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MAJOR ARTICLE

Live Respiratory Syncytial Virus (RSV) Vaccine Candidate Containing Stabilized Temperature-Sensitivity Mutations Is Highly Attenuated in RSV-Seronegative Infants and Children

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(See the Editorial Commentary by Polack on pages 1335–7 and the Major Article by McFarland et al on pages 1347–55.)

Background. Respiratory syncytial virus (RSV) is the most important viral cause of severe respiratory illness in young children and lacks a vaccine. RSV cold-passage/stabilized 2 (RSVcps2) is a modification of a previously evaluated vaccine candidate in which 2 major attenuating mutations have been stabilized against deattenuation.

Methods. RSV-seronegative 6–24-month-old children received an intranasal dose of $10^{5.3}$ plaque-forming units (PFU) of RSVcps2 (n = 34) or placebo (n = 16) (International Maternal Pediatric Adolescent AIDS Clinical Trials protocol P1114 and companion protocol CIR285). RSV serum neutralizing antibody titers before and 56 days after vaccination, vaccine virus infectivity (defined as vaccine virus shedding detectable in nasal wash and/or a ≥4-fold rise in serum antibodies), reactogenicity, and genetic stability were assessed. During the following RSV transmission season, participants were monitored for respiratory illness, with serum antibody titers measured before and after the season.

Results. A total of 85% of vaccinees were infected with RSVcps2 (median peak titer, 0.5 $\log_{10}$ PFU/mL by culture and 2.9 $\log_{10}$ copies/mL by polymerase chain reaction analysis); 77% shed vaccine virus, and 59% developed a ≥4-fold rise in RSV-serum neutralizing antibody titers. Respiratory tract and/or febrile illness occurred at the same rate (50%) in the vaccine and placebo groups. Deattenuation was not detected at either of 2 stabilized mutation sites.

Conclusions. RSVcps2 was well tolerated and moderately immunogenic and had increased genetic stability in 6–24-month-old RSV-seronegative children.

Clinical Trials Registration. NCT01852266 and NCT01968083.

Keywords. Respiratory syncytial virus; reverse genetics; pediatric vaccine; recombinant live-attenuated vaccine; respiratory virus infection.

Human respiratory syncytial virus (RSV) is the most important viral cause of severe respiratory disease in infants and children worldwide, and it lacks a vaccine or antiviral drug suitable for routine use [1]. Globally, among children aged <5 years, RSV infection results in 34 million episodes of acute lower respiratory tract infections annually [2], highlighting the need for an effective vaccine. Use of a commercial RSV-neutralizing monoclonal antibody as prophylaxis for high-risk (eg, premature) infants substantially reduces the incidence of hospitalization due to RSV [3]. However, a pediatric vaccine for universal use in healthy infants is needed. In the 1960s, a clinical trial of a formalin-inactivated RSV vaccine resulted in a high incidence of

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enhanced RSV disease when immunized children subsequently experienced natural RSV infection [4]. Animal studies showed that this disease enhancement is linked to nonreplicating RSV vaccines but does not occur with replicating RSV or vectors [5]. Clinical studies over the past 20 years confirmed that live-attenuated RSV vaccines are not associated with enhanced RSV disease [6–12].

Live-attenuated RSV vaccines are attractive candidates for young children because they can be administered intranasally, elicit a local mucosal and a systemic response, and are immunogenic even in the presence of maternal antibodies, potentially allowing immunization of very young infants at greatest risk of severe disease [13]. Attenuation of RSV for use in vaccines has historically been achieved by serial passage or mutagenesis in cell culture, processes that are laborious and poorly controlled. The availability of reverse genetics makes possible the creation of new attenuating mutations, such as gene deletions, and the introduction of combinations of specific mutations to produce candidate vaccines with a range of phenotypes.

