MAJOR ARTICLE

Respiratory Syncytial Virus Load, Viral Dynamics, and Disease Severity in Previously Healthy Naturally Infected Children

Chadi M. El Saleeby,[1] Andy J. Bush,[2] Lisa M. Harrison,[3,4] Jody A. Aitken,[3,4] and John P. DeVincenzo[3,4,5,6]

[1]Division of Pediatric Infectious Diseases, Department of Pediatrics, Harvard Medical School, Massachusetts General Hospital for Children, Boston; [2]Department of Preventive Medicine, and [3]Department of Pediatrics, University of Tennessee School of Medicine, [4]The Children's Foundation Research Center, [5]Le Bonheur Children's Hospital, and [6]Department of Molecular Sciences, University of Tennessee Graduate School of Health Sciences, Memphis

Background. Respiratory syncytial virus (RSV) disease severity was thought to be a result of host immunopathology but alternatively may be driven by high-level viral replication. The relationships between RSV load, viral clearance dynamics, and disease severity have not been carefully evaluated.

Methods. Previously healthy RSV-infected children <2 years old were recruited. RSV load was measured in respiratory secretions by fresh quantitative culture over 3 hospital days. Measures of disease severity were hospital admission, duration of hospitalization, requirement for intensive care, and respiratory failure.

Results. Multivariate logistic regression models revealed independent predictors of increased duration of hospitalization: male sex, lower weight, and higher viral load on any day. Viral loads at day 3 were more significantly associated with requirement for intensive care and respiratory failure than were viral loads at earlier time points. Faster RSV clearance was independently associated with shorter hospitalization.

Discussion. These observations challenge the immunopathology-based pathogenesis paradigm. They also have major therapeutic implications, suggesting that application of antiviral agents early in the disease course, even at a time when viral replication is at its highest, might improve subsequent morbidity by significantly lowering viral load and direct viral cytopathic effects, and aborting the potential downstream immunopathology.

Respiratory syncytial virus (RSV) is the most common cause of serious lower respiratory infection in infants [1–3]. Although premature infants, and those with lung or heart disease or immunodeficiencies, are at increased risk for severe RSV disease, most infants with RSV requiring hospitalization are previously healthy [4, 5], and the most important risk factor has been young age. RSV disease was thought to be largely immunopathogenic, resulting from exaggerated Th2 cellular responses [6], immune complex formation [7], and bystander killing effects of activated cytotoxic T cells [8]. However, these disease mechanisms are predominantly derived from animal models rather than from infected infants. Alternatively, quantitative differences in RSV replication may induce varying degrees of direct cytopathic effects. We hypothesized that viral load, and the ability of the immune system to control viral replication over time, predicts the severity of RSV disease in immunocompetent children. We therefore investigated the effects of viral load and dynamics on clinical disease through quantitative measurements over 3 days of observation.

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Correspondence: John DeVincenzo, MD, The Children's Foundation Research Center, Le Bonheur Children's Hospital, Rm 433 WPT, 50 North Dunlap St, Memphis, TN 38103 (jdevincenzo@uthsc.edu).

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METHODS

Patients

Previously healthy children aged ≤24 months were prospectively enrolled if they had RSV detected from respiratory secretions at some time within the past

48 hours. Subjects were excluded if they had a diagnosed immunodeficiency, had received systemic corticosteroids within the preceding month, or had diagnosed chronic lung disease of prematurity, hemodynamically significant congenital heart disease, or positive blood cultures. The study cohort represented a convenience sample collected over 5 consecutive RSV seasons. Plaque assay plates were scheduled so that 4 or 5, 12-well plates were 80% confluent on each day in anticipation of patients being identified on those days. On many days, most plates were discarded as no patients meeting study criteria were identified. On other days, >5 eligible patients were identified in which case preference was given to those exhibiting extremes of illness (eg, intubated patients and outpatients) for greatest statistical power. Data collected included patient demographics, risk factors for RSV disease and clinical information surrounding the time of RSV diagnosis. Four measures of disease severity were documented: (1) hospital admission, (2) duration of hospitalization, (3) admission to the intensive care unit, and (4) need for mechanical ventilation. These were selected and defined before data analysis for their ease of characterization and clinical relevance. The conduct of this research followed the IRB guidelines including appropriate informed consent.

