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MAJOR ARTICLE

Safety and Immunogenicity of the Respiratory Syncytial Virus Vaccine RSV/ΔNS2/Δ1313/I1314L in RSV-Seronegative Children

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(See the Editorial Commentary by Ramilo et al., on pages 4–6.)

Background. Respiratory syncytial virus (RSV) is the leading global cause of severe pediatric acute respiratory tract illness, and a vaccine is needed. RSV/ΔNS2/Δ1313/I1314L contains 2 attenuating elements: (1) deletion of the interferon antagonist NS2 gene and (2) deletion of codon 1313 of the RSV polymerase gene and the stabilizing missense mutation I1314L. This live vaccine candidate was temperature-sensitive, genetically stable, replication restricted, and immunogenic in nonhuman primates.

Methods. A single intranasal dose of RSV/ΔNS2/Δ1313/I1314L was evaluated in a double-blind, placebo-controlled trial (vaccine-placebo ratio, 2:1) at 10^6 plaque-forming units (PFU) in 15 RSV-seropositive children and at 10^5 and 10^6 PFU in 21 and 30 RSV-seronegative children, respectively.

Results. In RSV-seronegative children, the 10^5 PFU dose was overattenuated, but the 10^6 PFU dose was well tolerated, infectious (RSV/ΔNS2/Δ1313/I1314L replication detected in 90% of vaccinees), and immunogenic (geometric mean serum RSV plaque-reduction neutralizing antibody titer, 1:64). After the RSV season, 9 of 20 vaccinees had increases in the RSV titer that were significantly greater than those in 8 of 10 placebo recipients (1:955 vs 1:69, respectively), indicating that the vaccine primed for anamnestic responses after natural RSV exposure.

Conclusion. Rational design yielded a genetically stable candidate RSV vaccine that is attenuated yet immunogenic in RSV-seronegative children, warranting further evaluation.

Clinical Trials Registration. NCT01893554.

Keywords. RSV; vaccine; pediatric; live-attenuated RSV vaccine.

Respiratory syncytial virus (RSV) is the most important cause of severe acute lower respiratory illness (LRI) in infants and children worldwide [1, 2], and the relative importance of RSV has increased as the burden of bacterial pneumonia has declined with vaccine implementation [3]. According to global estimates, RSV caused approximately 33 million cases of LRI and approximately 118 000 deaths in children <5 years of age in 2015 [2]. More than 80% of all RSV-associated LRIs (RSV-LRIs) and more than half of the RSV-associated deaths in low- and middle-income countries were estimated to occur in infants ≥6 months old, highlighting the importance of developing RSV vaccines for active immunization of infants and children [4].

Live attenuated intranasal RSV vaccines are attractive for pediatric immunization because they mimic mild natural infections and induce durable cellular, humoral, local and systemic immunity. Furthermore, candidate live attenuated RSV vaccines [5–12] have not caused the vaccine-associated enhanced RSV disease that was observed in children who received formalin-inactivated RSV [13] and that also seemed to be associated with administration of RSV subunit vaccines in experimental animals [14–16].

Progress in the elucidation of RSV gene function [17] and the use of reverse genetics systems [18] led to the development of rationally designed attenuated RSV strains, including strains attenuated through deletion of the NS2 gene. NS2 is a virally encoded type I and III interferon antagonist that interferes with interferon induction and signaling [19–21]. Deletion of the NS2 gene diminished RSV replication in chimpanzees [22]. The increased interferon response to infection may enhance the adaptive immune response, as has been demonstrated for bovine RSV with NS1 or NS2 deletion in calves [23]. NS2 also functions as a pathogenicity factor, promoting epithelial cell shedding in vitro and in the hamster model, potentially contributing to small airway obstruction [24]. Thus, deletion of NS2 may be beneficial for

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vaccine safety. Recently, a candidate vaccine was developed that contains the NS2 deletion and the attenuating deletion of codon 1313 in the polymerase (L) gene, which also confers mild temperature sensitivity (shutoff temperature of 38°C–39°C [24];). This deletion was genetically and phenotypically stabilized by substitution of leucine (L) for isoleucine (I) at codon 1314. The resultant virus, RSV/ΔNS2/Δ1313/I1314L, was attenuated and immunogenic in nonhuman primates [25].

Based on this preclinical profile, we conducted a stepwise phase I evaluation of RSV/ΔNS2/Δ1313/I1314L in RSV-seropositive and RSV-seronegative children. In RSV-seronegative children, RSV/ΔNS2/Δ1313/I1314L was restricted in replication, well tolerated, immunogenic, and primed for potent antibody responses after natural exposure to wild-type (wt) RSV.

MATERIALS AND METHODS

Vaccine

RSV/ΔNS2/Δ1313/I1314L was derived from a recombinant version of wt RSV strain A2 (rD46 [22]; GenBank accession no. KT992094) with the further modification of a 112 nucleotide phenotypically silent deletion in the SH noncoding sequence that stabilizes the complementary DNA (cDNA) during propagation in bacteria [26]. RSV/ΔNS2/Δ1313/I1314L contains 2 independent attenuating elements: (1) a 523 nucleotide deletion of the NS2 gene and (2) a codon deletion in the L gene (Δ1313; deletion of S1313) plus the adjacent missense mutation I1314L that prevents the compensatory deattenuating mutation I1314T [25]. The virus was generated from cDNA on World Health Organization Vero cells by reverse genetics [18], and clinical trial material (CTM) was prepared at Charles River Laboratories. Sequence analysis confirmed that the seed virus and CTM were free of detectable adventitious mutations. The CTM had a mean infectivity titer of $10^{7.3}$ plaque-forming units (PFU)/mL. CTM was stored at −70°C and diluted to dose on site using Leibovitz L15 medium. L15 medium was used as placebo.

