Viral Load Drives Disease in Humans Experimentally Infected with Respiratory Syncytial Virus

John P. DeVincenzo[1–3], Tom Wilkinson[4], Akshay Vaishnaw[5], Jeff Cehelsky[5], Rachel Meyers[5], Saraswathy Nochur[5], Lisa Harrison[1,2], Patricia Meeking[6], Alex Mann[6], Elizabeth Moane[6], John Oxford[7], Rajat Pareek[1], Ryves Moore[1], Ed Walsh[8], Robert Studholme[9], Preston Dorsett[1,9], Rene Alvarez[5], and Robert Lambkin-Williams[6]

[1]Departments of Pediatric and Department of Molecular Sciences, University of Tennessee College of Medicine, [2]Children's Foundation Research Center, and [3]Le Bonheur Children's Medical Center, Memphis, Tennessee; [4]Southampton School of Medicine, Southampton, United Kingdom; [5]Alnylam Pharmaceuticals, Cambridge, Massachusetts; [6]Retroscreen Ltd. and [7]Queen Mary's School of Medicine and Dentistry, London, United Kingdom; [8]University of Rochester, Rochester, New York; and [9]Viral Antigens/Meridian Life Science, Inc., Memphis, Tennessee

***Rationale*: Respiratory syncytial virus (RSV) is the leading cause of childhood lower respiratory infection, yet viable therapies are lacking. Two major challenges have stalled antiviral development: ethical difficulties in performing pediatric proof-of-concept studies and the prevailing concept that the disease is immune-mediated rather than being driven by viral load.**

***Objectives*: The development of a human experimental wild-type RSV infection model to address these challenges.**

***Methods*: Healthy volunteers (n = 35), in five cohorts, received increasing quantities (3.0–5.4 log plaque-forming units/person) of wild-type RSV-A intranasally.**

***Measurements and Main Results*: Overall, 77% of volunteers consistently shed virus. Infection rate, viral loads, disease severity, and safety were similar between cohorts and were unrelated to quantity of RSV received. Symptoms began near the time of initial viral detection, peaked in severity near when viral load peaked, and subsided as viral loads (measured by real-time polymerase chain reaction) slowly declined. Viral loads correlated significantly with intranasal proinflammatory cytokine concentrations (IL-6 and IL-8). Increased viral load correlated consistently with increases in multiple different disease measurements (symptoms, physical examination, and amount of nasal mucus).**

***Conclusions*: Viral load appears to drive disease manifestations in humans with RSV infection. The observed parallel viral and disease kinetics support a potential clinical benefit of RSV antivirals. This reproducible model facilitates the development of future RSV therapeutics.**

Keywords: RSV; pneumonia; bronchiolitis; pathogenesis; viral load

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The pathogenesis of respiratory syncytial virus (RSV) infection has been studied primarily in rodent systems and indicates that disease is produced by an aberrant delayed and pathogenic immune response. Studies of pathogenesis in humans are largely lacking.

What This Study Adds to the Field

This study provides a human experimental infection model that can be used to study the pathogenesis of the disease in humans themselves, and that can also be used for evaluation of therapeutics and vaccines. The pathogenesis of RSV infection in humans shows that viral load is associated with disease itself and that the viral load and disease track together. This indicates that the previously held view of a delayed and aberrant immune response causing the disease is likely more applicable to animal models rather than to human RSV infection. This research also supports the development of antiviral therapeutics for RSV infection.

Respiratory syncytial virus (RSV) infects more than 68% of the birth cohort annually (1) and is the most common cause of lower respiratory infections in children less than 1 year of age resulting in significant morbidity (2, 3). RSV also causes life-threatening disease (4), with mortality rates approximately 10-fold higher than for influenza in those less than 1 year of age (5). Worldwide, as many as 1 million children may die annually of RSV infection (6, 7). RSV also produces significant morbidity and mortality in adult immunocompromised and debilitated populations (8). Despite the high disease burden, there is no vaccine for RSV and the only approved therapy (ribavirin) is rarely used because of its potential teratogenicity and its limited effectiveness (9–11). Although successful, passive monoclonal antibody prevention strategies are currently applied to less than 5% of the at-risk childhood population (12, 13). Thus, there is a major unmet medical need for effective therapies for RSV infection.

There are two main reasons for the unavailability of RSV therapeutics. The first is inadequate existing evidence of RSV pathogenesis that might support the putative effectiveness of RSV antivirals. Disease caused by RSV has long been thought to be the result of the viral triggering of an exuberant, aberrant, and long-lasting pathogenic immune response. This immunopathogenic concept has been largely developed through experimentation with rodent models of RSV infection (14–16). It is thought to result from exaggerated helper T-cell type 2 (Th2)

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***Disclaimer*:** The conduct of this research follows the human experimentation guidelines of the U.S. Department of Health and Human Services. This study was also conducted in accordance with ICH GCP guidelines (directive CPMP/ICH/135/95), local regulatory requirements and the declaration of Helsinki, and all relevant local laws and regulations. The protocol and informed consent document were reviewed and approved by a properly constituted ethics committee. All aspects of the study were explained in detail to prospective subjects and they were informed of the voluntary nature of their participation. Written informed consent was obtained from each subject. Portions of the data in this article have been presented as Abstract V-1257 at the Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, September 18, 2007.

Correspondence and requests for reprints should be addressed to John DeVincenzo, M.D., Children's Foundation Research Center, Le Bonheur Children's Medical Center, Room 433, 50 North Dunlap Street, Memphis, TN 38103. E-mail: jdevincenzo@uthsc.edu

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cellular immune responses (17, 18) through bystander killing effects of activated cytotoxic T cells (19) and by RSV-induced chemokine mimicry (20, 21). The failed RSV vaccine experience of the 1960s (22–24), which produced a prominent and fatal eosinophilic pulmonary infiltrate after natural RSV infection in the unfortunate vaccine recipients, further solidified this immunopathogenic concept of RSV disease (25). In a disease process that begins days after the start of a short period of viral replication, and that is followed by a prolonged immune-mediated disease continuing after viral elimination, applying an antiviral agent would likely be clinically ineffective. However, the relevance of this immunopathogenic concept for human RSV disease is not understood (26).

The second main reason for the current unavailability of effective RSV therapeutics is the understandable reluctance to study experimental therapies directly in the vulnerable pediatric naturally infected population before proof-of-concept studies can show antiviral effect in adults, who are able to understand and provide informed consent for research (27). The naturally infected immunocompromised adult population does not offer a suitable alternative, as researchers must contend with widely varying degrees of immunosuppression, major differences in elapsed times between initial infection and RSV detection, the difficulties produced by administration of multiple concomitant medications, and uncontrollable occurrences of death and other serious adverse events that may be rightly or wrongly attributed to the drug being studied. Furthermore, RSV-infected immunocompromised adult populations are small, geographically dispersed, and difficult to recruit.

