. From day 1 to day 5 post-inoculation, subjects underwent daily nasal lavage. From days 0 –5, subjects recorded daily symptom scores as described (E3, E4). The presence or absence of HRV-16 was assessed in nasal lavage, and serum neutralizing antibodies to HRV-16 were assayed prior to, and 20-30 days after, inoculation. Subjects were considered infected with HRV-16 if rhinovirus was determined to be present in at least one of their nasal lavage samples collected at the Day 1-5 visits and if it was determined that HRV-16 was present in the collective set of samples from the Day 1-5 visits -OR- if there was at least a 4-fold increase in the titer of serum neutralizing antibody to HRV-16 in their blood samples 3 – 4 weeks after inoculation compared to that prior to inoculation. Subjects were considered to have experienced a cold if they were infected with HRV-16 (as defined above) AND if they met either one of the

following criteria: a total symptom score > 6 and the presence of rhinorrhea for > 3 days , or a total symptom score > 6 and a response of "yes" to the question "did you develop a cold after you were inoculated and/or do you currently have a cold?" on day 5 (E5).

Microarray Analysis: Total RNA was extracted from nasal scrapings using the Trizol Reagent and protocol provided by supplier. Extracted RNA was purified further using Qiagen (Valencia, CA) RNeasy Micro columns. Purified RNA was quantified using a Nanodrop spectrophotometer and quality was determined using the Agilent (Santa Clara, CA) 2100 Lab-on-a-Chip System. Purified RNA was converted to GeneChip target using the NuGEN (San Carlos, CA) Ovation procedure. GeneChip target was then hybridized to Affymetrix (Santa Clara, CA) U133plus2.0 human GeneChips for analysis of over 47,000 transcripts and then washed, stained, and scanned using the protocol described by Affymetrix. All Affymetrix chips used were from the same lot number. A separate chip was used for each sample from each subject. Since each subject served as their own control, all 3 samples from one subject were run sequentially. All samples were run over a 2 day period, with nearly identical representation of infected and control subjects each day. Day 1 started with a rhinovirus infected subject and ended with a rhinovirus infected subject, and day 2 started with a control subject and ended with a control subject, such that, on each day, the average scan order rank was the same for both treatment groups. Data from all genomics samples underwent a rigorous quality control procedure to detect potential outliers due to processing, instrumentation, or other reasons. This process included examination of the GeneChip level Affymetrix QC metrics: Raw Q, Scaling Factor, Noise Average, Background Average, Percent Present, and Percent Absent. Probe set level QC metrics, such

as Prediction Interval Analysis, Principal Components Plots, and Pairs Plots were also considered.

Natural Rhinovirus Colds Study: One important function of gene array analysis is to identify candidate genes for further validation and study. Viperin was the most highly induced gene among the category of potential antiviral agents. We therefore selected this novel gene for further validation. To determine if this gene is induced during naturally occurring infections, which presumably would be due to serotypes other than just HRV-16, we performed a prospective, cross-sectional study in which subjects reported to the Clinic within 36h of the onset of cold symptoms. To be included in the study, subjects had to be between 18 and 60 years of age. As for the experimental infection study, exclusion criteria included the presence of significant co-morbid diseases, abnormal nasal anatomy, history of chronic headaches, cold symptoms within the previous 4 weeks and any use of tobacco products within the previous 6 months. Subjects also were excluded if they took any intranasal medications in the prior 2 weeks. None of the 9 subjects included in the study had a history of asthma or allergic rhinitis. Upon entering the clinic, nasal lavage was performed, and nasal scraping was collected from one nostril. Subjects returned for a retrospective baseline visit 4 weeks later, when lavage was repeated and a nasal scraping was taken from the other nostril. Total RNA was extracted from epithelial scrapings as described above. All subjects gave informed consent and the protocol was approved by the Conjoint Health Ethics Board of the Faculties of Medicine, Nursing and Kinesiology, University of Calgary. To determine which subjects had colds due to rhinovirus infections, nasal lavage supernatants from all study subjects were screened for human rhinovirus, using a recently developed quantitative real-time RT-PCR technique. Viral RNA from nasal lavage samples was isolated with the QIAamp Viral RNA Mini Kit (Qiagen, Mississauga, ON,

