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MAJOR ARTICLE

Live-Attenuated Respiratory Syncytial Virus Vaccine Candidate With Deletion of RNA Synthesis Regulatory Protein M2-2 is Highly Immunogenic in Children

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(See the Editorial Commentary by Polack on pages 1335–7 and the Major Article by Buchholz et al on pages 1338–46.)

Background. Live respiratory syncytial virus (RSV) candidate vaccine LIDΔM2-2 is attenuated by deletion of the RSV RNA regulatory protein M2-2, resulting in upregulated viral gene transcription and antigen expression but reduced RNA replication.

Methods. RSV-seronegative children ages 6–24 months received a single intranasal dose of 10^5 plaque forming units (PFU) of LIDΔM2-2 (n = 20) or placebo (n = 9) (NCT02237209, NCT02040831). RSV serum antibodies, vaccine infectivity, and reactogenicity were assessed. During the following RSV season, participants were monitored for respiratory illness and pre- and post-RSV season serum antibodies.

Results. Vaccine virus was shed by 95% of vaccinees (median peak titers of 3.8 $\log_{10}$ PFU/mL by quantitative culture and 6.3 $\log_{10}$ copies/mL by PCR); 90% had ≥4-fold rise in serum neutralizing antibodies. Respiratory symptoms and fever were common in vaccine (95%) and placebo (78%). One vaccinee had grade 2 rhonchi concurrent with vaccine shedding, rhinovirus, and enterovirus. Eight of 19 vaccinees versus 2 of 9 placebo recipients had substantially increased RSV antibody titers after the RSV season without medically attended RSV disease, indicating anamnestic vaccine responses to wild-type RSV without significant illness.

Conclusion. LIDΔM2-2 had excellent infectivity and immunogenicity, encouraging further study of vaccine candidates attenuated by M2-2 deletion.

Clinical Trials Registration. NCT02237209, NCT02040831.

Keywords. respiratory syncytial virus; live attenuated viral vaccine; pediatric RSV vaccine; neutralizing antibodies; immunogenicity; RNA regulatory protein M2-2.

Respiratory syncytial virus (RSV) is the most common viral cause of serious lower respiratory illness (LRI) in infants and children under 5 years of age worldwide [1]. Globally, RSV causes an estimated 33 million episodes of acute lower respiratory infection, 3.2 million hospital admissions, with up to 118 200 RSV-attributable deaths each year [1]. Although passive immune prophylaxis with the commercial RSV monoclonal antibody palivizumab is effective for high-risk infants [2], this approach is not feasible for general use [3]. Thus, there is a strong rationale to prioritize RSV vaccine development [4].

A major effort has been directed at development of a live-attenuated RSV vaccine [5, 6] to avoid the RSV disease enhancement previously observed with the formalin-inactivated RSV vaccine [7]. Disease enhancement has not been observed with live-attenuated RSV vaccine candidates or replicating vaccine vectors [8, 9]. Important potential advantages of intranasal live-attenuated RSV vaccines include induction of a spectrum of protective and immunoregulatory mucosal and systemic immune responses [10].

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Use of reverse genetics systems [11] and improved understanding of RSV gene function [12] allow for rational design of attenuated RSV candidate vaccines. Reverse genetics methods were used to develop a number of cDNA-derived candidate vaccines that have been evaluated in children and infants as young as 4 weeks old [13, 14]. A previous vaccine candidate (rA2cp248/404/1030ΔSH) constructed with several temperature sensitivity and cold passage point mutations and deletion of the RSV *SH* gene appeared to be sufficiently attenuated for use in young infants (age 1–2 months) [14]. A closely related vaccine virus, MEDI-559, had a high vaccine "take" in a larger trial enrolling infants 5–24 months of age [15]. However, in both studies, there was concern for inadequate genetic stability, as reversion of individual point mutations and reduced temperature sensitivity were observed in more than one-third of vaccine virus isolates [14, 15].

An alternative attenuation strategy employs deletion of most of the open reading frame (ORF) encoding the RNA synthesis regulatory protein M2-2. Since this approach is based on deletion of the coding sequence for most of a viral protein, the attenuation phenotype of RSV ΔM2-2 is expected to be very stable. The RSV M2-2 protein is a small, nonabundant protein encoded by the second, downstream ORF in the M2 mRNA, which slightly overlaps the 5′-proximal, upstream M2-1 ORF [16]. Deletion of M2-2 results in a shift in the viral RNA synthesis program such that gene transcription and antigen expression are increased whereas genome replication is decreased [17], which might lead to greater immunogenicity despite lower replication.

Indeed, the candidate vaccine RSV MEDIΔM2-2 in a previous study was highly restricted in replication yet more immunogenic than prior attenuated RSV vaccine candidates in RSV-seronegative children [18]. To gain additional information about safety and immunogenicity of M2-2 deletion mutants, the current study evaluated LIDΔM2-2, a virus bearing an M2-2 deletion in a different RSV strain A2 backbone, in RSV-seronegative children aged 6–24 months.

