MAJOR ARTICLE

Identification of a Recombinant Live Attenuated Respiratory Syncytial Virus Vaccine Candidate That Is Highly Attenuated in Infants

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(See the editorial commentary by Englund, on pages 1036–39.)

Background. Recombination technology can be used to create live attenuated respiratory syncytial virus (RSV) vaccines that contain combinations of known attenuating mutations.

Methods. Two live attenuated, recombinantly derived RSV vaccine candidates, rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH, were evaluated in 31 adults and in 95 children ⩾6 months old. rA2cp248/404/1030ΔSH was subsequently evaluated in 44 infants 1–2 months old. These vaccine candidates share 4 attenuating genetic elements and differ only in a missense mutation (1030) in the polymerase gene.

Results. Both vaccines were highly attenuated in adults and RSV-seropositive children and were well tolerated and immunogenic in RSV-seronegative children. Compared with that of rA2cp248/404ΔSH, replication of rA2cp248/404/1030ΔSH was restricted in RSV-seronegative children (mean peak titer, $10^{4.3}$ vs. $10^{2.5}$ plaque-forming units [pfu]/mL), indicating that the 1030 mutation had a potent attenuating effect. Although rA2cp248/404/1030ΔSH was well tolerated in infants, only 44% of infants who received two $10^{5.3}$-pfu doses of vaccine had detectable antibody responses. However, replication after administration of the second dose was highly restricted, indicating that protective immunity was induced. At least 4 of 5 attenuating genetic elements were retained in recovered vaccine viruses.

Conclusions. rA2cp248/404/1030ΔSH is the first RSV vaccine candidate to be sufficiently attenuated in young infants. Additional studies are needed to determine whether rA2cp248/404/1030ΔSH can induce protective immunity against wild-type RSV.

Respiratory syncytial virus (RSV) is the most important cause of viral lower respiratory tract illness (LRI) in infants and children [1], but a vaccine is not available because of several obstacles, including the need to vaccinate early in life [2–4] and the history of immune-mediated enhancement of naturally occurring RSV disease among RSV-naive recipients of a formalin-inactivated RSV vaccine [5–7]. Enhanced RSV disease has never been observed after natural infection or administration of candidate live attenuated RSV vaccines [2, 8–10]. Live virus vaccines administered intranasally also afford better mucosal immunity than do inactivated virus vaccines administered parenterally [11, 12].

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For these reasons, live attenuated vaccines are being developed for RSV-naive populations.

Live attenuated RSV vaccines have been in development for several decades. A cold-passaged *(cp),* non–temperature sensitive *(ts)* derivative of RSV, *cp*RSV, caused mild respiratory illness in young children [13]. Chemical mutagenesis of *cp*RSV produced several *ts* mutants [14–16] that were evaluated in clinical trials during the 1990s. *cpts*248/955 was insufficiently attenuated in RSV-seronegative children and older infants, precluding further evaluation in younger infants [9]. However, *cpts*248/404 was highly attenuated in RSV-seronegative children and was the first RSV vaccine to be administered to 1–2-month-old infants. Unfortunately, *cpts*248/404 caused nasal congestion in these infants, an unacceptable adverse effect in this population. The nasal congestion was temporally associated with vaccine virus shedding, and the mean peak titers shed were $10^{4.0}$ and $10^{4.9}$ pfu/mL at the 2 dose levels tested [2]. Although *cpts*248/404 was insufficiently attenuated in this target population, this study provided important information regarding (1) the level of attenuation necessary for infants, (2) the ability of live attenuated RSV to replicate and induce antibody responses in infants who have maternally derived RSV antibody, and (3) preliminary evidence of protection against illness associated with wild-type (wt) RSV infection [2].

Efforts were next made to develop a live RSV vaccine that was slightly more attenuated than *cpts*248/404 in RSV-naive infants and children. The mutations present in *cp*RSV and 6 *cpts* derivatives were identified by means of cDNA technology [17], permitting the generation of recombinant RSV (rRSV) vaccine candidates that contained new combinations of attenuating *cp* and *ts* point mutations [18–21] and deletions (Δ) of nonessential genes (e.g., the SH, NS1, NS2, and M2-2 genes) [22, 23]. On the basis of preclinical studies, 1 deletion mutation (ΔSH) and 1 *ts* mutation (1030) were selected for addition to *cpts*248/404, to generate rRSVs that might be more attenuated in humans. ΔSH attenuated wt RSV in mice [22] and chimpanzees. Although the levels of attenuation of 248/404ΔSH and *cpts*248/404 were similar in chimpanzees [23], ΔSH was chosen because studies in infants might show an additional attenuating effect, given that the young infant is a more permissive host for RSV than the chimpanzee [2]. The 1030 mutation was added to create rA2cp248/404/1030ΔSH, because rA2cp248/404/1030 was more *ts* in vitro and more attenuated in mice than was *cpts*248/404 [24].

rA2cp248/404ΔSH contains 4 independent attenuating genetic elements: *cp,* which is based on 5 missense mutations in the N and L proteins and the F glycoprotein that together confer the non-*ts* attenuation phenotype of *cp*RSV and that are considered to be a single attenuating genetic element [25]; *ts*248, a missense mutation in the L protein [16, 19]; *ts*404, a nucleotide substitution in the gene-start transcription signal of the M2 gene [20]; and ΔSH, complete deletion of the SH gene [20, 23, 25]. rA2cp248/404/1030ΔSH contains 5 independent attenuating genetic elements: those present in rA2cp248/404ΔSH and *ts*1030, another missense mutation in the L protein [24]. Here, we report the evaluation of the safety, immunogenicity, and phenotypic stability of rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH in adult and pediatric populations.

PARTICIPANTS, MATERIALS, AND METHODS

Vaccines. Construction, rescue, and biological cloning of infectious rA2 RSV strains were conducted at the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD); Wyeth-Ayerst Research (Gosport, United Kingdom); and Wyeth Vaccines (Pearl River, NY) [18, 24, 25]. rA2cp248/404ΔSH was rescued and biologically cloned by means of 3 successive plaque-to-plaque purifications and was amplified twice, all in HEp-2 cells. Virus seed was passaged once in Vero cells and was purified by means of 3 successive plaque-to-plaque isolations in Vero cells. The final virus clone was amplified by means of 1 passage in Vero cells and 1 passage in Vero cell microcarrier culture at 30°C.

rA2cp248/404/1030ΔSH was rescued in HEp-2 cells and biologically cloned by means of 2 additional terminal dilutions. The final virus clone was amplified by means of 2 passages in Vero cells followed by 1 passage in Vero cell microcarrier spinner culture. This material was used to prepare the vaccine in microcarrier culture at 30°C.