Canada), according to the manufacturers protocol. RNA was reverse transcribed using oligo(dT) and Superscript II. Real-time primer and probe sets were designed to target the highly-conserved 5' untranslated region (UTR) of the HRV genome. After analysis of all available 5' UTR sequences for human rhinovirus serotypes, two forward and two reverse primers were designed to cover minor variations between different HRV serotypes and used with a single fluorescent probe, as follows: Forward primer #1: 5'-AGCCTGCGTGGC**T**GCC-3'; Forward primer #2: 5'-AGCCTGCGT GGC**G**GC-3'; Reverse primer #1: 5' ACACCCAAAGTAGT**C**GGTCCC-3'; Reverse primer #2: 5'ACACCCAAAGTAGT**T**GGTCCCA-3'; Probe: 5' FAM-TCCGGCCCCTGAAT-MGB-3'. PCR analysis was performed in a reaction in which both sets of forward and reverse primers were combined. Based on analyzed HRV sequences, these primer and probe combinations should detect all known HRV sequences. To confirm this, a range of HRV serotypes (HRV-37, -39, -51, -89, -1A, 2, -14 and –16) showing sequence diversity were purchased (American Type Culture Collection, Rockville, MD). The designed primer and probe sets detected all serotypes tested with comparable sensitivity (not shown).

Total RNA from the nasal scrapings of nine subjects with established rhinovirus colds were used to confirm induction of viperin gene expression during rhinovirus infections by real-time RT-PCR. Gene expression for viperin was quantified using the Applied Biosystems Model 7900 Sequence Detector (Foster City, CA). Forward and reverse primers and a fluorescently labeled probe was designed using Primer Express software (Applied Biosystems). Primers and probe for viperin were: forward primer 5'CCTGCTTGGTGCCTGAATCT-3'; reverse primer 5'GCGCA TATATTCATCCAGAATAAGG-3'; probe 5'FAM-ACCAGAAGATGAAAGACT-MGB-3'. To permit absolute quantification, a first strand cDNA oligonucleotide of the amplicon incorporating the primer sequences was synthesized (DNA Core Facility, University of Calgary)

and used a standard. Analysis of the housekeeping gene GAPDH was performed on each sample using a primer and probe kit obtained from Applied Biosystems. For each gene of interest, we confirmed that the efficiency of amplification was comparable to that of GAPDH (data not shown). Viperin mRNA levels were normalized for minor variations in GAPDH levels.

In vitro Rhinovirus studies: Human rhinovirus type 16 (HRV-16) and WI-38 cells were purchased from the American Type Culture Collection (Rockville, MD). Viral stock solutions of HRV-16 were generated by passage in WI-38 cells and were purified by centrifugation through sucrose to remove ribosomes and soluble factors, as previously described (E6). Viral titers were determined using WI-38 cells grown in 96-well plates, as previously described (E7).

Epithelial Cell Cultures: Normal human lungs not used for transplantation were obtained from a tissue retrieval service (IIAM, Edison, NJ). Primary human airway epithelial cells were obtained by protease digestion of these normal human lungs as described (E8). Cells were grown in 6 well-plates in serum-free epithelial growth medium (BEGM, Lonza, Allendale, NJ). Twenty-four hours before stimulation, cells were cultured in BEGM from which hydrocortisone had been withdrawn, and this hydrocortisone-free medium was used for all experimental exposures.

Viral infection of epithelial cells: Cells were infected with $10^{5.5}$ $TCID_{50}$ units/ml of HRV-16 and incubated at 34°C for appropriate times. Total RNA was extracted with TRlzol. To ensure there was no contribution of genomic DNA to amplification, samples were treated with DNase I (Ambion, Austin, TX) before use. Gene expression for viperin was quantified by real-time RT-PCR as described above.

A specific Stealth siRNA (sense sequence: GGUGUAGGGAUUAUAGAGUCGCUUU), and matched scrambled control (sense sequence: GGUGAGGUAAUAUAGUGGCCUGUUU),

for viperin were obtained from Invitrogen (Burlington, Ontario, Canada). To assess the effects of siRNA-induced knockdown of viperin on viral titers, subconfluent monolayers were transfected with 100 nM of specific or control (scrambled) siRNA for 24 h using RNAimax transfection reagent (Invitrogen). After a 24 h recovery period, cells were exposed to HRV-16 for 2 h. Cells were then extensively washed and incubated for 24 h at 34°C, and viral titers were assessed in recovered supernatants.