Virology

Subjects were identified as RSV-infected using either direct fluorescent antibody detection (Bartels, Trinity Biotech, Wicklow, Ireland) or chromatographic rapid antigen detection (Directigen, Becton, Dickinson, MD, USA) [9]. All enrolled subjects had at least one respiratory specimen collected for viral load quantification. Nasal aspirates were obtained quantitatively at enrollment using a standardized technique [10] by trained members of the study team and placed immediately into RSV stabilization media (EMEM, 7.5% sucrose, 25 mmol/L HEPES, 1% L-glutamine, 1% penicillin/streptomycin) on ice. If the subject was being mechanically ventilated, a deep tracheal aspirate was also simultaneously obtained using the same quantitative technique. Hospitalized subjects had subsequent aspirates collected at 24-hour intervals until discharge. Aspirates were transported on ice and placed into culture within 3 hours of collection. Viral load was determined by plaque assay as described elsewhere [10] and expressed as log plaque-forming units (PFU) per mL. To assure assay precision, RSV quantitative standards (RSV A-long ATCC # VR-26) of known quantity were run in parallel with each subject's aspirate.

To determine the appropriateness of upper respiratory viral load as a marker of lower respiratory tract viral load, aspirates from both sites were compared using standard collinearity studies in intubated subjects when specimens from both upper and lower respiratory tracts were available at the same time from the same patient. Simultaneous viral loads from nasal aspirates and tracheal aspirates from the same subjects correlated well with each other (Figure 1*A* and *B*). In this population and at the time points studied, nasal aspirates appear to be a reasonable surrogate marker for lower respiratory tract viral load and were therefore used for all analyses in this study.

Statistical Analyses

Simple descriptive statistics were used to characterize the study population and parameters of RSV disease severity. Subjects left the study as they improved and were discharged.

First, we tested the association between viral load and disease severity on each study day (days 1, 2, and 3). Multivariate analyses were applied using the 4 measures of disease severity (hospital admission, duration of hospitalization, requirement for intensive care, and development of respiratory failure) as dependent outcome measures. Univariate analyses were initially applied to potential risk factors for RSV disease severity. These independent variables (Table 1) were selected based on biological plausibility and prior association with disease severity. Univariate analyses (χ^2 and Fisher exact tests for dichotomous variables, and *t* tests for continuous variables) were then applied. Variables found to have a $P \leq .1$ in the univariate analyses were placed into multivariate logistic regression models. The variables included in the final models were those associated with the outcomes measures at $P < .05$ that independently predicted RSV disease severity. One final model for each study day was constructed. Finally, RSV load, as an independent variable, was tested in these final best-fit models. To identify significant predictors of viral load, the effects of these variables on viral load (now as a dependent variable) were evaluated using similar techniques and were repeated for the 3 time points of observation.

The rate of RSV clearance as a predictor of disease severity was also analyzed by multiple logistic regression comparing $\Delta\log \text{PFU/mL}_{\text{d1-d3}}$ from the nasal aspirates of the subjects. If day 3 viral load data were not collected, Δday_{1-2} was used.

We further investigated the role of viral clearance on disease severity by dichotomizing duration of hospitalization (<4 days versus ≥4 days) and comparing the mean daily viral loads for both groups. This duration of hospitalization definition was made empirically so as to distribute our hospitalized patient population into 2 near equal groups for proper statistical comparison (Table 1). Additionally, viral clearance, which was based on viral load measurements obtained from the first 3 days of hospitalization, would be unaffected by this definition.

All analyses were 2-tailed with $\alpha < 0.05$ set for significance. The statistical analyses were performed using the SAS software package (version 9.0 for windows, SAS Institute Inc). GraphPad Prism 4 (GraphPad Software Inc) was used to generate figures and graphs.

