Evaluation of a Live, Cold-Passaged, Temperature-Sensitive, Respiratory Syncytial Virus Vaccine Candidate in Infancy

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A live-attenuated, intranasal respiratory syncytial virus (RSV) candidate vaccine, *cpts*-248/404, was tested in phase 1 trials in 114 children, including 37 1–2-month-old infants—a target age for RSV vaccines. The *cpts*-248/404 vaccine was infectious at 104 and 105 plaque-forming units in RSV-naive children and was broadly immunogenic in children >6 months old. Serum and nasal antibody responses in 1–2 month olds were restricted to IgA, had a dominant response to RSV G protein, and had no increase in neutralizing activity. Nevertheless, there was restricted virus shedding on challenge with a second vaccine dose and preliminary evidence for protection from symptomatic disease on natural reexposure. The *cpts*-248/404 vaccine candidate did not cause fever or lower respiratory tract illness. In the youngest infants, however, *cpts*-248/404 was unacceptable because of upper respiratory tract congestion associated with peak virus recovery. A live attenuated RSV vaccine for the youngest infant will use *cpts*-248/404 modified by additional attenuating mutations.

Respiratory syncytial virus (RSV) is the leading cause of severe viral respiratory disease in infants and children [1]. It is also an important cause of severe respiratory disease in the elderly [2], immunocompromised patients of all ages [3, 4], and infants with underlying cardiopulmonary disease [5]. It is considered a major infectious trigger for reactive airway disease [6]. RSV infections are estimated to account for ~90,000 pediatric hospitalizations and 4500 deaths yearly in the United States [7]. RSV causes a yearly epidemic during the winter months, with a high penetrance in the first years of life. Of the 2 serologically distinct subgroups, RSV A and B, RSV A viruses appear to be slightly more virulent and are more commonly isolated [8]. To be effective, a vaccine must protect against RSV-associated lower respiratory tract disease in very young infants, because the peak age of hospitalization is in the second and third months of life [9].

To introduce an RSV vaccine into the pediatric immunization schedule, the following properties of the vaccine must be assessed: (1) its safety in infancy, (2) the effect of maternal antibody on its infectivity, (3) the effect of immunological immaturity or transplacental maternal antibody on its immunogenicity [10, 11], and (4) its efficacy against natural RSV infection. By analogy with vaccines given in infancy against other pathogens, multiple doses are expected to be required.

Live, attenuated, intranasally administered RSV vaccines have been under development since the late 1960s. At that time, the parent strain of the lineage under current investigation (cold-passaged [*cp*] RSV) was evaluated in seronegative children as young as 2 years old [12]. In seronegative children, *cp* RSV caused mild respiratory illness that was temporally associated with virus shedding [13].

In parallel, there was an effort to develop a formalin-inactivated vaccine. This inactivated vaccine failed to protect vaccine recipients and led to enhanced illness on natural exposure to virus [14]. That enhanced illness has profoundly influenced the

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Informed consent was obtained from parents or guardians of volunteers, and the human experimentation guidelines of the US Department of Health and Human Services and of the relevant institutions were followed in the conduct of the clinical research.

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development of an RSV vaccine by delaying the evaluation of other inactivated or subunit vaccines that might follow similar antigen processing and presentation pathways and have attendant safety concerns [15]. Consequently, live vaccines that mimic natural infection have been pursued as the safest strategy for immunizing young children. Wild-type (wt) RSV infection does not exhibit enhanced disease during reinfection. Intranasal infection should induce mucosal immunity, which contributes to protection against RSV [16, 17] and should lead to a balance between Th1 and Th2 immunological responses without the risk of enhanced illness [18].

Recently, a series of further attenuated candidate vaccines [19–21] were derived by chemical mutagenesis of *cp* RSV. *cp* RSV was subjected to 2 rounds of chemical mutagenesis, and temperature-sensitive *(ts)* mutant derivatives were generated. The non–*ts*-attenuating mutations of the *cp* RSV parent virus were anticipated to work in concert with the subsequently introduced *ts* mutations, to yield further attenuated, genetically stable viruses. The *ts* phenotype of the *cpts* vaccine is more stable than that of a prototype *ts* virus, RSV *ts*-1, which also was evaluated in children [22, 23]. The mutations in the *cpts* vaccines are distinct from mutations in the overly attenuated RSV *ts*-2 that block replication in the human respiratory tract [24]. The first 2 vaccines evaluated in the *cpts* lineage, *cpts*-248/955 and *cpts*-530/1009, were either insufficiently attenuated or were transmitted among seronegative children 6–36 months old [25].

The present candidate, *cpts*-248/404, is among the most attenuated of the current series of *cpts* vaccines on the basis of in vitro and in vivo analysis. Because its shut-off temperature for plaque formation is between 35°C and 36°C, it is unlikely to replicate efficiently at human core body temperature, 37°C. The *cpts*-248/404 vaccine is highly restricted in its replication in chimpanzees. Only $10^{1.3}$ pfu/mL was recovered from the upper respiratory tract (levels >1000-fold lower than that of wt RSV), and virus was not recovered from the lungs, despite the fact that infection was initiated by direct intratracheal instillation. When passive RSV antibodies were given before immunization of 2 chimpanzees to mimic transplacental maternal antibodies, an inapparent infection with *cpts*-248/404 occurred without documented virus shedding and a limited antibody response that, nevertheless, protected these animals against challenge with wt RSV 28 days later. There was an enhanced neutralizing antibody response after challenge with wt RSV [19].

In the present study, *cpts*-248/404 underwent testing in progressively younger children, which resulted in the first administration of a live, attenuated RSV vaccine to the target age group for vaccination of infants—namely, those <2 months old. In the youngest age group, *cpts*-248/404 vaccine caused mild-to-moderate upper respiratory congestion, precluding it from being a candidate for efficacy trials in early infancy. Nevertheless, valuable information was derived from this study, including the quantity of vaccine virus required to infect a 1–2-month old infant, the level of vaccine virus replication in the presence of maternal antibodies, immunogenicity in the presence of maternal antibodies, ability of the first dose of vaccine to restrict replication of a second dose, transmissibility, phenotypic stability of the *cpts*-248/404 vaccine, the level of attenuation of RSV vaccines that is needed for the very young infant, and preliminary evidence of protection from symptomatic illness during natural reexposure to wt RSV.

Materials and Methods

Viruses. Isolation and characterization of *cpts*-248/404 (shut-off temperature for plaque formation of 35°C–36°C) has been described elsewhere [19, 20]. A viral suspension for clinical trials was produced in Vero cells and found to be free of adventious agents by Dr. Louis Potash (lot RSV A-25; Nova Vax, Bethesda, MD). The titer of *cpts*-248/404 (lot RSV A25) prepared for clinical evaluation was $10^{5.3}$ pfu/mL. When necessary to achieve the planned titer for inoculation, this virus suspension was diluted in L-15 medium (BioWhittaker, Walkersville, MD). Nineteen vaccine and 11 placebo recipients in the 6–24-month-old group received *cpts*-248/404 vaccine (lot LRSV-404-301) prepared by Wyeth-Lederle Vaccines and Pediatrics (Pearl River, NY) that had a titer of 10^8 pfu/mL. To achieve the planned titer for inoculation, this virus suspension was diluted in phosphate buffered saline with sucrose-phosphate-glutamate. The results with both vaccine lots are similar, and the results are combined.