These techniques were used to develop the strain A2 vaccine candidate RSV rA2cp248/404/1030ΔSH [8], which contains 5 independent attenuating elements: (1) a set of 5 cold-passage (cp) amino acid substitutions in the nucleoprotein (N), fusion (F), and large polymerase (L) proteins from a cold-passaged RSV strain [14]; (2) the attenuating and temperature sensitivity (ts) 404 nucleotide point mutation in the gene-start transcription signal of the gene encoding the M2-1 and M2-2 proteins [15, 16]; (3 and 4) the 248 and 1030 attenuating and ts amino acid substitutions, respectively, in the L protein [16, 17]; and (5) deletion of the gene encoding the small hydrophobic (SH) protein [18]. This combination of mutations yielded a highly attenuated virus with an in vitro shut-off temperature for replication of 35°C. The 404, 248, and 1030 mutations specified most of the attenuation phenotype. Temperature sensitivity is thought to restrict vaccine virus replication to the upper respiratory tract, increasing vaccine safety. Indeed, rA2cp248/404/1030ΔSH was well tolerated, safe, moderately immunogenic, and in a 2-dose study in 1-2 month-old RSV-naive infants, the first vaccine dose was protective against infection with the second dose of vaccine [8]. A second vaccine virus, MEDI-559, contained the same 5 attenuating elements but an RSV A2 that differed by 37 silent point mutations from the RSV A2 used to make rA2cp248/404/1030ΔSH. MEDI-559 also was generally safe and immunogenic in 5–24-month-old seronegative infants and children [10]. However, evaluation of nasal wash (NW) isolates from recipients of either rA2cp248/404/1030ΔSH or MEDI-559 identified a number of specimens exhibiting a partial loss of the ts phenotype, associated with loss of 1 (usually) or 2 (rarely) attenuating mutations, primarily the 248 or 1030 mutations [8, 10]. Thus, further stabilization of these 2 ts mutations would be desirable.

To stabilize the 248 and 1030 mutations, each site was evaluated separately by creating recombinant RSVs bearing each of the possible amino acid substitutions at either site. Attenuation and ts phenotypes specified by all possible amino acid assignments were characterized, and attenuating codons were selected that were relatively refractory to deattenuation [19, 20]. This resulted in the introduction of 5 nucleotide changes and 1 amino acid change into MEDI-559 (see Methods) to yield RSVcps2. A preclinical study in seronegative chimpanzees indicated that the level of attenuation of RSVcps2 was very similar to that of MEDI-559 [21]. In the present study, RSVcps2 was evaluated in a phase 1 clinical trial to determine its safety and immunogenicity in RSV-seronegative, healthy 6–24-month-old children.

METHODS

Vaccine

RSVcps2, bearing the stabilized 248 (248s) mutation and the 1030s mutation, contains the following changes relative to MEDI-559: (1) the original 248 mutant assignment of 831L(TTA) was changed to 831L(TTG; change underlined) [20], (2) the 1030 assignment of asparagine [1321N(AAT)] was changed to lysine [1321K(AAA)] [19], and (3) the nearby codon 1313S(AGC) was changed to 1313S(TCA) to prevent a second-site mutation that compensated for 1321K(AAA) [19].

RSVcps2 was generated from complementary DNA by reverse genetics [22] on Vero cells from the World Health Organization (WHO) Cell Bank. Clinical trial material–grade live RSVcps2 was manufactured under current good manufacturing practices and stored at −80°C. Shortly before immunization, vaccine was diluted to a dose of $10^{5.3}$ plaque-forming units (PFU) in a 0.5-mL volume, and a single dose was administered intranasally (approximately 0.25 mL per nostril). Leibovitz L15 medium (Lonza) was used as a placebo.

Study Design

This study was conducted at 4 sites in the International Maternal Pediatric Adolescent AIDS Clinical Trials (IMPAACT) Network (clinical trials registration NCT01852266) and at the Center for Immunization Research (CIR), Johns Hopkins Bloomberg School of Public Health (NCT01968083), between October 2013 and April 2015. This study adhered to guidelines of the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH); was approved by each institution's institutional review board; and was sponsored by the NIAID Office of Clinical Research Policy and Regulatory Operations.

Study participants were healthy children ≥6 and <25 months of age who were RSV seronegative, defined as having a reciprocal complement-enhanced 60% RSV-neutralizing serum antibody titer ($PRNT_{60}$) of <1:40 (<5.3 $\log_2$). Accrual occurred from 1 April to 15 October (31 October at 1 site), to avoid the RSV transmission season. Eligible children were randomized at a ratio of 2:1 to receive vaccine or placebo and, after intranasal administration, were monitored daily for 28 days (acute phase) for fever, respiratory illness, and other adverse events. In-person assessments and nasal washes (NWs) for virologic testing were

performed on days 0, 3, 5, 7, 10, 12, 14, 17, 19, 21, and 28 (±1) and on any illness days, as previously described [7, 9]. From days 29 through 56 (after the acute phase), children were monitored only for serious adverse events (SAEs) or lower respiratory tract illness (LRTI). During the subsequent RSV season, defined as 1 November through 31 March, children were monitored by weekly communication with parents or guardians for medically attended acute respiratory illness (MAARI), including fever (with grade 1 denoting a temperature of ≥38°C but ≤38.6°C; grade 2, ≥38.7°C but ≤39.1°C; and grade 3, ≥39.2°C but ≤40.5°C), upper respiratory tract symptoms, acute otitis media, or LRTI, each of which prompted an examination, a NW, and testing for adventitious agents. Sera were collected at baseline (within 42 days of enrollment), on day 56 after immunization, and at the beginning and end of the RSV season.