RESULTS

Two hundred nineteen subjects were enrolled over 5 RSV seasons. Baseline characteristics and RSV disease descriptors are

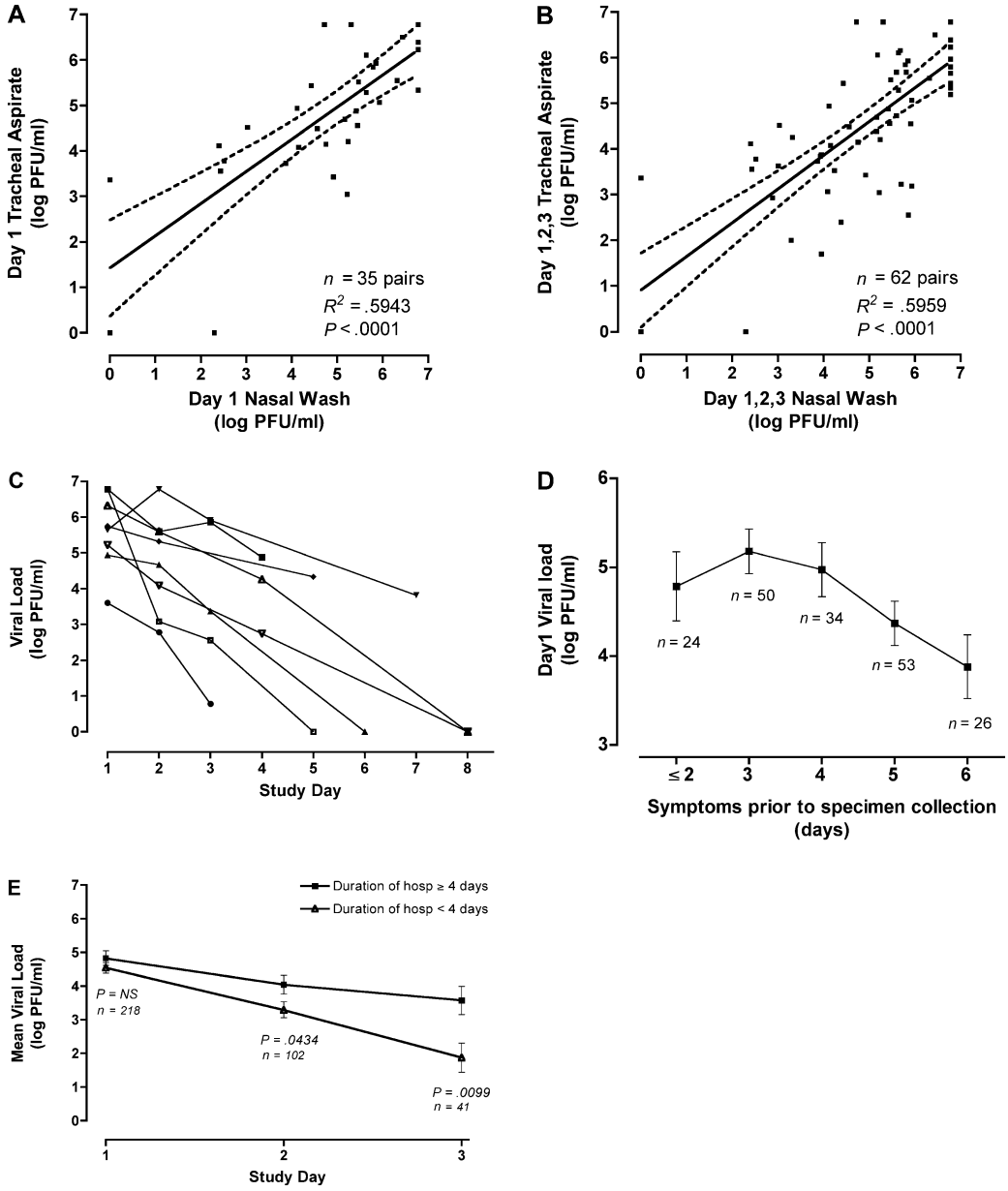

Figure 1. Viral load and viral dynamics. *A*, Viral loads on study day 1 measured simultaneously from both deep tracheal aspirates and nasal aspirates ($n = 35$ pairs). Each data point represents a single study subject. *B*, Viral loads on study days 1, 2, and 3 measured simultaneously from deep tracheal aspirates and nasal aspirates ($n = 62$ pairs). *C*, Representative viral load curves from individual subjects; *D*, Mean viral load at enrollment as a function of duration of symptoms prior to specimen collection. Individual observations are mean RSV viral loads. Error bars represent the standard error of the mean. *E*, Mean viral loads on study days 1, 2, and 3 dichotomized by duration of hospitalization (<4 days; ≥4 days). Error bars represent the standard error of the mean.