METHODS

Vaccine

The vaccine, LIDΔM2-2, is a cDNA-derived version of RSV subgroup A, strain A2 (the recombinant wt parent is GenBank KT992094), in which 241 nucleotides (nts) were deleted from the M2-2 ORF (nt 8189–8429 relative to GenBank KT992094) and the 3 potential translation initiation codons of the M2-2 ORF were silenced (ATG to ACG; the T > C mutations were positions 8161, 8167, and 8179) by site directed mutagenesis. In addition, 112 nts of the downstream nontranslated region of the *SH* gene (nt 4499–4610 relative to GenBank KT992094) were deleted and 5 translationally silent nt changes were present in the 3′ end of the *SH* open reading frame (4489C > T, 4492C > T, 4495A > T, 4497A > G, 4498G > A). These changes in the *SH* gene were described previously and were made in order to stabilize RSV full-length cDNA plasmids during propagation in bacteria, and appeared phenotypically inconsequential based on replication in mice [19]. LIDΔM2-2 was recovered from cDNA in qualified Vero cells, and clinical trial material (CTM) was manufactured under current good manufacturing practice (cGMP) in these Vero cells at Charles River Laboratories, Malvern, PA. Sequence analysis confirmed that the seed virus and final drug product were identical except for a single polymorphism (nt 4327; G/A in the *SH* ORF, resulting in a 50% mixed population with a phenylalanine-to-lysine mutation [amino acid 9 of the SH protein]). CTM was stored at −80°C and was diluted on site prior to dosing using Leibovitz L15 medium to a dose of 10^5 plaque forming units (PFU) in a 0.5 mL volume. This was administered intranasally as a single dose divided between nostrils. Leibovitz L15 medium was used as placebo.

Study Design

This randomized (2:1 vaccine to placebo), double-blind, placebo-controlled study (ClinicalTrials.gov identifiers: NCT02237209, NCT02040831, https://clinicaltrials.gov) was conducted at 7 clinical trials sites (Johns Hopkins Center for Immunization Research [CIR], Baltimore, MD; and 6 International Maternal Pediatric Adolescent AIDS Clinical Trials [IMPAACT] sites), with accrual between 1 September and 14 October 2014 and surveillance for RSV-like illness from 1 November 2014 through 31 March 2015. Eligible children were ≥6 and <25 months of age, healthy, with no history of current or past lung disease. Children born to women living with human immunodeficiency virus (HIV) but proven to be uninfected were permitted to enroll. Eligible children were RSV seronegative at screening, defined as having a serum RSV 60% plaque reduction neutralizing titer ($PRNT_{60}$) <1:40. Study accrual was stopped at 29 of a planned 51 participants due to a decision to close accrual early based on an interim analysis.

Clinical assessments and nasal washes (NWs) were performed on study days 0 (prior to intranasal inoculation), 3, 5, 7, 10, 12, 14, 17, 19, 21, and 28, with study site telephone contact to collect symptom information on all the intervening days. Additional physical examinations and NWs were obtained in the event of respiratory illness (upper respiratory illness [URI]: rhinorrhea, pharyngitis, or hoarseness; cough; acute otitis media [OM]; and LRI) or fever [14]. Data for all adverse events and reactogenicity events were collected through day 28. Following day 28, children were monitored until day 56 for serious adverse events (SAE) or LRI [14]. During RSV season surveillance, families were contacted weekly to determine whether medically attended acute respiratory illnesses (MAARI) had occurred, which were defined as fever, URI, LRI, or OM. Within 3 days of each illness episode, a clinical assessment and NW was obtained. Sera to measure antibodies to RSV were obtained

prior to inoculation, 56 days after inoculation (also served as pre-RSV season sample for this study), and after the RSV surveillance period (1–30 April).

Written informed consent was obtained from the parents/guardians of participants prior to enrollment. These studies were approved by each site's institutional review board, conducted in accordance with the principles of the Declaration of Helsinki and the Standards of Good Clinical Practice (as defined by the International Conference on Harmonization), and monitored by the independent data safety and monitoring board of the National Institute of Allergy and Infectious Diseases, Division of Clinical Research.

Laboratory Assays

Nasal wash specimens from days of illness were evaluated for the presence of common adventitious respiratory agents by reverse transcription-quantitative polymerase chain reaction (Respiratory Pathogens 21 multiplex RT-qPCR, Fast-track Diagnostics, Luxembourg). Vaccine virus in NW was quantified by immunoplaque assay on Vero cells and by RT-qPCR specific for the RSV matrix (M) protein, as previously described [18]. To determine the stability of the M2-2 deletion, vaccine virus from NW specimens from the time of each participant's peak vaccine shedding was amplified and sequenced. Specifically, the NW specimens were passaged once on Vero cells, total RNA isolated, a 649-bp fragment spanning the M2-2 deletion of LIDΔM2-2 amplified by RT-PCR, and the consensus nucleotide sequence determined.

Serum RSV $PRNT_{60}$ were determined by complement-enhanced 60% plaque reduction neutralization assay [20]. Serum IgG antibody titers to the RSV F glycoprotein (anti-RSV F IgG) were determined by an IgG-specific enzyme-linked immunosorbent assay (ELISA) using a purified baculovirus-expressed F protein [21, 22], kindly provided by Novavax, Inc. (Gaithersburg, MD), as previously described [18, 22, 23].

