Parainfluenza Virus Type 3 Expressing the Native or Soluble Fusion (F) Protein of Respiratory Syncytial Virus (RSV) Confers Protection from RSV Infection in African Green Monkeys

Roderick S. Tang,* Mia MacPhail, Jeanne H. Schickli, Jasmine Kaur, Christopher L. Robinson, Heather A. Lawlor, Jeanne M. Guzzetta, Richard R. Spaete, and Aurelia A. Haller†

MedImmune Vaccines, Inc., Mountain View, California

Respiratory syncytial virus (RSV) causes respiratory disease in young children, the elderly, and immuno-compromised individuals, often resulting in hospitalization and/or death. After more than 40 years of research, a Food and Drug Administration-approved vaccine for RSV is still not available. In this study, a chimeric bovine/human (b/h) parainfluenza virus type 3 (PIV3) expressing the human PIV3 (hPIV3) fusion (F) and hemagglutinin-neuraminidase (HN) proteins from an otherwise bovine PIV3 (bPIV3) genome was employed as a vector for RSV antigen expression with the aim of generating novel RSV vaccines. b/h PIV3 vaccine candidates expressing native or soluble RSV F proteins were evaluated for efficacy and immunogenicity in a nonhuman primate model. b/h PIV3 is suited for development of pediatric vaccines since bPIV3 had already been evaluated in clinical studies in 1- and 2-month-old infants and was found to be safe, immunogenic, and nontransmissible in a day care setting (Karron et al., Pediatr. Infect. Dis. J. 15:650–654, 1996; Lee et al., J. Infect. Dis. 184:909–913, 2001). African green monkeys immunized with b/h PIV3 expressing either the native or soluble RSV F protein were protected from challenge with wild-type RSV and produced RSV neutralizing and RSV F-protein specific immunoglobulin G serum antibodies. The PIV3-vectored RSV vaccines evaluated here further underscore the utility of this vector system for developing safe and immunogenic pediatric respiratory virus vaccines.

Human respiratory syncytial virus (RSV) infection is the most frequent cause of hospitalization of infants in developed countries (39). In the United States alone, ~100,000 infants with RSV infections are hospitalized annually (13). RSV is the causative agent of acute respiratory diseases of infancy and early childhood, resulting in 20 to 25% of pneumonia and 45 to 50% of bronchiolitis cases in hospitalized children (13). Premature birth in conjunction with chronic lung disease, congenital heart disease, and T-cell immunodeficiency were identified as conditions that predispose infants to more severe forms of RSV infection (39). RSV also represents a health threat for the elderly and immunocompromised individuals (11, 14).

Protection against disease following RSV infection has been attributed to secretory and virus-neutralizing antibodies as well as cellular immunity (1). Therefore, effective vaccines for RSV should stimulate mucosal and cellular immune responses. Intranasal, live, attenuated vaccines that mimic the natural route of infection will most likely achieve this. At present, no vaccine is available to protect children or adults at risk from infections with RSV. Hospitalization and immunoglobulin treatment are often necessary to alleviate complications associated with serious RSV infections. Synagis, a commercially available RSV F monoclonal antibody, is prescribed prophylactically to high-risk premature infants to prevent complications of RSV infection (24).

For decades, approaches to generate an effective RSV vaccine with virus subunits or inactivated virus vaccines have failed due to either lack of immunogenicity or the potential of causing enhanced pulmonary disease upon reinfection with naturally occurring wild-type RSV. The development of a reverse genetics system for RSV has provided for the generation of a number of genetically designed RSV vaccine candidates that harbor mutations in essential RSV genes or deletions of nonessential RSV genes in an effort to attenuate virus replication without compromising immunogenicity (2). However, to date, all genetically designed RSV vaccine candidates evaluated in humans were either over- or underattenuated.