Study Population, Study Design, and Clinical Trial Oversight

This phase 1 trial was conducted at the Center for Immunization Research (CIR), Johns Hopkins Bloomberg School of Public Health, between June 2013 and April 2018 (ClinicalTrials.gov NCT01893554). A single dose of RSV/ΔNS2/Δ1313/I1314L was evaluated sequentially in randomized, double-blind, placebo-controlled studies in RSV-seropositive children aged 12–59 months at a dose of 10^6 PFU and in RSV-seronegative children at a dose of 10^5 or 10^6 PFU (Figure 1). Children were randomized 2:1 to receive vaccine or placebo, administered as nose drops (0.5 mL; approximately 0.25 mL per nostril). Randomization, blinding, and unblinding were performed as described elsewhere [10].

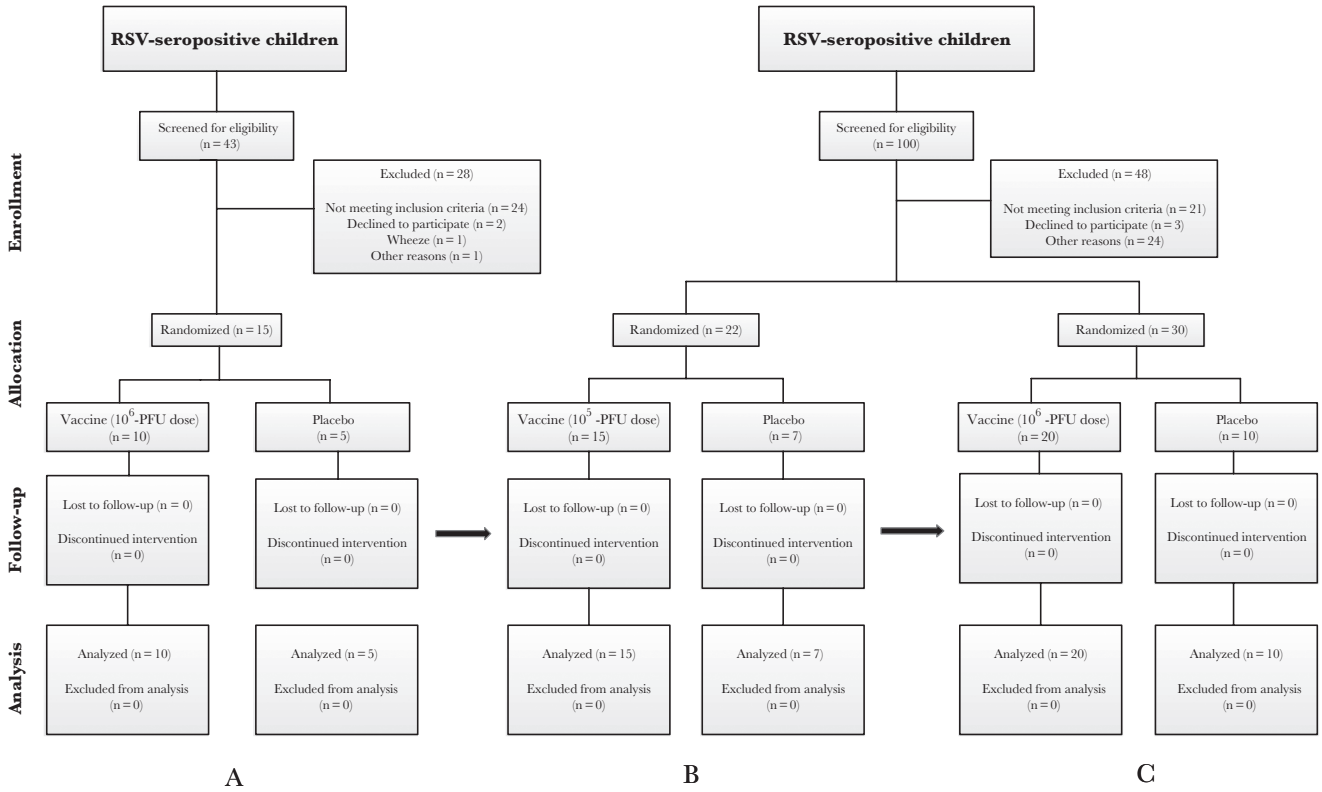

Figure 1. Screening, enrollment, and follow-up of RSV-seropositive children (panel A) and RSV-seronegative children (panels B and C) in the phase I clinical trial of the RSV/ΔNS2/Δ1313/I1314L vaccine. As described in Methods, enrollment in each of the cohorts depicted in A, B, and C occurred sequentially following a satisfactory safety review of data from the previous cohort.

Written informed consent was obtained from parents of study participants before enrollment. The study was 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) under the National Institute of Allergy and Infectious Diseases (NIAID)–held Investigational New Drug application (IND15465), reviewed by the US Food and Drug Administration. The clinical protocol, consent forms, and Investigator's Brochure were developed by CIR and NIAID investigators and approved by the Western Institutional Review Board and the NIAID Office of Clinical Research Policy and Regulatory Operations. Clinical data were reviewed by CIR and NIAID investigators and by the Data Safety Monitoring Board of the NIAID Division of Clinical Research.

Clinical Assessment: Acute Phase

Children were enrolled between April 1 and October 31 each year, outside the RSV season. Clinical assessments were performed and nasal wash (NW) samples obtained as described elsewhere (RSV-seropositive children, study days 0, 3–7 and 10; RSV-seronegative children, study days 0, 3, 5, 7, 10, 12, 14, 17, 19, 21, 28 ± 1 day at each time point) [10]. Adverse events were collected through day 28 for RSV-seropositive and RSV-seronegative children; serious adverse events and LRIs were collected through day 56 for RSV-seronegative children, with additional physical examinations performed and NW samples obtained in the event of LRI. Fever, upper respiratory illness (URI; including rhinorrhea, pharyngitis, and hoarseness), cough, LRI, and otitis media were defined as described elsewhere [27]. When illnesses occurred, NW samples were tested for other viruses and mycoplasma by means of real-time reverse-transcription polymerase chain reaction (RT-PCR) (Respiratory Pathogens 21 kit; Fast Track Diagnostics, Luxembourg).

Clinical Assessment: Surveillance

RSV-seronegative participants were monitored for medically attended acute respiratory illness (MAARI), which included medically attended acute LRI (MAALRI), during the first RSV season after inoculation [6]. After the first RSV season, parents of children in the 10^6 PFU dose cohort were asked to participate in an optional second season of RSV surveillance.