Clinical studies in children. Because the parental strains from which the further attenuated *cpts*-248/404 was derived had very limited infectivity in adults [12, 25], adult studies were not performed, and initial studies were done in seropositive children 15–59 months old. Children were enrolled in this randomized, double-blind, placebo-controlled phase I study at 3 study sites, with a 2:1 ratio of vaccine to placebo recipients. Children were eligible to participate if they were healthy and if all other family members and day care contacts were ⩾6 months old. Before enrollment, children were screened for the presence of RSV serum-neutralizing antibodies by a complement-enhanced, 60% plaque reduction neutralization test [26]. Those with titers >1:40 were considered RSV seropositive. Each study subject received 0.5 mL of vaccine or placebo as intranasal drops. Children were examined daily for 10 days after vaccine administration. Each study group of 4–6 children was unblinded after clinical observations were completed, so that continuous monitoring of safety could be maintained among sites.

Because of the high level of attenuation of a dose of 10^5 pfu in 17 seropositive children, studies were then performed in 74 seronegative children 6–24 months old at a dose of 10^5 or 10^4 pfu. The first 31 children immunized with 10^5 pfu of RSV *cpts*-248/404 vaccine were examined on days 0–10 and day 14. Subsequently, the remaining children immunized with 10^5 pfu and all children immunized with 10^4 pfu were examined and had samples taken on days 0, 4 or 5, 7 or 8, and 10 or 11. Using the latter schedule, studies then were done in infants 3–5 months old at a dose of 10^5 pfu and then in 1–2-month old infants at a dose of 10^5 pfu or 10^4 pfu. Although some infants had maternally derived antibodies in their serum, these study subjects had not been previously infected with RSV, because they were born after the previous annual RSV epidemic. One month after the first dose of *cpts*-248/404, all avail-

able 1–2-month-old vaccine and placebo recipients were given a second dose of the vaccine or placebo to which they were originally randomized.

Children were observed for 1–2 h on each study day in an outpatient setting. Febrile illness was defined as a rectal temperature ≥38.1°C. Respiratory illness was categorized as upper respiratory tract infection (URI), defined as rhinorrhea or pharyngitis of ≥2 consecutive days duration, or lower respiratory tract infection (LRI), defined as persistent rhonchi, rales, or wheezing. Cough for ≥2 consecutive days was recorded without assignment as to the site of involvement of the respiratory tract [23].

Isolation, quantitation, and identification of virus. Nasal wash specimens for virus isolation were obtained on each day of observation from all subjects and from those with illness reported in the 3 weeks after immunization, as described elsewhere [25]. Fresh or snap-frozen samples were inoculated into 2 sets of tissue culture tubes that contained either Vero or HEp-2 tissue culture cell monolayers and were incubated at 32°C. Viral isolates from these cultures were identified as RSV by use of an indirect immunofluorescence assay (IFA; Bartels Microscan; Baxter Healthcare, Bellevue, WA). RSV in fresh or snap-frozen specimens was titered by plaque assay on HEp-2 cell monolayer cultures maintained under a semisolid overlay at 32°C, as described elsewhere [24], and results were expressed as $\log_{10}$ pfu/mL. For purposes of calculation, samples in which virus was not detected were assigned a titer of $10^{0.6}$ pfu/mL.

Phenotypic characterization of viral isolates. The level of temperature sensitivity of virus present in snap-frozen nasal wash specimens was determined by plaque titration in HEp-2 cell monolayers at 32°C, 36°C, 37°C, 38°C, and 39°C, as described elsewhere [25]. Virus isolates from nasal wash samples that showed a significantly altered *ts* phenotype (an increase in shut-off temperature for plaque formation ≥2°C) were prepared for analysis by either 2 passages on HEp-2 monolayers or by 1 round of plaque purification on HEp-2 cell monolayers. The level of attenuation of isolates with altered *ts* phenotype was assessed by examining their level of replication in BALB/c mice, as described elsewhere [27].

Genetic characterization of viral isolates. Viral isolates that showed an altered *ts* phenotype were further characterized by sequence analysis. Reverse transcription–polymerase chain reaction (RT-PCR) amplification of viral RNA was performed for regions of *cpts*-248/404 known to contain determinants of the *ts* and attenuation phenotypes, as described elsewhere [25]. The nucleotide sequence of mutations specific to *cp* RSV and *cpts*-248/404 was determined by use of Sequenase 2.0 (USB, Cleveland, OH), as described by Whitehead et al. [27].

Immunological assays. Sera and nasal wash specimens were obtained before and either 4 or 8 weeks after initial immunization for measurement of RSV-specific antibodies. A third serum and nasal wash specimen was obtained 1 month after the second dose from study subjects who received either vaccine or placebo at 1–2 months old. For comparison, additional sera and nasal wash samples were available from 18 children 1–2 months old who were not enrolled in these trials but were hospitalized for illness caused by wt RSV infection. Sera were tested for antibodies to RSV by a plaque neutralization assay [26] and for IgG and IgA antibodies to RSV fusion (F) and attachment (G) proteins by an end-point titration in a modification of an ELISA described elsewhere [25]. In brief, Nunc polysorb plates were coated with 20 ng/well of either purified F or G protein in carbonate buffer and were blocked with 0.5% gelatin in phosphate buffered saline with 0.05% Tween (PBST). Sera were diluted in PBST with 0.5% gelatin and 2% fetal calf serum on antigen-coated and noncoated wells. After 1 h of incubation, the plates were washed and were incubated with goat anti–human IgG or IgA alkaline phosphate conjugate (Jackson ImmunoResearch, West Grove, PA) for 1 h. Color development used 1 mg/mL of D-nitrophenylphosphate (Sigma, St. Louis) in diethanolamine buffer. Optical densities (OD) were read at 405 nm wavelength, the OD was subtracted for the corresponding blank well, and the end-point dilution at 0.2 OD was calculated. The results were expressed as $\log_2$ of end point, with a positive response defined as a ≥4-fold increase in antibody titer.

Nasal wash samples were tested for the presence of IgA antibodies to purified RSV F and G proteins by a kinetic ELISA (kELISA) originally developed for influenza [28]. The increase in absorbance in milliOD/min of each nasal wash IgA value was expressed as a dilution of a standard serum curve run in the assay that gave the same increase in absorbance. kELISA values <5 milliOD/min were defined as negative. For pre- and postsamples with RSV-specific antibody, the RSV-specific results were adjusted for their total IgA concentration, as measured by a radial immunodiffusion assay using secretory IgA standards (Binding Site, San Diego, CA). Replicate experiments determined that a ≥4-fold difference in the standardized, adjusted result was at the 95% confidence interval (CI) for the test. Therefore, pre- to postimmunization changes of this magnitude or paired samples that went from negative to positive were considered to demonstrate a mucosal antibody response.