A chimeric bovine/human parainfluenza virus type 3 (b/h PIV3) is currently under development as a vector for a number of pediatric vaccines (15, 36). b/h PIV3 harbors the F and HN genes of human PIV3 (hPIV3) in a bovine PIV3 (bPIV3) genetic backbone (15). bPIV3 was shown in human clinical trials to be attenuated, immunogenic, nontransmissible in a day care center setting, and genetically stable in children as young as 2 and 6 months old (20, 25). The capacity of the bPIV3 vaccine to replicate in the nasal cavity without causing respiratory illness demonstrated its attenuation in young sero-negative children (20). With a rhesus monkey attenuation model, it was shown that b/h PIV3 retained the attenuation phenotype despite the presence of the hPIV3 F and HN genes (33, 35).

Previously we showed that b/h PIV3 could efficiently express RSV F protein from PIV3 genome position 1 (upstream of the PIV3 N gene) or 2 (juxtaposed between the N and P genes of PIV3) (36). Syrian golden hamsters immunized intranasally with b/h PIV3/RSV F were protected from both RSV A2 and hPIV3 challenge. Hamster sera collected 4 weeks postvaccina-

* Corresponding author. Mailing address: MedImmune Vaccines Inc., 297 N. Bernerdo Ave., Mountain View, CA 94043. Phone: (650) 919-6633. Fax: (650) 919-6611. E-mail: tangr@medimmune.com.
† Present address: GlobeImmune Inc., Aurora, CO 80010.

tion displayed RSV-neutralizing and hPIV3 hemagglutination-inhibiting (HAI) serum antibodies.

These results showed that b/h PIV3/RSV F was efficacious in a small-animal model, and therefore as a step toward human vaccine trials, we wanted to test whether nonhuman primates would also be protected from RSV infection upon vaccination with b/h PIV3/RSV F. In order to evaluate the replication, immunogenicity, and efficacy of b/h PIV3/RSV F2 and b/h PIV3/sol RSV F2, we selected African green monkeys (AGMs) as the nonhuman primate model because they support efficient replication of RSV (17) as well as hPIV3 (7). This allowed us to assess the replication of the PIV3/RSV chimeric viruses and observe the effects of a robust RSV challenge after immunization. However, AGMs are not an attenuation model for bPIV3. Coelingh et al. compared the replication of hPIV3 to bPIV3 in owl, squirrel, and rhesus monkeys as well as chimpanzees and showed that the host range restriction of bPIV3 replication was observed only in chimpanzees and the more readily available rhesus monkeys (38). Using the rhesus monkey model, Schmidt et al. showed that titers of a similar b/h PIV3 virus expressing RSV F was reduced by 2 $\log_{10}$ in the upper respiratory tract and 1.1 $\log_{10}$ in the lower respiratory tract compared to hPIV3, indicating that the PIV3/RSV chimeric virus was attenuated (33). However, the immunized animals were not challenged with wild-type RSV because RSV does not replicate efficiently in the respiratory tract of rhesus monkeys.

b/h PIV3/RSV F2 that expresses the native RSV F protein from PIV3 genome position 2 was described previously (36). b/h PIV3/RSV F2 was chosen for further analysis in primates rather than b/h PIV3/RSV F1, which harbors the RSV F gene in genome position 1, because this virus replicated to higher titers in tissue culture, which is important for vaccine manufacture.

In infected cells, the chimeric viruses express three surface glycoproteins originating from different viruses, the native RSV F protein and the hPIV3 F and HN surface glycoproteins. All three proteins are integral membrane proteins and have the potential to be inserted into the virion envelope. A potential change in tissue tropism due to expression of both hPIV3 and RSV surface glycoproteins by b/h PIV3 is a concern that we addressed experimentally in this study. b/h PIV3/sol RSV F2, the second RSV vaccine candidate evaluated here, produces an RSV F protein lacking the transmembrane domain and cytoplasmic tail, rendering the truncated RSV F protein incapable of inserting into the virion envelope. Both of the vaccine viruses evaluated here expressed the RSV F proteins efficiently from PIV3 genome position 2. Furthermore, soluble RSV F was secreted and could be readily detected in the infected cell medium as early as 27 h postinfection (36; J. Schickli, unpublished observation).