Laboratory Assays

NW specimens from days on which illness occurred were evaluated by real-time quantitative polymerase chain reaction (qPCR) analysis (Fast-track Diagnostics, Luxembourg) for the presence of common adventitious respiratory agents. Vaccine virus in NW specimens was quantified by the immunoplaque assay on HEp-2 cells and by real-time qPCR specific for the RSV matrix (M) protein as previously described [7]. To evaluate vaccine genetic stability, NW specimens obtained at time points during peak vaccine shedding were amplified once on Vero cells, and total RNA was prepared, RSVcps2 RNA was amplified by reverse-transcription PCR, and analyzed by consensus sequencing of regions spanning the 404, 248s, and 1030s mutations and the 2 cp mutations, C319Y and H1690Y, of the polymerase open reading frame. A complement-enhanced plaque reduction neutralization assay was used to determine the serum $PRNT_{60}$ [23]. Serum immunoglobulin G (IgG) antibody titers to the RSV F glycoprotein were determined by enzyme-linked immunosorbent assay, using a purified baculovirus-expressed F protein [9, 24] kindly provided by Novavax (Gaithersburg, MD), as previously described [7, 24, 25].

Data Analysis

The reciprocal serum $PRNT_{60}$ and anti-RSV F IgG titers were transformed to $\log_2$ values. Medians and interquartile ranges were used to summarize peak titers in NW specimens and antibodies to RSV. These data also were summarized by determining means and standard deviations (Supplementary Tables 1 and 2) to facilitate comparisons with findings from published studies. Summaries of titers in NW specimens were calculated for infected children, with infection defined as (1) detection of vaccine virus by culture and/or real-time qPCR and/or (2) a ≥4-fold rise in RSV $PRNT_{60}$ or serum anti-RSV F antibody titer. Rates of illness among vaccinees and placebo recipients were compared by the Fisher exact test. All analyses were performed using SAS, version 9.4 (SAS Institute, Cary, NC), and graphs were produced using the R software.

RESULTS

Study Conduct

Fifty-one RSV-seronegative children were enrolled, and 50 (98%) received vaccine (n = 34) or placebo (n = 16). One child withdrew at its parent's request prior to being immunized and was excluded from further analysis. Baseline characteristics of vaccine and placebo groups were similar (Table 1); the mean age at enrollment was 12 months (range, 6–23 months). Only 4 of 600 in-person visits scheduled for days 0–56 did not occur as scheduled (3 of these visits occurred but on a different day), and only 7 of the expected 950 telephone contacts were missed. During the RSV season, only 4 children missed 1 weekly contact each, 1 child missed 3 contacts, and 1 child missed 6 contacts.

Safety, Adverse Events, and Detection of Adventitious Agents

During the 28-day postimmunization period, respiratory and febrile illness was common and occurred at the same rate in vaccinees and placebo recipients (50%; Table 2). Upper respiratory tract illness (defined as the presence of rhinorrhea, pharyngitis, or hoarseness) was the most frequent event, occurring in 14 vaccinees (41%; 90% confidence interval [CI], 27%–57%) and 7 placebo recipients (44%; 90% CI, 23%–67%). Fever and cough each occurred in 5 vaccinees (15%; 90% CI, 6%–29%) and 1 placebo recipient (6%; 90% CI, .3%–26%; $P = .65$), and acute otitis media occurred in 1 vaccinee. Of the 5 febrile vaccinees, 3 experienced grade 1 fever, 1 had grade 2 fever, and 1 had grade 3 fever; vaccine virus was detected at the time of fever (grade 1) only in 1 subject, together with rhinovirus. In total, 15 vaccinees had ≥1 respiratory symptom, which was always concurrent with shedding of ≥1 of the following adventitious respiratory viruses: rhinovirus (13 vaccinees), adenovirus (2), bocavirus (1), metapneumovirus (1), and parainfluenza virus type 3 (1); 3 vaccinees shed >1 adventitious virus. RSV cps2 was detected (with the identity confirmed by sequence analysis) concurrently with respiratory illness in only 3 of these 15 children and always with at least 1 adventitious respiratory virus. In a fourth ill child, RSV was detected (together with rhinovirus and adenovirus) by adventitious-agent testing, but the level of RSV shedding was too low to confirm it as RSVcps2. Thus, adventitious viruses were detected in all vaccinees with respiratory illnesses, and codetection of RSV vaccine virus during illness was infrequent (occurring in a maximum 4 of 17 vaccinees with respiratory and/or febrile illness), suggesting that the vaccine was well tolerated. Illness episodes in placebo recipients were associated with human metapneumovirus (1 placebo recipient), rhinovirus alone (4) or together with bocavirus (1), showing that adventitious agents were common in both groups.

