MAJOR ARTICLE

Evaluation of Combined Live, Attenuated Respiratory Syncytial Virus and Parainfluenza 3 Virus Vaccines in Infants and Young Children

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We evaluated a combination respiratory syncytial virus (RSV) and parainfluenza 3 virus (PIV3) live, attenuated intranasal vaccine for safety, viral replication, and immunogenicity in doubly seronegative children 6–18 months old. RSV *cpts*-248/404 and PIV3-cp45 vaccines were combined in a dose of 10^5 plaque-forming units of each per 0.5-mL dose and compared with monovalent vaccines or placebo. The virus shedding pattern of RSV was not different between monovalent RSV *cpts*-248/404 vaccine and combination vaccine. Modest reductions in the shedding of PIV3-cp45 vaccine virus were found after the administration of RSV *cpts*-248/404 and PIV3-cp45 vaccine, relative to monovalent PIV3 vaccine; 16 (76%) of 21 children given combination vaccine shed PIV3-cp45 versus 11 (92%) of 12 of those given monovalent PIV3 vaccine. Both vaccines were immunogenic, and antibody responses were similar between the monovalent groups and the combination group. Combined RSV/PV3 vaccine is feasible for simultaneous administration, and further studies are warranted.

Respiratory syncytial virus (RSV) is the most important respiratory viral pathogen of infancy and childhood [1–3]. Most primary RSV infections are symptomatic [4, 5]; many RSV infections manifest as bronchiolitis and/or pneumonia, and severe infections occur in younger infants, with peak incidence of lower respiratory-tract disease (LRI) occurring in infants 2–6 months old, but a substantial portion of serious RSV illness and hospitalization still occurs in children >6 months old [6–9].

Human parainfluenza viruses (PIVs) are also important causes of serious respiratory-tract diseases in infants and young children <5 years old [10–12]. According to the Institute of Medicine, 25% of children <5 years old will have a clinically significant PIV infection annually, and ~2% will require hospitalization, most commonly for croup [12]. PIV is an important cause of or cofactor in acute otitis media (AOM) in children. Type 3 PIV (PIV3) is of particular significance in that, in addition to causing croup and bronchitis, it ranks second only to RSV as a cause of bronchiolitis and pneumonia in infants <6 months old. The virus causes severe disease throughout the first 2 years of life, and virtually all children have had primary PIV3 infections by 3–4 years of age. Overall, PIV3 is considered to be responsible for ~11% of hospitalizations for pediatric respiratory disease [11].

Significant progress is being made in developing live, attenuated intranasally (inl) administered vaccines for

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influenza, RSV, and PIV3 [13–17]. A cold-passage, temperature-sensitive strain of RSV (designated *cpts*-248/404) and a cold-passage, temperature-sensitive strain of PIV3 (designated -cp45) have been separately evaluated in phase 1 clinical trials in seronegative children 6–36 months old [15, 16]. Among children >6 months old, both vaccines were well tolerated and immunogenic at doses of 10^5 pfu and showed evidence of infection with vaccine virus (by isolation of virus and/or increase in antibody level) in >75% of children who were given inl vaccine [14, 16]. In studies of infants <6 months old who received 2 doses of PIV3-cp45 vaccine, there was evidence that the first dose provided some protection from shedding of vaccine virus after the second dose was administered, 1 month later [15]. Studies with PIV3-cp45 vaccine are progressing in infants [15], and a phase 2 study in 388 children 6–18 months old has confirmed the safety of this vaccine candidate for seronegative subjects [14]. The RSV candidate vaccine *cpts*-248/404 was evaluated in infants 4–12 weeks old and was found to cause nasal congestion that interfered with breast-feeding; therefore, additional attenuating mutations are being introduced into the vaccine [16]. However, for children >6 months old, RSV *cpts*-248/404 deserves further study.

In practice, it would be efficient to be able to administer these vaccines simultaneously. The present report summarizes the results of a phase 1 study evaluating the simultaneous administration of the RSV and PIV3 vaccines, each at a dose of 10^5 pfu, combined before inl administration. The objectives of the study were to (1) describe the infection rate, magnitude, and duration of shedding of RSV and PIV3 after 1 dose of a combined RSV/PIV3 vaccine administered inl; (2) determine the tolerability and safety of a combined RSV/PIV3 vaccine; (3) determine whether interference occurs when RSV and PIV3 are administered simultaneously; and (4) describe the antibody response as measured in serum and nasal-wash specimens.