given in Table 1. Nasal aspirates from 218 subjects were available for analysis on day 1, from 102 subjects on day 2, and from 41 subjects on day 3, reflecting natural dropout from the study as the disease improved. Mean nasal viral loads ± SEM were 4.63 ± 0.13 log PFU/mL, 3.63 ± 0.18 log PFU/mL, and 2.91 ± 0.33 log PFU/mL on days 1, 2, and 3, respectively. Fewer tracheal aspirates were collected, reflecting the lower number of intubated subjects ($n = 36$, 18, and 14 on days 1, 2, and 3, respectively). In those patients where simultaneous sampling was available from both the upper and the lower respiratory tracts, there were no significant differences between nasal wash and tracheal aspirate mean viral loads (4.76 and 4.42, respectively, $n = 62$ pairs, $P = .290$). In this population, nasal aspirates were found to be a reasonable surrogate marker for lower respiratory tract viral load (Figure 1*A* and *B*) and were used for all subsequent analyses. Viral loads generally declined smoothly over time in individual subjects (Figure 1*C*). When mean viral loads were plotted against the time since symptom

Table 1. Characteristics of the Study Population

Baseline characteristics	
No. of study subjects (*n*)	219
Age (days): mean ± SD	115.0 ± 124.1
Sex (%)	
Female	110 (50.2)
Male	109 (49.8)
Weight on admission (g): mean ± SD	5568 ± 2089
Ethnicity[a] (%)	
African-American	133 (60.7)
Caucasian	85 (38.8)
Other	1 (0.5)
Birth weight (g): mean ± SD	2956 ± 680
Gestational age at birth (weeks): mean ± SD	38.0 ± 2.9
No. of persons living in home[b]: mean ± SD	4.8 ± 2.1
Day care attendance[c] (%)	25.2
Breastfeeding[d] (%)	39.7
Tobacco exposure[e] (%)	30.1
Congenital anomalies[f] (%)	5.4
RSV disease descriptors	
Duration of symptoms prior to enrollment (days) mean ± SD	3.9 ± 2.2
Duration of symptoms prior to specimen collection (days)[g] mean ± SD	4.7 ± 2.4
Requiring hospitalization (%)	192 (87.7)
Duration of hospitalization[h] (days) mean ± SD	6.6 ± 12.8
Duration of hospitalization[h] (%)	
<4 days	102 (53.1)
≥4 days	90 (46.9)
Requiring intensive care (%)	59 (26.9)
Respiratory failure[i] (%)	51 (23.3)

NOTE. SD, standard deviation.

[a] Subject's caregivers were asked to report the patient's racial/ethnic classification as ''African-American,'' ''Caucasian,'' or ''other.''

[b] Excluding the study subject.

[c] Present attendance, prior to illness, in a group care setting with at least 4 other children.

[d] Any breastfeeding occurring for any duration after discharge from the birth hospitalization.

[e] Any current use of a smoked form of tobacco by a person living with subject, regardless of whether or not such use occurred within the subject's house.

[f] Study subjects with congenital anomalies included 3 with gastroschesis, 2 with trisomy 21, and 1 subject each with hypospadia, isolated duodenal atresia, cystic kidney disease, congenital hydrocephalus, cerebral palsy, and Pierre-Robin syndrome. Subjects with hemodynamically significant congenital heart disease were excluded from study.

[g] Calculated as the number of days between the onset of the earliest reported symptoms and specimen collection.

[h] Of those subjects hospitalized.

[i] Subjects who required mechanical ventilation at any time during the acute RSV illness except those intubated for apnea only. (The need for hospitalization, ICU admission and mechanical ventilation were decided upon by the treating physician and not influenced by the study or the investigators).

onset, the natural viral dynamics occurring during infection were depicted (Figure 1*D*).

In univariate analyses, multiple dichotomous and continuous variables were tested for their association with 4 measures of disease severity (Table 1). Factors relating to smaller subject size were significantly associated with worse disease. Whether these factors acted independently (or were, for example, simply surrogate markers of smaller airway size) was tested in multivariate analyses.

In multivariate analyses, the statistically significant independent predictors of hospitalization during an RSV infection were African-American ethnicity, younger age, lower admission weight, and lack of breastfeeding (Table 2). Measures of prematurity, household crowding, or viral load measured at the time of enrolment were not significantly associated with disease severity.