Vaccine Virus Infectivity, Replication, and Immunogenicity

A total of 29 of 34 vaccinees (85%; 90% CI, 71%–94%) were infected with RSVcps2 (Table 2); RSVcps2 was detected in NW specimens from 15 (44%; 90% CI, 29%–59%) by culture and

Table 1. Baseline Characteristics of Vaccine and Placebo Recipients

Characteristic	Recipients, No. (%)		
	Vaccine (n = 34)	Placebo (n = 16)	Overall (n = 50)
Sex			
Male	22 (65)	7 (44)	29 (58)
Female	12 (35)	9 (56)	21 (42)
Ethnicity			
Hispanic or Latino	19 (56)	8 (50)	27 (54)
More than one ethnicity	0 (0)	1 (6)	1 (2)
Race			
African American	9 (26)	5 (31)	14 (28)
American Indian	0 (0)	1 (6)	1 (2)
Native Hawaiian or other Pacific Islander	1 (3)	0 (0)	1 (2)
White	17 (50)	7 (44)	24 (48)
>1 race	4 (12)	2 (13)	6 (12)
Unknown	3 (9)	1 (6)	4 (8)
Residence			
California	8 (24)	2 (13)	10 (20)
Colorado	7 (21)	3 (19)	10 (20)
Illinois	15 (44)	9 (56)	24 (48)
Maryland	4 (12)	2 (13)	6 (12)
HIV exposed, uninfected	16 (47)	8 (50)	24 (48)
Age, mo	9.5 (7–15)	13.5 (9.5–17)	11.5 (7–15)

Data are no. (%) of children or median value (interquartile range).

Abbreviation: HIV, human immunodeficiency virus.

in NW specimens from 26 (77%; 90% CI, 62%–88%) by real-time qPCR (Table 2). All NW specimens that had virus detected by culture also had virus detected by real-time qPCR. Median peak titers in NW specimens were low (0.5 $\log_{10}$ PFU/mL by culture and 2.9 $\log_{10}$ copies/mL by real-time qPCR). The mean duration (±SD) of shedding (based on the last day virus was detected) among those with detectable virus was 12 ± 5 days and 13 ± 4 days for culture and real-time qPCR, respectively. No RSV shedding was detected during the acute phase in placebo recipients. The daily titers of vaccine virus shed by the 29 infected vaccinees are shown in Supplementary Table 3 (for culture) and Table 4 (for real-time qPCR).

Analysis of sera obtained on day 56 after immunization showed that 20 of 34 vaccine recipients (59%; 90% CI, 44%–73%) developed ≥4-fold increases in serum neutralizing antibody titers, although titers were generally low (median, 5.3 $\log_2$, or 1:39), and 23 (68%; 90% CI, 53%–81%) developed a ≥4-fold rise in ELISA titers to RSV F (median, 11.6 $\log_2$, or 1:3104; Table 3). Of these, 3 vaccinees had no detectable vaccine shedding but had ≥4-fold rises in serum antibody titers by both $PRNT_{60}$ and ELISA (n = 2) or by ELISA only (n = 1). Six vaccinees had detectable vaccine virus shedding but without ≥4-fold rises in serum antibodies. Of note, 2 placebo recipients, one enrolled in April and one in October, had ≥4-fold rises in titers in both antibody assays. These 2 children presumably were exposed to wild-type RSV between the prevaccination visit and the day 56 visit.