SUBJECTS, MATERIALS, AND METHODS

Vaccines

The preparation and derivation of the RSV *cpts*-248/404 vaccine, a derivative of the A2 strain of RSV (subgroup A), and the PIV3-cp45 vaccine have been described elsewhere [15, 16]. RSV *cpts*-248/404 vaccine was prepared by Wyeth Vaccines at a titer of 1×10^6 pfu/mL. To achieve the planned dose for inoculation, this virus suspension was diluted in PBS with sucrose-phosphate-glutamate (PBS-SPG) to a titer of 4×10^5 pfu/mL. PIV3-cp45 vaccine was prepared by Wyeth Vaccines at a titer of 1×10^6 pfu/mL. Vaccine was diluted in PBS-SPG to a titer of 4×10^5 pfu/mL.

Combination vaccine was made by mixing equal volumes of the diluted monovalent vaccine, which resulted in titers of 2×10^5 pfu/mL for each vaccine strain. A dose, administered as 0.5-mL inl drops, delivered 10^5 pfu of each vaccine virus (RSV *cpts*-248/404 and PIV3-cp45). Monovalent vaccines were diluted to a titer of 2×10^5 pfu/mL, which resulted in a dose of 1×10^5 pfu/0.5 mL of nasal drops. Placebo consisted of PBS-SPG.

Study Design

The study was a multicenter, randomized, double-blinded comparison of bivalent vaccine, monovalent RSV *cpts*-248/404 vaccine, monovalent PIV3-cp45 vaccine, and placebo. Approximately 60 children, 6–18 months old, who were doubly seronegative for RSV and PIV3, were randomized in a 2:1:1:1 ratio to receive 1 of the following regimens: RSV *cpts*-248/404 and PIV3-cp45 combination vaccine (24 children), RSV *cpts*-248/404 monovalent vaccine (12 children), PIV3-cp45 monovalent vaccine (12 children), or placebo (12 children).

Study Subjects

Healthy children 6–18 months old whose parent(s) or guardian(s) gave informed consent to participate were enrolled. Children who were seronegative for RSV (defined as neutralizing antibody titer <1:40) and for PIV3 (defined as a hemagglutination inhibition [HAI] antibody titer ⩽1:8) were selected. Children were screened by medical history and physical examination, to ensure that they had normal health and development. The human experimentation guidelines of the US Department of Health and Human Services and those of the authors' institutions were followed in the conduct of this clinical research.

Children were excluded if they had known or suspected impairment of immunological function or were receiving immunosuppressive therapy. Conditions for exclusion included systemic corticosteroid therapy, major congenital malformations, cytogenic abnormalities or serious chronic disorders, cardiac or respiratory illness, and any prior episode of wheezing confirmed by a physician (including illnesses diagnosed as asthma, wheezing, or reactive airway disease, whether attributed to environmental agents such as allergens or exposure to chemical irritants or to physical agents such as exercise- or cold-induced asthma, or infection). Also excluded were children with tympanostomy tubes and members of a household that contained a pregnant woman or an infant <6 months old or any immunocompromised individual. Children who attended day care and were in contact with infants <6 months old were excluded. They were also excluded if they exhibited a current febrile (temperature, ⩾38°C) or other acute illness, including upper or lower respiratory symptoms (including nasal congestion that was considered significant enough to reduce the likelihood of successful immunization) or AOM at the time of enrollment. Infants born at <37 weeks gestation were deferred from study participation until they were at least 1 year old.

Procedures

Serum samples were obtained before inl vaccination and tested for antibody to RSV and PIV3, to select doubly seronegative children. Seronegative children were randomized to receive either a vaccine or the placebo by nose drops in a volume of 0.25 mL/nostril, for a total dose of 0.5 mL. The parents were asked to keep track of any illness or symptoms on a parent diary card each day for 14 days after vaccination and whenever the child felt warm during the 42-day postvaccine study period. Children were examined by study personnel, and nasal-wash samples were collected for the quantitation and phenotyping of shed virus on days 3–7, 8 or 9, 10, 12, 14, 17 or 18, and 28 after vaccination. These brief visits (20–30 min each) allowed the study staff to examine the child closely for any signs of a rhinorrhea, pharyngitis, fever, cough, respiratory illness, or ear infections. Six weeks after the first vaccination visit, all enrolled children returned to the clinic for a brief physical examination and a blood sample and nasal wash to measure antibody responses to vaccination.