Viral suspensions for clinical trials were produced in Vero cells and were found to be free of adventitious agents by Wyeth Vaccines. The titers of rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH were $10^{7.0}$ pfu/mL and $10^{7.5}$ pfu/mL, respectively. To achieve the necessary titers, vaccines were diluted as described elsewhere [2]. Diluent was also used as placebo.

Study design. Both rRSV vaccines were evaluated in open-label trials in adults and in randomized, double-blind, placebo-controlled trials in RSV-seropositive and RSV-seronegative children (table 1). rA2cp248/404/1030ΔSH was also evaluated in infants. The studies were conducted between April and November over several years. One RSV-seronegative placebo recipient and 2 recipients of $10^{5.3}$ pfu of rA2cp248/404/1030ΔSH were naturally infected with community-acquired wt RSV, as determined by sequence analysis of recovered virus. Their data were excluded from the analysis of safety and immunogenicity (tables 1 and 2) but were included in comparisons of phenotypic stability (table 3).

Eligible individuals were healthy and had no contact with immunosuppressed individuals or infants <6 months old [2, 9]. Each 0.5-mL dose of vaccine or placebo was intranasally administered. After inoculation, physical examinations were performed, and nasal wash specimens were obtained for viral culture

Table 1. Clinical and virologic responses of adults, children, and infants to rA2cp248/404ΔSH, rA2cp248/404/1030ΔSH, and placebo.

Participants, virus given	Dose, $\log_{10}$ pfu	No. of participants	Participants infected, %	Virus isolation, nasal wash			Participants with indicated illness, %						
				Participants who shed virus, %	Duration of shedding, mean (±SD), days	Peak titer, mean (±SD), $\log_{10}$ pfu/mL	Fever	URI	Nasal congestion	LRI	Cough	OM	Any respiratory or febrile illness
Adults													
rA2cp248/404ΔSH	5.0	15	13	13	5.5 (0.7)	1.6 (0.1)	0	7	NT	0	0	7	13
rA2cp248/404ΔSH	4.3	16	0	0	0	≤0.3	0	0	NT	0	0	0	0
Children													
RSV seropositive													
rA2cp248/404ΔSH	5.0	10	10	10	4.0	1.6	10	10	NT	0	0	10	20
rA2cp248/404/1030ΔSH	4.3	12	8	8	12.0	1.4	17	8	NT	0	8	0	25
rA2cp248/404/1030ΔSH[a]	5.3	13	8	8	8.0	2.4	38	38	NT	8[b]	23	8	69
Placebo[c]	…	5	0	0	0	≤0.3	0	60	NT	0	20	0	60
Placebo[d]	…	10	0	0	0	≤0.3	0	30	NT	0	30	20	40
RSV seronegative													
rA2cp248/404ΔSH	5.0	8	100	100	14.1 (2.7)	4.3 (1.1)	50	75	NT	0	25	25	88
rA2cp248/404/1030ΔSH	4.3	13	69	62	13.4 (8.8)	2.6 (1.3)	62	77	NT	0	31	23	92
rA2cp248/404/1030ΔSH	5.3	8	100	100	15.8 (7.2)	2.5 (0.7)	38	38	NT	0	25	13	50
Placebo[c]	…	3	33	0	0	≤0.3	33	33	NT	0	0	0	67
Placebo[d]	…	13	0	0	0	≤0.3	46	54	NT	8	23	0	69
Infants													
rA2cp248/404/1030ΔSH													
First dose	4.3	16	63	63	10.1 (2.7)	2.4 (1.3)	6	38	19	13[b]	38	0	69
Second dose	4.3	14	29	29	11.8 (4.6)	1.5 (0.8)	7	29	43	0	29	7	64
rA2cp248/404/1030ΔSH													
First dose	5.3	16	94	94	10.6 (3.7)	3.5 (0.8)	19	19	44	0	6	0	56
Second dose	5.3	16	44	44	12.7 (7.1)	1.3 (0.6)	13	19	25	0	25	13	31
Placebo													
First dose	…	12	0	0	0	≤0.3	8	8	8	0	8	0	17
Second dose	…	9	0	0	0	≤0.3	33	33	22	0	0	11	44

NOTE. Nasal congestion was assessed only in infants. Children were tested for levels of serum antibodies to respiratory syncytial virus (RSV) by 60% plaque reduction neutralization assay [26]. Those with titers >1:40 were considered to be RSV seropositive, and those with titers ≤1:40 were considered to be RSV seronegative [9]. Physical examinations were performed and nasal wash specimens were obtained on study days 0, 3–7, and 10 for adults and RSV-seropositive children and on study days 0, 3, 5, 7; day 8 or 9; day 10 or 11; and days 12, 14, 21, and 28 for RSV-seronegative children. For infants, physical examinations were performed and nasal wash specimens were obtained on study days 0; 4 or 5; 7, 8, or 9; 11 or 12; 16, 17, or 18; and 28. Interim history was obtained by telephone on the study days on which the participants were not evaluated. Studies were conducted at the Johns Hopkins University Center for Immunization Research (Baltimore, MD), the Saint Louis University Vaccine Evaluation Unit (St. Louis, MO), and the Vanderbilt Vaccine Clinic (Nashville, TN). Duration of shedding was defined as the time between inoculation and the last day on which vaccine virus was recovered. Participants were considered to be infected if at least 1 of the following criteria was met: vaccine virus was isolated, a ≥4-fold increase in neutralizing antibody titer occurred, or ≥4-fold increases in ≥2 of the ELISA titers (serum IgG to RSV F glycoprotein, serum IgG to RSV G glycoprotein, serum IgA to RSV F glycoprotein, and serum IgA to RSV G glycoprotein) occurred. LRI, lower respiratory tract illness; NT, not tested; OM, otitis media; URI, upper respiratory tract illness.

[a] Includes 3 children who were found to be RSV seronegative at the initial screening but were found to be RSV seropositive when serum specimens were retested with a neutralization assay that included complement.

[b] Three vaccine recipients had LRI; none shed vaccine virus, but other viruses—enterovirus (1 participant), rhinovirus (1 participant), and parainfluenza virus type 3 (1 participant)—were detected in nasal wash specimens by culture or polymerase chain reaction.

[c] Placebo recipients in studies of rA2cp248/404ΔSH vaccine.

[d] Placebo recipients in studies of rA2cp248/404/1030/ΔSH vaccine.

Table 2. Serum antibody responses of adults, children, and infants to rA2cp248/404ΔSH, rA2cp248/404/1030ΔSH, and placebo.