Genetic Stability of RSVcps2

Sequence analysis of subsets of attenuating mutations was successful on peak titer isolates from the 26 vaccinees with detectable vaccine shedding (Table 4). Low viral shedding precluded sequence analysis of some sites in 12 of the peak titer isolates. The majority of vaccine isolates had no change in any of the analyzed sites. The stability of the 248s site was confirmed in 17 isolates (Table 4). The 2 cp sites in the polymerase L gene, C319Y and H1690Y, were confirmed in isolates from 17 and 23 subjects, respectively. The 1030s site was successfully sequenced in isolates from 24 subjects: in 23 cases, there was no instability, while in the remaining case, a single nucleotide change, from

Table 2. Vaccine Respiratory Syncytial Virus (RSV) cps2 Shedding, Peak RSV cps2 Titers, and Clinical Symptoms During the First 28 Days After Inoculation Among Vaccine and Placebo Recipients

Group	Recipients, No.	Infected[a]	Shed Vaccine Virus[b]	Peak Virus Titer		Clinical Symptom[c]					
				By the Plaque Assay, $\log_{10}$ PFU/mL[d]	By Real-Time qPCR, $\log_{10}$ Copies/mL[e]	Fever	URTI	LRTI	Cough	OM	Respiratory or Febrile Illness
Vaccine	34	29 (85)	26 (77)	0.5 (0.5, 1.9)	2.9 (2.4, 4.1)	5 (15)	14 (41)	0 (0)	5 (15)	1 (3)	17 (50)
Placebo	16	2 (13)[f]	0	ND	ND	1 (6)	7 (44)	0 (0)	1 (6)	0 (0)	8 (50)

Data are no. (%) of children or median value (interquartile range).

Abbreviations: LRTI, lower respiratory tract illness; ND, not detected; NW, nasal wash; OM, acute otitis media; PFU, plaque-forming units; qPCR, quantitative polymerase chain reaction; URTI, upper respiratory tract illness.

[a]Defined as detection of vaccine virus by culture or real-time qPCR and/or a ≥4-fold rise in RSV serum neutralizing antibody titer or serum anti-RSV F antibody.

[b]Defined as detection of vaccine virus shedding in NW specimens by culture and/or by real-time qPCR.

[c]Lower respiratory tract illness (LRTI) was defined as wheezing, rhonchi, or rales or as having received a diagnosis of pneumonia or laryngotracheobronchitis (croup). Upper respiratory tract illness (UTRI) was defined as rhinorrhea, pharyngitis, or hoarseness.

[d]For each infected vaccinee, the individual peak (highest) titer, irrespective of day, was selected from among all titers measured on days when virus was detected in the NW specimen by plaque assay; the lower limit of detection was 0.5 $\log_{10}$ PFU/mL. For infected vaccinees with no virus detected, the value of 0.5 $\log_{10}$ PFU/mL was assigned.

[e]For each infected vaccinee, the individual peak (highest) titer, irrespective of day, was selected from among all titers measured on days when virus was detected in NW specimens by real-time qPCR; the lower limit of detection was 1.7 $\log_{10}$ copies/mL.

[f]Two placebo recipients were counted as infected, based on their ≥4-fold rises in serum antibody titers, detected on day 56 after receipt of placebo (Table 3). Infection of placebo recipients was presumed to have occurred by exposure to wild-type RSV; see the text.

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

Group	Recipients, No.	Serum RSV neutralizing Ab Titer,[a] by Time Point						Serum Anti–RSV F IgG Ab Titer,[b] by Time Point					
		Vaccination			RSV Surveillance Season			Vaccination			RSV Surveillance Season		
		Before	After[c]	≥4-Fold Rise	Before	After	≥4-Fold Rise	Before	After[c]	≥4-Fold Rise	Before	After	≥4-Fold Rise
Vaccine	34	2.3 (2.3–4.2)	5.3 (4.1–6.2)	20 (59)	5.1 (4.0–6.1)	7.2 (5.6–8.8)	17 (50)	5.6 (4.6–9.6)	11.6 (9.6–11.6)	23 (68)	11.6 (7.6–11.6)	13.6 (11.6–15.6)	18 (53)
Placebo	16	2.9 (2.3–4.9)	2.3 (2.3–5.6)	2 (13)	2.3 (2.3–5.2)	6.1 (3.5–8.1)	9 (56)	7.6 (4.6–10.6)	5.6 (4.6–11.6)	2 (13)	5.1 (4.6–11.6)	11.6 (7.1–14.6)	9 (56)

Data are no. (%) of children or median reciprocal titer $\log_2$ (interquartile range).

Abbreviations: Ab, antibody; IgG, immunoglobulin G; Ab, antibody.

[a]Determined by complement-enhanced 60% plaque reduction neutralization assay. RSV seronegativity was defined as a titer of <5.3 $\log_2$ (<1:40). Reciprocal titers of 2.3 $\log_2$ were assigned to participants with negative results.