Surveillance. By use of methods described elsewhere [25], RSV vaccine recipients, placebo recipients, and age-matched control subjects were monitored during the subsequent RSV season for the occurrence of wt RSV–associated illness, to determine whether immunization with live attenuated vaccine prevented RSV disease or modified the clinical response to subsequent infection with RSV. Parents were contacted on a weekly basis throughout the time when wt RSV was identified in the 3 groups, in which trials were conducted. If a child developed a respiratory illness that met one of the definitions used during the initial vaccine evaluation, the child had a clinical assessment and viral culture. Before and after the RSV epidemic season, children had serum drawn to measure the incidence of RSV infection in the study population, as judged by increases in serum neutralizing antibody titer. The surveillance was not blinded; participant families and investigators knew the child's vaccine status.

Data analysis. Infection with RSV *cpts*-248/404 vaccine was defined as the isolation of *cpts* RSV, a ≥4-fold increase in serum RSV neutralizing antibody titer, and/or a ≥4-fold increase in ≥2 of the ELISA-based assays. In infants with residual maternal antibodies at the time of immunization, an increase was calculated as being 4-fold above the anticipated 28 day half-life ($t_{1/2}$) of passive antibodies. All comparisons of antibody titer by age were made after the first dose of vaccine. Titers were expressed as reciprocal mean $\log_2$. The Mann-Whitney *U* test (2-tailed) was used to compare the mean titers among the groups. Comparison of tabulated data (e.g., comparison of frequency of illness among vaccine and placebo recipients and comparison of immunological responses) was made using 2-tailed Fisher's exact tests. The κ statistics were

computed to measure concordance of antibody responses. The associations among antibody response, age, and prevaccine antibody were estimated using logistic regression analysis.

Results

Response of seropositive 15–59-month-old children to cpts-*248/404.* In this age group, the frequency of illness was comparable between the 11 vaccine and 6 placebo recipients (table 1). Virus was not recovered from seropositive vaccine recipients given 10^5 pfu, and the only antibody response was seen in a single study subject who had an increase in mucosal IgA antibody to the RSV G protein (table 2). Therefore, RSV *cpts*-248/404 is the most restricted in the *cpts* series evaluated, to date, in regard to infectivity for seropositive children.

Response of 6–24-month-old seronegative infants and children to RSV cpts-*248/404.* The *cpts*-248/404 vaccine was highly infectious and immunogenic in 6–24-month-old seronegative vaccine recipients. After a single administration of either 10^4 or 10^5 pfu, ~90% of vaccine recipients exhibited evidence of infection (tables 1 and 2). There was no difference in the frequency of illness seen in the vaccine and placebo recipients, although the ability to detect differences was limited by the high frequency of minor illness seen in children of this age group over a 10-day period of close observation. Notably, 1 vaccine recipient had mild wheezing associated with shedding of *cpts* RSV, and 2 vaccine recipients had rhonchi (1 with evidence of RSV infection). One placebo recipient developed mild wheezing temporally associated with an adenovirus infection.

Vaccine recipients given the 10^5 dose of vaccine shed virus with high frequency (79%) and at a moderately high level. The mean peak titer was $10^{4.2}$ $\log_{10}$ pfu/mL of nasal wash. The lower mean peak titer of vaccine virus ($10^{2.4}$ $\log_{10}$ pfu/mL) shed by recipients of the 10^4 dose may be methodological, because the samples were collected by nasal swabs rather than by nasal washes. Thirty-four of the 45 6–24-month-old vaccine recipients tested who had been given 10^4 or 10^5 pfu developed a serum-neutralizing antibody response, with an increase in mean neutralizing titer to ~1:300. The dose of vaccine did not influence the frequency or magnitude of the antibody response (table 2). Serum IgG ELISA responses to F and G proteins were concordant with neutralizing antibody responses (κ values of .76 and .74, respectively).

Response of 3–5-month-old infants to RSV cpts-*248/404.* In the next study, 16 infants (10 vaccine and 6 placebo recipients) 3–5 months old were given 10^5 pfu of *cpts*-248/404. One vaccine recipient developed a fever of 38.2°C, but LRI was not seen. There was a suggestion that URI was more frequent in vaccine recipients (7 of 10) versus placebo recipients (2 of 6; $P = .30$; table 1). Vaccine virus was recovered from 3–5-month old vaccine recipients, including 5 with residual maternal neutralizing antibodies, at levels comparable with older RSV-naive infants (figures 1 and 2). This finding suggests that maternally acquired serum antibody did not restrict nasal replication of the attenuated *cpts* vaccine, which is an observation confirmed in the youngest age group (figure 2). Systemic and mucosal immune responses were lower in frequency and in magnitude than those observed for vaccine recipients >6 months old; however, the

Table 1. Clinical and virological responses of infants and children to respiratory syncytial virus (RSV) *cpts*-248/404 or placebo.

			Virus isolation (nasal wash)		Percentage with indicated illness				
Study subjects (age), dose in pfu	Virus given	*n*	Shedding, %	Mean peak titer, $\log_{10}$ pfu/mL[a]	Fever	URI	LRI	Cough	Otitis media
Seropositive children (15–59 months)									
10^5	*cpts*-248/404	11	0	0.6[b]	18	36	0	0	9
	Placebo	6	0	0.6[b]	33	33	17	17	0
Seronegative children (6–24 months)									
10^5	*cpts*-248/404	38	79	4.2	29	68	8	24	11
	Placebo	20	0	0.6[b]	40	70	5	30	30
10^4	*cpts*-248/404	11	55	2.4	18	100	0	36	9
	Placebo	5	0	0.6[b]	20	60	0	20	20
Infants (3–5 months)									
10^5	*cpts*-248/404	10	70	4.2	10	70	0	10	0
	Placebo	6	0	0.6[b]	0	33	0	0	17
Infants (1–2 months)									
Dose 1									
10^5	*cpts*-248/404	17	76	4.0	0	65	0	18	6
	Placebo	8	0	0.6[b]	0	0	0	0	0
10^4	*cpts*-248/404	7	100	4.9	14	86	0	43	0
	Placebo	3	0	0.6[b]	0	33	0	33	0
Dose 2									
10^5	*cpts*-248/404	15	27	3.8	7	40	0	7	0
	Placebo	7	0	0.6[b]	14	0	0	0	0
10^4	*cpts*-248-404	7	0	0.6[b]	0	33	0	0	0
	Placebo	3	0	0.6[b]	0	33	0	0	0

NOTE. LRI, lower respiratory tract infection; URI, upper respiratory tract infection.

[a] Calculated for infected volunteers only.

[b] Lowest limit of detection of the assay was 0.7 $\log_{10}$ pfu/mL. For samples without detectable plaques, a titer of 0.6 was assigned.

Table 2. Immunological responses of infants and children to respiratory syncytial virus (RSV) *cpts*-248/404 or placebo.