During RSV surveillance (1 November through 31 March), families were contacted weekly to determine whether MAARI had occurred [6, 10]. For each illness, a clinical assessment was performed and a NW sample was obtained for adventitious agent testing. RSV-positive specimens were typed as RSV A or B [10].

Table 1. Clinical Responses and Shedding of Vaccine Virus Among Recipients of RSV/ΔNS2/Δ1313/I1314L or Placebo

			Vaccine Detection in NW Samples[a]				Children With Indicated Symptoms, %[b]						
Children	Dose, Log_{10} PFU/mL	No. of Children	Children Infected With Vaccine Virus, %[c]	Children Shedding Vaccine Virus, %[d]	Plaque Assay Titer, Mean (SD), Log_{10} PFU/mL[e]	RT-qPCR Titer, Mean (SD), Log_{10} Copies/mL[f]	Fever	URI	LRI	Cough	Otitis Media	Respiratory or Febrile Illness	Other
RSV seropositive													
Vaccinees	6.0	10	0	0	0.5 (0.0)	1.7 (0.0)	0	20	0	10	0	30	0
Placebo recipients	Placebo	5	0	0	0.5 (0.0)	1.7 (0.0)	0	0	0	0	0	0	0
RSV seronegative													
Vaccinees	5.0	15	80	73	0.6 (0.3)	3.0 (0.6)	20	73	0	13	7	73	67
Placebo recipients	Placebo	7	14[g]	0	0.5 (0.0)	1.7 (0.0)	0	57	0	29	0	57	14
Vaccinees	6.0	20	100	90	1.8 (0.9)	3.5 (0.5)	10	50	0	5	0	55	45
Placebo recipients	Placebo	10	0	0	0.5 (0.0)	1.7 (0.0)	40	40	20	30	10	80	70

Abbreviations: LRI, lower respiratory infection; NW, nasal wash; OM, otitis media; PFU, plaque-forming units; RT-qPCR, quantitative reverse-transcription polymerase chain reaction; RSV, respiratory syncytial virus; SD, standard deviation; URI, upper respiratory infection.

[a]For each child, the individual peak (highest) titer, irrespective of day, was selected from among all titers measured in the NW sample. Geometric means were calculated for participants who were infected with vaccine (see note c below on the definition of infection with vaccine virus).

[b]Illnesses are as described in the text. URI was defined as rhinorrhea, pharyngitis, or hoarseness, and LRI as wheezing, rhonchi, rales, or diagnosis with pneumonia or laryngotracheobronchitis (croup). Other symptoms included rash, conjunctivitis, nasal congestion, diarrhea, and vomiting.

[c]Infection with vaccine virus was defined as the detection of vaccine virus by means of 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.

[d]Shedding of vaccine virus as detected by means of culture and/or real-time RT-qPCR. The limit of detection of vaccine virus was 0.5 log_{10} PFU/mL for culture and 1.7 log_{10} copies/mL for RT-qPCR.

[e]The lower limit of detection for the RSV immunoplaque assay was 0.5 log_{10} PFU/mL.

[f]The lower limit of detection for RT-qPCR was 1.7 log_{10} copies/mL.

[g]A >4 fold-rise in serum RSV neutralizing antibody was detected in a placebo recipient in the 10^5 PFU dose cohort, probably indicating infection with wild-type RSV between study days 28 and 56.

Isolation, Quantitation, and Characterization of Virus

Vaccine virus in NW fluid was quantified by immunoplaque assay using a mix of 3 monoclonal antibodies (mAbs) to RSV F (mAbs 1129, 1243, and 1269 [28]) and by quantitative RT-PCR (RT-qPCR) [10]. To verify the presence and genetic stability of the attenuating elements at time of peak vaccine shedding, viral RNA was obtained from a single passage of NW fluid on Vero cells. The presence of the NS2 gene deletion was verified by agarose gel electrophoresis, confirming an 855 base pair RT-PCR amplicon spanning the deletion. The presence of the 1313 deletion and the I1314L mutation was confirmed by sequence analysis of a 758 base pair PCR fragment of the L gene [12].

Immunologic Assays

Serologic Specimens

Serum samples were obtained before inoculation and approximately 1 month after inoculation of RSV-seropositive participants and 2 months after inoculation of RSV-seronegative participants. To measure serum antibody responses after exposure to wt RSV, serum samples were obtained from RSV-seronegative participants in October of the calendar year in which the child was enrolled and in April of the following calendar year; for participants enrolled in September or October, the postvaccination serum samples also served as pre-RSV season serum samples. Of 30 RSV-seronegative children enrolled in the 10^6 PFU dose cohort, 22 participated in second-year RSV surveillance, with serum samples obtained before and after the second surveillance season.

Antibody Assays

Serum samples were tested for RSV neutralizing antibodies using a complement-enhanced 60% RSV plaque-reduction neutralization assay [29], and for immunoglobulin G (IgG) antibodies to the RSV F glycoprotein using an enzyme-linked immunosorbent assay [10]. The plaque reduction neutralization titer (PRNT) and RSV F IgG titer are expressed as reciprocal $\log_2$ values. Antibody responses were defined as ≥4-fold increases in titer in paired specimens.

Data Analysis

Infection with vaccine was defined as detection of vaccine virus by culture or RT-qPCR and/or a ≥4-fold rise in RSV PRNT or in RSV F IgG. The mean peak titer of vaccine virus shedding (in $\log_{10}$ PFU/mL) was calculated for infected vaccinees only. PRNT and RSV F IgG titers were transformed to $\log_2$ values for calculation of means, and the Student *t* test was used to compare means between groups. Rates of illness and antibody responses were compared using the 2-tailed Fisher exact test.