These two main reasons have served to inhibit the preclinical and clinical development of RSV antiviral therapeutics. Other than ribavirin, numerous small molecules and novel therapeutics have been discovered with proven RSV antiviral activity in submicromolar concentrations both *in vitro* and *in vivo* (28–38). However, only one of these has been tested in a proof-of-concept study within an RSV-infected population (39). This trial, which involved adults undergoing hematopoietic stem cell transplantation, failed to show an antiviral effect because of many of these aforementioned logistic, immunologic, and virologic factors.

To reevaluate the prevailing immune-based model of RSV pathophysiology and to provide a practical means to conduct proof-of-concept RSV therapeutic trials, we sought to develop a safe, reproducible, and well-characterized human experimental RSV infection model in adult volunteers that paralleled natural RSV infection and disease. Some of the results of these studies have been previously reported in the form of abstracts (40, 41).

METHODS

Subjects

The study included healthy males and females 18–45 years of age. Exclusion criteria included the following: any history during adulthood of asthma of any etiology or any use of a bronchodilator within the past year; chronic (or acute within the last 7 d) use of any medication or other product (prescriptive or over the counter) for symptoms of rhinitis or nasal congestion or for any chronic nasopharyngeal complaint, or chronic use of any intranasal medication for any indication; presence of any febrile illness or symptoms suggestive of viral respiratory infection within the last 2 weeks; history of seasonal hay fever or seasonal allergic rhinitis, including the use of symptomatic prescriptive and nonprescriptive medication; contact with people at risk for severe RSV infections; steroid use in the past month; chronic sinusitis; and the presence of known immunosuppressive conditions. All subjects' nasal washes tested negative by real-time polymerase chain reaction (PCR) on Study Day −1 for viral respiratory pathogens (RSV-A and -B; influenza A and B; parainfluenza 1, 2, and 3; and human metapneumovirus). Appropriate local regulatory, institutional review board (University of Tennessee, Memphis, TN), and Ethics Committee (East London and the City Local Research Ethics Committee 3, London, UK) approval was obtained as appropriate. All subjects provided written informed consent.

Inoculating Virus (RSV)

RSV-A (Memphis 37 strain) was isolated and manufactured according to Good Manufacturing Practice in Food and Drug Administration–approved Vero cells from the respiratory secretions of an infant hospitalized for bronchiolitis and who had known high viral loads of RSV-A. The isolate was plaque-picked and passaged five more times to prepare the inoculum. The identity of the inoculating virus (RSV) was confirmed by an immunofluorescent antibody assay, electron microscopy, and N-gene sequencing. It was determined at several steps in the selection and manufacturing process to be free of adventitial agents and human pathogens by four methodologies: (*1*) 28-day culturing in five indicator cell lines (MRC-5, Vero, MDBK, HeLa, and MEF) while under specific RSV neutralization conditions (high concentrations of RSV-specific monoclonal antibody). Routine cytopathic effect observations, hemabsorption, and hemagglutination at 14 days were performed, followed by blind passage and identical repeat evaluations after an additional 14 days (Bioreliance Inc., Rockville MD); (*2*) a product-enhanced reverse transcriptase assay for the general detection of retrovirus reverse transcriptase (Bioreliance Inc.); (*3*) an expansive series of individual PCR assays designed to detect human pathogens; and (*4*) electron microscopy. The Memphis 37 virus preparation was diluted in 25% sucrose immediately before inoculation of subjects to create the desired target inoculum for the specific cohort (Figure 1b). Each volunteer in a cohort received an aliquot of the same inoculum. Inoculation was by intranasal drops (0.5 ml/naris). To calculate the amount of RSV inoculated into each volunteer within a cohort, a quantitative culture (plaque assay in HEp-2 cells) was performed in parallel on duplicate aliquots at the exact time of inoculation of the first volunteer within a cohort and again in duplicate at the exact time of inoculation of the last volunteer within a cohort. The mean of these parallel aliquot quantitative culture results represents the cohort's inoculum.

Study Design

The study was conducted at a single quarantine unit. Thirty-six subjects were enrolled through five sequential cohorts (A–E). Viral assays and assessments were not completed on one subject because she voluntarily withdrew from the study. Statistical analyses did not include this one subject. Subjects were admitted to the quarantine unit for 13 days and were observed for at least 1 day before RSV inoculation, which occurred on Day 0. Nasal washes (5 ml of normal saline instilled and withdrawn per naris) for viral assays and cytokine measurements were obtained on the day of admission to the quarantine unit (Day −1) and twice daily on Days 1 through 12. Pulmonary function tests were performed daily on all volunteers, using a Viasis Micro-lab calibrated spirometer. For assessment of upper respiratory signs and symptoms, a physician's daily directed physical examination (Days −1 to 12) and a twice-daily subject-reported RSV symptom score card (Days −1 to 12) were completed. Details of these scoring instruments can be found in the online supplement. Mucus weights for each 24-hour period were recorded from Days −1 through 12. Adverse events were recorded through the Day 28 follow-up visit.

Typical signs and symptoms of RSV infection captured in the symptom or physical examination scores were not counted as adverse events. No concomitant medications were allowed during quarantine except acetaminophen for headache.

Specimen Collection

Nasal washes were collected into cold RSV stabilization medium containing 25% sucrose, transported on ice, and placed onto HEp-2 cell monolayers within 30 minutes of collection. Parallel aliquots were snap-frozen and stored at −80°C until use.

RSV Assays

RSV quantitative cultures in HEp-2 cell plaque assays were performed in 12-well plates with triplicate 10-fold dilutions of nasal wash as

previously described (42). Plates were fixed and stained at 5 days with hematoxylin and eosin. RSV quantitative standards (RSV-A Long) (VR-26; American Type Culture Collection [ATCC], Manassas, VA) were run in parallel with each plaque assay to ensure precision. Plaque-counting rules were preestablished. Units are reported as log (base 10) plaque-forming units per milliliter (log PFU/ml). The lower limit of detection of the assay was set at 1.7 log PFU/ml.

The quantitative real-time reverse transcription-polymerase chain reaction assay (qPCR) employed an ABI 7900ht sequence detection system (Applied Biosystems International, Foster City, CA) amplifying a portion of the N-gene as previously described and validated (43). Each specimen was run in duplicate in 96-well plates with internal standards of duplicate pairs of six 10-fold dilutions of RSV RNA extracted from parallel aliquots containing a known quantity of RSV-A Long (VR-26; ATCC) as defined by and as used in the plaque assay. Results are presented as means of duplicates in log (base 10) plaque-forming unit equivalents per milliliter (log PFUe/ml). The RSV concentration in a single nasal wash collection is termed a viral load. Viral loads below the level of detection were arbitrarily set at a value of zero. Peak viral load is the highest viral concentration for a given volunteer in any nasal wash. Area under the curve (AUC) viral load is the area under the curve defined by a single volunteer's multiple viral loads collected twice daily. AUC was calculated by the trapezoidal rule, using exact times of collection of each nasal wash.