Fever (rectal temperature, ⩾38°C), upper respiratory-tract illness (rhinorrhea or pharyngitis), cough, and LRI were defined as described elsewhere [17]. AOM was defined as findings of inflamed, immobile tympanic membrane, with or without bulging, observed by a physician or nurse practitioner and confirmed by tympanometry or by a second observer. All children were examined at the end of the study, on days 35–49 after vaccination.

In the event of acute respiratory illness, additional nasal-wash samples were obtained and cultured for RSV, PIV3, and a variety of other common respiratory viruses, to help determine illness etiology. The nursing assessment completed during these visits included review of the diary card and transcription of the information, to determine the occurrence of any adverse events. A postimmunization blood sample and a nasal-wash sample were obtained from each child on day ~42 (±7 days) after immunization.

Laboratory Methods

Serum antibody response. Serum samples collected before and ~6 weeks after vaccination were evaluated for the presence of antibody to RSV and PIV3. Antibody to RSV was measured by the plaque reduction neutralization (PRN) assay, as described elsewhere [18]. Antibody to PIV3 was assessed by the HAI and by ELISA (IgA and IgG) to purified hemagglutinin-neuraminidase (HN) protein, as described elsewhere [19, 20].

Nasal-wash antibody response. Nasal-wash samples were evaluated by kinetic ELISA, as described elsewhere [21, 22], to determine the antibody response to RSV (glycoproteins F and Ga—i.e., the G attachment protein of RSV subgroup A) and to PIV3 (purified HN protein). The IgA concentration (in micrograms per milliliter) was determined similarly by use of a capture method. An increase in the ratio of ⩾4 when prevaccination samples were compared with postvaccination samples from a child or seroconversion from 0 to any value was considered to be an increase in the level of nasal-wash antibody.

Vaccine virus quantitation and genetic stability. Vaccine virus shedding levels and temperature-sensitive *(ts)* phenotypic stability were determined from fresh or frozen nasal-wash samples by plaque assay. For the quantitation of RSV in nasal-wash samples, it was necessary to neutralize any PIV3 that might be present in the sample, to prevent any PIV3 cytopathic effect (CPE) from obscuring RSV plaques. Each sample to be tested for RSV was incubated for 1 h at 32°C in the presence of 5% anti-PIV3 horse serum, then serial 10-fold dilutions of PIV3-neutralized samples were inoculated onto HEp2 cell monolayers in duplicate 24-well plates and overlaid with 0.75% methylcellulose in minimum essential medium. The duplicate plates were incubated for 5 days at 32°C for virus quantitation or at 39°C for assessment of the *ts* phenotypic stability of the virus after replication within the human host. RSV plaques were stained in an indirect immunoperoxidase (IP) assay by use of RSV-specific monoclonal antibodies (MAbs) directed against F and G proteins and horseradish peroxidase (HRP)–labeled goat anti–mouse IgG, as described elsewhere [23]. Virus titers were expressed as $\log_{10}$ plaque-forming units per milliliter of nasal-wash fluid. To determine the *ts* phenotypic stability of the RSV recovered from the children, the infectivity titers of virus grown at 32°C and 39°C were compared. The *ts* phenotype of the RSV present in the sample was considered to be stable if the titer of RSV detected at 39°C was at least 100-fold lower that the titer of the RSV observed at 32°C.

The method to determine the titer and phenotype of the PIV3 in nasal-wash samples was modified from a plaque assay described elsewhere [24]. Plaques were visualized by use of PIV3-specific MAbs and HRP-labeled goat anti–mouse IgG in an indirect IP assay. Briefly, the agarose plugs were gently removed, and the monolayers were fixed with acetone: methanol (50%:50%). A mixture of PIV3 MAbs to the HN glycoprotein was added to each monolayer [25, 26]. After incubation for 1–2 h at 37°C or overnight at 2°C–8°C, the plates were washed with PBS, the substrate (Enhance Orange DAB-C/H_2O_2; Kirkegaard and Perry) was added, and the monolayers were incubated on a rocker platform for ~10–15 min at room temperature. The monolayers were rinsed with tap water and air-dried, and plaques were enumerated.