Subjects (age), dose in pfu	Virus given	*n*	Percentage infected[a]	RSV serum neutralizing antibody response			Antibody response to indicated RSV protein													
				Titer, reciprocal mean $\log_2$			Serum IgG ELISA, reciprocal mean $\log_2$						Serum IgA ELISA, reciprocal mean $\log_2$						Nasal wash IgA ELISA	
							F protein			G protein			F protein			G protein			F protein	G protein
				Before	After	≥4-Fold increase, %	Before	After	≥4-Fold increase, %	Before	After	≥4-Fold increase, %	Before	After	≥4-Fold increase, %	Before	After	≥4-Fold increase, %	≥4-Fold increase, %	≥4-Fold increase, %
Seropositive children (15–59 months)																				
10^5	*cpts*-248/404	11	0	9.3	8.8	0	14.1	13.9	0	12.0	11.5	0	ND	ND	ND	ND	ND	ND	0	9
	Placebo	6	0	8.0	8.1	0	13.4	13.3	0	9.3	9.2	0	ND	ND	ND	ND	ND	ND	0	0
Seronegative children (6–24 months)																				
10^5	*cpts*-248/404	38	87	3.3	7.3	68	8.1	12.5	71	6.5	11.6	77	3.0	6.4	71	2.7	6.4	74	78	72
	Placebo	20	10	3.5	3.6	0	8.5	8.2	12.5	6.8	6.6	12.5	3.6	3.2	6.3	2.7	2.8	0	0	11
10^4	*cpts*-248/404	11	91	4.6	8.5	91	8.7	13.0	91	6.5	11.6	91	3.5	5.9	55	2.0	5.8	82	33	55
	Placebo	5	0	4.6	4.6	0	9.3	8.3	0	7.2	6.2	0	3.2	3.8	20	2.0	2.0	0	25	20
Infants (3–5 months)																				
10^5	*cpts*-248/404	10	90	5.0	5.8	44	9.8	10.2	78	9.0	8.9	56	2.7	3.5	22	2.3	4.2	56	22	56
	Placebo	6	0	4.8	3.8	0	10.2	8.2	17	10.2	8.2	17	3.1	3.5	17	2.5	3.0	17	0	17
Infants (1–2 months)																				
Dose 1																				
10^5	*cpts*-248/404	17	76	5.9	4.4	0	14.7	13.2	0	12.2	11.6	7	1.9	3.0	33	21	5.5	73	11	27
	Placebo	8	0	6.6	4.7	0	14.2	12.9	0	11.9	11.2	0	2.2	2.2	0	2.1	2.1	0	0	14
10^4	*cpts*-248/404	7	100	5.8	3.7	0	12.5	12.1	0	11.3	10.9	0	2.3	4.5	57	2.4	7.3	100	50	100
	Placebo	3	0	6.0	5.1	0	12.2	12.3	33	10.8	10.1	0	2.3	2.3	0	2.5	2.5	0	0	0
Dose 2[b]																				
10^5	*cpts*-248/404	15	33	4.6	4.7	13	13.2	12.9	20	11.6	11.6	20	3.0	4.0	33	5.5	6.3	47	18	45
	Placebo	7	0	4.7	3.8	0	12.9	11.7	0	11.2	10.2	0	2.0	2.2	0	1.9	2.1	0	0	0
10^4	*cpts*-248/404	7	14	3.7	4.4	29	12.1	11.4	0	10.9	11.1	29	7.3	7.3	29	7.3	7.3	29	17	17
	Placebo	3	0	5.1	4.3	0	12.3	12.5	33	10.1	10.9	33	2.3	2.4	0	2.5	2.4	0	0	0

NOTE. Data indicate no. of volunteers enrolled in the specified treatment group. Serology results and the calculated percentages with ≥4-fold increases in the associated columns indicate data obtained from testing the available paired samples, which, in all cases, represented ≥90% of the volunteers in that group. Paired nasal wash specimens were available in ≥75% of each group. After, after vaccination; Before, before vaccination; ND, not determined.

[a] Percentage infected was calculated using evidence by either serology or virus shedding, as described in Materials and Methods.

[b] Percentage of increases for these specimens are calculated to reflect increases specifically occuring with dose 2.

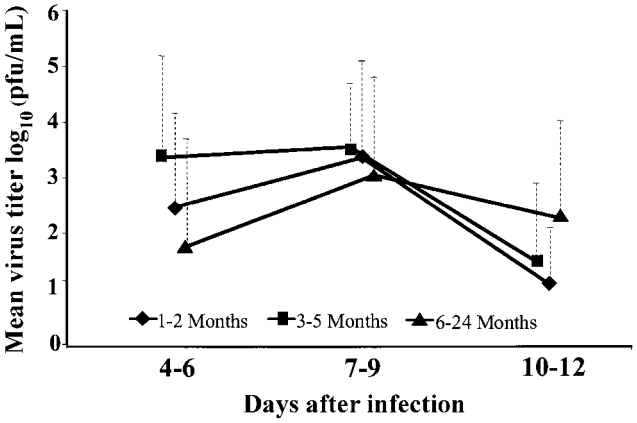

Figure 1. Recovery of respiratory syncytial virus (RSV) *cpts*-248/404 from the upper respiratory tract of RSV-naive infants and young children on selected days after vaccine administration. Mean titer of virus shed by children 1–3 months old (♦), 3–5 months old (■), and 6–24 months old (▲). SD is shown by upward deflection lines.

small number of 3–5-month-old children did not allow for statistical comparisons.

Response of 1–2-month-old infants to initial immunization. In a 2-dose regimen, 10^5 and 10^4 doses of vaccine were given to infants 1–2 months old, which is the target age group for RSV vaccination. Twenty-five children were studied; 17 received 10^5 pfu of vaccine, 7 received 10^4, and 11 received placebo (table 1). Seventeen of the vaccine recipients developed a clinical syndrome characterized by nasal congestion that occurred most typically between days 8 and 12. There were no signs of LRI on repeated examinations, although 6 mothers of vaccine recipients reported that their infants had a mild cough. Symptoms of congestion were linked temporally with the peak of virus shedding. Fifteen of 18 infants who shed $>10^3$ pfu of RSV per milliliter of nasal wash experienced congestion, fussiness while trying to sleep, and mild difficulty with feeding, which lasted ~24 h. Virus shedding was not accompanied by the profuse rhinorrhea typically seen with wt RSV infection, nor did any of the infants have LRI, otitis media, or fever. One of 11 placebo recipients and 1 of 4 vaccine recipients who did not shed virus had mild congestion on days 2 and 3 after receiving vaccine B, a significant difference in the rate of illness, compared with that of vaccine recipients from whom *cpts*-248/404 was recovered ($P = .0002$). The same pattern and frequency of congestion was seen in 10^5 and 10^4 vaccine recipients.

Thirteen of 17 vaccine recipients shed virus after the first 10^5 dose. All 7 vaccine recipients receiving 10^4 pfu shed virus in an amount equivalent to that observed for vaccine recipients receiving 10^5 pfu. In figure 1, the magnitude of virus shedding is compared among RSV-naive infants 6–24 months, 3–5 months, and 1–2 months old on days 4–6, 7–9, and 10–12 (constant days of sampling throughout the study). Peak virus shedding was similar for all ages. The lack of a correlation of the peak virus shedding in each infant <6 months old with his/her age (figure 2*A*) or level of maternal derived antibody (figure 2*B*) demonstrates that virus replication in the nasopharynx was independent of these variables.