Spin-enhanced cultures were produced, using 80% confluent HEp-2 cell monolayers on coverslips within shell vials, and were inoculated with 200 μl of fresh nasal wash and centrifuged at 700 × *g* for 60 minutes. Monolayers were acetone-fixed after 2 days. Coverslips were evaluated for RSV by direct fluorescent antibody techniques using RSV-specific mouse monoclonal antibodies (Bartels; Trinity Biotech, Wicklow, Ireland).

Serum RSV-neutralizing antibodies were measured in an HEp-2 cell RSV 50% microneutralization assay as previously described (44) but performed with the Memphis 37 strain. To maximize successful infection, only volunteers with a relatively low serum RSV microneutralization titer were included, representing approximately the lower third of the normal distribution in healthy adults evaluated during study screening (Figure 1a).

Cytokine Assays

Cytokine and chemokine concentrations of IL-4, IL-6, IL-8, IL-10, macrophage inflammatory protein (MIP)-1α, RANTES (regulated upon activation, normal T-cell expressed and secreted), tumor necrosis factor (TNF)-α, and IFN-γ were measured in nasal washes from all volunteers in cohorts B and C (n = 15) on Days 1, 2, 4, 6, 8, 10, and 12. Nasal wash aliquots were analyzed at neat, 1:10 and 1:50 dilutions using chemiluminescent multiplexed sandwich enzyme–linked immunosorbent assay cytokine arrays (SearchLight, Aushon BioSystems, Billerica, MA). Concentrations of cytokines/chemokines from an individual volunteer measured over all days were summed so as to represent the total amount of cytokine/chemokine produced during the volunteer's infection.

Statistical Analysis

Statistical methods were based on International Conference on Harmonization guidelines. Continuous values below the lower limit of quantification were set at zero. Normally distributed continuous variables were evaluated by two-sample *t* test or by linear regression as appropriate. Continuous data that were not normally distributed, or noncontinuous data, were analyzed via the Wilcoxon rank-sum test. All statistical analyses were performed with SAS (SAS Institute, Cary, NC) version 8.2 or higher for Windows. Linear regression graphs were created with Prism version 4.0a for the Macintosh (GraphPad Software, San Diego, CA).

RESULTS

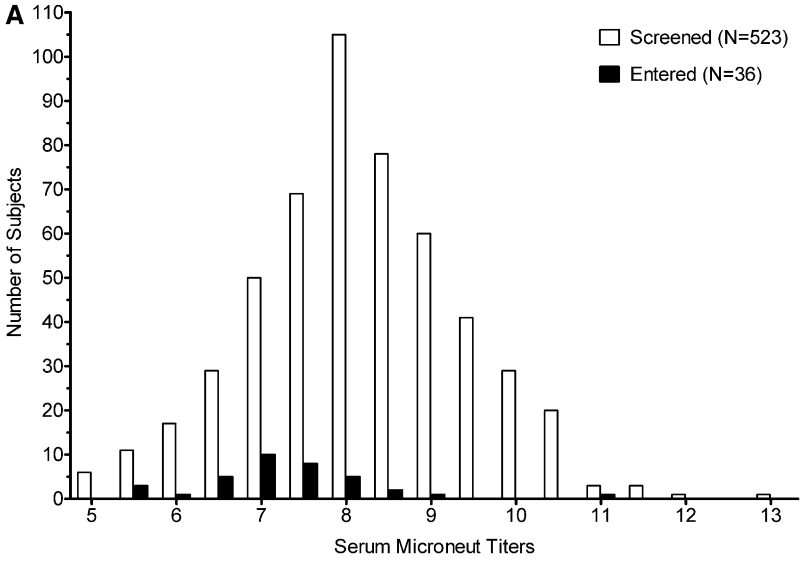
Subjects and Safety

Thirty-six healthy volunteers without signs, symptoms, or a history of recent upper respiratory infection and with low serum RSV-neutralizing antibody titers were selected from 523 persons screened (Figure 1a). Volunteers were entered into quarantine and confirmed to be clear of RSV-A and -B, influenza A and B, parainfluenza virus 1, 2, and 3, and human metapneumovirus, based on RT-PCR testing of nasal washes. They were then inoculated with a wild-type strain of RSV-A (Memphis 37). Volunteers, mean age 26.7 years (range, 19–43 yr; 16 of 36 [44%] women), were inoculated in five sequential cohorts (A through E). Each volunteer in a cohort received the same quantity of inoculum of RSV. Subsequent cohorts were inoculated with increasing quantities of RSV (Figure 1b). One volunteer withdrew from the study and virologic assessments were stopped, although safety assessments continued on this individual. Disease was restricted to the upper respiratory tract with no medically significant pulmonary function changes occurring. No serious adverse events occurred; all adverse events were mild or moderate in severity and no trend toward increasing frequency or severity of adverse events was noted with increasing RSV inoculum. No medically significant ECG or pulmonary function abnormalities occurred in any volunteers. One volunteer developed bilateral otitis media. No conjunctivitis, sinusitis requiring antibiotics, or clinical pneumonia occurred.

Characteristics of Viral Infection and Disease

RSV was quantified by two independent assays: a quantitative viral culture assay (plaque assay) measured as log plaque-forming units per milliliter (log PFU/ml) and a quantitative reverse transcriptase-polymerase chain reaction technique (qPCR) amplifying the RSV N-gene, measured as log PFU equivalents per milliliter (log PFUe/ml). RSV was also detected nonquantitatively in a spin-enhanced culture and by RSV antigen detection. For two representative individual volunteers, RSV infection and disease are shown in Figures 2a and 2b. A volunteer was defined as infected if RSV was detected in respiratory secretions at two or more sequential time points, the first occurring between Days 2 and 8 (inclusive) after inoculation. Of the 35 volunteers evaluated virologically, 27 of 35 (77%) were defined as infected by using the more sensitive qPCR assay and 21 of 35 (60%) were found to be infected as defined by quantitative culture. This infection rate was consistent between cohorts and did not vary by quantity of inoculum received (Figure 1b). As assayed by qPCR, the incubation period was 3.1 (±1.5) days and the mean duration of viral shedding was 7.4 (±2.5) days. Duration of shedding was lower as assessed by quantitative culture and spin-enhanced culture (Table 1). Of those infected as defined by qPCR, 40.7% were still RSV qPCR positive on Study Day 12. All volunteer nasal washes were negative for RSV by qPCR on follow-up evaluation approximately 1 month after inoculation. There was no correlation between amount of RSV inoculum received and any quantitative measures of infection (Figure 1b), or between inoculum and disease (data not shown). Because of the lack of different outcomes between the cohorts, the rest of the analyses were conducted on all cohorts combined. There were 35 fully evaluated volunteers in the combined cohorts.