Participants, virus given	Dose, log_{10} pfu	Participants		RSV neutralizing antibody titer, mean (±SD)			ELISA IgG titer, mean (±SD)						ELISA IgA titer, mean (±SD)					
							F glycoprotein			G glycoprotein			F glycoprotein			G glycoprotein		
		Total	With any antibody response, %	Before	After	⩾4-fold increase, %[a]	Before	After	⩾4-fold increase, %[a]	Before	After	⩾4-fold increase, %[a]	Before	After	⩾4-fold increase, %[a]	Before	After	⩾4-fold increase, %[a]
Adults																		
rA2cp248/404ΔSH	5.0	15	0	8.5 (1.0)	8.6 (1.1)	0	14.1 (0.8)	14.1 (0.8)	0	13.4 (1.3)	13.4 (1.1)	0	8.8 (1.5)	8.9 (1.5)	0	9.6 (2.0)	9.8 (2.1)	0
rA2cp248/404/1030ΔSH	4.3	16	6	7.9 (0.7)	7.9 (0.6)	0	14.3 (1.2)	14.2 (1.3)	0	13.0 (1.5)	13.1 (1.9)	6	8.8 (2.3)	9.0 (2.0)	0	8.8 (7.2)	8.6 (2.2)	0
Children																		
RSV seropositive																		
rA2cp248/404ΔSH	5.0	10	0	7.8 (1.7)	8.0 (1.9)	0	15.3 (0.9)	15.1 (1.4)	0	12.4 (0.9)	12.3 (1.2)	0	9.7 (1.6)	9.8 (1.6)	0	7.4 (1.5)	6.6 (1.8)	0
rA2cp248/404/1030ΔSH	4.3	12	8	8.6 (1.4)	8.6 (1.3)	0	15.1 (1.6)	15.0 (1.3)	0	12.2 (1.9)	12.0 (1.5)	8	8.0 (1.7)	8.0 (1.6)	0	4.8 (2.7)	4.7 (2.5)	0
rA2cp248/404/1030ΔSH	5.3	13	15	8.7 (1.2)	8.5 (1.3)	0	16.4 (2.3)	15.8 (1.8)	0	11.3 (3.9)	11.6 (4.1)	15	10.3 (2.4)	9.5 (2.5)	0	5.8 (3.1)	5.7 (3.3)	0
Placebo[b]	…	5	0	8.3 (1.0)	7.7 (1.9)	0	15.0 (1.0)	14.8 (1.0)	0	11.8 (2.3)	11.8 (2.6)	0	9.4 (1.9)	9.5 (1.7)	0	6.2 (1.3)	6.3 (1.0)	0
Placebo[c]	…	10	0	8.1 (1.5)	8.0 (1.3)	0	15.9 (2.1)	15.9 (1.9)	0	11.4 (3.9)	10.9 (3.7)	0	9.4 (2.2)	9.5 (2.0)	0	5.4 (2.9)	5.7 (2.9)	0
RSV seronegative																		
rA2cp248/404ΔSH	5.0	8	88	3.3 (0.0)	6.6 (1.5)	88	9.1 (2.7)	13.3 (1.6)	88	7.0 (2.2)	11.7 (1.1)	88	3.0 (2.4)	5.4 (2.9)	63	1.9 (0.7)	2.9 (1.3)	38
rA2cp248/404/1030ΔSH	4.3	13[d]	33	3.4 (0.3)	5.2 (2.6)	33	7.7 (3.0)	10.9 (4.2)	36	6.6 (2.0)	8.9 (3.2)	36	2.8 (1.0)	5.2 (3.8)	36	2.3 (0.4)	3.7 (3.0)	18
rA2cp248/404/1030ΔSH	5.3	8	100	3.3 (0.0)	7.2 (1.6)	88	6.9 (1.7)	14.2 (1.4)	100	5.0 (0.8)	10.9 (2.5)	88	3.3 (1.9)	6.7 (4.2)	63	2.2 (0.4)	4.2 (2.4)	55
Placebo[b]	…	3	33[e]	5.1 (1.6)	3.9 (1.0)	0	9.1 (1.8)	10.5 (4.1)	33	7.1 (3.6)	8.4 (3.9)	33	1.3 (0.0)	3.5 (3.0)	50	1.8 (0.0)	1.8 (0.0)	0
Placebo[c]	…	13	0	3.4 (0.3)	3.3 (0.0)	0	7.5 (2.6)	7.1 (2.2)	0	5.9 (1.5)	5.3 (1.5)	0	3.2 (1.5)	3.0 (1.2)	0	2.2 (0.8)	2.1 (0.5)	0
Infants																		
rA2cp248/404/1030ΔSH																		
First dose	4.3	16	6	7.7 (1.0)	7.0 (1.1)	0	13.6 (1.2)	12.7 (1.4)	0	13.3 (1.6)	12.4 (1.5)	0	3.0 (1.5)	3.0 (1.4)	0	2.5 (1.3)	2.6 (1.0)	6
Second dose	4.3	14	6	7.0 (1.1)	6.6 (1.2)	0	12.7 (1.4)	11.6 (0.9)	0	12.4 (1.5)	10.7 (1.3)	0	3.0 (1.4)	3.0 (1.0)	6	2.6 (1.0)	2.3 (0.8)	0
rA2cp248/404/1030ΔSH																		
First dose	5.3	16	31	7.4 (1.7)	6.8 (1.6)	0	13.4 (1.3)	12.5 (1.4)	0	12.7 (1.1)	12.1 (1.2)	0	2.1 (0.5)	3.3 (2.4)	25	2.9 (0.5)	4.4 (2.6)	31
Second dose	5.3	16	44	6.8 (1.6)	7.1 (1.5)	19	12.5 (1.4)	12.6 (1.8)	13	12.1 (1.2)	11.7 (1.4)	6	3.3 (2.4)	4.5 (3.5)	31	4.4 (2.6)	4.9 (2.7)	13
Placebo																		
First dose	…	12	0	7.7 (1.1)	7.0 (1.6)	0	13.3 (1.4)	12.7 (1.7)	0	12.4 (1.6)	11.4 (1.7)	0	2.4 (0.8)	2.4 (0.8)	0	2.6 (0.7)	2.5 (0.6)	0
Second dose	…	9	0	6.8 (1.6)	5.5 (1.8)	0	12.4 (1.5)	11.8 (1.4)	0	11.1 (1.7)	10.5 (1.7)	0	2.4 (0.8)	2.5 (0.8)	0	2.5 (0.6)	2.5 (0.6)	0

NOTE. All antibody titers are expressed as mean reciprocal log_2 values. Serum specimens were obtained before immunization and 4 weeks (for adults and respiratory syncytial virus [RSV]–seropositive children) or 8 weeks (for RSV-seronegative children) after immunization. Serum specimens were obtained from 1–2-month-old infants before the first dose was administered, before the second dose was administered, and 4 weeks after the second dose was administered.