RESULTS

Study Participants

RSV/ΔNS2/Δ1313/I1314L was sequentially evaluated in 15 RSV-seropositive children (10 vaccinees and 5 placebo recipients), 22 RSV-seronegative infants and children at 10^5 PFU (15 vaccinees and 7 placebo recipients), and 30 RSV-seronegative infants and children at 10^6 PFU (20 vaccinees and 10 placebo recipients; Figure 1 and Table 1) between April 2013 and April 2018. None were lost to follow-up or excluded from analysis. The mean age was 30.1 months (range, 13–51 months) for RSV-seropositive participants; 11.6 months (6–22 months) for RSV-seronegative participants in the 10^5 PFU dose cohort, and 12.8 months (6–23 months) for RSV-seronegative participants in the 10^6 PFU dose cohort. Of the 67 participants, 49% were female, 69% white, 8% black, 3% Asian, and 20% described as of mixed racial heritage; 8% were Hispanic, and 92% were non-Hispanic.

Adverse Events and Vaccine Infectivity

In RSV-seropositive participants, URI was observed in 2 and cough was observed in 1 of 10 vaccinees during the 28-day postimmunization reporting period (Table 1); in each case, rhinovirus was detected in NW samples at the time of illness. None of the vaccinees shed vaccine virus, indicative of attenuation.

In RSV-seronegative participants, URI (rhinorrhea or pharyngitis), cough, and febrile illnesses occurred frequently. Overall, respiratory or febrile illnesses were observed during

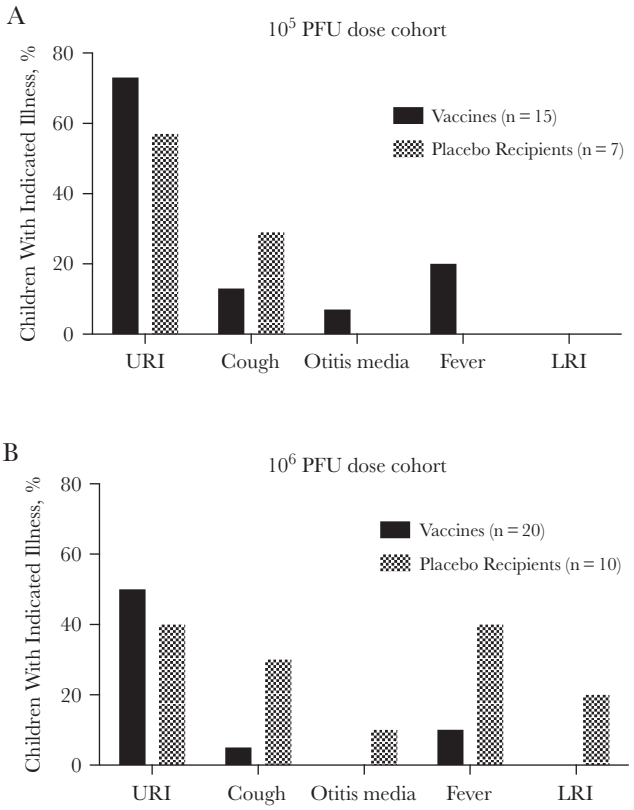

Figure 2. Proportions of RSV-seronegative vaccinees and placebo recipients with indicated illnesses in the 105 PFU cohort (panel A) or the 106 PFU cohort (panel B). Vaccinees are shown in black; placebo recipients are shown in gray.

the acute phase in 11 of 15 vaccinees (73%) versus 4 of 7 placebo recipients (57%) for the 10^5 PFU dose cohort and in 11 of 20 vaccinees (55%) versus 8 of 10 placebo recipients (80%) for the 10^6 PFU dose cohort, including 2 LRIs in placebo recipients (with onset on days 33 and 45 after placebo administration) (Table 1 and Figure 2]. Although URI (rhinorrhea) occurred more frequently in vaccinees (in 73% vs 57% of placebo recipients in the 10^5 PFU cohort, and in 50% vs 40%, respectively, in the 10^6 PFU cohort) (Figure 2 and Table 1), these differences were not statistically significant, and the incidence of rhinorrhea did not increase with increased vaccine dose. One vaccinee and 1 placebo recipient had otitis media.

Other viruses were detected in 10 of 22 and 7 of 12 symptomatic RSV-seronegative vaccine and placebo recipients, respectively, including rhinovirus, enterovirus, adenovirus, coronavirus, bocavirus, and parainfluenza virus (PIV) type 3. The NW samples obtained within 3 days after illness onset from the 22 symptomatic vaccinees revealed other respiratory viruses only (5 children), other respiratory viruses and vaccine virus (5 children), vaccine virus alone (5 children), or neither vaccine virus nor other respiratory viruses (7 children).

Interestingly, during the genetic stability testing of the vaccine isolates from NW sample, the presence of wt RSV A was detected, together with vaccine, on day 10 after immunization in a child who had received 10^6 PFU of vaccine. This vaccinee developed grade 1 rhinorrhea 4 days after detection of both viruses in the NW. However, there were many instances in which a potential causative agent was not detected. For example, only 7 of 12 symptomatic RSV-seronegative placebo recipients had other viruses detected by RT-PCR; of the 5 in whom none were detected, 2 had LRI (1 episode of croup and 1 episode of croup and stridor).