Composite infection dynamics, with the incubation periods arbitrarily normalized to Day 1, showed that viral dynamics were significantly different when measured by qPCR as compared with quantitative culture (Figures 2c and 2d). The time elapsed until peak viral concentrations in respiratory secretions (peak viral load) was similar between assays. The peak viral loads occurred 3.5 days after RSV became detectable by qPCR and 3 days after first detection by quantitative culture (Figures 2c and 2d). This corresponded to Postinoculation Days 7 and 6, respectively. However, the rate of viral clearance after peak viral loads had occurred was noticeably less rapid by qPCR as compared with quantitative culture (Figures 2a–2d). Volunteers



B

Cohort	A N=4	B N=7	C N=8	D N=7	E N=9	All N=35
Inoculum	3.01	4.09	4.00	4.95	5.38	-
% infected	75.0%	71.4%	75.0%	85.7%	77.8%	77.1%

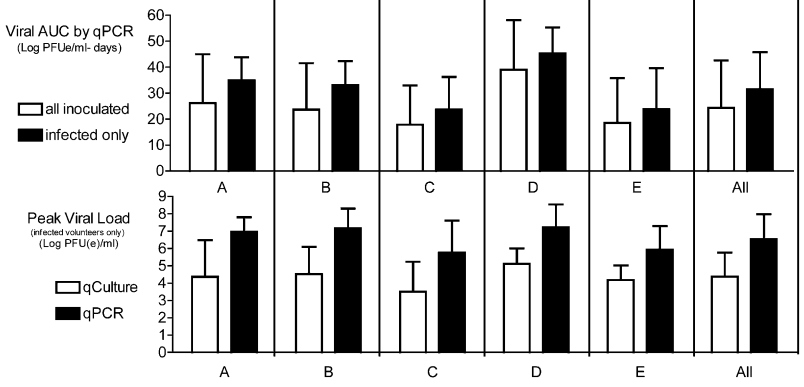


Figure 1. Screening immunology and viral outcomes of cohorts. (*A*) Histogram of serum respiratory syncytial virus (RSV)–neutralizing antibody titers to Memphis 37 strain in screened and entered volunteers. (*B*) Each successive cohort of volunteers was inoculated with increasing quantities of RSV. The mean quantity of RSV administered to volunteers within individual cohorts was measured as log plaque-forming units (PFU) in an HEp2 cell quantitative culture assay (plaque assay). A volunteer was defined as infected if two successive respiratory secretion collections contained detectable RSV by quantitative reverse transcriptase-polymerase chain reaction (qPCR). The mean amounts of RSV in each cohort are illustrated here in two ways. The *top row* of bar graphs plots the mean area under the curve (AUC) of the viral loads for each of the volunteers in each cohort. All volunteers were inoculated with RSV but only 71–86% in each cohort produced detectable virus, meeting the definition of infection. The viral AUC (calculated by including only volunteers who were defined as infected) are represented by *solid columns* and the viral AUC calculated from all volunteers in the cohort are represented by *open columns*. The *bottom row* of bar graphs plots the mean peak viral loads of the volunteers in each cohort. These peak viral loads were measured by quantitative culture (qCulture) (log PFU/ml; *open columns*) or by qPCR (log PFU equivalents/ml [log PFUe/ml]; *solid columns*). No significant differences in mean viral AUC or peak viral loads existed between cohorts. *Error bars* represent the SD.

defined as infected demonstrated significantly higher subjective and objective measures of disease compared with those defined as uninfected (Figures 3a–3f, $P = 0.021$, $P = 0.18$, and $P = 0.0056$ for symptom scores, physical examination scores, and mucus weights, respectively).

Relationships between Virus and Disease

The onset of infection as measured by culture and qPCR coincided with the onset of subjects' disease. Within the day after the first detection of RSV (as defined by quantitative culture) the symptom and physical examination scores had significantly risen above their pre-RSV detection baselines ($P = 0.0003$ and $P = 0.015$, respectively). This was also true for symptom and physical examination scores if infection was detected by qPCR ($P = 0.06$ and 0.0052, respectively). The timing of peak viral load also coincided closely with the timing of peak disease severity. These temporal relationships can be seen in Figure 2e. Statistically, the peak viral load (qPCR) occurred 1.17 (95% confidence interval, 0.11–2.22) days after peak symptom scores had occurred and 1.07 (95% confidence interval, 0.07–2.08) days after peak mucus weights occurred. As viral load (qPCR) declined, the severity of disease subsided in parallel (Figures 2 and 3). Viral loads as assessed by quantitative culture declined much more rapidly, nearly all becoming abruptly culture negative on Day 6 after first culture detection.

As quantity of virus detected in respiratory secretions increased, disease severity also increased in a statistically significant manner (Figures 4a–4f). This direct association was consistent, being demonstrated across both viral quantification assays (qPCR and quantitative culture) and all methods of calculation of viral quantity (peak viral load, duration of shedding, and area under the curve [AUC] viral load) and was statistically significant (for all, $P < 0.05$). This direct association was also significant when the evaluations were restricted to include only those volunteers who were defined as RSV-infected (Figures 4d–4f). A further evaluation of the effect of viral load on disease severity was undertaken by examining the viral loads and disease severity measurements at individual time points within individual volunteers. Within the individual 27 RSV-infected volunteers (defined by PCR), 22 of 27 (81%) showed a statistically significant association between their viral loads and at least one of their measures of disease severity (symptom score, physical examination score, or mucus weight). When all individual time points for all infected individual volunteers were evaluated together, viral loads (as measured either by qPCR or by quantitative culture) correlated significantly with symptom score, physical examination score, and mucus weight ($P < 0.0001$ for all disease severity markers).