[b]Determined by enzyme-linked immunosorbent assay. Reciprocal titers of 4.6 $\log_2$ were assigned to participants with negative results.

[c]At study day 56.

1321K(AAA) to 1321I(ATA), was detected. This did not represent reversion to the wild-type assignment of tyrosine (which would have required 2 nucleotide changes); rather, previous analysis indicated that this specific change to an isoleucine residue would reduce the temperature sensitivity specified by 1030s only marginally, from 38°C to 39°C [19]. This particular isolate was confirmed to retain the 248s and 404 mutations and the 2 cp mutations in L and thus would remain highly attenuated; the single change in 1 of 5 attenuating elements would be unlikely to significantly reduce the attenuation of RSVcps2.

The 404 site was sequenced in isolates from 18 subjects: 17 had no evidence of instability, whereas 1 had a direct reversion from the mutant C to the wild-type T (Table 4). Since the 404 site involves an RNA transcription signal, it was not possible to genetically stabilize this site. This was the site of the only direct reversion detectable in any of the isolates. The resultant virus was confirmed to contain the 248s and 1030s mutations, as well as the 2 cp mutations in the L gene, and would also remain highly attenuated.

Surveillance Season

During the RSV surveillance period, MAARI was reported in 19 children (38%; 90% CI, 27%–51%), with similar distributions between the vaccine group (12 of 34 [35%; 90% CI, 22%–51%]) and the placebo group (7 of 16 [44%; 90% CI, 23%–67%]). Only 5 of these events were associated with detection of RSV in NW; 3 (9%) were in the vaccine group (2 associated with RSV subgroup A [RSV-A] and 1 associated with RSV subgroup B [RSV-B]), and 2 (13%) were in the placebo group (both associated with RSV-B). Both RSV subgroups are highly related antigenically, and a subgroup A vaccine would be expected to induce an immune response effective against both subgroups [26].

Seventeen of 34 vaccinees (50%; 90% CI, 35%–65%) and 9 of 16 placebo recipients (56%; 90% CI, 33%–77%) exhibited ≥4-fold rises in RSV neutralizing serum antibody titers during the RSV surveillance period (Figure 1), suggesting that in both groups numerous RSV infections occurred during the RSV seasons that did not produce illness severe enough to require medical attention.

DISCUSSION

The development of a pediatric RSV vaccine has been difficult. Inactivated and subunit RSV vaccines pose strong safety concerns because of their association with enhanced RSV disease in RSV-naive infants and experimental animals [4, 27–32]. Live-attenuated RSV strains are not associated with enhanced RSV disease [6]. However, their development by conventional means has been laborious. In particular, immunogenicity and genetic/phenotypic stability have been concerns. Reverse genetics has aided RSV vaccine development, allowing design of specific mutations (eg, gene deletions), combination of mutations to adjust the level

Table 4. Genetic Stability of Respiratory Syncytial Virus (RSV) cps2 Vaccine Isolates

Nucleotide Position[a]	Site[b]	Amino Acid[c]	Nucleotide Assignment		Sequence Obtained, No. of Vaccinees	Change Observed, No. (Nucleotide; Amino Acid Assignment)
			WT RSV	RSV cps2[d]		
7606	404	NA	T	C	18	1 (C; NA)
9453–5	cp	C319Y	TGT	TAC	17	0
10 989–91	248	Q831L	CAA	TTG	17	0
12 435–7	1030	S1313	AGC	TCA	24	0
12 459–61	1030	Y1321K	TAT	AAA	24	1 (ATA; I1321)
13 566–8	cp	H1690Y	CAT	TAC	23	0

To evaluate the genetic stability of RSVcps2, nasal wash specimens collected at time points during peak vaccine shedding were amplified once on Vero cells. Total RNA was prepared, RSV cps2 RNA was amplified by reverse-transcription quantitative polymerase chain reaction, and analyzed by consensus sequencing of regions spanning the 404, 248s, and 1030s mutations and of the 2 cp mutations C319Y and H1690Y of the L open reading frame.

Abbreviations: NA, not applicable; WT, wild type.

[a]Values are genomic position in reference to wild-type recombinant RSV strain A2 [22] (Genbank accession number KT992094). All sequences are positive sense.