[a] Percentage of participants who experienced a ⩾4-fold increase in the indicated antibody titer.

[b] Placebo recipients in studies of rA2cp248/404ΔSH.

[c] Placebo recipients in studies of rA2cp248/404/1030ΔSH.

[d] Serum specimens were available for measurement of neutralizing antibody titer from 12 participants and for measurement of RSV F and G glycoprotein antibody titers from 11 participants; this is reflected in the given percentages of participants who experienced antibody responses.

[e] A single placebo recipient experienced increases in IgG and IgA antibody to RSV F and G glycoprotein, as measured by ELISA. This participant was inoculated in October and may have been infected with wild-type RSV before collection of the postinoculation serum specimen in December.

Table 3. Phenotype of respiratory syncytial virus (RSV) shed by recipients of rA2cp248/404/1030ΔSH and by those naturally infected with wild-type (wt) RSV.

Virus recovered from vaccine recipients	No. of participants who shed virus	No. of nasal wash specimens with virus that formed plaques at 32°C	No. of nasal wash specimens with virus that formed plaques at indicated temperature			
			35°C	36°C	37°C	38°C
Vaccine virus	45	141	48	27	1	0
wt RSV	3	9	9	9	9	9

NOTE. Original nasal wash specimens were used to perform phenotype characterization. Naturally acquired wt RSV was recovered from 2 vaccine recipients and 1 placebo recipient, as described in Results, and was included as a control. The parent virus, rA2cp248/404/1030ΔSH, can form plaques at 36°C and 37°C.

(table 1) [2, 9]. Fever, respiratory illnesses, and otitis media (OM) were defined as described elsewhere [9]. We also monitored nasal congestion in infants <6 months old [2]. In our analysis, we included nasal congestion that interfered with eating or sleeping or that resulted in obligatory mouth breathing.

Surveillance. To determine whether immunization with a live attenuated RSV vaccine was associated with enhanced disease, RSV-seronegative children and infants who received either vaccine or placebo were monitored for wt RSV infection during the subsequent RSV season [2, 9].

Virus isolation, quantitation, and phenotypic characterization. Virus was isolated from snap-frozen nasal wash specimens and identified as RSV, as described elsewhere [9]. Infectivity was quantitated by plaque assay [9]. Specimens that were negative by culture were assigned a titer of $10^{0.3}$ pfu/mL.

To determine the level of temperature sensitivity of virus present in nasal wash, specimens from recipients of rA2cp248/404ΔSH were tested for efficiency of plaque formation (EOP) at 32°C, 36°C, 37°C, 38°C, and 39°C, and specimens from recipients of rA2cp248/404/1030ΔSH were tested for EOP at 32°C, 35°C, 36°C, 37°C, and 38°C. On the basis of comparison with titers observed at 32°C, the EOP of rA2cp248/404ΔSH was ⩾100-fold reduced at 38°C, and the EOP of rA2cp248/404/1030/ΔSH was ⩾100-fold reduced at 35°C.

Genetic characterization. Sequence analysis was performed on 9 viruses recovered from 5 recipients of rA2cp248/404/1030/ΔSH. These viruses plaqued efficiently at 36°C and, in one case, at 37°C. Sequencing was performed directly on 1 specimen and otherwise on uncloned, passaged preparations, as described elsewhere (table 4).

Immunologic assays. Serum specimens were tested for titers of antibodies to RSV by 60% plaque reduction neutralization assay [26] and for IgG and IgA antibodies to RSV F and G glycoproteins by ELISA [9]. All titers are expressed as mean reciprocal $\log_2$ values.

Data analysis. Participants were considered to be infected if at least 1 of the following criteria was met: vaccine virus was isolated, a ⩾4-fold increase in neutralizing antibody titer occurred, or ⩾4-fold increases in ⩾2 of the ELISA titers (serum IgG to RSV F [fusion] glycoprotein, serum IgG to RSV G [attachment] glycoprotein, serum IgA to RSV F glycoprotein, and serum IgA to RSV G glycoprotein) occurred (table 1). Mean peak titers of vaccine virus shed ($\log_{10}$ pfu/mL) were calculated for infected vaccine recipients. To calculate means, neutralizing antibody and ELISA reciprocal titers were $\log_2$ transformed. The Mann-Whitney *U* test was used to compare titers. Rates of illness were compared by Fisher's exact test (2-tailed). $P < .05$ was considered to be statistically significant.

RESULTS

Response of adults and RSV-seropositive children. In adults and RSV-seropositive children, rA2cp248/404/ΔSH and rA2cp248/404/1030ΔSH were well tolerated and highly restricted in replication (table 1). One seropositive recipient of rA2cp248/404/1030ΔSH experienced pneumonia on study day 5; this child did not have evidence of infection with vaccine virus, but enterovirus was detected on study days 4 and 5 (table 1). After vaccination, ⩾4-fold increases in antibody titers rarely occurred (table 2), suggesting that these vaccines are minimally infectious or immunogenic in non–RSV-naive populations.

Response of RSV-seronegative children. In RSV-seronegative children, rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH were infectious and immunogenic. The frequency of illnesses was similar in vaccine recipients and placebo recipients (table 1). OM was observed slightly more often in RSV-seronegative vaccine recipients than placebo recipients, but it occurred sporadically throughout the study period and was not consistently associated with vaccine virus shedding. LRIs were not observed in vaccine recipients.

Although both rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH readily infected RSV-seronegative children, the level of viral replication differed significantly. The mean peak titer shed by recipients of the $10^{5.0}$-pfu dose of rA2cp248/404ΔSH was ~50-fold greater than that shed by recipients of the $10^{5.3}$-pfu dose of rA2cp248/404/1030ΔSH ($10^{4.3}$ vs. $10^{2.5}$ pfu/mL, respectively; $P = .009$) (table 1), and this difference was observed throughout the study period (figure 1). The dose of rA2cp248/404/

Table 4. Phenotypic and genetic characterization of selected viruses recovered from nasal wash specimens from recipients of rA2cp248/404/1030ΔSH.