Volunteers with shorter incubation periods had greater quantities of virus ($P = 0.0001$ and $P < 0.0001$ for viral AUC

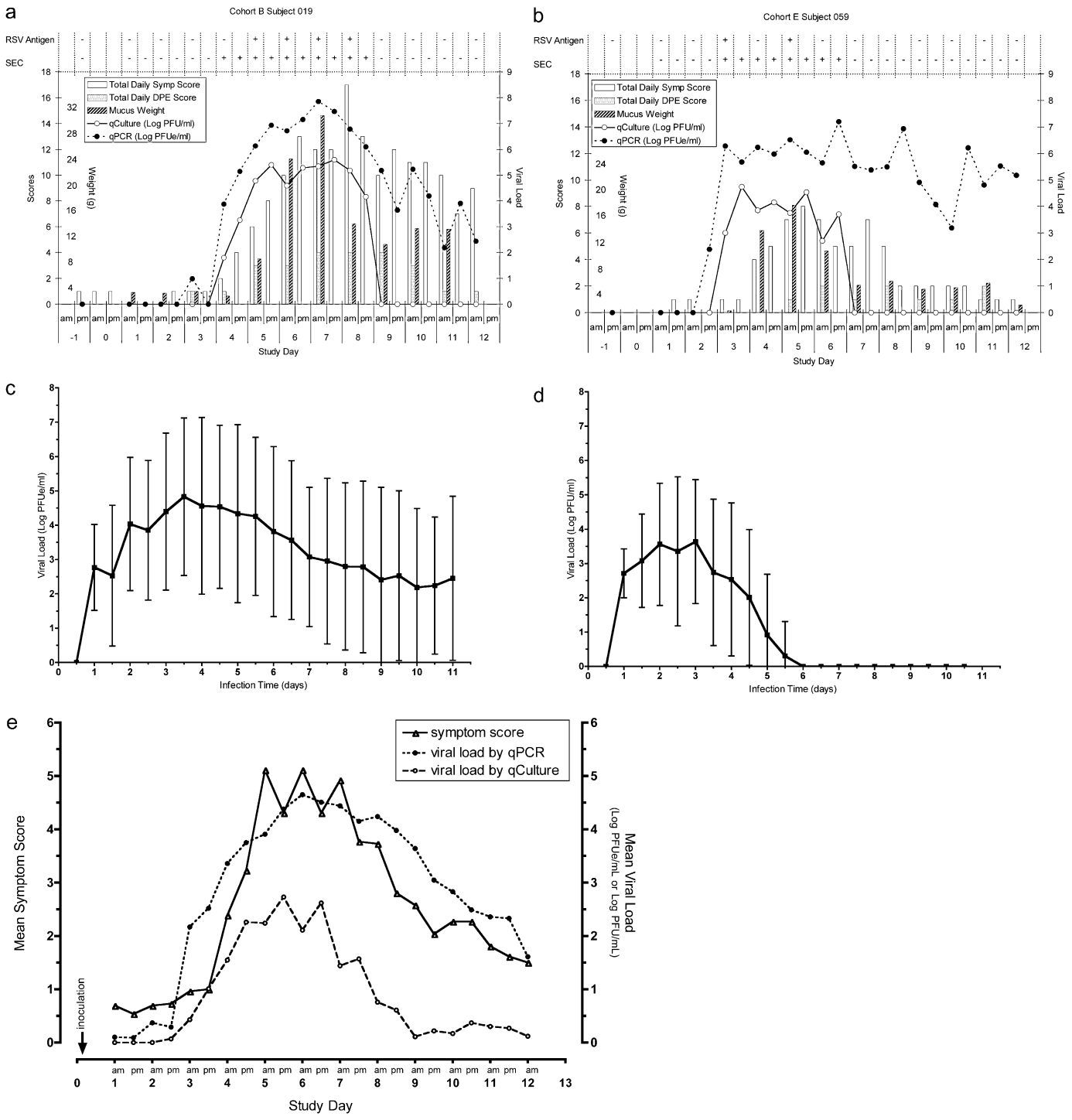


Figure 2. Viral load and disease over time in human volunteers. (*a* and *b*) Viral and disease measurements for selected individual volunteers over time. Volunteers were inoculated with respiratory syncytial virus (RSV) on Day 0 and evaluated in quarantine until Day 12. Twice-daily symptom scores (*open columns*), once-daily directed physical examination (DPE) scores (*solid columns*), and daily quantification of nasal mucus weight (*striped columns*) are measures of RSV disease severity. Viral testing was performed on nasal washes collected twice daily. The nonquantitative twice-daily spin-enhanced culture (SEC) and daily RSV antigen test results are indicated as either + or − in the top rows of graphs. Quantitative real-time reverse transcriptase PCR (qPCR, *dashed line*) and quantitative culture (qCulture; plaque assay on HEp-2 cells, *solid line*) were measured twice daily. (*c* and *d*) Mean daily viral loads (qPCR and quantitative culture) with normalized incubation periods. The timing of individual volunteer viral load curves was normalized by inserting a common incubation period so that their first positive viral measurement occurred arbitrarily on Infection Day 1. (*c*) Viral load measured by qPCR; (*d*) viral load measured by quantitative culture. *Error bars* represent the SD. (*e*) Timing of mean viral load and symptomatic disease. Mean data from all infected volunteers from each collection time point starting from Day 1 postinoculation are shown. No adjustment for the variable incubation periods is included in this panel. This makes the breadth of the curves wider and the magnitude of the curves lower than when incubation periods are normalized [as in (*c*) and (*d*)]. Initial rises in mean viral loads (qPCR and quantitative culture) correlate with the timing of onset of the rise in mean symptom scores. The timing of peak viral load correlates with the occurrence of peak symptom severity. log PFUe/ml = log plaque-forming unit equivalents per milliliter.

TABLE 1. MEAN VIRAL INFECTION MEASURES THROUGHOUT STUDY: COHORTS A–E COMBINED

	Spin-enhanced Culture	Quantitative Culture	qPCR
AUC viral load,* $\log_{10}$ PFUe/ml	N/A	10.1 (±7.2)	31.5 (±14.3)
Duration of shedding,* d	5.2 (±1.4)	3.6 (±1.1)	7.4 (±2.5)
Incubation period,* d	3.4 (±1.3)	4.0 (±1.5)	3.1 (±1.5)

Definition of abbreviations: AUC = area under the curve; $\log_{10}$ PFUe/ml = log (base 10) plaque-forming unit equivalents per milliliter; N/A = not applicable; qPCR = quantitative reverse transcriptase-polymerase chain reaction.

Numbers in parentheses represent the SD.

* Calculated only for volunteers who met the definition of infection as determined by the specific assay listed.

and peak viral load, respectively, by qPCR, and $P = 0.0021$ and $P = 0.0020$ for AUC and peak viral load, respectively, as measured by quantitative culture).