[b]Attenuating sites: 404, nucleotide point mutation in the gene-start transcription signal of the M2 gene [15, 16]; cp mutations, C319Y and H1690Y, located in the polymerase open reading frame (part of a set of five cold-passage (cp) amino acid substitutions from a cold-passaged RSV strain in the nucleocapsid (N), fusion (F), and large polymerase (L) proteins [14]); 248 and 1030, the genetically stabilized 248s [20] and 1030s [19] attenuating and ts amino acid substitutions in the L protein [16, 17], respectively. The 1030s site involves a stabilizing change of codon S1313 of the polymerase open reading frame (from AGC to TCA) and the attenuating and ts mutation Y1321K [19].

[c]Amino acid changes from recombinant WT RSV to RSVcps2 are indicated.

[d]Amino acid changes from recombinant WT RSV to RSVcps2 are underlined.

of attenuation, and development of mutations refractory to deattenuation. The present study evaluated a version of a previous lead candidate in which 2 of the major attenuation mutations had been redesigned for increased genetic stability.

The previous candidate, rA2cp248/404/1030ΔSH, was well tolerated, moderately immunogenic, and protective against a second dose of vaccine in 1–2-month-old infants [8], but a moderate level (approximately 33%) of genetic instability was discovered in vaccine isolates, and similar observations were made for the version MEDI-559 [10]. MEDI-559 also was generally well tolerated, but an imbalance in medically attended LRTIs was observed in the 28-day period after dosing, although this was based on small numbers and did not involve contemporaneous vaccine shedding. This imbalance might have been attributable to atypically low rates of medically attended LRTI in the placebo group of that study, but further evaluation would be needed to establish safety. In the present study, the stabilized version of MEDI-559, RSVcps2, was safe and well tolerated in 6–24-month-old RSV-seronegative children, and medically attended LRTI was not observed.

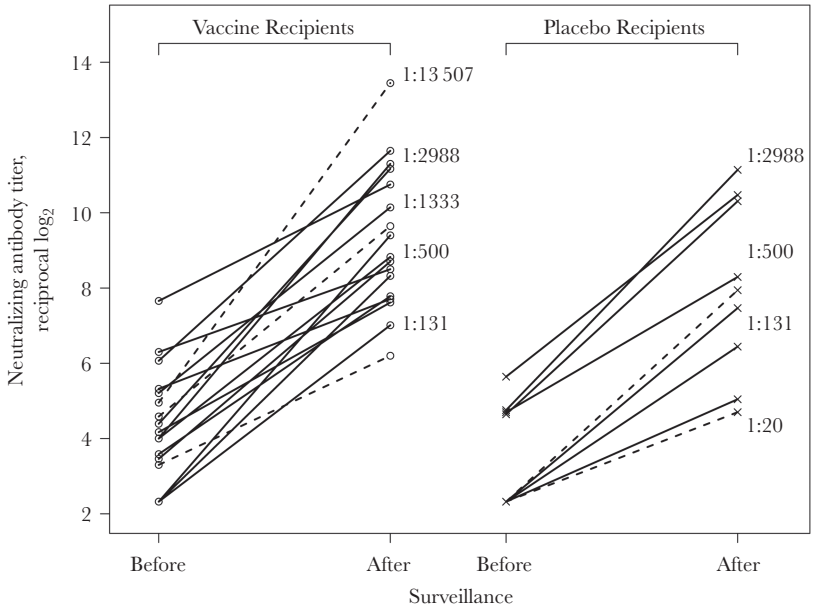

Figure 1. Increases in serum respiratory syncytial virus (RSV) 60% plaque reduction neutralization assay titers during the RSV surveillance period. A total of 17 vaccinees and 9 placebo recipients developed a ≥4-fold increase between the presurveillance and postsurveillance periods. Dashed lines denote participants in whom RSV medically attended, acute respiratory illness (MAARI) episodes were observed. Solid lines denote participants without reported RSV MAARI during the surveillance season. Titers are expressed as reciprocal $\log_2$ values. Selected arithmetic values are shown on the right.

Vaccine take, as measured by vaccine shedding in NW and/or serum immune response to RSV, was good but not universal; 5 of 34 (15%) remained uninfected by RSVcps2 vaccine, whereas vaccine take of rA2cp248/404/1030ΔSH was 100% [8]. The serum antibody response to RSVcps2 was somewhat lower than that to rA2cp248/404/1030ΔSH, which induced ≥4-fold increases in the $PRNT_{60}$ in 88% of vaccinees and ≥4-fold rises in the level of serum IgG to RSV F in 100% of vaccinees [8]. MEDI-559 also exhibited a somewhat lower level of immunogenicity than rA2cp248/404/1030ΔSH [8, 10]. We believe that the higher level of replication and immunogenicity of rA2cp248/404/1030ΔSH was linked to the 37 silent nucleotide differences between the RSV A2 version used to derive rA2cp248/404/1030ΔSH, and the RSV A2 version used to derive both RSVcps2 and MEDI-559. This strongly suggests that importing the 248s and 1030s mutations into the rA2cp248/404/1030ΔSH backbone would provide a virus that combines the genetic stability of RSVcps2 with the greater replication and immunogenicity of rA2cp248/404/1030ΔSH.