Even when the expected decay in maternal antibody was considered in the calculations, 1–2-month-old vaccine recipients receiving either dose of vaccine rarely developed an increase in serum neutralizing antibody or an IgG ELISA response to F or G protein (table 2). In 18 infants of the same age hospitalized with wt RSV, infection serum neutralizing responses were also seen infrequently; only 30% had a ⩾4-fold increase. In contrast, 29 of 35 6–24-month-old vaccine recipients who shed virus developed a neutralizing antibody response, which is significant when compared with the 1–2-month-old vaccines ($P < .001$).

In contrast to their failure to develop a serum neutralizing and IgG-based ELISA antibody responses, the 1–2-month-old vaccine recipients frequently developed a serum IgA response to RSV G and F glycoproteins (table 2 and figure 3). The frequency and magnitude of the IgA antibody response to the G glycoprotein of the 1–2-month-old vaccine recipients did not differ from that of 6–24-month-old vaccine recipients. In contrast, the frequency of the IgA response to the F glycoprotein, combining the results of 10^4 and 10^5 doses by the 1–2-month-old vaccine recipients (41%), was less than that of the 6–24-month-old vaccine recipients (67%) and also less than that to the G glycoprotein (82%) in the 1–2-month-old vaccine recipients. Therefore, these findings indicate that 1–2-month-old vac-

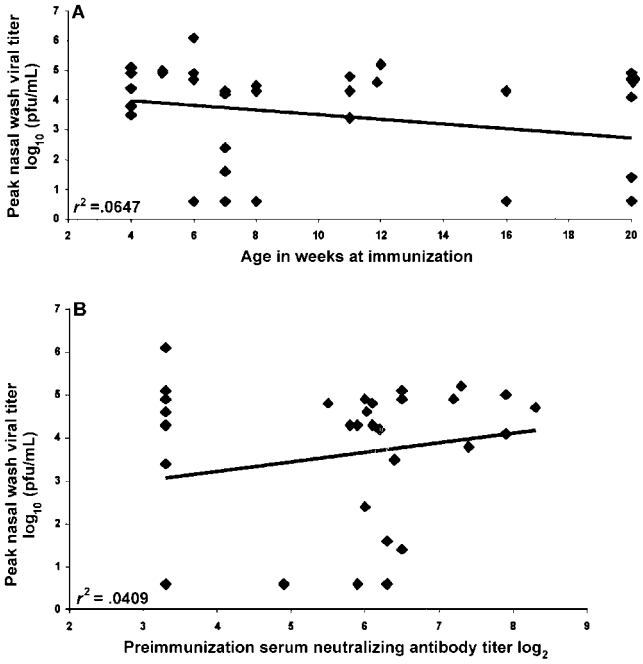

Figure 2. Peak recovery of respiratory syncytial virus (RSV) *cpts*-248/404 from the upper respiratory tract is independent of age *(A)* or level of maternally acquired serum antibody *(B)*. ♦, Individual volunteers.

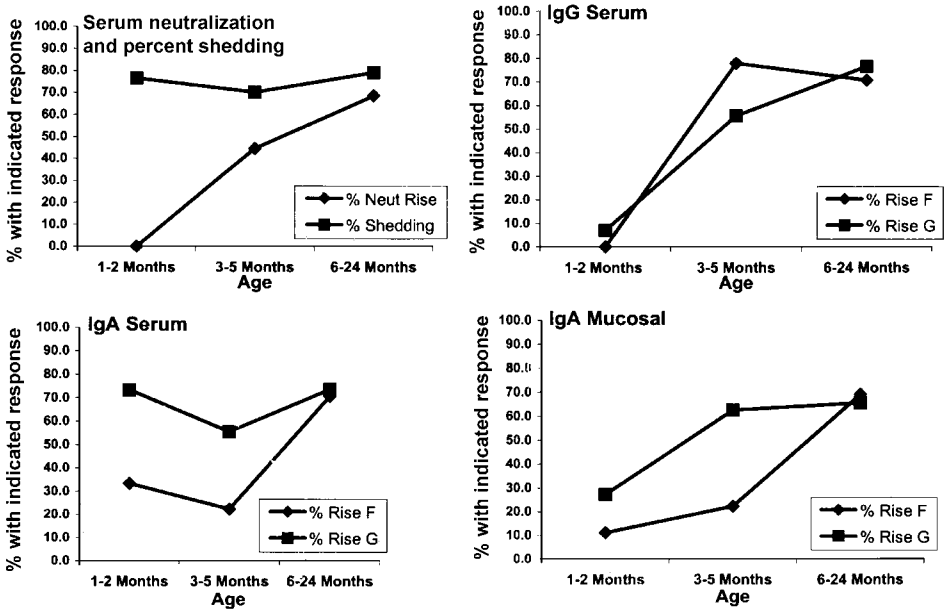

Figure 3. Maturation of the systemic and mucosal immune responses to respiratory syncytial virus *cpts*-248/404 vaccine with age. Percentage of children with a systemic or mucosal antibody response to the indicated immunoglobulin isotype is shown graphically by age. F, fusion protein; G, attachment protein.

cine recipients preferentially respond to the RSV G protein with an IgA response and that this response was similar to that of older vaccine recipients and was not influenced by titer of maternally derived antibodies.

Mucosal IgA responses were also seen in the 1–2-month-old infants (table 2 and figure 3). The mucosal response to G protein was also more frequent than that to F protein. Study of 18 infants of the same age hospitalized with wt RSV detected IgA mucosal responses in 61% to the F protein and 50% to the G protein, which are frequencies slightly higher than that observed for 1–2-month-old vaccine recipients. Neutralizing activity to RSV could be not detected in 10 available postvaccination nasal washes from 1–2-month-old vaccine recipients when tested at a dilution of 1:4.

Response of 1–2-month-old infants to second dose of vaccine. The relatively mild nature of the symptoms associated with the first dose allowed us to give the planned second dose of vaccine or placebo 4–6 weeks later to 22 available vaccine recipients (15 who received 10^5 and 7 who received the 10^4 dose) and to 10 placebo recipients (table 1). Virus was recovered from only 2 of 19 vaccine recipients who had shed virus after the first dose. Two of 3 who had not shed virus after the first dose of vaccine were infected, leaving 1 child who did not shed virus after either dose. Nasal congestion was observed in each of the vaccine recipients who shed RSV, but only in 2 of 18 vaccine recipients from whom virus was not recovered and in 1 of 10 placebo recipients ($P = .002$). The inverse relationship between peak virus shedding with the first and second dose of vaccine is shown in figure 4. Infection with the first dose provided a high level of resistance to replication of the second dose. There was an absolute correlation of protection from reinfection with detection of serum IgA antibody to the G protein ($P < .0001$), although, whenever an immune response to the first dose was documented by any assay in either serum or nasal wash, reinfection did not occur (table 3). Antibody increases after the second dose of vaccine were largely restricted to those vaccine recipients not infected with the first dose (table 2).