The RSV candidate vaccines were analyzed for levels of replication in the respiratory tract of AGMs and for the ability to elicit a protective immune response against wild-type RSV challenge. Antibodies produced in response to expression of the RSV F protein by b/h PIV3 are expected to result in cross-neutralization and cross-protection against infection by all strains of RSV because the RSV F genes are highly conserved between RSV subgroups A and B. The most promising

b/h PIV3/RSV F vaccine candidate will be evaluated further for safety and efficacy in human clinical trials.

MATERIALS AND METHODS

Cells and viruses. Vero cells were maintained in modified Eagle's medium (MEM) (JRH Biosciences) supplemented with 2 mM L-glutamine, nonessential amino acids (NEAA), antibiotics, and 10% fetal bovine serum. b/h PIV3/RSV F2, b/h PIV3/sol RSV F2, RSV A2, RSV B 9320, and hMPV/NL/1/00 were propagated in Vero cells. Cells were infected with the viruses at a multiplicity of infection (MOI) of 0.1 PFU/cell. Three to 5 days postinfection, the cells and supernatant were collected and stabilized by adding 10× SPG (10× SPG is 2.18 M sucrose, 0.038 M KH$_2$PO$_4$, 0.072 M K$_2$HPO$_4$, and 0.054 M L-glutamate) to a final concentration of 1×. The virus stocks were stored at −70°C.

Virus titers were determined by plaque assays on Vero cells. Vero cells were infected with 10-fold serially diluted virus samples and overlaid with L-15 medium containing 1% methylcellulose and 2% fetal bovine serum. After 5 to 6 days of incubation at 35 or 37°C, the overlay was removed and the cells were fixed with methanol. Plaques were enumerated following immunostaining with a primary RSV polyclonal goat antibody (Biogenesis) or primary PIV3 polyclonal goat antibody (VMRD), and a secondary rabbit anti-goat immunoglobulin G (IgG) conjugated with horseradish peroxidase (Dako Corporation).

Human metapneumovirus (hMPV) plaque assays were performed in the same way except that the overlay contained OptiMEM (Invitrogen) and the infected cells were incubated at 37°C for 7 days prior to immunostaining. Following methanol fixation, hMPV plaques were stained with a primary ferret anti-hMPV antibody (MedImmune Vaccines, Inc.) and a secondary horseradish peroxidase-conjugated goat anti-ferret IgG (Immunology Consultants Laboratory). All primary and secondary antibodies were used at a 1:1,000 dilution except the ferret anti- hMPV antibody, which was used at a 1:500 dilution. The virus stocks were stored at −70°C.

Generation of full-length b/h PIV3/sol RSV F2 cDNA and recombinant virus. The b/h PIV3/sol RSV F2 cDNA harbored the fusion (F) and hemagglutinin-neuraminidase (HN) genes derived from human PIV3 and the RSV F gene from RSV A2, while the rest of the viral genome originated from bPIV3. The previously described plasmid 1–5 bPIV3/RSV F2 was used as a DNA template for PCR (36). This plasmid contained bPIV3 sequences from nucleotides (nt) 1 to 5200 and the RSV F gene inserted at nt 1774. A PCR fragment containing a partial RSV F gene without the 150 nucleotides from the 3′ end was generated. The PCR fragment was digested with HpaI and SalI and introduced into 1–5 bPIV3/RSV F2 treated with HpaI and SalI to yield plasmid 1–5 bPIV3/sol RSV F2. The bPIV3 subclone harboring the sol RSV F gene in the second position was digested with SphI and NheI, and a 6.3-kb DNA fragment was isolated. This fragment was ligated to a 14-kb NheI-SphI DNA fragment containing the remaining b/h PIV3 genome to generate the full-length b/h PIV3/sol RSV F2 cDNA plasmid. The recombinant virus was recovered by reverse genetics as described previously (36). High-titer virus stocks were generated and quantified by plaque assays on Vero cells.

Primate studies. RSV- and PIV3-seronegative AGMs (*Cercopithecus aethiops*) (3.5 to 6.5 years old, 2.6 to 5.8 kg) were identified with an RSV F IgG enzyme-linked immunosorbent assay (ELISA) (Immuno-Biological Laboratories) and a hemagglutination inhibition (HAI) assay (described below) on primate "presera" collected 14 days prior to the study start date. The study protocol was approved by the IAACUC committee at the primate facility. The primates were housed in individual microisolator cages. The primary enclosures were as specified in the U.S. Department of Agriculture Animal Welfare Act and as described in the Guide for the Care and Use of Laboratory Animals (National Academy Press).