Statistical Analysis

Reciprocal serum $PRNT_{60}$ and anti-RSV F IgG titers were transformed to $\log_2$ values. Even though log transformed, some data deviated from normality; thus, nonparametric methods were used for testing for statistical differences and assessing correlations. Medians and interquartile ranges (IQR) were used to summarize peak nasal wash titers and serum antibody titers to RSV. Mean and standard deviation values were presented to allow descriptive comparisons with other studies (Supplementary Tables 1 and 2). The summaries of vaccine virus shed in NW detected by culture and RT-qPCR were restricted to the 19 (of 20) vaccine recipients who were infected with vaccine. Infection was defined as the detection of vaccine virus by culture and/or RT-qPCR and/or a ≥4-fold rise in serum RSV $PRNT_{60}$ or anti-RSV F IgG titer. When comparing the vaccinated and placebo groups, 1-tailed tests were used when there was a clear biological prediction for directionality and 2-tailed tests when we tested for differences but there was no definite directional hypothesis. The Wilcoxon rank sum test was used to compare peak viral titers and antibody titers between vaccine and placebo recipients. Rates of illness among vaccinees and placebo recipients were compared by the Fisher's exact test. Spearman correlation analyses were performed to assess the strength of associations and test their statistical significance. All analyses were performed using SAS Version 9.4 (SAS Institute Inc., Cary, NC) and the graphs were produced using R software.

RESULTS

Accrual and Participant Characteristics

The study accrued 20 vaccine and 9 placebo recipients. The distribution of sex, age, ethnicity, and racial characteristics was similar for vaccine and placebo recipients (Table 1). All children received their assigned study treatment. One child did not have serum obtained at the post-RSV surveillance visit and happened to be the one vaccinee that did not shed vaccine virus.

Safety and Adverse Events

During the 28 days postinoculation, respiratory and/or febrile illness was frequent in both vaccine and placebo recipients, with 95% (90% confidence interval [CI], 78%–99.7%) and 78% (90% CI, 45%–96%) ($P = .22$) having 1 or more illness episodes, respectively (Table 2). The median time to onset of a respiratory illness or fever was 5 days for both groups. Among the 19

Table 1. Baseline Characteristics of Vaccine and Placebo Recipients

	Number (%)		
	Vaccine N = 20	Placebo N = 9	Total N = 29
Gender			
Female	11 (55%)	3 (33%)	14 (48%)
Male	9 (45%)	6 (67%)	15 (52%)
Ethnicity			
Hispanic or Latino	7 (35%)	1 (11%)	8 (28%)
Not Hispanic or Latino	13 (65%)	8 (89%)	21 (72%)
Race			
Asian	0 (0%)	1 (11%)	1 (3%)
African American	8 (40%)	6 (67%)	14 (48%)
White	8 (40%)	1 (11%)	9 (31%)
American Indian	2 (10%)	0 (0%)	2 (7%)
More than 1 race	2 (10%)	1 (11%)	3 (10%)
Residence[a]			
California	5 (25%)	0 (0%)	5 (17%)
Colorado	7 (35%)	3 (33%)	10 (34%)
Illinois	4 (20%)	2 (22%)	6 (20%)
Maryland	2 (10%)	1 (11%)	3 (10%)
Tennessee	2 (10%)	3 (33%)	5 (17%)
HIV exposed, uninfected	8 (40%)	3 (33%)	11 (38%)
Age in months[b]	11 (7–18)	8 (7–12)	9 (7–16)

[a]Of the 7 sites, 2 were in California and 2 were in Illinois.

[b]Age expressed as median (IQR).

Table 2. Vaccine Virus Shedding, Peak Virus Titers, and Clinical Assessment During the First 28 Days After Inoculation

			Viral detection[a]		Number (%) with indicated symptom[b]					
Group	No. of children	% Shedding vaccine virus[c]	Plaque assay $\log_{10}$ PFU/mL[d]	RT-qPCR $\log_{10}$ copies/mL[e]	Fever	URI	LRI	Cough	OM	Respiratory or Febrile illness
Vaccine	20	95	3.8 (2.2, 4.3)	6.3 (4.6, 6.9)	8 (40)	19 (95)	1 (5)	13 (65)	2 (10)	19 (95)
Placebo	9	0	0.5 (0.5, 0.5)	1.7 (1.7, 1.7)	4 (44)	7 (78)	0 (0)	5 (56)	2 (22)	7 (78)

Abbreviations: LRI, lower respiratory illness, defined as wheezing, rhonchi, or rales, or having been diagnosed with pneumonia or laryngotracheobronchitis (croup); NW, nasal wash; OM, acute otitis media; PFU, plaque forming unit; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; URI, upper respiratory illness, defined as rhinorrhea, pharyngitis, or hoarseness.

[a]Median (25th, 75th percentile) peak viral titers detected in NWs. For the vaccine group, these summaries were calculated only for the 19 children who were infected with vaccine virus; infection was defined as the detection of vaccine virus by culture and/or RT-qPCR and/or a ≥4-fold rise in RSV serum neutralizing antibody titer and/or serum anti-RSV F antibody titer. As expected, no placebo recipients shed vaccine virus.

[b]Number (percentage) of children with indicated respiratory symptoms occurring in the 28 days after inoculation.

[c]Percentage of children with vaccine virus detected in NW by culture and/or RT-qPCR. All 19 children had vaccine virus detected by both culture and PCR.