Relationships between Immunity and Viral Loads

The mean preinoculation (Day −2) RSV microneutralization titer of the volunteers was 7.11 MU/ml, and all but one titer fell within the range of 5.29–8.79 MU/ml (Figure 1a). Within this relatively narrow range of systemic immunity evaluated there were no statistically significant correlations between preexisting RSV microneutralization titers and the percentage of volunteers who became infected, the quantity of virus, the duration of shedding, or any disease measures. However, greater quantities of RSV detected in the volunteers did produce significantly greater rises in their RSV microneutralization titer (ΔDay −2 to Day 28, $P < 0.0001$, $r^2 = 0.61$; $P < 0.0001$, $r^2 = 0.65$ for viral AUC by qPCR and viral AUC by quantitative culture, respectively). Longer durations of shedding and higher peak viral loads also correlated significantly with the production of a greater rise in RSV microneutralization titer (for all, $P \leq 0.007$; data not shown).

Cytokines and Chemokines as Biomarkers

Cytokine and chemokine concentrations were measured in parallel aliquots of nasal washes. The concentrations of these individual cytokines and chemokines at all collected time points were summed to assess their overall contribution to disease. IL-6, MIP-1α, RANTES, IL-8, and TNF-α concentrations appeared to function as biomarkers of disease, because their concentrations correlated most significantly and consistently with all disease severity measures ($P < 0.05$ for symptom score, physical examination score, and mucus weight; Figures 5a and 5b). Cytokine production appeared to be driven by the viral loads in these respiratory secretions at the time of their collection. IL-6 and IL-8 nasal secretion concentrations correlated with quantitative viral measures ($P = 0.004$, $r^2 = 0.486$, and $P = 0.04$, $r^2 = 0.284$, respectively; Figure 5c). The concentrations of the Th2 cytokine IL-4 did not correlate with disease severity and the concentrations of this cytokine actually tended to be lower with greater degrees of illness.

DISCUSSION

This experimental model contains features that mimic natural infection: being initiated by a relatively low inoculum of a clinical strain, having a defined incubation period, generating sustained viral replication in an appropriate anatomic site, producing typical disease manifestations, and then self-resolving in immunocompetent adults without sequelae. Limited prior experiences with experimental RSV infection models primarily using laboratory-adapted RSV strains were safe in healthy adults (45–47).

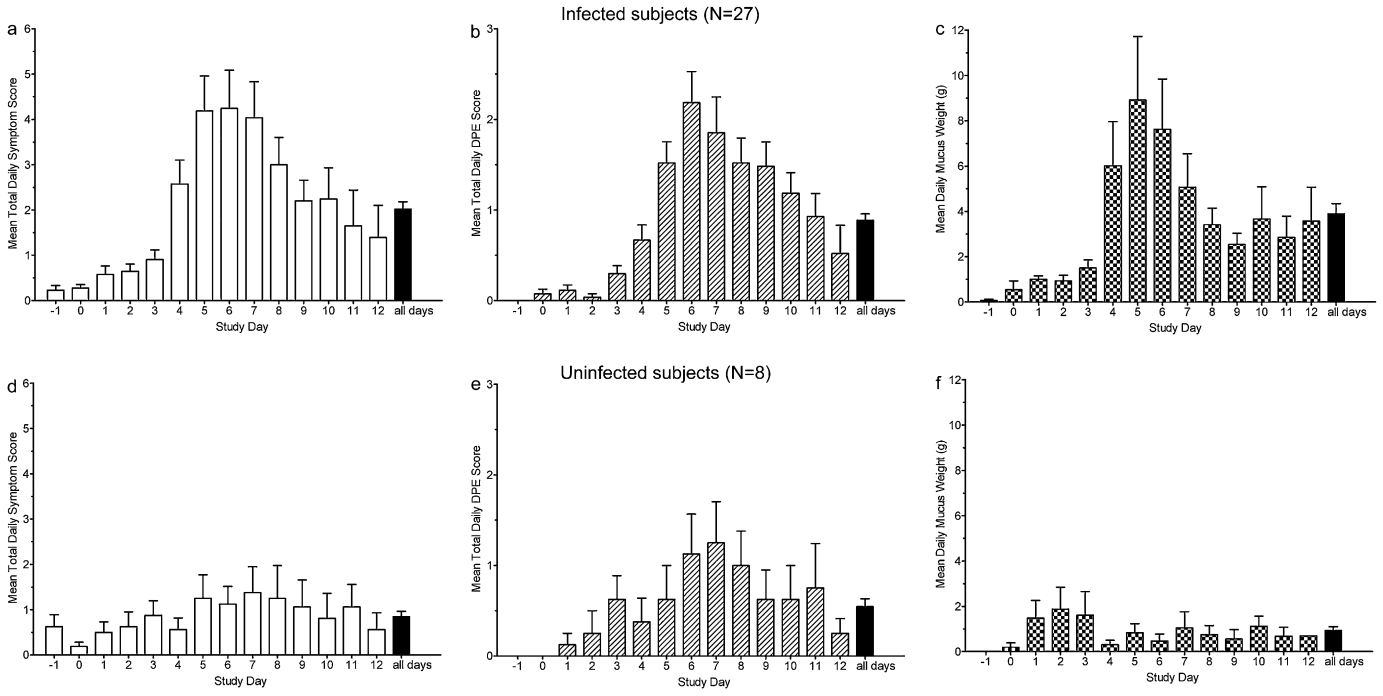


Figure 3. Disease measures over time in infected and uninfected volunteers. Volunteers were defined as infected if two successive samples of nasal washes, collected between Study Days 2 and 8 inclusive, contained detectable respiratory syncytial virus (RSV). (*a–c*) Volunteers who became infected; (*d–f*) volunteers who failed to reach the definition of infected. Infected volunteers showed significantly higher measures of disease than did uninfected volunteers (all days combined). Mean total daily symptom scores, $P = 0.021$ (*a* vs. *d*), mean total daily directed physical examination (DPE) scores, $P = 0.181$ (*b* vs. *e*) and mean daily nasal mucus weights, $P = 0.0056$ (*c* vs. *f*). *Error bars* represent the SD.

