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Unrecognized prolonged viral replication in the pathogenesis of human RSV infection

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ABSTRACT

Background: Respiratory symptoms in RSV persist long after the virus is no longer detected by culture. Current concepts of RSV pathogenesis explain this by RSV inducing a long-lasting pathogenic immune cascade. We alternatively hypothesized that prolonged unrecognized RSV replication may be responsible and studied this possibility directly in a human wild-type RSV experimental infection model.

Objective: The objective of the current report was to define the duration of true human RSV replication by studying it directly in immunocompetent adults experimentally infected with a clinical strain of RSV utilizing this previously established safe and reproducible model.

Study Design: 35 healthy adult volunteers were inoculated with RSV-A (Memphis-37, a low11 passage clinical strain virus, manufactured from a hospitalized bronchiolitic infant) and evaluated over 12 days. Viral load by culture, parallel quantitative PCR (genomic, message) and RSV-specific IgA, were measured twice daily from serially collected nasal washes.

Results: After inoculation, 77% (27/35) of volunteers became RSV infected. As expected, culture-detectable RSV ceased abruptly by the 5–6 t h 15 infection day. However, infected volunteers demonstrated prolonged RSV presence by both genomic and message PCR. RSV-specific IgA rose within respiratory secretions of infected volunteers during same time frame.

Conclusions: RSV replication appears to continue in humans far longer than previously thought. The rise in nasal RSV-specific IgA shortly after infection likely neutralizes culture detectable virus producing misleadingly short durations of infection. Prolonged viral replication helps explain RSV's extended disease manifestations and increases the potential utility of antivirals.

1. Background

RSV is a major cause of lung disease in adults and children accounting for as many as one million pediatric deaths annually [1]. RSV is a leading cause of infant hospitalizations in the US accounting for nearly 50% of all pneumonia and up to 90% of reported cases of bronchiolitis in infancy with mortality rates approximately ten times higher than those for influenza in infants [2–4]. RSV mortality rates in adults approach those for influenza and the disease causes significant morbidity and mortality in immunocompromised and frail, elderly adult patient populations [5–7].

The lack of available RSV interventions is attributed to our inadequate understanding of RSV pathogenesis, over-reliance on data from non-parallel animal models, and limited human studies [8].

RSV lower respiratory tract infections in early life can predispose to increased rates of asthma and airway hyper reactivity [9,10]. Cough can linger for around 4 weeks after infant RSV infection and nearly 50% of patients experience prolonged wheezing following RSV-associated illness [11,12]. Measurable pulmonary function abnormalities can occur up to 8 weeks following RSV infection in normal, healthy adults [13].

The exact mechanism of this prolonged disease process after apparent RSV clearance, as assessed by viral culture, is unclear. Current concepts of RSV pathogenesis attempt to explain this by the virus inducing a pro-inflammatory pathogenic immune cascade, a concept which is supported mainly by small animal models [14–16] and by

Abbreviations: RSV, respiratory syncytial virus; qPCR, quantitative polymerase chain reaction; PFU, plaque forming units; qCulture, quantitative culture; RT, reverse transcriptase

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vaccine-induced immunopathology observed in children [17,18]. A unique wild-type human experimental RSV infection model has been established to study the disease pathogenesis. Previous experience with this model has shown that disease severity and viral loads peaked at similar time points [19–21].

2. Objectives

The objective of the current report was to define the duration of true human RSV replication by studying it directly in immunocompetent adults experimentally infected with a clinical strain of RSV utilizing this previously established safe and reproducible model [19,23]. We hypothesized that prolonged unrecognized RSV replication is responsible for driving a prolonged clinical disease state. We, therefore, studied this possibility directly in human wild-type RSV experimental infections through parallel quantification of both genomic and message RSV RNA by qPCR at multiple time points before and after viral clearance (defined by culture). We also postulated that nasal RSV-specific IgA might be inhibiting RSV culture detection giving falsely short durations of human viral replication. We, therefore, studied the dynamics and quantity of nasal RSV-specific IgA over time in the respiratory secretions of these volunteers and correlated the same with the dynamics of RSV replication and viral clearance.

3. Study design

3.1. RSV inoculum

Wild type RSV A (Memphis 37 strain), a low passage virus isolated from a hospitalized infant with bronchiolitis was used for inoculation [22]. Safety and reproducibility of Memphis 37 has been established [19,23].