The monkeys were infected intranasally and intratracheally with b/h PIV3/ RSV F2, b/h PIV3/sol RSV F2, RSV A2, and hMPV/NL/1/00. The nasal dose volume was 0.5 ml per nostril, and the tracheal dose volume was 1 ml. On day 1, each animal received a dose of 2 ml containing 2 × 10^5 to 3 × 10^5 PFU of virus in OptiMEM (Invitrogen) containing 1 × SPG. The placebo animal group received the same dose of OptiMEM supplemented with 1 × SPG. The dosing of the monkeys was performed in the following manner. The monkeys were lightly sedated with a 1:1 (vol/vol) mixture of ketamine and diazepam at 10 mg of ketamine and 0.5 mg of diazepam per kg. For intranasal dosing, a syringe was used to slowly expel the inoculum into both nostrils. For intratracheal dosing, a flexible latex tube was inserted through the mouth and advanced into the trachea with the help of a laryngoscope. After the tube was in place, the dosing syringe was attached to the exposed end of the tube, and the inoculum was slowly dripped into the tracheal region, followed by an air flush prior to withdrawal of the tube.
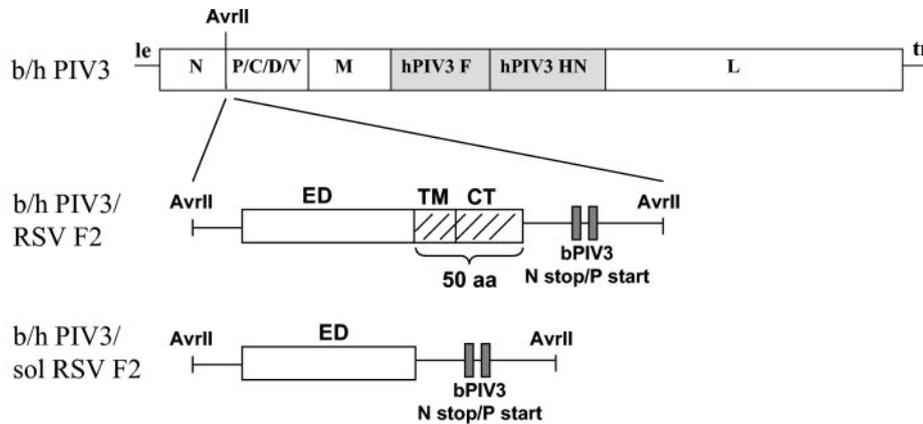
FIG. 1. Diagram of the viral RNA genomes of the b/h PIV3-vectored RSV F vaccine candidates, showing the open reading frames of the constructs. b/h PIV3/RSV F2 contained the native RSV F gene in PIV3 genome position two, while b/h PIV3/sol RSV F2 expressed a soluble RSV F lacking the transmembrane and cytosolic domains. The removal of the transmembrane domain and cytosolic tail of the RSV F protein was accomplished by deleting 50 amino acids at the C terminus. The PIV3 gene stop and gene start sequences of the native RSV F and sol RSV F gene cassettes were identical. le, leader; tr, trailer; ED, ectodomain; TM, transmembrane domain; CT, cytoplasmic tail; AvrII, restriction enzyme site used for RSV F gene insertions.

On day 28, all animals were challenged intratracheally and intranasally with 7 × 10⁵ PFU of RSV A2 (1 ml at each site). Nasopharyngeal (NP) swabs were collected daily for 11 days postimmunization and postchallenge from each monkey with sterile cotton swabs moistened in OptiMEM containing 1 × SPG. Each cotton swab was placed into a tube containing 1.0 ml of OptiMEM with 1 × SPG and immediately frozen on dry ice. Bronchoalveolar lavage (BAL) specimens were collected on days 1, 3, 5, 7, and 9 postimmunization and postchallenge. The monkeys were sedated with a ketamine-valium mixture to allow passage of a sterile laryngoscope and endotracheal tube. The feeding tube was passed through the endotracheal tube, and 5 to 10 ml (~2 ml/kg) of warm sterile saline was instilled and gently suctioned back into the syringe promptly. The BAL samples were adjusted to a final concentration of 1 × SPG, and two aliquots of the samples were frozen on dry ice and stored at −70°C.