Analysis of prolonged RSV hospitalization revealed that lower weight was independently and significantly associated with this outcome, as well as predicting nearly all other measures of more severe disease. Being male and having congenital anomalies significantly increased the duration of hospitalization. Importantly, higher viral loads strongly predicted prolonged hospitalization. An initial viral load increase of 1 log PFU/mL predicted ~1 additional day of hospitalization. Higher viral loads were also associated with an increased risk of requiring intensive care (Table 2).

Independent predictors of respiratory failure were analyzed. Lower admission weight was a strong predictor of respiratory failure on all days (days 1, 2, and 3; $P < .001$, $<.001$, and .02, respectively). Congenital anomalies and younger age were independently associated with respiratory failure on day 1 ($P < .001$). Higher viral load also predicted the development of respiratory failure on day 3 ($P = .009$) and was the only independent predictor of this outcome on day 3 in addition to admission weight.

Associations between specific risk factors and viral load were also assessed using multivariate analyses. None of the following risk factors were independently associated with viral load on any study day: breastfeeding, household crowding, use of day care, birth weight, gestational age, sex, ethnicity, or age at admission. However, higher admission weight was significantly associated with higher viral load on day 1 ($P = .026$). Shorter durations of symptoms prior to specimen collection were independently associated with lower viral loads (day 1, $P = .032$).

We also assessed viral dynamics. The mean RSV load on days 1, 2, and 3 was dichotomized by duration of hospitalization: <4 days (53.1% of hospitalized subjects) versus ≥4 days. By univariate analysis, viral loads on day 1 were not different between these groups, but successive days showed significant differences in viral load (Figure 1*E*). The rate of viral clearance (Δ $PFU_{D1\text{-}3}$) was evaluated using multivariate techniques. Less rapid clearance was independently associated with prolonged

Table 2. Factors Independently Predicting Disease Severity Among RSV-Infected Children

Outcome measure	Independent variables associated with outcome	*P* value
Predicting increased likelihood of hospitalization[a]	Ethnicity (Caucasian)	.040
	Younger age	.022
	Lower admission weight	< .001
	Presence of breastfeeding	.014
Predicting prolonged duration of hospitalization	Sex (male)	.036
	Lower admission weight	.044
	Presence of congenital anomalies	.016
	Longer duration of symptoms prior to specimen collection	.010
	Higher viral load[b]	**<.001**
Predicting increased likelihood of requiring intensive care (day 1)	Younger age	.001
	Lower admission weight	<.001
	Presence of congenital anomalies	.017
	Higher viral load[b]	**.055**
Predicting increased likelihood of requiring intensive care (day 2)	Lower admission weight	<.001
	Higher viral load[b]	**.046**
Predicting increased likelihood of requiring intensive care (day 3)	Lower admission weight	.016
	Higher viral load[b]	**.009**

NOTE. Models were generated using multivariate stepwise techniques to determine factors that best predict the outcome (measures of disease severity) in question. Viral load was tested last. Factors not listed here were not independently significant predictors.

[a] The effects of congenital anomalies on hospitalization could not be assessed because no subject with congenital anomalies remained an outpatient.

[b] Measured from nasal aspirates. Viral load estimates are highlighted in bold.

hospitalization ($P = .035$). There was no difference in viral clearance rates between RSV A and B strains (by univariate or multivariate analyses). Likewise, there were no significant differences between strains A and B in disease severity that were independent of viral load.

DISCUSSION

Among children naturally infected with RSV, we have shown that viral load and dynamics predict the clinical severity of disease. Increased viral load is independently associated with increased risk for requiring intensive care or prolonged hospitalization, or developing respiratory failure. Also, delayed viral clearance is independently associated with greater disease severity. The validity of this study is confirmed by its prospective methodology and its consistency. The nonviral risk factors associated with more severe RSV disease in this study are consistent with previous reports [5, 11], including the independent factors of breastfeeding, congenital anomalies, and, most importantly, admission weight. Because of the small number of outpatients enrolled, the power of this study to detect factors predicting hospitalization was limited. Nevertheless, even with a small sample size, previously accepted and other statistically significant risk factor associations were made (Table 2).