		Original nasal wash isolate						Amplified nasal wash isolate						Sequence of amplified nasal wash isolate at the indicated attenuating mutation site (gene)				
		Virus titer at indicated temperature, log_{10} pfu/mL					Fold reduction,	Virus titer at indicated temperature, log_{10} pfu/mL					Fold reduction,					
Participant or virus	Study day	32°C	35°C	36°C	37°C	38°C	36°C vs. 32°C	32°C	35°C	36°C	37°C	38°C	36°C vs. 32°C	*cp*	404 (M2)	248 (L)	1030 (L)	SH
A	10	3.2	2.5	2.3	<1.0	<1.0	7.9	6.0	NT	5.6	<1.0	<1.0	2.5	*cp*	C	**CTG/CAG Leu/Gln**	AAT Asn	–
B	5	2.3	1.3	1.5	<1.0	<1.0	6.3	6.3	5.8	5.7	<1.7	<1.7	4.0	*cp*	C	CTG Leu	AAT Asn	–
	7	3.3	2.9	2.6	<1.0	<1.0	5.0	5.7	5.6	5.2	<1.7	<1.7	3.2	*cp*	C	CTG Leu	**TAT Tyr**	–
	9	2.7	2.3	1.9	<1.0	<1.0	6.3	5.5	5.2	5.0	<1.7	<1.7	3.2	*cp*	C	CTG Leu	**TAT Tyr**	–
	10	1.3	1.0	1.5	<1.0	<1.0	<1.0	6.3	6.2	5.9	<1.7	<1.7	2.5	*cp*	C	CTG Leu	**TAT Tyr**	–
C	7	3.6	2.5	2.3	<1.0	<1.0	20.0	5.5	5.2	4.0	<1.7	<1.7	31.6	*cp*	C	CTG Leu	AAT Asn	–
D	10	3.1	2.5	2.3	1.3	<1.0	6.3	NT	NT	NT	NT	NT	NT	*cp*	C	CTG Leu	AAT Asn	–
E	12	3.1	2.9	2.8	<1.0	<1.0	2.0	6.1	NT	6.0	5.7	<1.0	1.3	*cp*	C	CTG Leu	**TAT Tyr**	–
F	11	3.5	2.5	1.7	<0.7	<0.7	63.0	6.5	5.1	4.9	3.5	<1.7	39.8	*cp*	C	CTG Leu	**AAT/TAT/CAT Asn/Tyr/His**	–
rA2cp248/404/1030/ΔSH		5.7	<1.7	<1.7	<1.7	<1.7	>10,000.0	…	…	…	…	…	…	*cp*	C	CTG Leu	AAT Asn	–
RSV A2 wt		6.6	6.6	6.6	6.5	6.6	0	…	…	…	…	…	…	wt	T	CAA Gln	TAT Tyr	+

NOTE. Participants A–E were respiratory syncytial virus (RSV)–seronegative children, and participant F was an infant. Specimens were selected for sequence analysis as described in Participants, Materials, and Methods. For participant D, virus was sequenced directly from nasal wash. All other consensus sequences were determined after 1–2 passages in Vero cells at 32°C without biological cloning (Y. Lin, A. Deatly, and W. Chen, unpublished data) [27]. After passage, the viral isolate from participant E was less temperature sensitive than the original nasal wash virus, raising the possibility that additional changes occurred during the postisolation passages. The number designations for the RSV mutants and mutations refer to the plaque numbers of the original biologically derived mutants and do not indicate amino acid or nucleotide sequence positions. Assignments that reverted to that of wild-type (wt) RSV are boldfaced. In some cases, a particular position appeared to contain a mixture of 2–3 assignments; the various assignments of these mixtures are boldfaced. +, positive; –, negative; Asn, asparagine; *cp,* cold passaged; Gln, glycine; His, histidine; Leu, leucine; NT, not tested; Tyr, tyrosine.

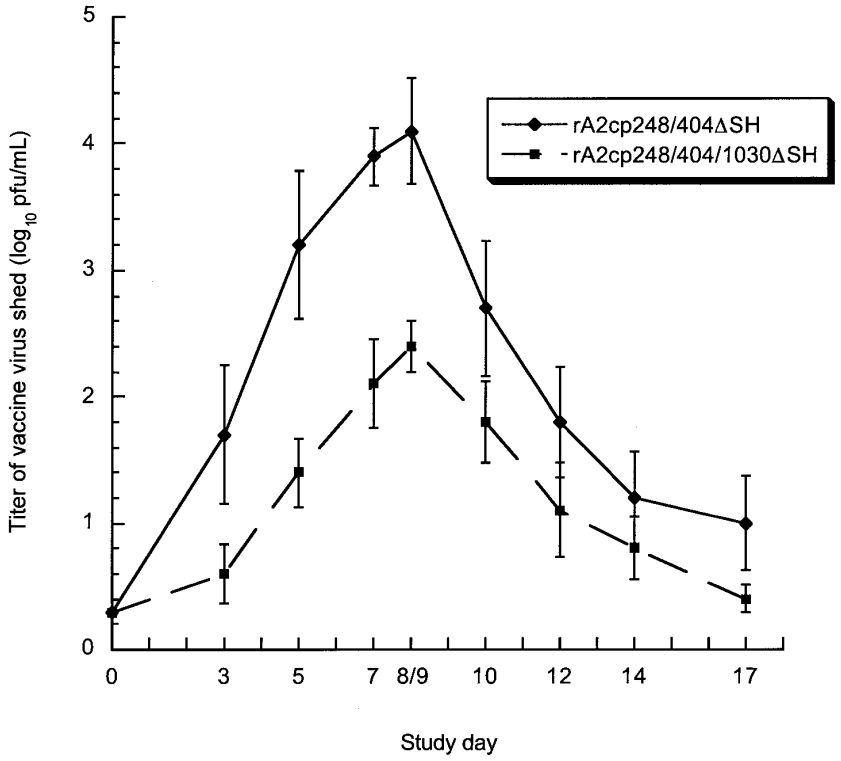

Figure 1. Replication of 10^5 pfu of rA2cp248/404ΔSH and $10^{5.3}$ pfu of rA2cp248/404/1030ΔSH in respiratory syncytial virus–seronegative children (for each vaccine, $n = 8$). Log_{10} mean titer of vaccine virus detected in nasal wash specimens is shown for each study day; bars indicate SE.

1030ΔSH influenced the frequency of infection (69% for the $10^{4.3}$-pfu dose vs. 100% for the $10^{5.3}$-pfu dose) but not the mean peak titer shed (table 1).