Factors influencing the immune responses of infants <24 months old to cpts*-248/404 vaccine.* Multivariate analysis of

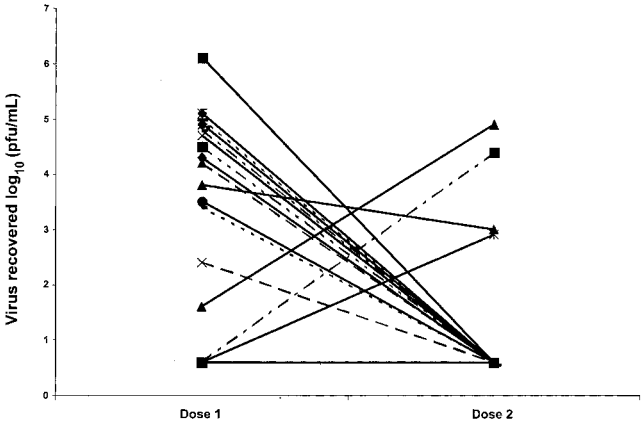

Figure 4. Influence of peak virus shedding on virus recovery, with the initial dose of respiratory syncytial virus *cpts*-248/404 given at 1–2 months old and a second dose of vaccine given 1 month later.

Table 3. Correlates of protection against a second dose of respiratory syncytial virus (RSV) *cpts*-248/404 vaccine when the initial dose was given at 1–2 months old.

Specimen tested, assay used	Response	Shed virus	No response	Shed virus
Serum				
RSV neutralizing antibody	0	0	22	4
IgA ELISA for RSV F protein	9	0	13	4
IgA ELISA for RSV G protein	18	0	4	4
IgG ELISA for RSV F protein	0	0	20	4
IgG ELISA for RSV G protein	1	0	19	4
Nasal wash				
RSV neutralizing antibody	0	0	9	1
IgA ELISA for RSV F protein	3	0	13	1
IgA ELISA for RSV G protein	8	0	7	1

NOTE. Data are no. of vaccine recipients with indicated antibody response to first dose who shed virus after the second dose.

the correlation of increase in antibody titer at 4–8 weeks after immunization with dose of vaccine and level of preexisting maternal antibodies in the infants <24 months old showed that preexisting neutralizing antibodies inhibited or masked an increase in neutralizing antibodies (odds ratio [OR], 4.0; 95% CI, 1.7–9.5; $P = .035$). IgG antibody responses to the F protein ($P < .001$) and the G protein ($P < .001$) were significantly decreased or masked in a direct relationship to the level of preexisting homologous antibodies but were not influenced by age. The frequency (OR, 0.85; 95% CI, 0.72–0.99; $P = .03$) and magnitude ($P = .02$) of IgA responses to F, but not to G, were significantly depressed by maternally acquired IgG antibody level to the homologous protein but not by age.

Evidence for lack of transmission of cpts-*248/404.* Vaccine and placebo recipients often came into close contact with one another for brief periods during follow-up examinations. However, virus was not recovered from placebo recipients, and only 1 of 52 seronegative control subjects developed a serological response. Transmission had been shown under similar circumstances with earlier vaccines in this lineage [25]. A more formal demonstration of the level of transmission will be performed with this and future candidates in a day care setting, as described elsewhere [23].

Effect of breast-feeding on magnitude of virus shedding. Seventeen of 18 children who were exclusively breast-fed shed virus with a mean peak titer of $10^{4.5}$, whereas 7 of 12 exclusively bottlefed shed virus with a lower mean peak titer of $10^{3.5}$.

Stability of ts *and attenuation phenotype of the vaccine.* Of the 176 specimens tested, 173 had a level of temperature sensitivity of plaque formation within 1°C of that of the *cpts*-248/404 vaccine virus, which indicates the high level of phenotypic stability of this virus after replication in susceptible infants and children. Specimens obtained from a single 11-week-old vaccine recipient on days 15, 16, and 17, however, contained an RSV isolate that produced plaques at 38°C, which is 2°C higher than the highest temperature at which *cpts*-248/404 produces plaques. This vaccine recipient experienced a pattern of congestion similar to that seen in other infants receiving this vaccine. Virus from these 3 nasal wash samples was amplified at permissive temperature (32°C), and isolates then were amplified further at both 32°C and 37°C. The higher shut-off temperature of these amplified virus suspensions was confirmed, and the recovered virus was shown to be of vaccine origin by nucleotide sequence analysis. The nucleotide sequences of the genomic regions of these recovered viruses that contain the attenuating mutations present in *cpts*-248/404—namely, the set of 5 *cp* mutations [29], the 248 mutation [30], and the 404 mutation in the M2 gene start sequence [31]—were determined. The recovered virus was found to have sustained a single C→A nucleotide substitution at nucleotide position 9 of the M2 gene start sequence. The mutation in the recovered vaccine occurred at the same position that specifies the 404-M2 attenuating mutation (table 4). It is interesting to note this substitution is not a reversion to the wt RSV T nucleotide at this position.

To assess the effect of this nucleotide substitution on the attenuation phenotype of the resulting virus, virus isolated from the vaccine recipient on day 15 was studied in mice and compared with wt RSV A2, *cpts*-248 (the parent of *cpts*-248/404), and *cpts*-248/404 viruses that were evaluated at the same time in mice. The viruses from the vaccine recipient replicated to the same level in the upper and lower respiratory tract of mice as *cpts*-248, which indicates that the loss of the distinguishing M2-404 mutation returned the virus to the same level of attenuation as *cpts*-248 (table 4). In a previous study, a recombinant virus counterpart of *cpts*-248/404, constructed by site-directed mutagenesis to lack the M2-404 mutation, was shown to have the same temperature sensitivity and attenuation phenotype as *cpts*-248 virus [27].

Surveillance. To examine whether infection with live attenuated *cpts*-248/404 RSV vaccine modified the frequency or severity of wt RSV–associated illness, young infants and seronegative children (i.e., RSV-naive individuals) immunized in these trials and age-matched control subjects in the same clinical care setting were closely followed through the subsequent RSV epidemic season. As in previous studies with live attenuated RSV vaccines, enhanced illness did not occur during reinfection of vaccine recipients with wt RSV [22, 25].

Indeed, the surveillance provided preliminary evidence that the live attenuated RSV vaccine induced a measure of protection (table 5). The rate of subsequent wt RSV infection, as judged by a serological increase, was very high and did not differ between 6–24-month-old vaccine recipients and age-matched control subjects, with 37% overall becoming infected. Indeed, in those <6 months old, there was a significantly higher seroconversion rate in the vaccine recipients than in the age-matched control subjects, which we suggest might result from the vaccine priming the immune system to mount a booster response. There was a marginally significant lower rate of children with subsequent symptomatic wt RSV infection in 6–24-month-old vaccine recipients (4%) than in age-matched control

Table 4. Respiratory syncytial virus (RSV) shed by a vaccine recipient on days 15, 16, and 17.