Because lung disease is presumably a reflection of viral replication in the lung, it is likely that disease severity would be more closely associated with lower rather than upper respiratory tract viral load. By necessity, lower respiratory viral loads could not be measured unless respiratory failure had occurred. Therefore, nasal aspirates were relied upon as providing a surrogate marker for viral load in the lower respiratory tract. Upper and lower respiratory tract viral loads are tightly correlated when measured at the same time in individuals (Figure 1*A* and *B*). This correlation between upper and lower respiratory tract viral load has also been seen using different RSV-measuring techniques [12].

The measurement of differences in viral load over time in children experiencing different disease severity had to contend with study dropouts. Children who improved rapidly were discharged and were therefore not available for the multiple determinations of viral load required for calculations of clearance rates. Despite this, less rapid clearance rates were statistically significantly associated with prolonged disease.

That collected nasal secretions are reflective of actual differences in viral load is likely for several reasons. First, sampling was performed by one of 2 dedicated research personnel following a specific protocol [10, 12], thus controlling for sample collection differences. Any remaining collection differences would likely be unable to override the log differences seen in the measurement of viral load. Second, several steps were taken to limit the variability of the plaque assay itself, including the analysis of fresh specimens and the use of parallel quantitative RSV controls to maintain high assay precision (mean = 5.06 log PFU/mL, 95% confidence interval [CI], 5.00–5.11). The validity of the measurements is clearly demonstrated in the smooth curves generated by individual subjects over time (Figure 1*A*).

The relatively small differences in gestational age represented within this study population did not influence viral load. However, an increased weight (size) of the subject was independently associated with a higher viral load. This may be due to larger children requiring higher viral loads to produce the same quantifiable disease severity as that produced in smaller infants with smaller airways. Factors putatively relating to RSV inoculum (the number of children in the home or the use of day care) were not independently associated with increased viral load.

Along with the standard plaque assay, viral load quantification by polymerase chain reaction (PCR) could offer further insights to the viral dynamics and its association with disease severity. It should be noted, however, that viral clearance assayed by quantitative cultures (cessation of viral replication) is different than the viral clearance measured by PCR, which will continue to detect residual nucleic acid despite the cessation of active viral replication [13]. These functional assay differences suggest that the correlation between disease severity and PCR viral load will not necessarily be equivalent or similar to the analyses using plaque assay (quantitative culture). The use of RSV PCR quantification technique for clinical studies should only be solidified after its correlation with RSV disease severity is established.

Since the total number of hospitalized infants eligible for the study was not documented, and since the number of infants enrolled within each of the 5 separate seasons of the study was small, the study was not conducive to evaluating interseasonal differences in disease severity.

In a population of previously healthy infants, there are functional differences in the dynamics of RSV immune clearance (Figure 1*A*) that may be under some genetic control. Numerous single nucleotide polymorphisms (SNPs) have been associated with differences in RSV disease severity [14–19]. To date, these SNPs have been postulated to affect RSV disease through augmentation of a pathogenic immune response. However, our data suggest that other mechanisms, including those imparting functional differences in the limitation of viral spread or in viral clearance, may also be important [15, 20, 21]. Indeed, variations in innate immune response genes have now been associated with susceptibility to RSV bronchiolitis [22]. Because RSV clearance rates differ and because decreased clearance is associated with more prolonged and severe disease, it is possible that low-level viral persistence in the lower respiratory tract may be related to the maintenance of the prolonged post-acute sequelae of RSV. More study in this area is needed, but, provocatively, animal models indicate that RSV may persist in the lower respiratory tract [23] and that RSV is associated with markers of persistent reactive airway disease [24].

This study represents a comprehensive analysis of RSV viral load, dynamics, and the effects on several markers of disease severity. The findings help explain the broad range of RSV disease severity seen in healthy infants. The implications are that early intervention with antiviral therapeutics, even initiated at a time when viral replication is at its highest (day 3 of symptoms), might improve disease outcome. The underlying mechanism remains unclear, but it seems logical that reduction of the viral burden early in the course of the infection might reduce direct viral cytopathic effects and abort the downstream immune response that may contribute to later disease manifestations.

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Disclaimer: The conduct of this research follows the human experimentation guidelines of the US Department of Health and Human Services and those of the participating institutions including appropriate informed consent from the parents or guardians.

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