The genetic stability of PIV3 vaccine virus was assessed by measuring the *ts* phenotype of the shed virus in original nasal-wash samples at 39°C. Duplicate monolayers of LLC-MK2 cells were inoculated with test sample; 1 set was incubated at 32°C, and the other set was incubated at 39°C. The former was used to determine the titer of PIV3 shed by each child, and the latter was used to ascertain the genetic stability of the PIV3 being

recovered from the nasal-wash samples. The duration of viral shedding was defined as the last day of vaccine virus detection.

Diagnostic virologic testing on nasal-wash samples obtained during illness. Children who presented with respiratory symptoms during the study provided nasal-wash samples for virus isolation. Nasal-wash samples were collected and immediately diluted in 5× virus transport media. The samples were inoculated onto primary monkey kidney cells, as well as onto at least 2 other cells lines appropriate for the isolation of common respiratory viruses, including adenovirus, influenza A and B, PIV 1–4, and RSV. The tissue-culture cells were incubated at 32°C and observed for CPE for 14 days after inoculation.

If PIV3 was detected in association with illness and the result did not coincide with the detection of *ts* virus (vaccine) isolated from routinely scheduled nasal-wash samples, then the PIV3 isolate was identified by sequence analysis of reverse-transcription polymerase chain reaction (RT-PCR) products by use of primers specific for the amplification of a variable region of the F gene. The RT-PCR procedure used to detect wild-type *(wt)* PIV3 has been described elsewhere [15].

Statistical Analyses

Mean $\log_{10}$ titers of vaccine virus in nasal-wash samples were calculated for each day tested; nasal-wash samples in which virus was not detected (minimum amount of virus detectable, 0.7 $\log_{10}$ pfu) were considered to have a titer of 0.4 $\log_{10}$ pfu for our calculations. Days of virus shedding were compared between groups by use of the χ^2 text. Mean virus shedding in each monovalent group was compared with that in the combination vaccine group by use of analysis of variance (ANOVA).

RESULTS

A surprisingly high proportion of children screened, approximately one-half, were seronegative for both RSV and PIV3 at 6–18 months old. As expected, seronegative children tended to be younger than seropositive children. The average age of enrolled children was 10 months; 2 children were screened at 18 months of age but were not vaccinated until 19 months of age; they were included in the analysis. Fifty-four children were randomized—21 to the combined RSV *cpts*-248/404 and PIV3-cp45 group, 9 to placebo, and 12 each to RSV *cpts*-248/404 or PIV3-cp45 alone.

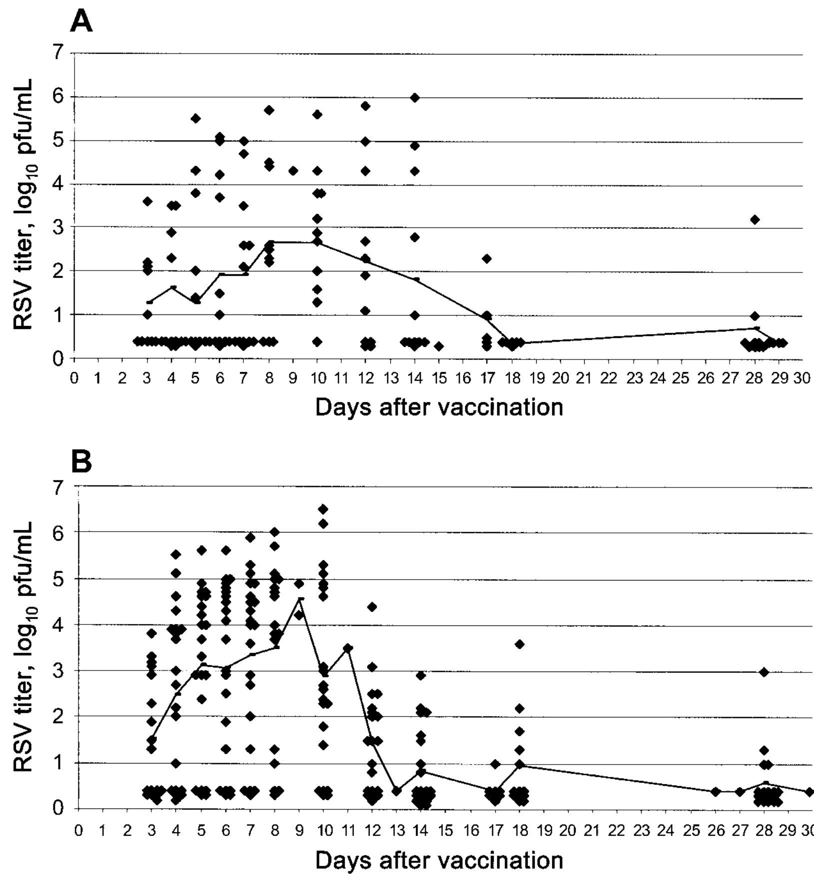
Shedding of RSV in the monovalent RSV vaccine group (figure 1*A*) and the combination vaccine group (figure 1*B*) was not significantly different in overall pattern, number of days of shedding (mean duration of RSV vaccine shedding, 14 days in the monovalent group and 15 days in the combination group; table 1), or mean peak titer shed (2.8 vs. 3.4 $\log_{10}$, monovalent vs. combination group; $P = .19$, ANOVA). In contrast, comparison of PIV3 shedding in the monovalent PIV3 group (figure 1*C*) and combination group (figure 1*D*) revealed modest viral interference, with PIV3-cp45 shedding in the combination group, compared with the PIV3 monovalent group. The mean peak titer of PIV3-cp45 shed was lower (3.8 vs. 2.1 $\log_{10}$, monovalent PIV3 vs. combination group; $P = .01$, ANOVA), and significantly fewer days of detection of PIV3-cp45 shedding were found in the combination group. The duration of PIV3-cp45 shedding (defined as the last day that vaccine virus was shed) was not significantly different between the 2 groups.