Blood samples obtained from the femoral vein were collected on days 0, 7, 14, 21, 28, 35, 42, 49, and 56 for serological analysis. The serum samples were stored at −70°C. The animals were monitored for body temperature changes indicating a fever, signs of a cold, runny nose, sneezing, loss of appetite, and body weight. Virus present in the frozen NP and BAL specimens was quantitated by plaque assays on Vero cells that were immunostained with goat polyclonal RSV antiserum.

PRNA. The plaque reduction neutralization assays (PRNAs) were carried out for sera obtained on days 1, 28, and 56 postdose from primates infected with b/h PIV3/RSV F2 and b/h PIV3/sol RSV F2. The primate sera were heat inactivated at 56°C for 1 h, twofold serially diluted, and incubated with 100 PFU of RSV A2 in the presence of guinea pig complement for 1 h at 4°C. The virus-serum mixtures were transferred to Vero cell monolayers and overlaid with 1% methyl cellulose in EMEM/L-15 medium (JRH Biosciences; Lenexa, Kans.) containing 2% fetal bovine serum and 1% antibiotics. After 6 days of incubation at 35°C, the monolayers were immunostained with RSV goat polyclonal antiserum for quantitation. Neutralization titers were expressed as the reciprocal log₂ of the highest serum dilution that inhibited 50% of viral plaques.

RSV F IgG ELISA. The heat-inactivated primate sera from days 1, 28, and 56 from the vaccinated animals were analyzed for the presence of RSV F IgG in an ELISA (Immuno-Biological Laboratories, Hamburg, Germany) according to the manufacturer's instructions. The secondary monkey antiserum (Rockland Inc.) was used at a 1:1,000 dilution.

hPIV3 microneutralization assays. Microneutralization assays were performed on Vero cells. Serial twofold dilutions of heat-inactivated primate serum, starting at 1:4, were incubated at 37°C for 60 min with 100 50% tissue culture infections doses (TCID₅₀) of hPIV3. Virus-serum mixtures were transferred to cell monolayers in 96-well plates and incubated at 37°C for 6 days, after which all wells were observed for cytopathic effect (CPE). Neutralization titers were expressed as the reciprocal of the highest serum dilution that inhibited CPE. Neutralization antibody titers of ≤1:4 (the lowest serum dilution tested) were assigned a reciprocal log₂ titer of 2.

PIV3 HAI assay. HAI assays were performed by incubating serial twofold dilutions of primate serum at 25°C for 30 min with 8 HA units/0.05 ml of either bPIV3 or hPIV3. Subsequently, guinea pig red blood cells were added to each well, incubation was continued for 90 min, and each well was observed for hemagglutination. HAI titers were expressed as the reciprocal of the highest dilution of antiserum that inhibited virus-mediated agglutination of erythrocytes.

RESULTS

Recombinant b/h PIV3 expressing native or soluble RSV F protein. We had previously shown that Syrian Golden hamsters immunized with b/h PIV3 expressing the RSV F protein were protected from both wild-type hPIV3 and RSV A2 challenge (36). These promising results warranted further analysis of b/h PIV3/RSV F2 efficacy in a nonhuman primate model as an RSV vaccine candidate.

Chimeric viruses displaying viral surface glycoproteins originating from two different pathogens, e.g., hPIV3 and RSV, may result in altered pathogenesis and disease because they may spread more extensively in the host, causing virus replication in tissues and cells not normally associated with a natural hPIV or RSV infection. To address this safety concern, in vitro neutralization studies were carried out for b/h PIV3/RSV F2 (36). RSV polyclonal and RSV F monoclonal antibodies were unable to neutralize b/h PIV3/RSV F2. However, the chimeric virus was readily neutralized with PIV3 polyclonal antibodies (36). These results indicated that the presence of the native RSV F protein did not alter the neutralization properties of b/h PIV3/RSV F2, although the finding cannot rule out the presence of small amounts of RSV F protein that may be associated with the virion envelope. However, any amount of RSV F protein associated with the virion was not able to functionally substitute for the PIV3 F protein in the immunological assay.