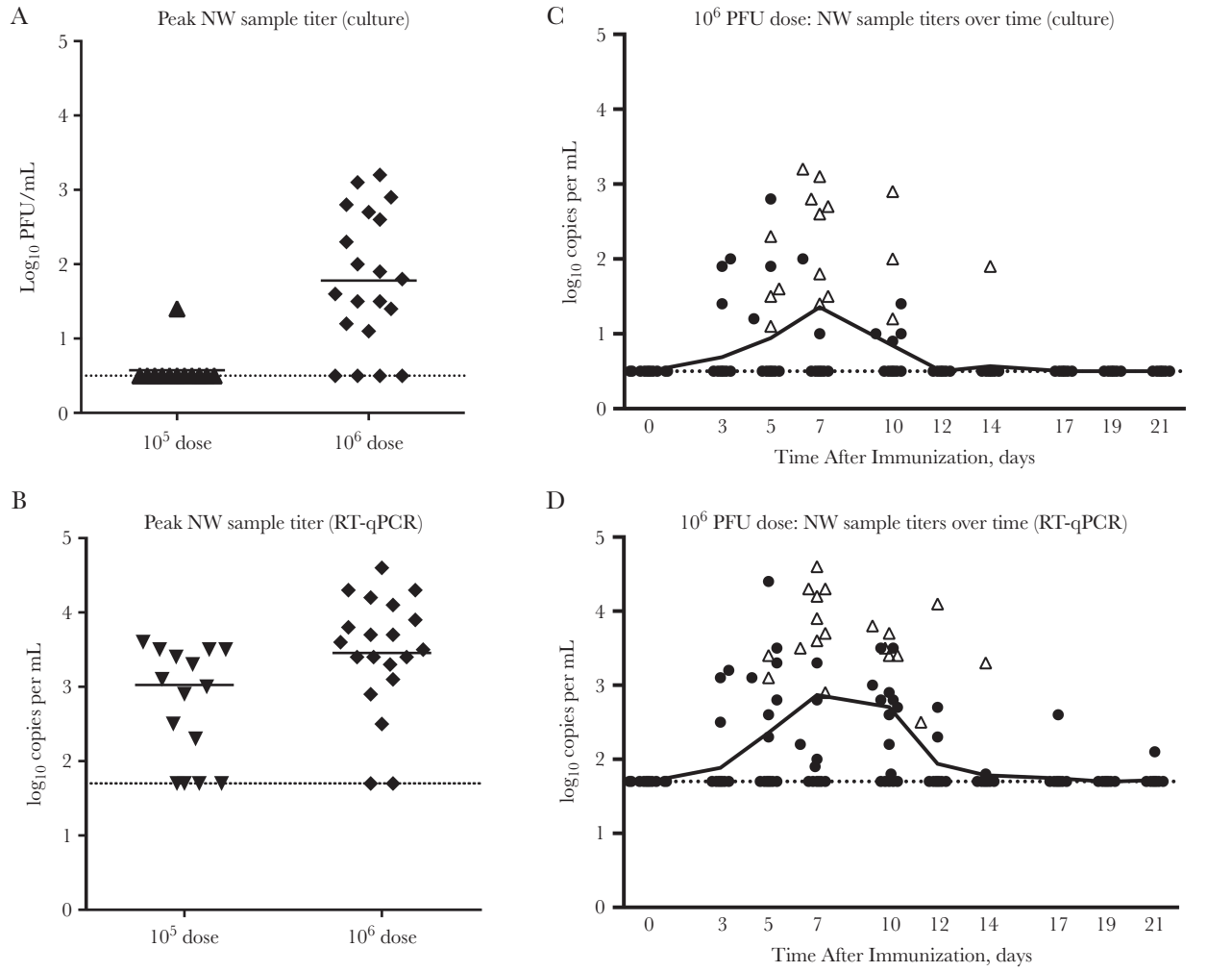

Figure 3. Peak titers of vaccine virus shed as detected by culture (*A*) and quantitative reverse-transcription polymerase chain reaction (RT-qPCR) (*B*) among RSV-seronegative recipients of 10^5 or 10^6 plaque-forming units (PFU) of vaccine. *C, D,* Individual daily titers by culture (*C*) and RT-qPCR (*D*) for RSV-seronegative recipients of the 10^6 PFU dose. Triangles represent peak titers from individual vaccine recipients; continuous lines, mean values; and dotted lines, titers assigned for culture-negative (0.5 $\log_{10}$ PFU/mL) (*C*) and RT-qPCR–negative (*D*) (1.7 $\log_{10}$ copies/mL) nasal wash (NW) samples.

Replication and Genetic Stability of RSV/ΔNS2/Δ1313/I1314L in RSV-Seronegative Children

At the 10^5 PFU dose, vaccine virus was detected in NW samples by culture on a single day in 1 of 15 vaccinees (titer, $10^{1.4}$ PFU) and by RT-qPCR in 11 of 15 vaccinees (mean peak copy number, $10^{3.0}$; Table 1 and Figure 3A and 3B). Infectivity and replication of this vaccine was significantly improved with an increased dose: at 10^6 PFU, vaccine virus was detected in 16 of 20 vaccinees by culture ($P < 0.0001$; Fisher exact test) and in 18 of 20 by RT-qPCR (Table 1 and Figure 3A and 3B), and most vaccinees shed vaccine virus over several days (Figure 3C and 3D). For most vaccinees, the peak of vaccine shedding was detectable by culture and by RT-qPCR between days 5 and 10 (Figure 3C and 3D; triangles).

At both the 10^5 PFU and 10^6 PFU doses, the magnitude of vaccine virus replication was highly restricted (mean peak titer by culture, $10^{0.6}$ and $10^{1.8}$ PFU; mean peak copy number by RT-qPCR, $10^{3.0}$ and $10^{3.5}$ among those who were infected) (Table 1 and Figure 3A and 3B), indicative of the substantial attenuation of this vaccine. RT-PCR and partial sequence analysis of NW isolates obtained at the peak of vaccine shedding from 18 of 20 RSV-seronegative vaccinees who received 10^6 PFU confirmed the presence of the NS2 deletion and the Δ1313 and I1314L mutations.

Antibody Responses to RSV/ΔNS2/Δ1313/I1314L

None of the RSV-seropositive vaccinees had a ≥4-fold rise in RSV F serum IgG titer or PRNT (Table 2). Eight of 15 RSV-seronegative children who received 10^5 PFU had RSV neutralizing antibody and F IgG responses; in contrast, RSV neutralizing antibody responses occured in 16 of 20 and F IgG responses in 17 of 20 RSV-seronegative children who received 10^6 PFU (18 of 20 total; Table 2 and Figure 4). For recipients of 10^5 PFU, the mean postvaccination PRNT was 5.2 $\log_2$, or 1:37 (Table 2); for recipients of 10^6 PFU, it was 6.0 $\log_2$, or 1:64 (Table 2 and Figure 4). There was no apparent correlation between the magnitude of viral shedding as measured by culture or RT-qPCR and neutralizing or RSV F IgG antibody responses (data not shown). One placebo recipient in the 10^5 PFU dose cohort had a rise in RSV PRNT at day 56 (4.8 $\log_2$, or 1:28); this child was presumed to have been infected with wt RSV between days 28 and 56 (Table 2).