[d]For each child, the individual peak (highest) titer, irrespective of day, was selected from among all titers measured in the NW by viral culture and expressed as $\log_{10}$ PFU/mL. The lower limit of detection was 0.5 $\log_{10}$ PFU/mL.

[e]For each participant, the individual peak (highest) titer, irrespective of day, was selected from among all titers measured in NW by RT-qPCR and expressed as $\log_{10}$ copies/mL. The lower limit of detection was 1.7 $\log_{10}$ copies/mL.

vaccinees with a respiratory illness, FastTrack qPCR of NW detected RSV alone in 6, RSV plus ≥1 other virus in 10, and no RSV but ≥1 other virus in 3. Other viruses detected were rhinovirus (n = 9), adenovirus (n = 4), parainfluenza (n = 3), enterovirus (n = 1), and bocavirus (n = 1). There was 1 grade 3 fever (39.3°C) in a vaccinee concurrent with shedding vaccine virus without other agents detected; all other events in both groups were grade 2 or lower in severity. One vaccinee had a grade 2 LRI on days 8–11 after inoculation, characterized by rhonchi, with rhinorrhea, mild cough, and watery eyes. The rhonchi cleared with inhaled bronchodilator therapy; a total of 3 doses were given. At the time of the illness, rhinovirus and enterovirus (days 2–16), in addition to vaccine virus (days 2–14), were detected in the child's NWs. No SAEs or LRIs were noted during the days 29 to 56 monitoring period.

Infectivity and Immunogenicity

Nineteen of 20 (95%; 90% CI, 78%–99.7%) vaccinees had vaccine virus detected by both quantitative culture and RT-qPCR (Table 2). Vaccine virus was shed for a median duration of 9 days (range 5–14, IQR 7–12) by culture and 13 days (range 10–17, IQR 10–14) by RT-qPCR. Median peak titers of shed vaccine virus were 3.8 $\log_{10}$ PFU/mL and 6.3 $\log_{10}$ copies/mL (Table 2) for the 19 vaccinees meeting vaccine infection criteria. Of note, 7 (37%) had a peak titer >4 $\log_{10}$ PFU/mL, and of those 3 had a peak titer exceeding 5 $\log_{10}$ PFU/mL. Sequence analysis of LIDΔM2-2 isolates from NW confirmed that the shed vaccine stably retained the deletion of the M2-2 ORF in all of the 16 participants with samples from which sequence analysis data could be obtained.

Serum antibody responses to the vaccine were assessed at approximately 2 months postinoculation (Table 3, Figure 1). Four-fold or greater rises in both serum RSV $PRNT_{60}$ and anti-RSV F IgG titer were detected in 90% of vaccinees and none of the placebo recipients ($P < .001$). Among vaccinees, 85% achieved a serum RSV $PRNT_{60}$ ≥6.0 $\log_2$ (1:64; Figure 2).

The peak titers of vaccine virus determined by culture correlated with the changes from baseline in both anti-RSV F IgG titer and RSV $PRNT_{60}$ (Figure 3A, C). The vaccine virus peak copy numbers by RT-qPCR correlated with the changes from baseline in anti-RSV F IgG titer but not RSV $PRNT_{60}$ (Figure 3B, D). A sensitivity analysis, excluding the single vaccinee that did not have detectable vaccine virus in NW, demonstrated a significant association for peak titer by culture and anti-RSV F IgG ($P = .01$). The associations between peak culture titer and RSV $PRNT_{60}$ ($P = .06$) and between peak RT-qPCR copy number and anti-RSV F IgG ($P = .06$) were marginally significant (data not shown).

RSV Surveillance

During the RSV surveillance period, rates of MAARI did not differ significantly between the vaccine and placebo recipient groups (35% [90% CI, 18%–56%] vs 67% [90% CI, 35%–90%], $P = .23$). None of the vaccinees had an RSV-associated MAARI; 1 placebo recipient had RSV-MAARI, which was a URI.

Comparison of RSV antibody titers before and after the RSV season following vaccination allowed an evaluation of boosting of responses after presumed exposure to wt RSV. Of the participants with post-RSV season data, 8 of the 19 (42%; 90% CI, 23%–63%) vaccinees and 3 of 9 (33%; 90% CI, 9.6%–65%) placebo recipients exhibited a ≥4-fold increase in either $PRNT_{60}$ or anti-RSV F IgG titer compared to pre-RSV season. The post-RSV season $PRNT_{60}$ in 3 of the vaccinees was >1:1000 and substantially higher than the $PRNT_{60}$ for the 3 placebo recipients (Figure 4).