	Mutation			Virus titer at indicated temperature, $\log_{10}$ pfu/mL					Mean titer in mice,[a] $\log_{10}$ pfu/g tissue		Nucleotide[b]							
											cp					248	404-M2[c]	404-L
Variable	*cp*	248	404-M2	32°C	36°C	37°C	38°C	39°C	Nasal turbinate	Lung	1938	6313	7228	9453	13565	10989	7605	12046
Compared virus																		
wt RSV A2	−	−	−	5.2	5.0	5.1	5.1	5.2	4.5	4.3	G	A	C	G	C	A	T	T
cpts-248	+	+	−	6.4	6.4	6.1[d]	≤0.7	<0.7	3.4	3.1	A	C	T	A	T	T	T	T
cpts-248/404	+	+	+	4.9	3.6[d]	≤0.7	<0.7	<0.7	2.0	2.4	A	C	T	A	T	T	C	A
Day RSV recovered																		
Day 15	+	+	−	6.5	6.3	6.1	6.0[d]	≤0.7	3.6	3.3	A	C	ND	A	ND	ND	A	ND
Day 16	+	+	−	6.4	6.2	6.1	6.0[d]	≤0.7	ND	ND	A	C	ND	A	ND	ND	A	ND
Day 17	+	+	−	6.5	6.3	6.0	6.0[d]	≤0.7	ND	ND	A	C	ND	A	ND	ND	A	ND
Passage temperature[e]																		
32°C	+	+	−	6.3	6.1	6.1	6.0[d]	≤0.7	3.6	3.5	A	C	T	A	T	T	A	A
37°C	+	+	−	5.5	4.9	5.0	4.8[d]	≤0.7	3.4	3.3	A	C	T	A	T	T	A	A

NOTE. RSV was recovered from a single vaccine recipient on days 15, 16, and 17 and was passaged twice on HEp-2 cells at 32°C. Virus has a nucleotide substitution at the 404 mutation site in the M2 gene start signal, which reduced its temperature sensitivity and attenuation in mice to a level similar to that of RSV *cpts*-248. Shut-off temperature is defined as the lowest restrictive temperature at which a ≥100-fold reduction of plaque titer is observed and is underlined. *cp,* cold-passaged; ND, no data; wt, wild type; +, presence of mutation; −, absence of mutation.

[a] Groups of 5 mice under light anesthesia were given 10^6 pfu of the indicated virus in a 0.1-mL inoculum. After 4 days, virus titer was determined in the nasal turbinate and lung tissues.

[b] Numbered from 3′ end of negative-sense (viral) RNA. Nucleotide assignments are given in the positive sense.

[c] M2 gene start signal (nucleotide 7605 is underlined): A2 wt, GGGGCAAATA; *cpts*-248/404, GGGGCAAACA; and isolate, GGGGCAAAAA.

[d] Pin-point plaque size.

[e] Virus present in the day 15 nasal wash sample was grown at either 32°C or 37°C on HEp-2 cells. Five plaques were picked at each temperature, and passage was continued at either 32°C or 37°C. Results are shown for only plaque no. 4 at each temperature, although remaining plaques were tested and had the same level of temperature sensitivity and the same nucleotide sequence as those shown here.

subjects (20%; table 5). There were too few LRIs to reach any conclusions of vaccine efficacy.

Discussion

The strategy of passaging virus at low temperature to generate vaccine candidates has been successfully used to derive topically administered vaccines for influenza [32] and parainfluenza type 3 [33] that are appropriately attenuated, immunogenic, genetically stable, and, as shown in the case of influenza, highly efficacious on exposure to wt influenza virus [32]. Similar *cp* derivation of a vaccine for RSV yielded a mutant that was only partially attenuated [13]. The current systematic approach to exploring the correlates of virulence and using animal models to determine attenuation and infectivity has been very predictive of behavior of candidate vaccine strains in humans and in establishing a rank-order attenuation of such vaccine mutants for the young seronegative child.

This is the first time that a live attenuated RSV vaccine candidate has been evaluated in 1–2-month-old infants, the target age for vaccination, which allows us to examine factors that influence safety, infectivity, and immunogenicity of an RSV vaccine in early infancy. The demonstration that *cpts*-248/404 retained sufficient residual virulence to cause mild symptomatic URI in 1–2-month-old infants and to readily infect the respiratory tract and to attain a titer $\geq 10^4$ pfu of virus per milliliter of nasal wash would not have been predicted from the chimpanzee studies [19, 20]. Therefore, the young infant is a more permissive host for RSV than the chimpanzee. The absence of lower respiratory tract illness in the vaccine recipients is consistent with the failure to recover the vaccine candidate from the chimpanzee's trachea and the shut-off temperature of *cpts*248/404, which is less than that of human core body temperature, 37°C.

The level of passively acquired maternal antibodies and the infant's age did not have an effect on replication of this attenuated RSV strain in the nasopharynx. This observation is similar to data with bovine parainfluenza [34] and rotavirus vaccines given at the same age [35] and indicates that the mucosal route of administration of a live attenuated vaccine virus permits replication in the upper respiratory tract or gastrointestinal tract, despite the presence of a moderately high titer of serum antibodies. Very high levels of infused RSV serum antibodies can limit upper respiratory tract replication in experimental animals, but this is not an efficient process. Also, it should be noted that the effectiveness of high-titered RSV antibody in prophylaxis against human disease is presumed to be primarily the result of passive transfer of antibody across the mucosal epithelium into the lumen of the lower respiratory tract [36]. Our study also suggests that breast-feeding does not interfere with RSV replication, although breast milk may have antibodies to RSV [37].

In contrast, maternal antibodies and/or age had an inhibitory effect on neutralizing antibody responses and inhibited or masked RSV IgG-binding antibody responses. The vaccine recipients in the youngest age group rarely developed serum antibody re-

Table 5. Surveillance of *cpts*-248/404 vaccine recipients and unvaccinated control subjects during the subsequent respiratory syncytical virus (RSV) epidemic.

		No. (%) who developed RSV-associated illness[a]				Serological response	
Age at immunization, subject	*n*	Any	URI	Otitis media	LRI	Total	RSV positive
<6 Months							
Vaccine recipients[b]	28	6 (21)	5 (18)	2 (7)	0 (0)	25	12[c]
Unimmunized age-matched controls[d]	51	14 (28)	13 (26)	5 (10)	2 (4)	37	4
6–24 Months							
Vaccine recipients[b]	28	1 (4)[e]	0 (0)[f]	1 (4)	1 (4)	24	7
Unimmunized age-matched controls[d]	70	14 (20)	9 (13)	6 (9)	1 (1)	52	21

NOTE. LRI, lower respiratory tract infection; URI, upper respiratory tract infection.

[a] Culture positive at time of illness. Serological response is ⩾4-fold increase in neutralizing antibody.

[b] One vaccine recipient 6–24 months and 4 vaccine recipients <6 months were not included in surveillance because they had no evidence of vaccine infection.