A total of 132 RSV-positive nasal-wash samples from 28 children and 123 PIV3-positive nasal-wash samples from 27 children were tested for *ts* phenotype. There was no change in the *ts* phenotype of RSV or PIV3 vaccine virus detected in postimmunization nasal-wash samples recovered from children enrolled in the study (data not shown.) These same samples contained RSV vaccine titers as high as 6.5 $\log_{10}$ pfu/mL or PIV3 vaccine titers as high as 5.4 $\log_{10}$ pfu/mL, which indicates multiple rounds of replication in the human nasopharynx without any change in the *ts* phenotype.

Clinical events occurring within 14 days of vaccination are summarized in table 2. LRI was not observed in any study subject. As expected, some children in the placebo group manifested fever, cough, rhinorrhea, or AOM, and these events reflect the high background rate of common upper respiratory-tract illnesses in young children. Although the rates of these minor illnesses did not differ significantly between vaccinees and placebo recipients, the study was not powered to examine the frequency of these events in placebo recipients versus vaccinees. However, these results provide background information for future studies to examine these questions; the sample size used in the present study has been used elsewhere [15, 16] to screen vaccine candidates and eliminate highly reactogenic or overly attenuated vaccines.

Children in the 3 vaccine groups manifested a similar spectrum of illnesses as the children who received placebo, with the possible exception of the occurrence of AOM. Evidence of AOM was observed in 4 (38%) of 12 children vaccinated with RSV *cpts*-248/404 (3/4 children with AOM shed a virus other than vaccine type, including 1 each of *wt* PIV, adenovirus, and influenza virus) and 7 (33%) of 21 children given the combination of RSV *cpts*-248/404 and PIV3-cp45 vaccines (0/7 shed a *wt* virus), compared with 1 (8%) of 12 in the PIV3-cp45 group (this child shed influenza A virus and PIV3-cp45 vaccine) and 1 (11%) of 9 in the placebo group. However, these rates were not statistically different. Other manifestations of viral respiratory disease were common in all study groups, including cough, rhinorrhea, and fever.

In addition to the concurring viral infections associated with AOM, as described above, concurrent viral infections were detected in study children and included 1 *wt* RSV infection in a child in the monovalent PIV3-cp45 vaccine group and 2 children with enterovirus infections in the combination vaccine group. *wt* PIV3 was circulating at some of the study sites at