Assessment after the RSV season allowed evaluation of the durability of antibody responses in vaccinees who did not have a boosted response and thus were presumed not to have been

Table 3. Respiratory Syncytial Virus (RSV) Specific Serum Antibody Responses Before and After Inoculation and After RSV Surveillance Season

		Serum RSV Neutralizing Antibodies[a]					Serum IgG ELISA RSV F Antibodies[a]				
Group	No. of Children	Pre[b]	Post[c]	Post-RSV[d]	≥4-fold Rise Pre vs Post No. (%)[e]	≥4-fold rise Post vs Post-RSV No. (%)[f]	Pre[b]	Post[c]	Post-RSV[d]	≥4-fold Rise Pre vs Post No. (%)[e]	≥4-fold Rise Post vs Post-RSV No. (%)[f]
Vaccine	20	2.3 (2.3, 3.4)	7.3 (6.6, 8.5)	7.3[g] (6.7, 9.3)	18 (90)	6[g] (32)	5.6 (4.6, 7.6)	11.6 (10.6, 13.6)	11.6[g] (11.6, 13.6)	18 (90)	8[g] (42)
Placebo	9	2.3 (2.3, 3.9)	2.3 (2.3, 2.3)	2.3 (2.3, 6.0)	0 (0)	3 (33)	9.6 (7.6, 9.6)	7.6 (5.6, 9.6)	9.6 (5.6, 11.6)	0 (0)	2 (22)

Abbreviations: ELISA, enzyme-linked immunosorbent assay; $PRNT_{60}$, 60% plaque reduction neutralizing titer.

[a]Serum RSV $PRNT_{60}$ was determined by complement-enhanced 60% plaque reduction neutralization assay; serum IgG titers to RSV F were determined by ELISA. Titer results are expressed as median reciprocal $\log_2$ with the 25th and 75th percentiles indicated in parenthesis, determined for all participants in each group. Specimens with titers below the limit of detection were assigned reciprocal titers of 2.3 $\log_2$ ($PRNT_{60}$) and 4.6 $\log_2$ (ELISA).

[b]Prior to inoculation.

[c]Postinoculation at study day 56, collected 1 October to 12 December.

[d]Post-RSV season surveillance, collected 1 to 30 April at approximately 6 months postinoculation.

[e]Number and percentage of vaccine and placebo recipients with ≥4-fold increase in antibody titers between preinoculation and postinoculation.

[f]Number and percentage of vaccinee and placebo recipients with ≥4-fold increase in antibody titers between postinoculation and post-RSV season.

[g]One child (the child lacking vaccine virus shedding) had missing data at this time point.

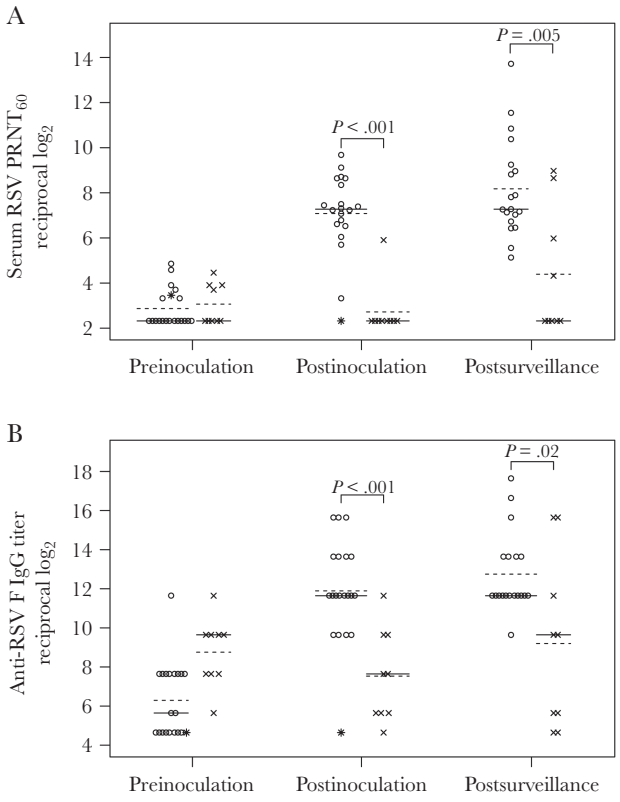

Figure 1. Serum respiratory syncytial virus (RSV) antibody titers in vaccine and placebo recipients. Serum RSV 60% plaque reduction neutralizing titers ($PRNT_{60}$) (*A*) and anti-RSV F IgG titers (*B*) were determined by complement-enhanced 60% plaque reduction neutralization assay and IgG-specific enzyme-linked immunosorbent assay (ELISA) against purified RSV F protein, respectively, for vaccine (open circles) and placebo (×) recipients in sera collected at preinoculation (screening), postinoculation (study day 56), and postsurveillance (after the RSV season, 1 to 30 April in the calendar year following the inoculation). Titers are expressed as the reciprocal $\log_2$. The lines indicate median (solid line) and mean value (dashed line). *P* values were determined by Wilcoxon rank sum test. One vaccine recipient who did not shed vaccine virus is indicated with an asterisk; this infant was lost to follow-up at the postsurveillance visit and the data for this time point are missing.

exposed to RSV during the surveillance period. In these 11 vaccinees, the pre- and post-RSV season median RSV $PRNT_{60}$ was minimally changed (median [IQR] 8.3 [7.1–8.7] vs 7.1 [6.4–7.8]). Similar results were observed for the pre- and post-RSV season anti-RSV F IgG titer (median [IQR] 13.6 [11.6–15.6] vs 11.6 [11.6–11.6]).