Statistical Analysis: All data related to in vivo studies were analyzed using SigmaStat software (SPSS, Chicago, IL). Levels of gene transcript expression at baseline (day –14) were compared between groups using ANOVA, with main effects for gender and group, and the 2-way interaction between gender and group. Analysis of covariance (ANCOVA), with main effects for gender and group, and the 2-way interaction between gender and group, was used to compare data between groups for each post-inoculation visit. The gene expression levels for the baseline visit (Day -14) were modeled as a continuous covariate, with different slopes for each combination of gender and group. The main effects for gender and group and the 2-way interaction between gender and group were evaluated at the respective covariate means for males and females. For all comparisons, the statistical hypotheses were two-sided and p-values were reported. Due to the exploratory nature of this study, no adjustments were made to control the experiment-wise type I error rate.

For *in vitro* studies, data were analyzed using Statview Software (SAS Institute, Cary, NC). Appropriate ANOVA with post hoc analysis using Fisher's least significant difference tests were used. The Wilcoxon Matched-pairs Signed-ranks test was used for nonparametric data.

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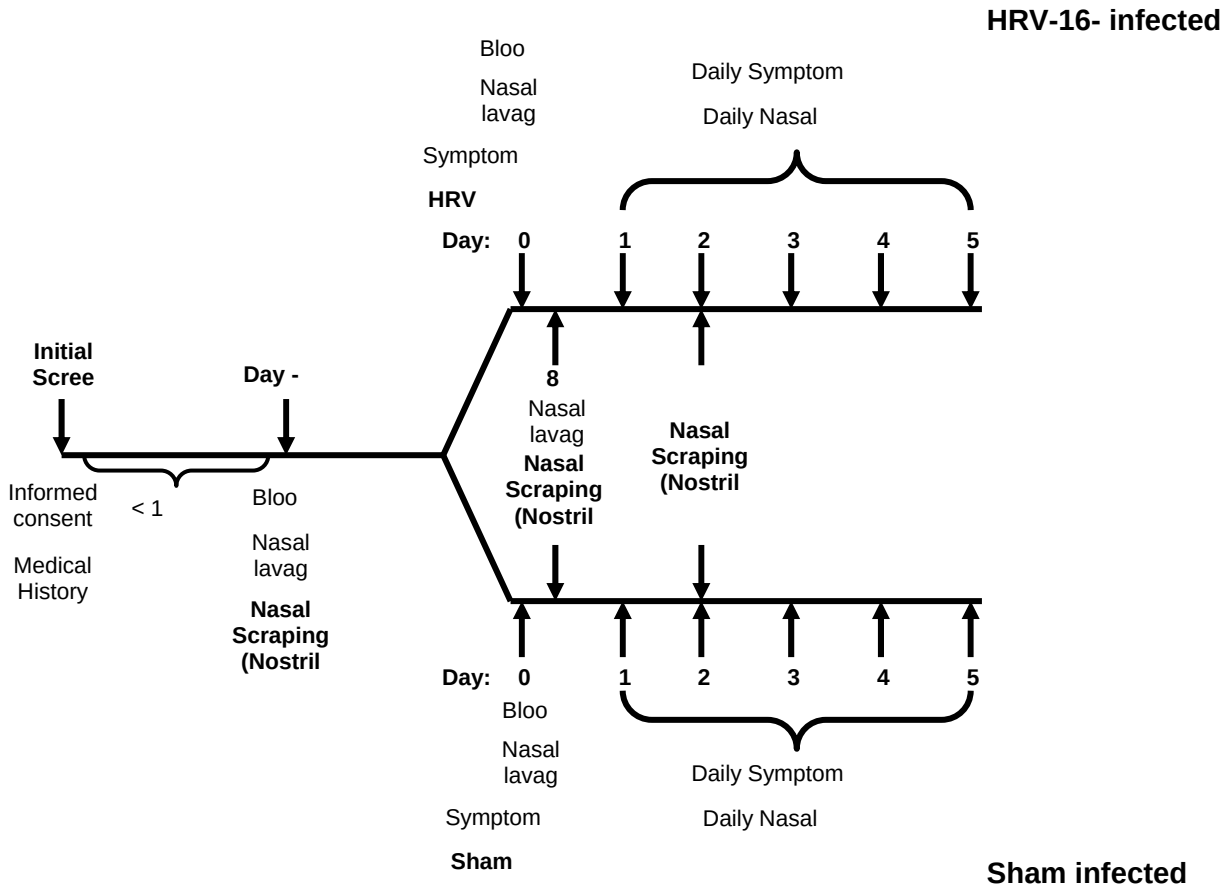
FIGURE LEGENDS

Figure E1: Study design for the experimental rhinovirus infection protocol.

Figure E1


HRV-16- infected
Bloo
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