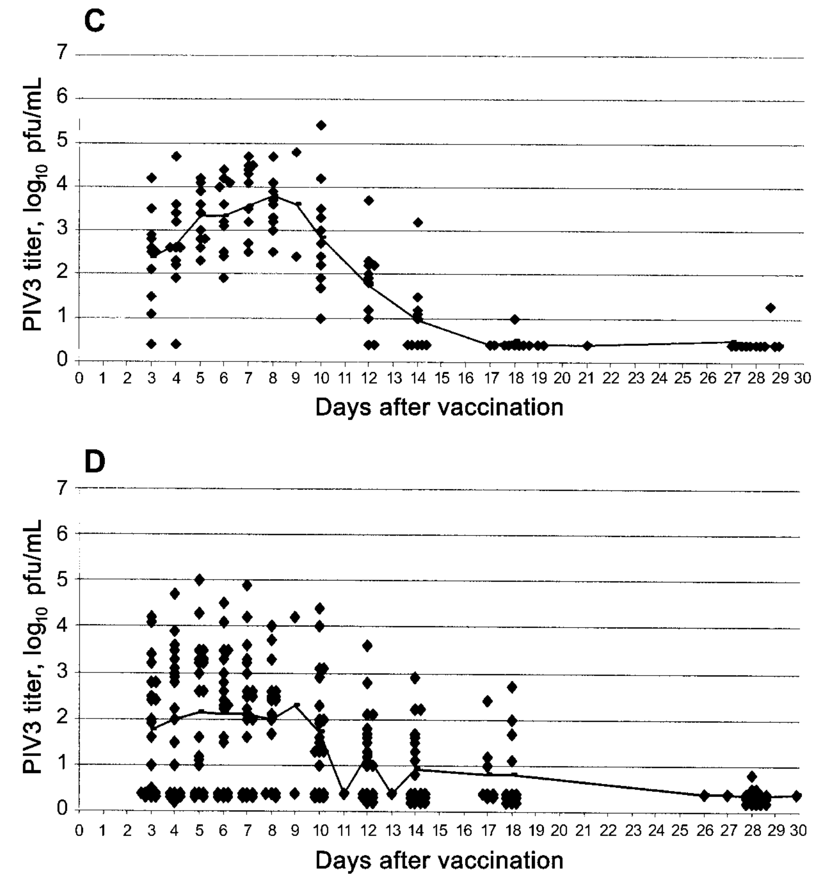


Figure 1. Virus shedding pattern of respiratory syncytial virus (RSV) *cpts*-248/404 in the monovalent RSV vaccine group *(A)* vs. the virus shedding pattern of RSV *cpts*-248/404 in the combination RSV/parainfluenza 3 virus (PIV3) vaccine group C *(B)* ($P = .19$, analysis of variance [ANOVA]). Virus shedding pattern of PIV3-cp45 in the monovalent PIV3 vaccine group *(C)* vs. the virus shedding pattern of PIV3-cp45 in the combination RSV/PIV3 vaccine group *(D)* ($P = .01$, ANOVA).

Table 1. Mean duration of virus shedding and mean quantity shed on the peak day of virus shedding of respiratory syncytial virus (RSV) *cpts*-248/404 or parainfluenza 3 virus (PIV3)–cp45 vaccine among children intranasally given 10^5 pfu of monovalent RSV vaccine, 10^5 pfu of monovalent PIV3 vaccine, 10^5 pfu each of RSV and PIV3 vaccines, or placebo.

	No. shedding/ no. vaccinated (%)		Duration of shedding,[a] mean, days		Virus titer on peak day of shedding,[b] mean, pfu/mL $\log_{10}$	
Group	RSV	PIV3	RSV	PIV3	RSV	PIV3
RSV *cpts*-248/404	11/12 (92)	…	14	0	2.8	<0.5
PIV3-cp45	…	11/12 (92)	0	15	<0.5	3.8
RSV *cpts*-248/404 and PIV3-cp45	19/21 (90)	16/21 (76)	16	16	3.4	2.1[a]
Placebo	0/9	1/9[c]	0	0	<0.5	<0.5

[a] Significantly more days with no shedding of PIV3-cp45 were seen in the combination group vs. the monovalent PIV3-cp45 group. Duration of shedding was calculated by use of the last day of virus isolation to indicate the total duration of virus replication.

[b] Mean virus titer on peak day was calculated by use of the peak titer of virus shed from each child on day 10 for the RSV monovalent group and on day 7 for the PIV3 monovalent group and the RSV/PIV3 combination group.

[c] Wild-type PIV3 was isolated from 1 control child.

the time of the study, and evidence of concurrent RSV or PIV3 infection was present in the serologic results. Specifically, 2 children in the PIV3 group had antibody increases to RSV, and antibody increases to PIV3 occurred in 2 children who received RSV vaccine and in 3 placebo recipients.