DISCUSSION

The RSV vaccine candidate LIDΔM2-2 had excellent immunogenicity in RSV-seronegative children ages 6–24 months, inducing substantial RSV-neutralizing serum antibodies in 90% of the vaccinees that persisted 6 months after vaccination. The postvaccine neutralizing antibody response in vaccinees

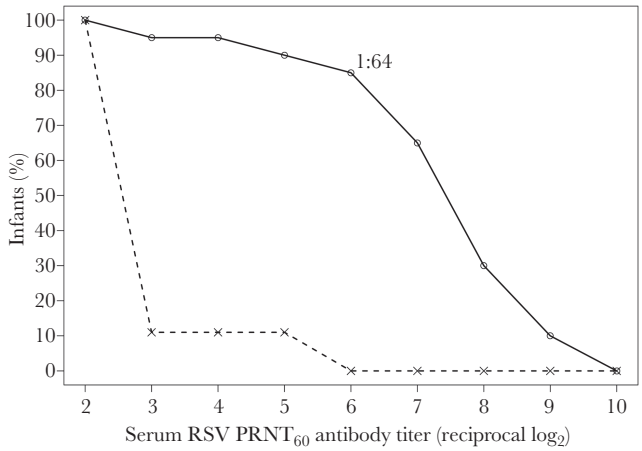

Figure 2. Reverse cumulative distribution of serum respiratory syncytial virus (RSV) neutralizing antibody titer postinoculation. Serum RSV plaque reduction neutralizing titers ($PRNT_{60}$) determined by complement-enhanced 60% plaque reduction neutralization assay in sera collected at 56 days after inoculation are expressed as reciprocal $\log_2$ for vaccine (solid line) and placebo (dashed line) recipients. For ease of interpretation, the arithmetic value (1:64) at the titer achieved by 85% of vaccinees is indicated.