[c] $P < .01$ when comparing serological responses in vaccine recipients and control subjects.

[d] Age-matched participants were <9 months old at the beginning of the winter surveillance (i.e., were RSV naive).

[e] $P = .059$ when comparing total illness in vaccine recipients and control subjects.

[f] $P = .056$ when comparing URI in vaccine recipients and control subjects.

sponses that could be detected by a sensitive plaque-reduction neutralization assay or an IgG ELISA assay to purified F and G protein. This lack of a detectable serum response is not unique to vaccine-induced immunity. As we report in this article and as has been documented elsewhere [38], <50% of young infants hospitalized with culture-documented wt RSV infection demonstrate a neutralizing response. It is also possible that a low-level IgG response is masked by residual maternal antibody, since the level of maternal IgG antibodies to RSV F and G proteins in the 1–2-month-old infants is comparable with that seen in the 6–24-month-old children after vaccination, but vaccination did not decrease the expected decay of maternally acquired RSV IgG antibodies, and neutralizing antibody titers were low enough that an increase might have been detected. Passive transfer of RSV antibodies in animals suppress the immune response to RSV F and G proteins expressed by vaccinia virus, despite the fact that the virus vector is not inhibited in its growth. These antibody-suppressed animals are susceptible to RSV reinfection [39]. Previous studies in human infants infected with wt RSV virus have suggested that both age, acting primarily on F protein responses, and the level of maternal antibodies, acting primarily on G protein responses, influence the response to infection with wt RSV [11]. Multivariate analysis suggested that, in this study, the suppression of the all IgG responses and the IgA antibody response to F from the vaccine virus were a function of the level of maternal acquired antibodies and not of age.

Serum IgA responses to the RSV G protein proved to be the most consistent response in the youngest infants. It is reasonable to suggest that the IgA serum antibody is a direct result of replication of vaccine virus in the mucosa of the respiratory tract and reflects a response that originated in the mucosal arm of the immune system. Mucosal IgA responses to RSV F and G protein were also seen, although with a lower frequency than that seen in serum, again more commonly to the G protein. IgA in the respiratory tract is inherently more difficult to measure, because of collection methodology and rapid clearance of IgA in secretions. The IgA responses were not accompanied by a measurable increase in nasal wash or serum neutralizing antibody in the 1–2-month-old vaccine recipients.

The unexpected focus of the infant's immune system on the RSV G protein strongly suggests that a live attenuated RSV vaccine will need to be a bivalent vaccine that contains RSV subgroups A and B, because the G proteins of the RSV subgroups are only ~50% related by amino acid sequence and only 5% related antigenically [40].

The *cpts*-248/404–infected vaccine recipients were highly resistant to infection with the second dose of vaccine. Vaccine virus was not recovered from any vaccine recipient who developed any immunological response to the first dose of vaccine. The second dose of vaccine induced a slight boost in immunity in vaccine recipients infected with the first dose but did not lead to the enhanced neutralizing antibody response seen in the chimpanzee model [19]. Because the second dose of vaccine did infect 2 of 3 children not infected by the first dose, an argument can be made for 2 doses of vaccine as a minimum schedule for immunization.

Each of the vaccine recipients protected against a second dose of vaccine developed a serum IgA response to the G protein after the initial dose, whereas all those infected with the second dose failed to mount such a response to the initial dose. Immunity induced by vaccinia virus that expresses RSV G has been demonstrated, although the degree of protection was less than that seen with a vaccinia virus F recombinant [41]. It is possible that the protection afforded in the present study by IgA antibodies to the RSV G protein were mediated by neutralizing IgA antibodies below the level of our detection, but it is also possible that mucosal IgA antibodies are mediating protection by a mechanism other than, or in addition to, viral

neutralization. Proposed mechanisms of antiviral action for IgA beyond neutralization include antibody-dependent cellular cytotoxicity [42] and intracellular interruption of virus replication during trancytosis of IgA across the epithelial mucosa [43]. The critical response may be mucosal IgA memory on secondary challenge, as reported for RSV in calves whose primary responses are inhibited by colostrums [44]. Others have reported cell-free and cell-bound IgA antibody in the nasopharynx in the course of primary RSV infection in children and its lack of correlation with neutralizing activity [45]. Work with other viral model systems has suggested that protection may be seen in the absence of a neutralizing antibody response that is mediated by components of the cellular immune response measured by lymphoproliferation, as shown in monkeys infected with measles [46]. An advantage of using a live attenuated vaccine is that it can theoretically stimulate a balanced immune response, including antibodies, MHC class I restricted $CD8^+$ cytotoxic T cell response, and a $CD4^+$ T cell memory.

Our inability to infect seropositive children with the attenuated *cpts*-248/404 is another indication of RSV immunity. The marked inhibition of vaccine virus replication by prior natural infection is similar to that seen with influenza and parainfluenza type 3 viruses, and, collectively, they are a strong indicator that effective immunity to respiratory viruses can be induced. It is, however, important to emphasize that the resistance observed to vaccine challenge was to an attenuated virus. Finally, the vaccine recipients 6–24 months old had decreased RSV-associated illness on subsequent exposure to wt RSV. The observed decrease in subsequent wt RSV illnesses by vaccine recipients is an encouraging signal that a similarly derived RSV vaccine may prove to be efficacious when tested for protection against LRI. It is clear from serological studies that wt RSV reinfection occurs frequently in vaccine recipients, as it does after natural infection, and thus cannot serve as a marker for vaccine efficacy.

Despite the high level of replication in fully susceptible young vaccine recipients, *cpts*-248/404 maintained the full *ts* phenotype in 173 of 176 isolates; however, 3 isolates from 1 vaccine recipient exhibited a decrease in their level of temperature sensitivity and in their attenuation in rodents. The 3 virus isolates retained the set of 5 *cp* mutations and the 248 mutation in the L protein of RSV and exhibited a level of temperature sensitivity and attenuation for rodents that were characteristic of viruses that contain these mutations. The alteration in the level of temperature sensitivity and partial loss of attenuation for rodents resulted from a single nucleotide substitution in the M2 gene start sequence at the same position as the original attenuating mutation. As indicated below, a more attenuated RSV vaccine than *cpts*-248/404 is being sought. It is anticipated that the introduction of an additional attenuating mutation will result in a concomitant increase in the stability of the attenuation phenotype.

The *cpts*-248/404 vaccine is not an acceptable RSV vaccine candidate for the youngest infant. It might have a role in seronegative children >6 months old to prevent the substantial burden of RSV-associated URI, otitis media, and milder LRI seen at that age. For the 1–2-month-old infant, ⩾1 additional attenuating mutations need to be introduced in *cpts*-248/404 to generate a vaccine that is slightly more attenuated. This can be readily achieved using reverse genetics [47, 48]. Equally important to the optimization of an RSV vaccine will be an understanding of the immune mechanisms by which resistance to reinfection with vaccine virus is conferred in the young child. Future studies will investigate whether protection against a second dose of vaccine continues to translate into protection against reinfection with wt virus and/or the amelioration of the severity of illness because of RSV in the young child, as seen in this study.

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