To further address this safety concern, a b/h PIV3 was generated that expressed a soluble form of the RSV F protein lacking the transmembrane and cytosolic domains, rendering the RSV F protein incapable of being inserted into the virion membrane (Fig. 1). The removal of the transmembrane and cytosolic domains was accomplished by deleting 50 amino acids at the C terminus of the RSV F protein. The bPIV3 gene end
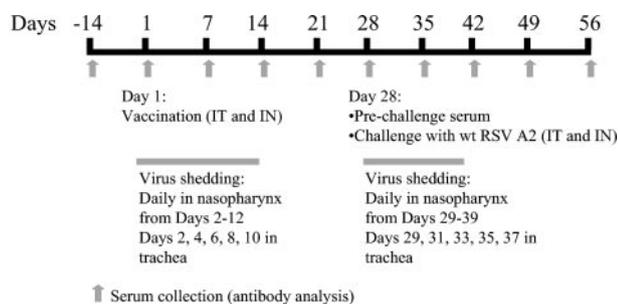
FIG. 2. Outline of the AGM primate study design from day −14 to day 56. Serum was collected at the indicated time points (arrows). Initial vaccinations on day 1 and RSV challenge administration on day 28 are indicated. wt, wild type.

and gene start sequences of the sol RSV F gene cassette remained identical to that of the full-length RSV F gene cassette (Fig. 1). Both chimeric b/h PIV3 viruses expressed the native and soluble RSV F proteins efficiently and replicated to high titers of 10^7 to 10^8 PFU/ml in tissue culture (36) (data not shown).

b/h PIV3/RSV F2 and b/h PIV3/sol RSV F2 replicated efficiently in the respiratory tract of AGMs. AGMs have been shown to support high levels of RSV A and RSV B replication in the lower (LRT) and upper respiratory tract (URT) (17). This nonhuman primate model was chosen to test the ability of the b/h PIV3/RSV F2 and b/h PIV3/sol RSV F2 vaccine candidates to protect against challenge with RSV. The study design is summarized in Fig. 2. Briefly, on day 1, RSV- and PIV3-seronegative AGMs were immunized intranasally and intratracheally with b/h PIV3/RSV F2 or b/h PIV3/sol RSV F2. A positive control group was infected with wild-type RSV A2, and the negative control groups were administered placebo medium or hMPV, a closely related paramyxovirus. On day 28, all animals were challenged with wild-type RSV A2. Virus replication in the URT and LRT of the animals following initial dosing and RSV challenge was quantitated by virus titration of the NP and BAL samples.

Following vaccination with b/h PIV3/RSV F2, monkeys shed vaccine virus for 7 days in the nasopharynx displaying a mean peak titer of 5.6 $\log_{10}$ PFU/ml and for 9 days in the trachea with mean peak titers of 7.0 $\log_{10}$ PFU/ml. Immunization of AGMs with vaccine virus expressing the soluble form of the RSV F protein, b/h PIV3/sol RSV F2, resulted in virus shed-

ding for 8 days in the nasopharynx, showing mean peak titers of 5.6 $\log_{10}$ PFU/ml, and for 7 days in the trachea, with peak titers of 6.8 $\log_{10}$ PFU/ml (Table 1). In contrast, infection of primates with wild-type RSV A2 resulted in 6 days of virus shedding in the nasopharynx, achieving mean peak titers of 3.3 $\log_{10}$ PFU/ml, and 8 days of virus shedding in the trachea, displaying peak titers of 5.0 $\log_{10}$ PFU/ml. The animals that were administered placebo medium did not shed virus (Table 1).