Despite differences in replication, both vaccines were immunogenic in RSV-seronegative children. Eighty-eight percent of recipients of the $10^{5.0}$-pfu dose of rA2cp248/404ΔSH had ⩾4-fold increases in neutralizing antibody and RSV F and G antibody titers (table 2); similarly, 100% of recipients of the $10^{5.3}$-pfu dose of rA2cp248/404/1030ΔSH developed antibody responses, with mean postvaccination titers similar to those achieved with rA2cp248/404ΔSH (table 2). The dose of rA2cp248/404/1030ΔSH also influenced the frequency and magnitude of the antibody response: only 4 of 12 RSV-seronegative children who received the $10^{4.3}$-pfu dose developed a neutralizing antibody response, compared with 7 of 8 who received the $10^{5.3}$-pfu dose ($P = .03$), and the postvaccination mean reciprocal log_2 titers were 5.2 and 7.2, respectively. This difference can be explained by the increased rate of infection with vaccine virus among the recipients of the $10^{5.3}$-pfu dose and suggests that increases in dose may be one strategy for enhancement of the immune response to highly attenuated RSV vaccines.

Response of infants. rA2cp248/404ΔSH was not evaluated further because its level of replication in RSV-seronegative children was similar to that of *cpts*248/404, which caused congestion in infants [2]. In contrast, replication of rA2cp248/404/1030ΔSH was highly restricted in RSV-seronegative children, making it suitable for evaluation in infants. Two doses of either $10^{4.3}$ or $10^{5.3}$ pfu of rA2cp248/404/1030ΔSH or of placebo were administered to infants. Of the 44 infants enrolled, 2 were withdrawn before the second $10^{4.3}$-pfu dose of vaccine was administered, and 3 were withdrawn before the second dose of placebo was administered.

Mild illnesses occurred frequently and at similar rates in infants who received vaccine or placebo (table 1). Rates of illness were highest in recipients of the first $10^{4.3}$-pfu dose (69%) (table 1), but illnesses occurred at similar rates in infected and uninfected vaccine recipients (70% vs. 67%, data not shown) and were not temporally associated with viral replication. LRI was observed in 2 infants who received vaccine: rhinovirus was recovered from 1 infant with bronchiolitis (study days 4–14), and parainfluenza virus type 3 was recovered from 1 infant with pneumonia (study days 5–12). Neither infant shed vaccine virus.

As was observed for the RSV-seronegative children, replication of rA2cp248/404/1030ΔSH was highly restricted in infants—mean peak titers after the first dose of vaccine was administered were $10^{2.4}$ pfu/mL for recipients of the $10^{4.3}$-pfu dose and $10^{3.5}$ pfu/mL for recipients of the $10^{5.3}$-pfu dose (table 1). Increasing the dose increased the percentage of infected infants, from 63% to 94%; similar increases were observed for the RSV-seronegative children (table 1).

Despite the infectivity of the higher dose of rA2cp248/404/1030ΔSH in infants, the antibody responses observed were not consistent: 44% of infants developed IgA antibody responses after two $10^{5.3}$-pfu doses of vaccine, whereas 100% of RSV-seronegative children developed IgA or IgG antibody responses after a single $10^{5.3}$-pfu dose ($P = .009$). However, the antibody response in infants did not predict resistance to infection with the second dose, as the rate of infection and the magnitude of vaccine virus shed were lower after the second dose than after the first dose. These differences were significant in infants administered the $10^{5.3}$-pfu dose (rate of infection, 94% vs. 44% with respect to the first and second doses [$P = .006$]; mean peak titer, $10^{3.5}$ vs. $10^{1.3}$ pfu/mL with respect to the first and second doses [$P < .001$]) (table 1 and figure 2). These data indicate that rA2cp248/404/1030ΔSH induced immune responses in these young infants but that the mediators of this immunity remain to be identified.

Surveillance. RSV-seronegative children and infants enrolled in the present vaccine studies also participated in surveillance for RSV disease during the winters after vaccination. Enhanced disease was not observed when children and infants initially infected with vaccine virus were naturally infected with wt RSV.

Phenotypic and genetic analysis. rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH are *ts* viruses, with ⩾100-fold reductions in EOP at 38°C and 35°C, respectively. In 42 nasal wash specimens from 8 recipients of rA2cp248/404/ΔSH, plaques were not detected at 39°C (data not shown), indicating stability of the *ts* phenotype. One hundred forty-one nasal wash specimens from 45 recipients of rA2cp248/404/1030ΔSH contained virus that formed plaques at 32°C (table 3). Of these specimens, 48 contained virus that formed plaques at 35°C, 27 contained virus that formed plaques at 36°C, and 1 contained virus that formed plaques at 37°C (table 3). In contrast, 9 specimens from 3 children naturally infected with wt RSV contained virus that formed plaques at 35°C, 36°C, 37°C, and 38°C (table 3).

The kinetics of the appearance of vaccine-derived viruses that formed plaques at 35°C, 36°C, and 37°C are shown in figure 3. These viruses were not detected before study day 5 (figure 3*A–E*), suggesting that they were unlikely to be present in the vaccine administered to study participants. Shedding of these viruses occurred during peak viral replication, and they sometimes predominated (figure 3*B–D*). However, shedding of these viruses diminished in parallel with the shedding of *ts* virus. Viruses that formed plaques at 36°C were present at $\leqslant 10^{3.0}$ pfu/mL, and virus that formed plaques at 37°C was present in a single child on a single day, at a titer of $10^{1.3}$ pfu/mL (figure 3*D*). In contrast, virus from a child naturally infected with wt RSV grew at titers of $10^{5.0}$–$10^{6.8}$ pfu/mL at all temperatures and on all study days tested (figure 3*F*).

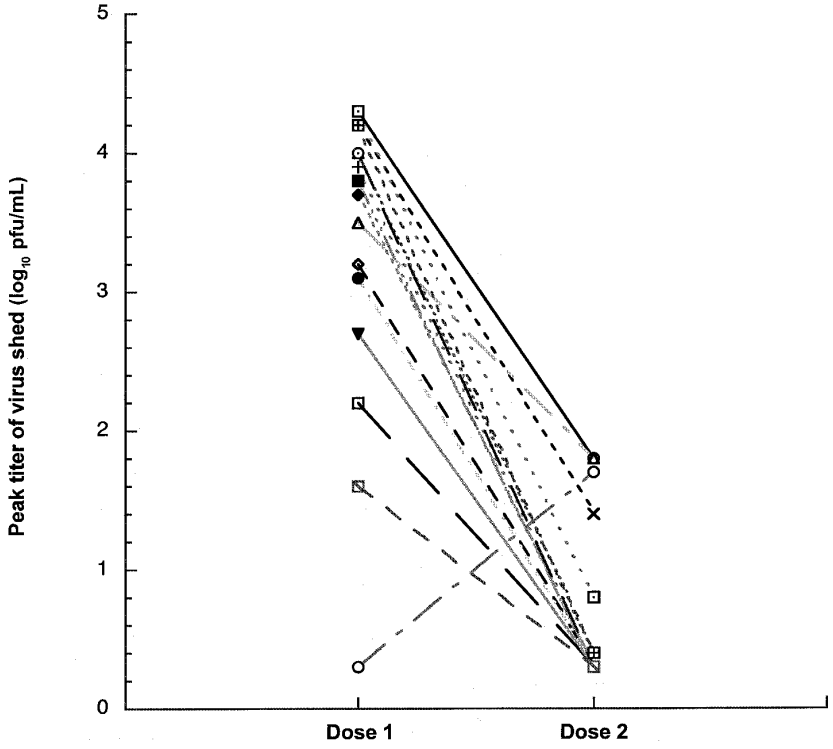

Figure 2. Relationship, in young infants, between peak virus shedding after administration of the first $10^{5.3}$-pfu dose of rA2cp248/404/1030ΔSH and peak virus shedding after administration of the second dose. The 2 doses of vaccine were administered 4–8 weeks apart, beginning at 4–12 weeks old.