Surveillance During 2 RSV Seasons: Illness and Antibody Response

All 52 RSV-seronegative children participated in RSV surveillance during the first season after inoculation, and 22 of 30 (16 vaccinees and 6 placebo recipients) in the 10^6 PFU dose cohort participated during the second RSV season.

First RSV Surveillance Season

All-cause MAARI was frequent, occurring in 52% of children (27 of 52), with many children experiencing multiple episodes (52 episodes in 27 children). All-cause MAALRI occurred in 19% (10 of 52). In the 10^5 PFU cohort, RSV-associated MAARI occurred in 2 of 15 vaccinees (1 RSV A and 1 RSV B) and in no placebo recipients. Four fold or greater increases in RSV PRNT after the first RSV surveillance season were detected in 7 of 15 vaccinees in the 10^5 PFU cohort (including 1 of 2 with RSV-associated MAARI [RSV-MAARI]), and 4 of 7 placebo recipients (Table 2). Unexpectedly, 1 vaccinee in the 10^5 PFU (not shown) and 1 in the 10^6 PFU dose cohort (Figure 5A, *right*) experienced laboratory-confirmed RSV-MAARI without an increase in PRNT. In each case, several other viruses were detected, and these may have reduced RSV replication or immunogenicity.

Table 2. Antibody Responses to Respiratory Syncytial Virus (RSV) Vaccination and Wild-Type RSV Infection Among Recipients of RSV/ΔNS2/Δ1313/I1314L or Placebo

			Serum RSV Neutralizing Antibody[a]						Serum IgG RSV F Antibody[a]		
Children	Dose, $\log_{10}$ PFU/mL	No. of children	Pre (SD)	Post (SD)	≥4 Fold Rise, %	Pre-SS (SD)	Post-SS (SD)	≥4 Fold Rise, %	Pre (SD)	Post (SD)	≥4 Fold Rise, %
RSV seropositive											
Vaccinees	6.0	10	7.7 (1.2)	7.5 (1.1)	0	ND	ND	ND	12.6 (1.1)	12.4 (1.0)	0
Placebo recipients	Placebo	5	7.4 (0.5)	7.0 (0.8)	0	ND	ND	ND	12.8 (1.1)	12.8 (1.1)	0
RSV seronegative											
Vaccinees	5.0	15	2.9 (1.0)	5.2 (1.7)	53	5.2 (1.6)	6.9 (2.4)	47	8.0 (2.9)	11.5 (2.2)	53
Placebo recipients	Placebo	7	2.8 (0.8)	2.7 (0.9)	14[b]	2.7 (0.9)	4.9 (2.4)	57	6.1 (2.3)	6.2 (2.3)	14
Vaccinees	6.0	20	2.4 (0.6)	6.0 (1.9)	80	5.6 (1.5)	7.1 (2.9)	40	7.2 (2.3)	13.0 (2.4)	85
Placebo recipients	Placebo	10	2.3 (0.0)	2.4 (0.4)	0	2.4 (0.4)	5.3 (1.7)	80	6.4 (1.6)	5.6 (1.4)	0

Abbreviations: IgG, immunoglobulin G; ND, not done; PFU, plaque-forming units; Post, after inoculation; Post-SS, after surveillance season; Pre, before inoculation; Pre-SS, before surveillance season; RSV, respiratory syncytial virus; SD, standard deviation.

[a]Antibody data are expressed as reciprocal mean $\log_2$ titers. Postinoculation antibody titers were measured at day 28 in RSV-seropositive children and at day 56 in RSV-seronegative children. For RSV-seronegative children, serum samples were also collected and assayed before and after the surveillance season; this was not done for RSV-seropositive children. RSV plaque reduction neutralizing titers (PRNT) were determined by means of complement-enhanced 60% plaque reduction neutralization assay; serum IgG titers to RSV F were determined by means of enzyme-linked immunosorbent assay (ELISA). Titers below the limit of detection were assigned values of 2.3 $\log_2$ (PRNT) and 4.6 $\log_2$ (ELISA).

[b]A >4 fold-rise in serum RSV neutralizing antibody was detected in a placebo recipient in the 10^5 PFU dose cohort, probably indicating infection with wild-type RSV between study days 28 and 56.

In the 10^6 PFU cohort, RSV-MAARI occurred in 4 of 20 vaccinees (3 RSV A and 1 RSV B) (Figures 4A and 5A, *left and right*; triangles) and in 3 of 10 placebo recipients (2 RSV A and 1 RSV B) (Figures 4B and 5B; triangles). However, in 3 of 4 cases of putative RSV-MAARI in vaccinees and 1 of 3 in the placebo group, additional viruses were isolated, so causality is unclear. The fourth RSV-MAARI in a vaccinee was associated with RSV B infection. The placebo recipient with a viral coinfection experienced MAALRI (grade 3 croup), with both RSV A and PIV type 2 detected.

Four fold or greater increases in RSV PRNT after the first RSV surveillance season were detected in 8 of 20 vaccinees (Table 2 and Figures 4A and 5A), including 3 of 4 with RSV-MAARI (Figure 5A, *left*; triangles), and in 8 of 10 placebo recipients (Table 2 and Figures 4B and 5B; triangles), including 3 with RSV-MAARI. The mean postsurveillance PRNT in these 8 placebo recipients (6.1 $\log_2$) (Figure 4B; circled) was comparable to the mean postvaccination PRNT in vaccinees in the 10^6 PFU dose cohort (6.0 log) (Figure 4A and Table 2), suggesting that the RSV neutralizing antibody response to the vaccine was comparable to that induced by primary wt RSV infection. There were no differences in the magnitude of the PRNT in children with medically attended or inapparent RSV infections (Figure 4; dots compared to triangles).

In all, 9 RSV-seronegative vaccinees who received 10^6 PFU had evidence of wt RSV infection during surveillance, as determined by rise in PRNT and/or viral detection. Of note, the postsurveillance PRNT in these 9 vaccinees was significantly greater than in the 8 placebo recipients (9.9 $\log_2$ vs 6.1 $\log_2$, or 1:955 vs 1:69; $P < 0.0001$) (Figure 4A and 4B) indicating that a single intranasal dose of RSV/ΔNS2/Δ1313/I1314L primed for potent anamnestic responses to wt RSV infection.