Both the RSV and PIV3 vaccine strains induced the production of serum and mucosal antibody. The frequency of developing serum neutralization antibody responses for RSV or serum HAI antibody responses for PIV3 was not significantly different in the monovalent groups versus the combination group (table 3). The RSV vaccine above induced antibody in 9 (90%) of 10 children, and the RSV/PIV3 combination vaccine induced RSV antibody in 18 (95%) of 19 children. There was a suggestion that PIV3 immunogenicity was reduced, but this did not achieve statistical significance; 9 (82%) of 11 children developed antibody in the monovalent PIV3-cp45 group, compared with 12 (60%) of 20 in the bivalent group ($P = .26$). Among those who were infected by a vaccine virus, the post-vaccine geometric mean PRN antibody titer to RSV or HAI antibody to PIV3 was not significantly different (table 3, footnotes c and d). Nasal-wash antibody responses to RSV for Ga antigens occurred in 6 (50%) and 8 (67%) of 12 children, respectively, in the monovalent vaccine group and in 7 (33%) and 14 (67%) of 21 children, respectively, in the combination vaccine group (table 3). After a single dose of vaccine, nasal-wash antibody response to PIV3 was found in only 2 (17%) of 12 children in the monovalent vaccine group and in 6 (29%) of 21 children in the combination group.

DISCUSSION

The development of a safe and effective vaccine for the prevention of respiratory disease caused by RSV and PIV3 represents an important but elusive objective. RSV is the most important cause of LRI in infants and young children, and it causes significant disease in elderly and immunocompromised patients. PIV3 is

Table 2. No. of children with the indicated sign or symptom of illness on days 0–14 after intranasal vaccination with respiratory syncytial virus (RSV) *cpts*-248/404, parainfluenza 3 virus (PIV3)–cp45, a combination of both vaccines, or placebo.

Group (no. of children)	Temperature ⩾38°C	Cough	Rhinorrhea	LRI	AOM
RSV *cpts*-248/404 (12)	2	7	11	0	4[d]
PIV3-cp45 (12)	1[a]	4	8	0	1[e]
RSV *cpts*-248/404 and PIV3-cp45 (21)	7[b]	7[c]	19	0	7[f]
Placebo (9)	4	3	5	0	1

NOTE. Data are no. of children. AOM, acute otitis media; LRI, lower respiratory-tract disease.

[a] One child with temperature ⩾38°C shed wild-type *(wt)* RSV.

[b] Four children with a temperature ⩾38°C shed both RSV and PIV3 (2 of the 4 also shed an enterovirus), and the other 3 shed only the RSV vaccine phenotype.

[c] Four children with cough shed RSV and PIV3, 2 shed RSV only, and virus was not isolated from 1.

[d] Concurrent *wt* infections occurred in 3 children—1 each of PIV, adenovirus, and influenza virus.

[e] Concurrent infection with influenza A occurred in this child.

[f] The children with fever, cough, and/or AOM were not the same 7 children; 14 children had various combinations of fever and/or cough and/or AOM.

Table 3. Development of ⩾4-fold serum and/or nasal-wash antibody responses to respiratory syncytial virus (RSV) or parainfluenza 3 virus (PIV3) among initially seronegative children after vaccination with RSV *cpts*-248/404, PIV3-cp45, both vaccines, or placebo.

	Increase in serum antibody, no. with increase/no. tested			Increase in nasal-wash antibodies, no. with increase/no. tested			
Group (no. of children)	RSV	PIV3	Both	RSV F	RSV Ga	PIV3	Both
RSV *cpts*-248/404 (12)	9/10	2/11[a]	1/10[a]	6/12	8/12	1/12	0/12
PIV3-cp45 (12)	2/12[a]	9/11[b]	2/11[a]	3/12	1/12	2/12	0/12
RSV *cpts*-248/404 and PIV3-cp45 (21)	18/19[c]	12/20[b,d]	11/18	7/21	14/21	6/21	5/21
Placebo (9)	0/5	3/9[a]	0/5	2/8	0/8	3/8	0/8

NOTE. Seronegative to RSV, serum neutralizing antibody titer <1:40; seronegative to PIV3, serum hemagglutination inhibition (HAI) antibody ⩽1:8.

[a] Wild-type RSV unexpectedly circulated in the community, and PIV3 was endemic, as expected.

[b] Nine of 11 vs. 12 of 20 ($P = .26$).

[c] Among those who were infected with RSV vaccine-type virus, as indicated by shedding of RSV *cpts*-248/404 or ⩾4 fold antibody increase to RSV, the log mean postvaccine neutralizing antibody to RSV was not significantly different in the monovalent vs. combination group, 8.8 ± 1.1 vs. 8.1 ± 1.0. Geometric mean titers were 504 and 276, respectively.