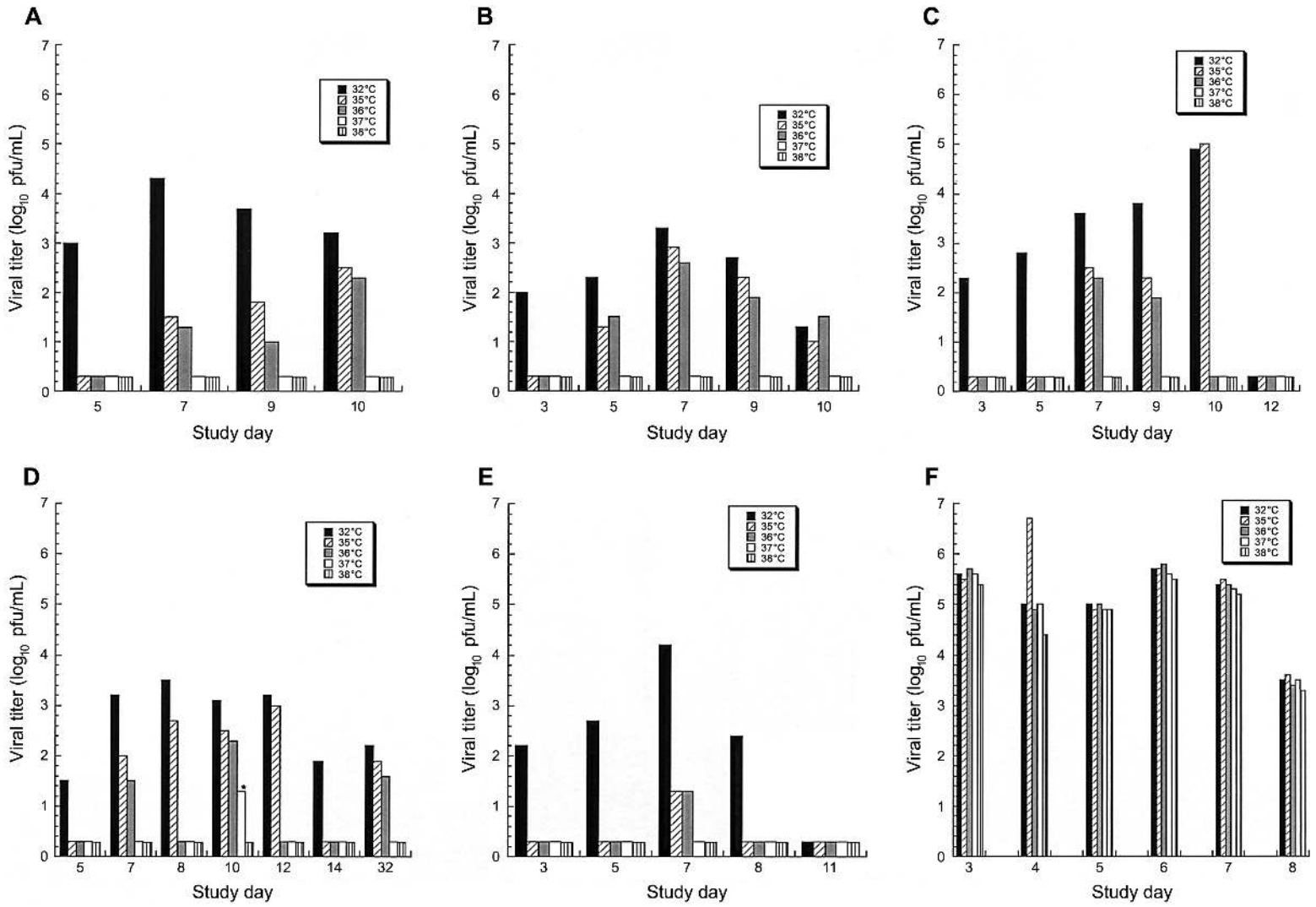

Figure 3. Phenotypic characterization of rA2cp248/404/1030ΔSH shed by 5 respiratory syncytial virus (RSV)–seronegative children *(A–E)* and of wild-type (wt) RSV shed by a naturally infected seronegative child (F) on the indicated study day. For each virus present in nasal wash specimens, efficiency of plaque formation at 32°C, 35°C, 36°C, 37°C, and 38°C is shown. Panel E depicts a representative pattern of plaque formation by virus that maintained the temperature-sensitive phenotype of the input vaccine; this was the predominant pattern observed. Panels A–D depict the pattern of shedding in which a large proportion of virus formed plaques at 35°C or 36°C (for 2 children, data not shown). A single nasal wash specimen (*), shown in panel D, contained vaccine virus that formed plaques at 37°C. Panel F depicts the pattern of plaque formation of wt RSV, for which efficient replication was observed at all temperatures, including 37°C and 38°C. Panels A–D correspond with participants A–D in table 4.

The genetic characterization of selected viruses is shown in table 4. The 5 *cp* mutations, the 404 mutation, and the ΔSH mutation were present in all isolates tested, demonstrating the stability of these mutations after replication in RSV-naive children. In 5 instances, single nucleotide substitutions were observed at either the 248 or 1030 codons, with reversion to the wt coding assignment. A sixth isolate had a mixed population of nucleotides at the 1030 codon (table 4). Each isolate had nucleotide substitutions at no more than 1 codon.

In some isolates, genetic changes were not detected, despite alteration in *ts* phenotype (table 4). In these isolates, it is likely either that mutations occurred at the known attenuating sites, but in a proportion of the uncloned virus population that was insufficient to be detected by sequence analysis, or that suppressor mutations occurred elsewhere in the genome.