The replication properties of hMPV in the respiratory tract of AGMs were described elsewhere (26). The daily mean replication titers showed that b/h PIV3 RSV F2 and b/h PIV3/sol RSV F2 achieved peak virus titers of ~5 $\log_{10}$ in the URT on day 4 postimmunization (Fig. 3A). In the LRT, b/h PIV3/RSV F2 peaked on day 4 postdose, and b/h PIV3/sol RSV F2 reached the highest level of replication on day 8 postvaccination (Fig. 3B). In contrast, the animals infected with RSV A2 did not display a pronounced peak in virus replication in the URT but shed smaller amounts of virus continuously (Fig. 3A). In the LRT, the highest level of RSV shedding was observed on day 6 postinfection (Fig. 3B). Thus, immunization of nonhuman primates with b/h PIV3/RSV F2 or b/h PIV3/sol RSV F2 resulted in similar high levels of replication and duration of virus shedding for both vaccine candidates tested.

The animals were observed for 11 days postvaccination and 11 days postchallenge for signs of RSV disease, such as rhinorrhea, cold, or fever. No signs of disease were noted during the first 11 days postvaccination, a period of acute virus replication, or the time following RSV challenge (data not shown). The lack of RSV disease signs in AGMs is not surprising because chimpanzees are the only nonhuman clinical model for RSV that display disease signs.

AGMs immunized with b/h PIV3/RSV F2 or b/h PIV3/sol RSV F2 were protected from RSV A2 challenge. In order to evaluate immune protection from RSV infection, the vaccinated primates were challenged with a high dose of wild-type RSV A2 4 weeks postimmunization. Efficacy was measured as a reduction in shed RSV challenge virus titer in the URT and LRT of the infected animals. Primates immunized with b/h PIV3/RSV F2 or b/h PIV3/sol RSV F2 were effectively protected from RSV A2 challenge (Table 1; Fig. 3C and 3D). Only one animal vaccinated with b/h PIV3/RSV F2 shed low levels of challenge virus (1.8 $\log_{10}$ PFU/ml) for 1 day in the nasopharynx and 1 day in the trachea (1.6 $\log_{10}$ PFU/ml). The mean

TABLE 1. AGMs immunized with b/h PIV3/RSV F2 or b/h PIV3/sol RSV F2 were effectively protected from challenge with RSV A2

| Immunizing virus[a] | No. of animals | Mean peak titer ($\log_{10}$ PFU/ml) ± SE[b] | | | |
| | | Prechallenge | | Postchallenge | |
		NP	BAL	NP	BAL
RSV A2	3	3.3 ± 1.5	5.0 ± 0.4	<1.2 ± 0.2	<1.0 ± 0.0
b/h PIV3/RSV F2	4	5.6 ± 1.0*	7.0 ± 0.4*	<1.2 ± 0.4	<1.2 ± 0.3
b/h PIV3/sol RSV F2	4	5.6 ± 0.2*	6.8 ± 0.4*	<1.1 ± 0.2	<1.0 ± 0.0
hMPV	3	ND	ND	4.0 ± 0.1	5.0 ± 0.2
Placebo	2	0.0 ± 0.0	0.0 ± 0.0	4.3 ± 0.3	5.7 ± 0.3