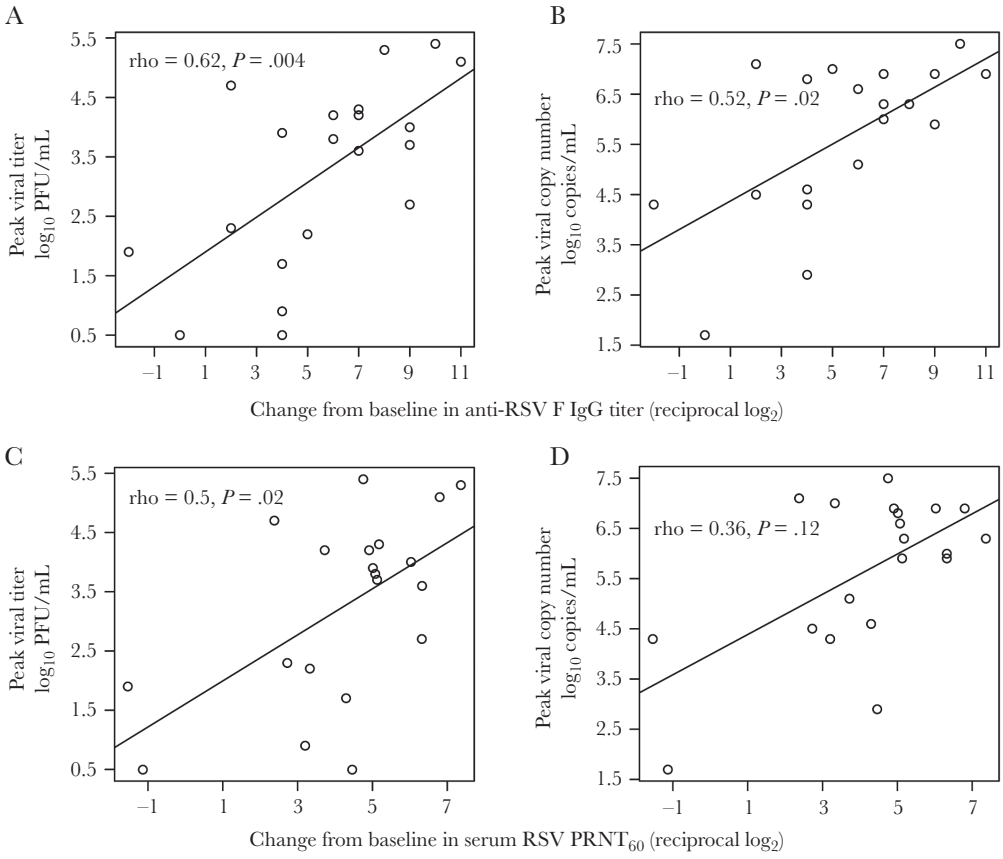

Figure 3. Correlation of the peak titer of vaccine virus in nasal washes and serum respiratory syncytial virus (RSV) antibody titer. Correlation of peak vaccine virus titers shed in nasal wash (NW), determined by culture (*A*, *C*) and RT-qPCR (*B*, *D*) with the change from baseline to study day 56 in anti-RSV F IgG titer (*A*, *B*) and RSV serum neutralizing antibody titer (*C*, *D*). Titers of vaccine virus determined by immunoplaque assay and expressed as $\log_{10}$ transformed number of plaque-forming units (PFU) per mL of NW. Log_{10} copy numbers of vaccine virus were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) specific for the RSV M gene. The limit of virus detection by titration and by RT-qPCR was 0.5 $\log_{10}$ PFU/mL and 1.7 $\log_{10}$ copy numbers/mL; samples below the limit of detection for the plaque assays and RT-qPRC were assigned a titer of 0.5 $\log_{10}$ PFU/mL and 1.7 $\log_{10}$ copy numbers/mL, respectively. Serum RSV 60% plaque reduction neutralizing titers (PRNT_{60}) and anti-RSV F IgG titers were determined by complement-enhanced 60% plaque reduction neutralization assay and IgG-specific enzyme-linked immunosorbent assay (ELISA) against purified RSV F protein, respectively, and titers expressed as reciprocal $\log_2$. Samples below the limit of detection were assigned the reciprocal titers of 2.3 $\log_2$ (PRNT_{60}) and 4.6 $\log_2$ (ELISA). Spearman correlation coefficients and *P* values are shown. The regression lines are included to give a descriptive summary of the directionality of the association, but statistical analysis consists of Spearman correlations, which avoid the assumption of normality.

(median PRNT_{60} 7.3 $\log_2$) was of similar magnitude to that in placebo recipients with primary wt RSV infection following RSV surveillance (median PRNT_{60} 8.7). The frequency of induction and magnitude of neutralizing antibody and anti-RSV F IgG responses was comparable to that observed in a previous study in a similar population vaccinated with RSV MEDIΔM2-2 [18] and was higher than that observed in studies of other live attenuated RSV candidate vaccines constructed with combinations of cold-passaged, temperature-sensitive, and RSV SH protein deletion mutations [14, 15, 24].

When participants were monitored during the subsequent RSV season, 42% of the vaccinees demonstrated 4-fold or greater increases in their RSV-specific serum antibody titers, suggesting that they had been exposed to wt RSV, but none of the exposures resulted in a medical visit. Several of these vaccine recipients had substantially greater PRNT_{60} after the RSV season than the placebo recipients, consistent with the interpretation that the vaccinees experienced anamnestic boosts compared to primary responses for the placebo recipients. Similarly strong post-RSV season boosting was observed with RSV MEDIΔM2-2 vaccine [18]. Furthermore, assessment of RSV-specific serum antibody titers alone provides an incomplete picture of the immune responses induced by a live intranasal RSV vaccine because, in contrast to passive antibody prophylaxis, the live vaccine results in local and systemic antibody responses as well as local and systemic cellular immunity, all of which may contribute to protection.

The median peak titer of vaccine virus shed by vaccinees in this study was approximately 100-fold higher than in recipients of MEDIΔM2-2, which ostensibly has a very similar attenuating mutation [15, 18]. Despite this, the rates of respiratory events following inoculation were similar for both vaccine and placebo recipients, and similar to studies with MEDI-559 [15] and MEDIΔM2-2 [15, 18]. As the detection of contemporaneous

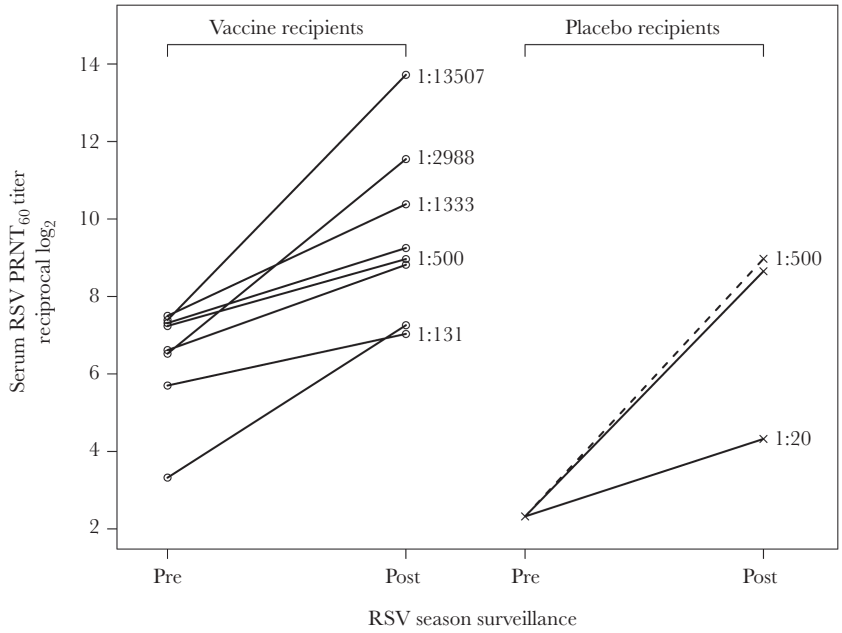

Figure 4. Rises in serum respiratory syncytial virus (RSV) neutralizing antibody titers, and incidences of medically attended RSV illness, during the RSV season surveillance. Serum RSV plaque reduction neutralizing titers ($PRNT_{60}$) in sera collected pre- and post-RSV season surveillance shown for vaccine (left) and placebo (right) recipients who had a 4-fold or greater increase in either serum RSV $PRNT_{60}$ or anti-RSV F IgG titer. Dashed and solid lines indicate subjects with and without a medically attended RSV-associated illness during surveillance. Note, 2 participants with ≥4-fold increase in anti-RSV F IgG titer had a smaller increase in serum neutralizing antibody titer. Titers are expressed as the reciprocal $\log_2$, but for ease of interpretation, titers corresponding to the arithmetic values of 1:20, 1:131, 1:500, 1:1333, 1:2988, and 1:13507 are indicated.