[d] Among those who were infected with PIV3 vaccine virus, as indicated by shedding of PIV3-cp45 or ⩾4 fold antibody increase to PIV3, the log mean postvaccine HAI titer to PIV3 was not significantly different in the monovalent vs. combination group, 4.1 ± 1.7 (±SD) vs. 3.6 ± 2.0; geometric mean HAI antibodies were 17 vs. 12, respectively.

the second most important cause of bronchiolitis and pneumonia during the first 6 months of life and is a common cause of febrile respiratory disease and AOM in older children. Recent experience with a cold-adapted, *ts,* attenuated influenza vaccine administered by the inl route demonstrated the feasibility of this technology for vaccine administration [13].

Several key issues in developing a bivalent RSV-PIV3 vaccine were addressed in the present pilot study. We sought to develop preliminary observations to assess whether combined vaccine would exhibit evidence of augmented reactogenicity. RSV and PIV3 frequently are involved in dual or mixed viral infections that occur in ~15% of respiratory illnesses in the community [27–30]. In general, the clinical syndromes associated with dual respiratory viral infections have appeared to be indistinguishable from single-agent infections, although one review [29] found that dual infections may be more severe and were more likely to result in hospitalization. Additionally, evidence of viral interference or decreased immunogenicity was sought to determine a preliminary strategy for the vaccination of children against both diseases.

The present results suggest that bivalent RSV/PIV3 vaccine is feasible to develop. A majority of children in the bivalent vaccine group responded to both the RSV and PIV vaccine components. The present results demonstrate only very modest interference by RSV with the PIV component. A simple strategy to overcome this interference would be to give 2 doses separated by an appropriate interval, to be determined by clinical investigations—a 2-month interval was successful with live, attenuated trivalent inl influenza vaccine. In previous studies in this age group [16], the RSV *cpts*-248/404 vaccine appeared to be satisfactorily attenuated, but it retained some reactogenicity in infants <6 months old. As a test-of-concept study, the acute safety of the combined RSV-PIV3 vaccine could not be differentiated from those of either monovalent vaccine or placebo. Although no significant differences were observed, the clinical events associated with the RSV component of bivalent vaccine may be significant, but confirmation of this observation will require a larger study, given the high frequency of concurrent virus infections in this age group.

Peak virus shedding titers in the monovalent RSV *cpts*-248/404 vaccine group were higher than have been previously reported [16]; the higher titers in the present study reflect a change in the laboratory assessment methods. In the present study, IP staining of plaques was used to determine titers, whereas, elsewhere, plaque assay without IP was used. Similarly, peak virus shedding titers in the monovalent PIV3-cp45 vaccine group were higher than those reported elsewhere because of our use of an IP stain to determine plaque count [15, 17].

The characteristics of PIV3-cp45 vaccine appear to be suitable for expanded trials. Recently, a phase 2 study was completed in 380 children 6–18 months old. No increase in rhinorrhea, cough, fever, or AOM was found when these findings were compared in seronegative, vaccinated recipients versus placebo control subjects. PIV3-cp45 vaccine induced vigorous HAI antibody responses (geometric mean titer [GMT], 1:24 after vaccination) that were within ~2-fold of the antibody level found in naturally infected children before vaccination (GMT, 1:50).

Genetic stability was assessed, and the vaccine viruses retained their *ts* phenotype, despite multiple cycles of replication in young seronegative children; the multiple genetic changes introduced into PIV3-cp45 and RSV *cpts*-248/404 provide a good means of safety to ensure that viruses with a virulent phenotype will not emerge during replication in children.

Significant progress is being made toward further attenuation

of the RSV component. Once a suitable RSV vaccine component is derived, it can be combined with PIV3-cp45 vaccine and evaluated for the safety, infectivity, and efficacy of each component. The present study has provided a rationale and a model protocol for proceeding with those future evaluations. The RSV *cpts*-248/404 component interfered with PIV3-cp45, but not the reverse; this suggests that further attenuated derivatives of RSV *cpts*-248/404 might not interfere or would interfere to a lesser degree and, therefore, would work well in combination with PIV3-cp45, but this will need to be tested in clinical trials and determined empirically. The results of the present test-of-concept trial provide the framework for future development of bivalent RSV/PIV3 vaccine.

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