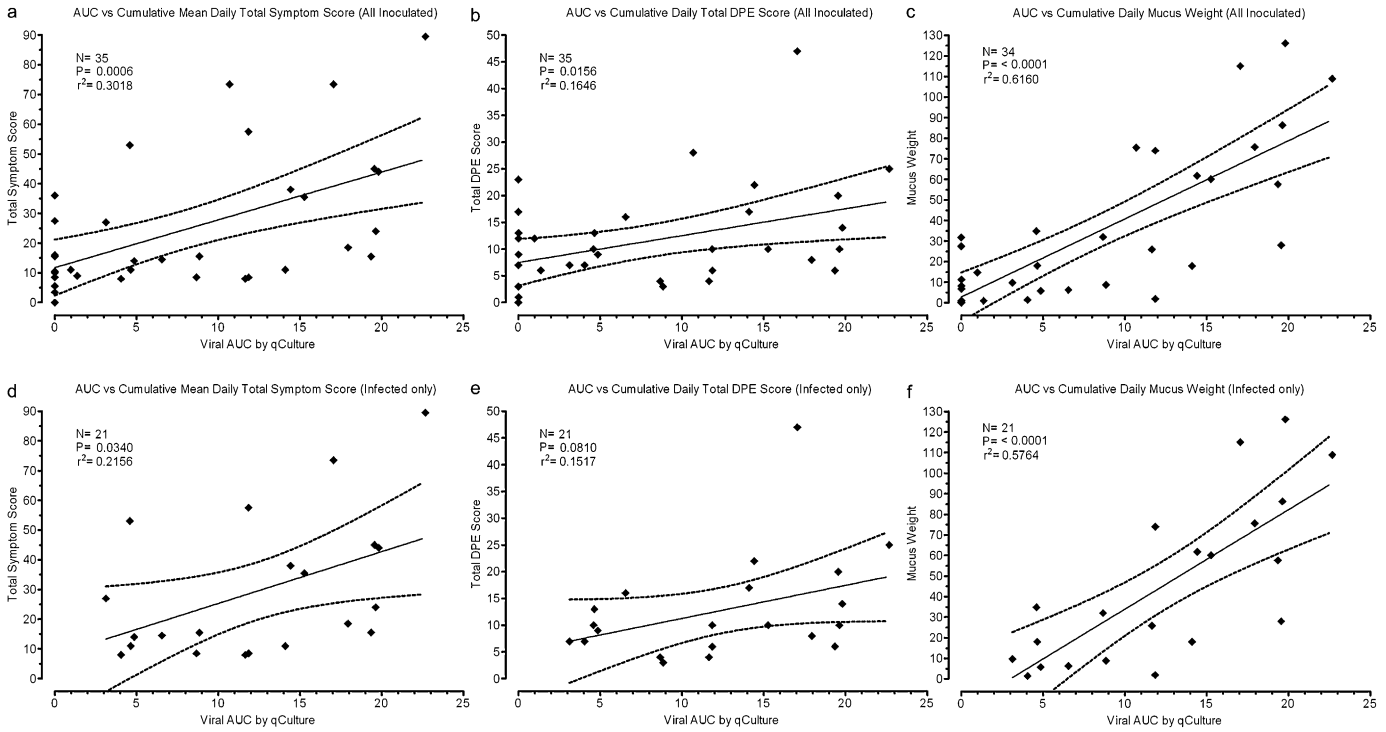


Figure 4. Relationships between quantitative viral and disease measures. All volunteers were inoculated with respiratory syncytial virus (RSV) and are represented in (*a–c*). (*d–f*) Analyses restricted to those volunteers who met the definition of infected. Disease measures (total symptom scores, total directed physical examination [DPE] scores, and mucus weights) for individual volunteers are plotted against their viral area under the curve (AUC). Viral AUC is the area under the viral load curves calculated for individual volunteers. *P* values represent the probability that the slopes of the regression lines do not include a slope of zero. The *dashed curved lines* indicate the 95% confidence interval of the slopes of the regression line (*solid line*). Similar statistically significant direct relationships were observed between viral and disease measures when viral AUC was measured by quantitative real-time reverse transcriptase-polymerase chain reaction. qCulture = quantitative culture.

The model reported here, using wild-type RSV, is safe and is also highly reproducible. Five separate cohorts (A through E) of volunteers studied over a 4-month period and inoculated with a range of about 500-fold different viral quantities, produced essentially the same quantitative viral and disease outcomes.

Faithfully reproducing natural RSV infection and disease makes this model useful in proving the antiviral concept that reducing RSV can reduce human disease and in evaluating the effectiveness of specific RSV therapeutics before progressing to studying such experimental therapeutics in the vulnerable pediatric population.

Animal models of RSV have several features that are likely not parallel to human disease. Mouse models require large nonphysiologic inoculums of RSV, approximately log 6–7 PFU per animal, aspirated directly into their lungs, and respiratory disease is not reliably produced. Conversely, the features of

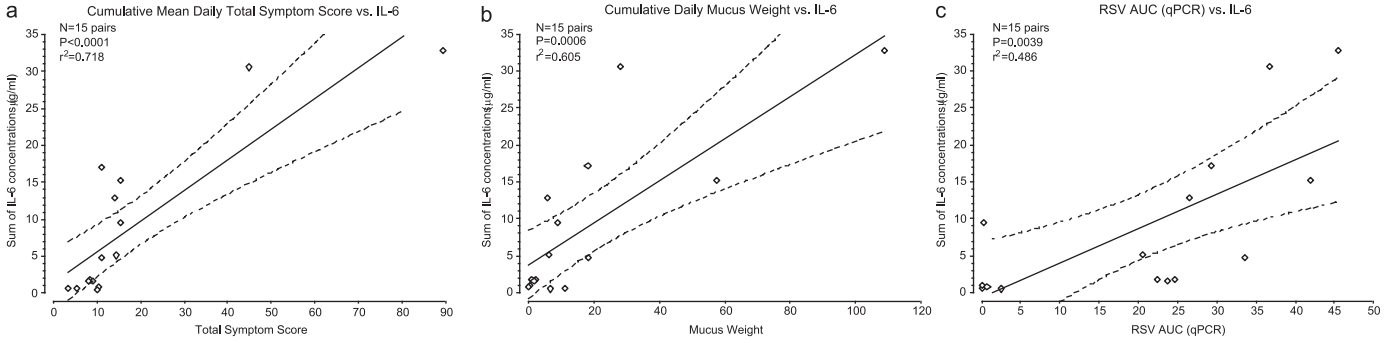


Figure 5. Relationships between IL-6 concentration, disease severity, and quantity of respiratory syncytial virus (RSV). Concentrations of IL-6 were measured in respiratory secretions of volunteers and were compared with disease measures and viral quantities. (*a*) The cumulative sum of the IL-6 concentrations versus disease severity as measured by individual volunteer cumulative symptom scores. (*b*) The cumulative sum of IL-6 concentrations versus disease severity as measured by individual volunteer cumulative nasal mucus weight. (*c*) Comparison of the cumulative sum of IL-6 concentrations versus area under the curve (AUC) viral load (quantitative real-time reverse transcriptase-polymerase chain reaction, qPCR). *P* values represent the probability that the slopes of the regression lines do not include a slope of zero. The *dashed curved lines* indicate the 95% confidence interval of the slopes of the regression line (*solid line*). Similar statistically significant direct relationships were observed when viral AUC was measured by quantitative culture.

natural human RSV infection (upper respiratory tract inoculation, appropriate incubation period, and typical upper respiratory symptomatic disease) are replicated in this experimental infection model of wild-type RSV. Studying RSV infection directly in humans reveals several important points, especially the quantitative and temporal correlation between viral replication and disease. Viral load has been shown to be an independent risk factor for RSV disease severity in children experiencing their first RSV infection (48, 49), but this association has been controversial and difficult to replicate (50, 51). Here we convincingly demonstrate the relationship between viral load and disease severity in this human experimental RSV infection model. Increased viral load (peak, duration of shedding, and viral AUC) is consistently and significantly associated with both subjective and objective measures of increased disease severity (Figure 4). Importantly, once infection is established in a volunteer, increased viral replication is significantly associated with both subjective (symptom scores) and objective (mucus weight) measures of increased disease severity (Figure 4).