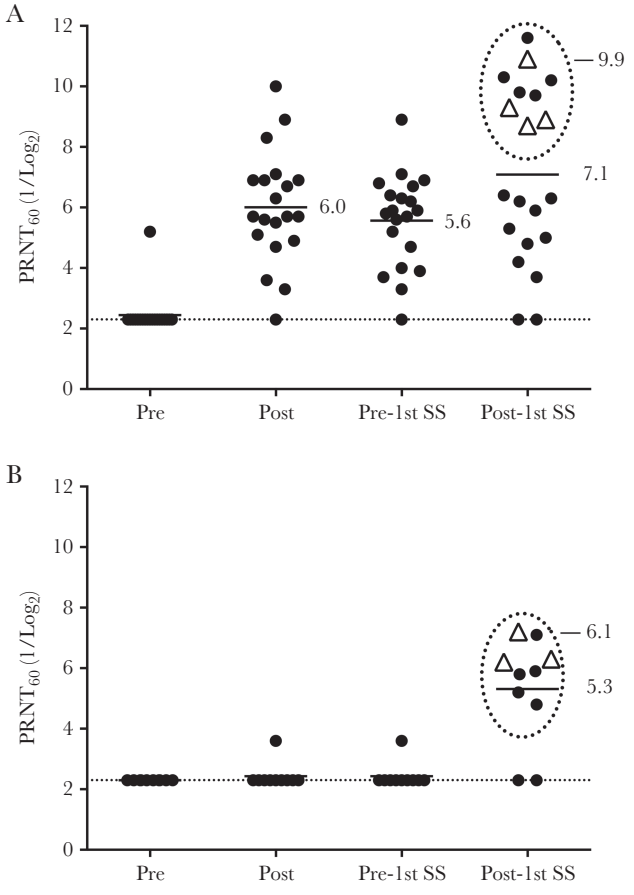

Figure 4. Respiratory syncytial virus (RSV) plaque reduction neutralization antibody titers (PRNT) in RSV-seronegative recipients of 10^6 plaque-forming units of vaccine (*A*) or placebo (*B*), measured before immunization (Pre), 56 days after immunization (Post), before the first RSV surveillance season (Pre–1st SS) and after the first RSV surveillance season (Post–1st SS). Triangles represent children with RSV-associated medically attended acute respiratory illness (RSV-MAARI); solid lines, means for the postinoculation, presurveillance, and postsurveillance time points; symbols enclosed by dotted line, titers from vaccinees with evidence of wild-type RSV infection during surveillance (≥4-fold increases in PRNT, RSV-MAARI, or both), with means from these subsets provided next to enclosed symbols.

Second RSV Surveillance Season

All-cause MAARI was again frequent (13 episodes in 9 of 22 children; 41%), including 1 mild episode of RSV-associated MAALRI in a 28-month-old vaccinee with congestion, cough, and posttussive emesis beginning 473 days after vaccination. At physical examination, she was afebrile and not in respiratory distress, but crackles were heard on auscultation. RSV A was detected as a single pathogen. Increases in RSV PRNT of ≥4-fold were detected in 6 of 16 vaccinees and 2 of 6 placebo recipients (Figure 5A, *middle,* and Figure 5B). No vaccinee had a ≥4-fold increase in PRNT during both seasons, but 2 placebo recipients did (Figure 5B).

DISCUSSION

RSV/ΔNS2/Δ1313/I1314L was created by reverse genetics, using knowledge of RSV gene function and known attenuating mutations engineered for genetic stability. The vaccine was attenuated by deletion of the interferon antagonist NS2 gene and the insertion and stabilization of an attenuating *ts* mutation, Δ1313/I1314L [25]. Sequence analysis of shed vaccine virus confirmed the stability of these mutations. Previously, RSV vaccine candidates containing the NS2 deletion and other nonstabilized *ts* mutations were evaluated in phase 1 studies; these vaccines were either underattenuated or overattenuated [7]. However, 10^6 PFU of RSV/ΔNS2/Δ1313/I1314L infected all RSV-seronegative children and induced a primary antibody response in 90%.

We did not observe LRI after immunization, and rates of fever, cough, and otitis media were comparable in RSV-seronegative vaccinees and placebo recipients. Rhinorrhea occurred more often in seronegative vaccinees than in placebo recipients, although the differences were not statistically significant when the 10^5 PFU and 10^6 PFU dose cohorts were considered singly or together. Rates of illness did not increase with the higher dose. Other respiratory viruses were detected