adventitious viral respiratory agents were common in both groups, the contribution of the higher than expected shed vaccine virus to the respiratory symptoms in the vaccinees is not clear. In the present study, 1 vaccinee recipient had a mild LRI that occurred during the time of vaccine virus shedding but was also concurrent with shedding of rhinovirus and enterovirus. Therefore, causality for this incident of LRI cannot be determined. However, the comparatively high level of LIDΔM2-2 vaccine shedding does raise potential safety concerns. In a previous study of a RSV vaccine virus (cpts248/404), young infants with peak vaccine shedding of 4.0–4.9 $\log_{10}$ PFU/mL had high rates of nasal congestion, resulting in difficulty feeding and sleeping [13]. In another study, a vaccine virus (cpts248/955) that replicated to a mean peak titer of 4.4 $\log_{10}$ PFU/mL was associated with LRI in 1 recipient [12]. In contrast, a vaccine with titers of 2.4–3.5 $\log_{10}$ PFU/mL was well-tolerated in infants [14]. In the current study, although the median peak virus titer of LIDΔM2-2 was 3.8 PFU/mL, peak titers exceeding 4 $\log_{10}$ PFU/mL were common. Higher vaccine shedding may be associated with more robust vaccine-induced immune responses, as suggested by our observed correlation of peak titers of shed vaccine virus and antibody responses and possible benefits of herd immunity. However, the mild reactogenicity among infants in prior studies with vaccines shed at higher titers raised concern that LIDΔM2-2 may be insufficiently attenuated for a younger population. This consideration led to the precautionary decision not to proceed with further evaluation of this vaccine.

The mechanism underlying the higher replication for LIDΔM2-2 versus MEDIΔM2-2 is not clear. The 2 viruses differ in several ways. They were made from 2 different laboratory stocks of RSV strain A2 (the wt parent of LID strain is GenBank KT992094) and also have differences in introduced restriction enzyme sites. This resulted in 21 nt differences scattered through the genome, 2 of which affect amino acid coding (K51R in the NS2 ORF, and T24A in the N ORF [25]). The design of the M2-2 deletion differed between the 2 viruses: the deletion is 241 nts in LIDΔM2-2 versus 234 nts in MEDIΔM2-2, and all 3 possible M2-2 translation initiation codons are silenced by point mutation in LIDΔM2-2, but not in MEDIΔM2-2. In addition, LIDΔM2-2 differs from MEDIΔM2-2 by a 112-nt deletion in the downstream noncoding region of the *SH* gene plus 5 silent mutations at the end of the *SH* ORF, which were introduced to increase cDNA stability in bacteria [19]. In particular the 112-nt deletion may confer a slight increase in replication, due to the slightly smaller genome size of this candidate. The systems available to evaluate replication, cell culture and in African green monkeys, were not sufficiently sensitive to detect phenotypic differences between these 2 vaccine candidates, but the present study indicates that they appear to be distinct in the natural human host. Thus, we conclude that RSV LIDΔM2-2 was less restricted in replication due to genetic differences previously not recognized as phenotypically relevant. Effects of the genetic differences are presently under evaluation.

This study has several limitations. The small sample size precludes firm conclusions regarding rates of vaccine-associated events. The immune assessment included only the most established correlates of protection against RSV disease, namely serum RSV neutralizing and serum anti-RSV F IgG titers. IgG antibodies to the prefusion conformation of RSV F, which are more likely to be neutralizing than antibodies to the postfusion conformation of RSV F [26], were not measured due to specimen volume limitations. Future studies could include assays to assess antibodies specific to the RSV prefusion protein, RSV-neutralizing antibodies in NW, and cellular immune responses.

In summary, the LIDΔM2-2 vaccine induced substantial titers of RSV-neutralizing serum antibodies that, during the subsequent RSV season, were boosted without medically attended RSV disease. The observations with serum antibody responses should also extend to local and systemic cellular and humoral responses. Unexpectedly, the LIDΔM2-2 virus appeared to be less restricted in replication compared to MEDIΔM2-2. Because a vaccine with a lower range of peak viral titers may be better tolerated, we have generated 2 alternative versions of LIDΔM2-2, 1 with addition of a temperature sensitivity mutation in the L protein and another with 5 point mutations in the N, F, and L proteins previously identified in a cold-passaged attenuated RSV strain. These vaccines are currently under investigation (NCT02794870; NCT02890381) to identify those with optimal levels of attenuation and immunogenicity. Overall, this study supports the hypothesis that the shift in viral RNA synthesis from RNA replication to gene transcription that results from deletion of the ΔM2-2 ORF increases RSV immunogenicity and fundamentally improves the possibility of an effective live-attenuated RSV vaccine [18].

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Disclaimer. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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Potential conflicts of interest. U. J. B. and P. L. C. are listed as inventors on patents related to live-attenuated RSV vaccines, including vaccines made by ablating expression of the M2-2 ORF with royalties paid by Sanofi Pasteur. U. J. B., P. L. C., and C. L. received research support from Sanofi Pasteur outside the submitted work. No additional conflicts of interests or financial disclosures are reported by any authors. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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