Largely through the study of animal models, disease caused by RSV infection has been thought to result from the viral triggering of an exaggerated Th2 immune response (17, 18). Our findings do not support this Th2-mediated disease hypothesis. The two Th2 cytokines evaluated, IL-4 and IL-10, did not correlate with disease severity, and IL-4 actually showed a trend toward correlating inversely with disease severity. Conversely, other non-Th2 proinflammatory cytokines and chemokines correlated strongly with disease severity and also with viral loads. This implies that Th2-driven immune responses may not be as important in human RSV disease as animal models have suggested. The correlation between viral load and proinflammatory cytokines IL-6 and IL-8 suggests that if viral load were reduced by a robust antiviral, then disease severity might also be decreased even if disease were mediated by these proinflammatory cytokines, but studies employing an effective antiviral therapy in humans need to be performed to directly prove this.

We have shown a close temporal association between onset, peak, and clearance of viral replication and the onset, peak, and resolution of the disease. This clearly argues against a pathogenesis hypothesis involving infectious triggering of a delayed pathogenic immune response and conversely supports the potential clinical effectiveness derived by achieving a robust reduction in viral load through antiviral compounds.

Human experimental wild-type RSV infection described here can inform us well regarding the pathogenesis of natural human infections, even those occurring in infants and children. However, when extrapolating these results to natural disease in the pediatric population, certain potential differences should be considered. First, infants are immune-naive with respect to RSV whereas the adults studied here were not. Because of the ubiquitous nature of RSV in all human populations, there is no such thing as an immunologically RSV-naive adult. We studied those adults with relatively low RSV-neutralizing antibodies against Memphis 37 (Figure 1a), but it is likely that even these adults had virologically significant serum neutralizing antibodies. The correlates of protection for RSV in adults have not been determined, but serum neutralizing antibodies (in the form of IgG) provide significant concentrations to the epithelial lining fluid of the lower respiratory tract, but not the upper respiratory tract, thus preferentially protecting the lower respiratory tract (52). We observed essentially an upper respiratory tract disease in this model, and this may, in part, be due to these neutralizing antibodies preventing infection and consequent disease in the lower respiratory tract.

Cell-mediated immune function, triggered and sustained by multiple natural exposures to RSV throughout life, also likely plays a role in limiting RSV replication and disease in the adults studied. Infants with natural RSV infection likely have limitations in both humoral and cell-mediated aspects of immune control of RSV. Therefore we expect that infants would have more extensive viral replication involving both upper and lower respiratory tracts. Indeed, using the same qPCR assay employed here, these adults had mean peak nasal viral loads of about 4.5 log PFUe/ml compared with an extrapolated mean peak of RSV-A in naturally infected infants of more than 6 log PFUe/ml (43). Extrapolation of the timing of disease from this model to infants also bears discussion. Pathology studies and studies of RSV-specific cell-mediated immune function in adults have not been performed. It is unlikely that preexisting RSV-specific cell-mediated immune function would produce earlier symptoms in adults than in RSV-naive infants. This is because even severe RSV disease (leading to respiratory failure and death) in infants is produced despite the relative absence of such immune effector cells in the lungs. Although difficult to perform, studies of natural infections of children should be done to verify these early viral and disease dynamics.

This study reveals a striking difference between the magnitude and duration of RSV infection quantified by traditional approaches (quantitative culture and spin-enhanced culture) as compared with real-time qPCR. The degree of prolonged RSV shedding evidenced by qPCR in our study has not been previously evaluated or described. It is possible that the previously accepted relatively short duration of culture-defined viral shedding is not reflective of true viral replication in the human respiratory tract. RSV has been shown to be neutralized by antibodies, including IgA, and the disappearance of culturable RSV corresponds to the onset of detectable RSV-specific IgA in respiratory secretions of naturally infected infants (53). Thus, it is possible that the previously recognized short course of RSV infection in humans is a reflection of the virus being neutralized within respiratory secretions despite its persistent replication as shown by qPCR. There are alternative explanations for the observed prolonged high levels of RSV detectable by qPCR as compared with quantitative culture. It is unlikely that the prolonged higher levels of RSV-specific nucleic acid are caused simply by increased molecular detection thresholds provided by PCR. If prolonged detectability were simply an improved molecular detection phenomenon, then the quantitative viral load curves for an infected patient over the time course of the infection would be expected to have the same ratio of culturable virus compared with virus quantified by PCR. That is, the viral load curves (qPCR vs. quantitative culture) would simply run in parallel (qPCR being similarly higher quantitatively than quantitative culture at all time points). Observations of the data do not support this (Figures 2a and 2b). Rather, the viral load curves of qPCR and quantitative culture appear to be parallel during the upswing of the infection, but then the two curves dissociate after peak viral load is achieved in an individual infected volunteer (Figures 2a and 2b). The ratios of N-gene copy number compared with culturable viral particles for *in vitro* cultures of RSV-A, using the same assays as employed here, has been measured as approximately 150:1 (43). Another explanation for the prolonged viral loads as quantified by PCR is that culturable (replication-competent) RSV ceases to be produced in these volunteers at a relatively early time point (before approximately Day 6 of infection) and that after being produced, this virus becomes replication incompetent or is somehow cleared faster from the nasal washes compared with the clearance of components of the virus detectable by nucleic acid amplification techniques (PCR). These and other explanations deserve to be evaluated as they have major pathogenesis, diagnostic, and transmission implica-

tions for RSV and other respiratory viruses. If true, this newly measured prolongation of likely RSV replication has implications for pathogenesis and adds impetus for the development of antiviral therapeutics.

In conclusion, a safe and reproducible human experimental model of RSV infection and disease has been developed. It alters the current thinking about the pathogenesis of RSV in humans by establishing a significant direct relationship between viral replication and resultant clinical disease in humans and a parallel timing between viral and disease kinetics. Importantly, this model can be used to test RSV antivirals and vaccines, thus proving their effectiveness before being tested in vulnerable RSV-infected pediatric populations. These findings and the future use of this wild-type experimental human infection model relieve major obstacles in the development of therapeutics for this important disease.

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