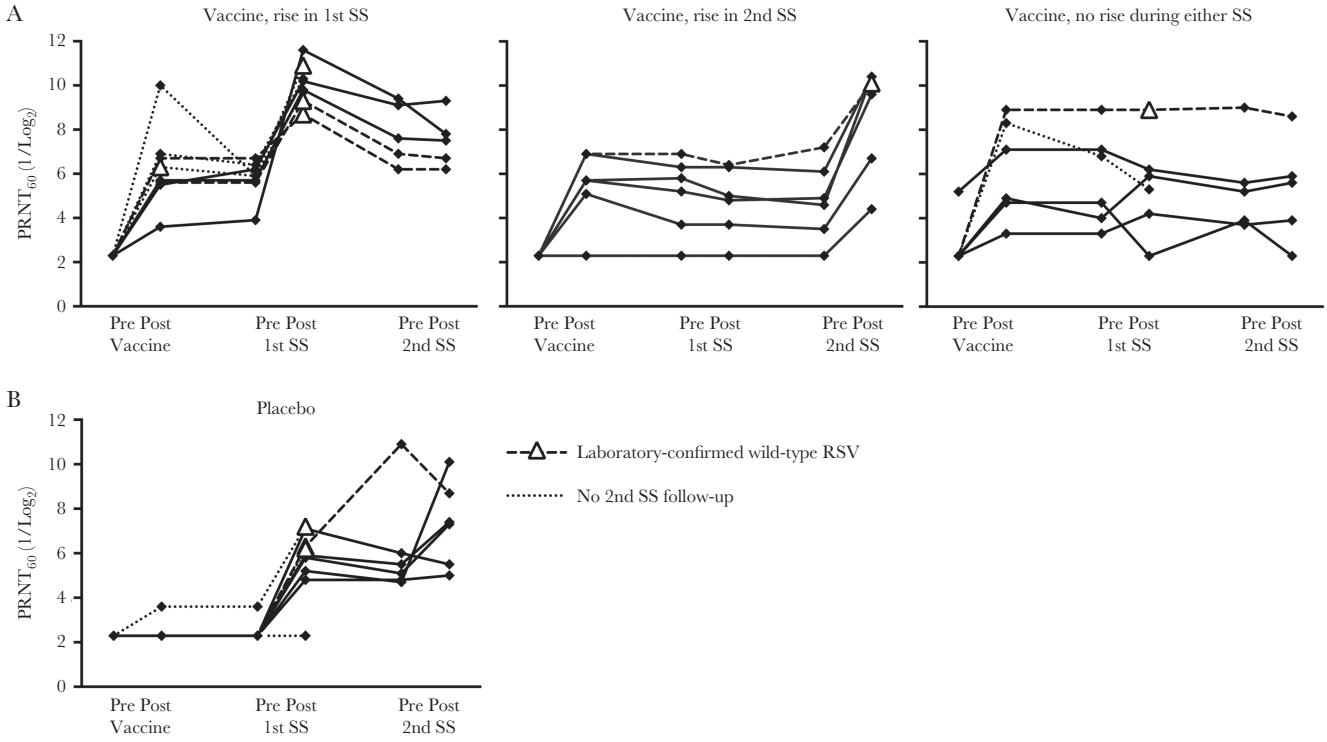

Figure 5. Respiratory syncytial virus (RSV) plaque reduction neutralization antibody titers (PRNT) in 20 RSV-seronegative recipients of 10^6 plaque-forming units of vaccine (*A*) and 10 placebo recipients (*B*), by individual child. All 30 children were followed during the first surveillance season (SS); 16 of 20 vaccinees and 6 of 10 placebo recipients were followed through 2 RSV surveillance seasons. Dotted lines represent data from the 4 vaccinees and 4 placebo recipients who participated only in the first season; dashed lines, participants who experienced an episode of RSV-associated medically attended acute respiratory illness or lower respiratory illness during a surveillance season; triangles, occurrence of such illness. *A,* Vaccinees with a ≥4-fold increase in the first season (*left*), the second season (*middle*), or neither season (*right*). *B,* Placebo recipients with a ≥4-fold increase in 1 or both seasons. Only 1 placebo recipient did not have a detectable increase in RSV PRNT, and this child participated in only the first surveillance season. Abbreviations: Post, after; Pre, before.

frequently in vaccinees and placebo recipients, consistent with previous studies [6, 30], but we also encountered a substantial number of respiratory events without concurrent detection of any pathogen. This high incidence of background respiratory illness, typical for this age group, and the inability to detect potential causative agents in some symptomatic children, indicate that larger studies will be needed to determine whether administration of RSV/ΔNS2/Δ1313/I1314L is associated with an increased risk of mild respiratory illness. Should administration of RSV/ΔNS2/Δ1313/I1314L be associated with transient rhinorrhea, this would probably be acceptable if efficacy against RSV-associated LRI was demonstrated.

As in previous studies [6–8, 10–12, 27], we conducted surveillance for RSV-associated MAARI in RSV-seronegative participants, with clinical assessment performed for each medically attended illness and NW samples obtained for viral identification. In the current study, we also conducted surveillance for the first time during a second RSV season in a subset of children to assess the durability of the immune response. As previously demonstrated for all live attenuated RSV vaccines evaluated to date, there was no evidence of enhanced RSV disease in any vaccine recipient. Of the children with presumed RSV-MAARI, 50% of vaccines and 33% of placebo recipients had ≥1 additional respiratory virus isolated, so attribution remains unclear. We note that the rates of RSV-MAARI in this study and in our previous studies [10–12] were low compared with some population-based epidemiologic studies, probably because we purposefully selected children without medical risk factors for serious RSV disease for enrollment in these phase 1 studies.

Comparison of RSV PRNT before and after each surveillance season indicated that RSV/ΔNS2/Δ1313/I1314L primed for substantial anamnestic serum antibody responses; for example, during the first surveillance season, titers among vaccinees with PRNT responses were approximately 14-fold higher than titers among placebo recipients with PRNT responses (1:955 vs 1:69, respectively). The antibody responses in the vaccinees were memory responses, whereas those in the placebo recipients generally were primary responses. These remarkably high memory responses are a consistent and important feature of recently evaluated live attenuated RSV vaccines [10–12]. Memory responses were also observed during the second season, indicating that priming is durable.

During the second RSV surveillance season, mild RSV-associated LRI was observed in 1 vaccinee. Although an isolated event, this information suggests that it may be desirable to offer a booster dose of RSV/ΔNS2/Δ1313/I1314L to protect during a second RSV season. Alternatively, the boost could consist of a PIV-vectored bivalent RSV/PIV vaccine [31]; in animal studies, the replication and immunogenicity of PIV-vectored RSV vaccines was unaffected by RSV-specific immunity from a prior immunization. These possibilities could be evaluated in future vaccine trials.

When administered to RSV-seronegative children, a 10^6 PFU dose of RSV/ΔNS2/Δ1313/I1314L was highly infectious yet restricted in replication, induced serum PRNT responses comparable to those observed after primary wt RSV infection, primed for substantial anamnestic serum antibody responses after natural exposure to wt RSV, and was genetically stable. These desirable vaccine characteristics have also been noted for live attenuated RSV vaccine candidates bearing the M2-2 deletion ([10] and unpublished data). Larger studies to directly compare the tolerability and immunogenicity of candidate vaccines bearing NS2 and M2-2 deletions will be needed; 1 such study (NCT03916185) is ongoing.

Notes

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Potential conflicts of interest. C. L., P. L. C., and U. J. B. are inventors on US patents pertaining to this vaccine candidate and its attenuating mutations. 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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