DISCUSSION

Recovery of infectious virus from cDNA clones of RSV [17] has profoundly influenced the development of live attenuated RSV vaccines, because it is now possible to develop new vaccine candidates by introducing combinations of attenuating mutations into rRSV by direct manipulation of the DNA intermediate. The effect of each mutation cannot be predicted precisely, because the phenotype associated with an individual mutation is not always additive in the context of other mutations. This was observed in the present study, in which deletion of the SH gene did not further attenuate *cpts*248/404. However, incremental increases in attenuation can be achieved by including additional mutations, as was observed with the addition of the 1030 mutation to rA2cp248/404ΔSH and with other vaccine candidates under clinical development [28]. The flexibility of this technology suggests that future live RSV vaccine candidates will be developed by means of recombination techniques rather than the classical methods of serial cold passage and chemical mutagenesis [28].

Here, rA2cp248/404ΔSH was evaluated in adults and in RSV-seropositive and -seronegative children. It was minimally infectious in adults and seropositive children, which was expected on the basis of our experience with *cpts*248/404 [2, 29]. Indeed, previous studies have suggested that minimal infectivity in these individuals is a necessary prerequisite for evaluation in RSV-naive infants and children [9]. However, the mean peak titer of rA2cp248/404ΔSH shed by RSV-seronegative children was $10^{4.3}$ pfu/mL, which is comparable to that observed in seronegative recipients of *cpts*248/404 [2]. Thus, in the context of the *cpts*248/404 mutations, deletion of the SH gene does not further attenuate RSV. Because the level of replication is a useful predictor of attenuation for live respiratory viral vaccines [28], it appeared unlikely that rA2cp248/404ΔSH would be more attenuated in young infants than *cpts*248/404 [2]. For this reason, rA2cp248/404ΔSH was not evaluated in young infants.

In contrast, replication of rA2cp248/404/1030ΔSH was significantly restricted in RSV-seronegative children, with a mean peak titer of $10^{2.4}$–$10^{3.5}$-pfu/mL in nasal wash specimens. These data indicated that the 1030 mutation conferred substantial attenuation in the context of the *cp,* 248, 404, and ΔSH mutations and that rA2cp248/404/1030ΔSH was sufficiently attenuated to merit evaluation in young infants. Excess respiratory and febrile illnesses were not observed in infants infected with vaccine virus. Specifically, the clinically significant nasal congestion observed after vaccination with *cpts*248/404 was not observed in infants who received rA2cp248/404/1030ΔSH. Also, the level of replication of rA2cp248/404/1030ΔSH was comparable in infants and RSV-seronegative children, indicating that replication can occur in the upper respiratory tract even in the presence of maternally derived antibody. This finding is consistent with those of previous studies of live attenuated influenza, parainfluenza, and RSV vaccines [2, 30–33] and suggests that this outcome should be expected when these vaccines are evaluated in infants.

rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH induced high titers of RSV antibodies in seronegative children. In contrast, only a minority of infants developed antibody responses to rA2cp248/404/1030ΔSH. We previously showed that a majority of infants developed IgA antibody responses after vaccination with *cpts*248/404 [2], suggesting that the decreased antigenic load associated with the highly restricted replication of rA2cp248/404/1030ΔSH may have diminished the response in this age group. Of note, IgA antibody responses in infants were directed toward RSV F and G glycoproteins with equal frequency, whereas studies in infants with *cpts*248/404 showed responses primarily directed toward RSV G glycoprotein [2]. Further studies will be needed to address this inconsistency.

Despite the modest antibody responses, young infants, after receipt of the first dose of rA2cp248/404/1030ΔSH, were protected against challenge with the second dose; significant reductions in the proportion of infants shedding virus and the amount of virus shed were observed after the second dose. This protection was likely mediated by RSV-specific immune responses that we were unable to measure but that were effective in preventing infection and in clearing virus from the respiratory tract. Future efforts should be directed toward determination of the correlates of protection in young infants.

Although both rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH are *ts* viruses, rA2cp248/404/1030ΔSH is more *ts* than is rA2cp248/404ΔSH. When the *ts* phenotype of viruses recovered from nasal wash specimens of children who received these vaccines was assessed, there was no evidence of reversion to wt virus. Virus present in specimens from children who received rA2cp248/404ΔSH maintained the *ts* phenotype of the parent virus (no plaques were detected at 39°C). Some change in the *ts* phenotype occurred in a fraction of the specimens obtained

from recipients of rA2cp248/404/1030ΔSH: 27 of 141 specimens contained virus that formed plaques at 36°C, and 1 specimen contained virus that formed plaques at 37°C. Genetic characterization revealed reversion of either the 248 or the 1030 mutation in several isolates, with preservation of the *cp*, 404, and ΔSH mutation in all recovered viruses.

It is perhaps not surprising that a subpopulation of viruses that could replicate at 36°C–37°C was generated after infection with rA2cp248/404/1030ΔSH, because selective pressure for generation of these viruses would exist in the upper airway. Although the clinical significance of these viruses cannot be determined from the present study, they remained highly *ts* and would likely be at least as attenuated as *cpts*248/404, which was attenuated even in young infants [2]. These viruses also retained all of the non-*ts* attenuating mutations and 2 of the 3 *ts* attenuating mutations present in the vaccine. Studies of live attenuated influenza and parainfluenza vaccines have shown that *ts* and non-*ts* mutations independently contribute to the stability of the attenuation phenotype [34–36]; therefore, it is likely that the viruses shed by recipients of rA2cp248/404/1030ΔSH would remain highly attenuated. Also, because the viruses were shed at $\leqslant 10^3$ pfu, they would not likely be transmitted to others, but transmissibility studies are needed to address this issue. The potential transmissibility of these highly attenuated vaccine-derived viruses should be considered in the context of wt RSV, which is highly contagious and infects virtually all children by 2 years of age [37].

These rRSV vaccine candidates are the first to be evaluated in clinical trials. rA2cp248/404ΔSH is not suitable for infants, given its level of replication in RSV-seronegative children. However, rA2cp248/404/1030ΔSH is the first RSV vaccine candidate that appears to be appropriately attenuated for young infants. rA2cp248/404/1030ΔSH shows limited phenotypic instability. It remains to be seen whether attenuating *ts* point mutations can be stabilized by means of molecular techniques [38] or whether some instability must be expected in highly *ts* live respiratory virus vaccines. In light of the limited replication after the second dose, multiple doses of rA2cp248/404/1030ΔSH might protect against RSV-associated LRI, despite the limited antibody response in young infants. Field trials should be conducted to address this issue. Strategies to augment the antibody response in infants, such as enhancement of RSV F and G glycoprotein expression by gene shift to promoter proximal positions [39], should also be explored.

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