RSVcps2 contains the same attenuating loci as its 2 predecessors, and its genomic sequence is identical to MEDI-559, except for 5 nucleotide changes and 1 amino acid change to stabilize the 248 and 1030 mutations against deattenuation (Table 4). Sequence analysis of RSVcps2 vaccine isolates indicated stability at the 248s site and detected only a single instance of instability at the 1030s site. The particular change that was involved should not have a significant deattenuating effect, based on previous characterization of effects of all possible amino acid assignments at this site [19]. This indeed validates the stabilization strategy of selecting a codon for which any possible single-nucleotide change would not result in substantial deattenuation. A single instance of direct reversion and deattenuation was detected at the 404 site, which involved a nucleotide change in a transcription signal rather than a missense mutation and, thus, could not be stabilized by this strategy. Thus, RSVcps2 had substantially greater genetic stability of the major attenuating 248s and 1030s mutations as compared to rA2cp248/404/1030ΔSH and MEDI-559. While instability occurred at the 404 site, as also was observed previously with MEDI-559, there was no compensatory increase in instability of RSVcps2 in response to stabilization of 248 and 1030. The 248s and 1030s mutations also can be used to develop other RSV candidates, such as LID/ΔM2-2/1030s (containing1030s), which is presently being evaluated in a clinical trial (clinical trials registration NCT02794870 and NCT02952339).

Recently, 2 other RSV vaccine candidates, MEDI/ΔM2-2 [7] and LID/ΔM2-2 [33], were evaluated in phase 1 studies in 6–24-month-old children (clinical trials registration NCT01459198 and NCT02040831/NCT02237209). These viruses contain the deletion of the RNA regulatory factor M2-2, resulting in a global upregulation of viral gene transcription and increased antigen expression, and differ from each other at a number of sequence positions in their backbones. These 2 ΔM2-2 viruses exhibited increased immunogenicity, compared with rA2cp248/404/1030ΔSH [7, 33]. Shedding of MEDI/ΔM2-2 was low, detectable by culture of NW specimens from 60% of vaccinees at a mean peak NW titer of 1.5 $\log_{10}$ PFU/mL, whereas both the infectivity and the mean peak NW titer of LID/ΔM2-2 were substantially higher (90% and 3.2 $\log_{10}$ PFU/mL, respectively). These ΔM2-2 viruses appear to be promising candidates for further development. Additional versions of ΔM2-2 viruses also are presently in clinical trials (analysis of D46/cp/ΔM2-2, clinical trials registration NCT02601612; LID/cp/ΔM2-2, NCT02890381/NCT02948127; and LID/ΔM2-2/1030s, NCT02794870/NCT02952339), as well as a virus that has a deletion of ΔNS2 (RSV/ΔNS2/Δ1313/I1314L, NCT01893554).

Although the ΔM2-2 viruses may have the advantage of increased immunogenicity, the lineage based on rA2cp248/404/1030ΔSH and MEDI-559 has undergone more-extensive clinical evaluation, including assessment in very young infants [8, 10]. Temperature sensitivity of these candidates may increase safety, as already noted. The member of this lineage that replicated to the highest titers, rA2cp248/404/1030ΔSH, appeared to be well tolerated in a phase 1 trial in 1–2-month-old infants [8]. The present study shows that the introduction of the 248s and 1030s stabilizing mutations into MEDI-559 did not change its replication, consistent with expectations and preclinical data. We therefore expect that rA2cp248/404/1030ΔSH could be similarly stabilized without changing its replication and immunogenicity. RSVcps2, or preferably a version of rA2cp248/404/1030ΔSH containing the 248s and 1030s mutations, would be suitable for further evaluation, including in the youngest target group for a live-attenuated RSV vaccine, young infants.

STUDY GROUP MEMBERS

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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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Potential conflicts of interest. U. J. B., C. L., and P. L. C. are listed as inventors on a patent application for genetically stable live-attenuated RSV vaccines. All other authors report no potential conflicts. 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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