3.2. Subjects and study protocol

Thirty – six healthy adult volunteers 18–45 years of age were intranasally inoculated with Memphis 37 after obtaining informed written consent, appropriate local regulatory, ethics committee and institutional review board approval.

Volunteers were admitted to the quarantine unit one day prior to inoculation and remained there for 13 days' post inoculation. Twice daily nasal washes were obtained, and previously described methods were used to measure disease severity [19,23].

3.3. Viral quantification assays

3.3.1. qCulture

RSV quantitative cultures (qCulture) were performed within 30 min of specimen collection in HEp-2 cell plaque assays as previously described and reported as $\log_{10}$ plaque forming units/ml ($\log_{10}$ PFU/mL) [19,24].

3.3.2. Nucleic acid extraction

Viral RNA was extracted from the patient samples using the automated EZ1 Advanced (QIAGEN, Valencia, CA) and stored at −70 °C to be used for creating the cDNA through a single reverse-transcription reaction using the Omniscript reverse transcription kit (QIAGEN, Valencia, CA) and custom primers (message, genomic) as described below.

3.3.3. qPCR- genomic and message

qPCR was performed on the viral RNA extracted from nasal washes. A previously designed and validated quantitative real time RT-PCR assay (qPCR) was used to amplify a portion of the negative strand (genomic) portion of the N gene of RSV [25]. Another primer was designed by reversing the polarity of the same single primer set described above to detect positive strand (message).

The resulting cDNA was amplified using identical techniques as previously described [25]. Results are expressed as mean of duplicates in $\log_{10}$ plaque forming unit equivalents/ml (log PFUe/ml) with the viral load representing the duplicate wells' geometric mean quantity of RSV nucleic acid detected in a single nasal wash collection. Viral loads below the level of detection were arbitrarily set at a value of zero.

3.4. Measurement of nasal RSV antibody, RSV specific IgA

A previously described ELISA assay was used to detect and quantify RSV specific IgA in the nasal washes [27]. All diluted samples were performed in duplicate and the IgA level assigned to a sample was the calculated mean (log ng/ml).

3.5. Statistical analysis

Statistical analyses and construction of figures were performed with GraphPad Prism Software v 5·0 (La Jolla, CA). All analyses were two tailed. Statistical significance was set at $\alpha = 0.05$.

4. Results

4.1. Subjects

Thirty-six volunteers were inoculated using a clinical strain of RSV-A (Memphis 37). One subject voluntarily withdrew from the study and was not included in the analysis. The subject characteristics, inclusion and exclusion criteria, and safety profiles have been described [19]. Subjects were defined as being "infected" if RSV was detected in respiratory secretions at ≥2 successive times between D2 – D8 (inclusive). Of the 35 volunteers included in the analysis, 27 (77%) met the study definition of becoming "infected" by the qPCR assay (genomic) while 21/35 (60%) volunteers were deemed "infected" by quantitative culture. All subjects defined as "infected" by qCulture also met the definition of "infected" by qPCR. The volunteers defined as infected by the genomic PCR assay were further studied by message qPCR.

4.2. Viral load and viral dynamics- qPCR (genomic, message), and qCulture

As assayed by genomic qPCR, the mean incubation period was 3.1 (± 1.5) days and the mean duration of viral shedding was 7.4 (± 2.5) days. Quantitative culture based detection revealed a longer mean viral incubation period (4.0, ± 1.5 days) and lower mean duration of shedding (3.6, +/−1.1) [19]. Mean daily viral loads of infected volunteers thus demonstrated prolonged RSV presence by genomic qPCR despite an abrupt decrease in culture-detectable RSV by the 5-6th infection day. Similarly, mean daily viral load as measured by the message qPCR assay demonstrated the continued viral presence beyond viral detection by culture (Figs. 1 and 4). The timing of the viral detection by qPCR, both genomic and message coincided with each other and with peak symptom scores.

The ratio of message to genomic virus was high at first (likely representing first rounds of replication) and then stayed relatively constant throughout the remainder of the serial assessments (Fig. 2). For further analysis of dynamics, we measured viral loads after the incubation periods were mathematically normalized to day 1 (Fig. 3). After incubation period normalization, the peak viral loads occurred 4 days after first detection of the virus by message qPCR, similar to that seen with the genomic qPCR (3.5 days). After incubation period normalization, symptoms peaked at the same time as the peak of both genomic and message qPCR (Fig. 3).

The mean duration of viral shedding by qPCR of all 27 infected volunteers was 8.3 days (SD = 2.9). One volunteer remained negative