[a] Animals were inoculated with the indicated virus at each site intranasally and intratracheally in a 1-ml volume.
[b] Mean peak virus titer is the mean of the highest titer of virus of each animal in the specific group during the course of the study. Animals were challenged on day 28 with 7×10^5 PFU of RSV A2. ND, not determined. *, $P = 0.0571$ (two-sided Wilcoxon exact P values) for NP and BAL compared to RSV A2. $P = 1.0$ (NP) and $P = 0.486$ (BAL) compared to each other.
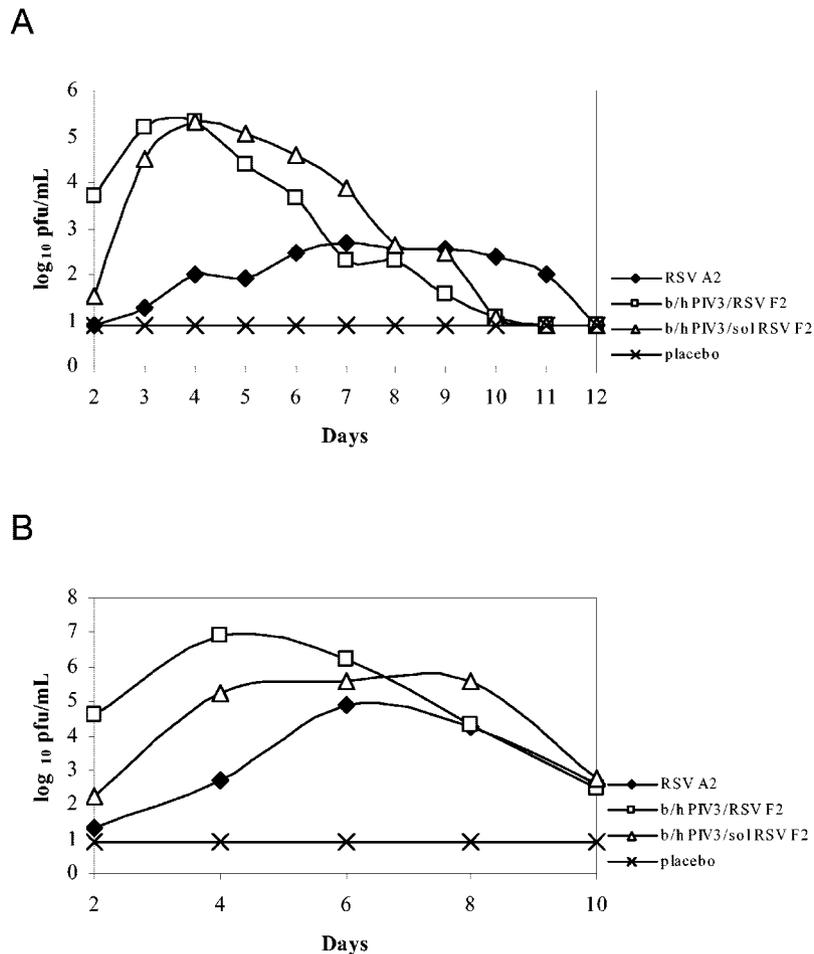
A

B

FIG. 3. Kinetics of daily b/h PIV3/RSV F2 and b/h PIV3/sol RSV F2 replication in the URT and LRT of AGMs for 11 days postimmunization and 11 days postchallenge. The daily virus titers were determined by plaque assays immunostained with RSV polyclonal antisera. The mean virus titers observed (A) in the URT of AGMs postvaccination determined from NP samples and (B) in the LRT determined from BAL samples are shown. The RSV challenge virus shedding in the URT (C) and LRT (D) of AGMs is shown for 11 days postchallenge. Viruses: RSV A2 (◆), b/h PIV3/RSV F2 (□), b/h PIV3/sol RSV F2 (△), hMPV/NL/1/00 (●), and placebo (×).

peak titers for this treatment group were 1.2 $\log_{10}$ PFU/ml in the URT and 1.2 $\log_{10}$ PFU/ml in the LRT.

The animals that were administered b/h PIV3/sol RSV F2 were also protected from wild-type RSV challenge (Table 1; Fig. 3C and 3D). One animal displayed low levels of challenge virus shedding (1.3 $\log_{10}$ PFU/ml) for 3 days in the nasopharynx, but this animal did not shed RSV in the trachea. The mean peak titers observed for the b/h PIV3/sol RSV F2-immunized primates were 1.1 $\log_{10}$ PFU/ml in the nasopharynx and 1.0 $\log_{10}$ PFU/ml in the trachea. Similar levels of immune protection were observed for the AGMs infected with wild-type RSV A2 (Table 1; Fig. 3C and 3D). This group showed levels of 1.2 $\log_{10}$ PFU/ml and 1.0 $\log_{10}$ PFU/ml of shed RSV challenge virus in the nasopharynx and trachea, respectively. In fact, only one animal that was administered RSV shed virus for 1 day and only in the nasopharynx. In contrast, treatment groups that received placebo medium displayed high levels of RSV challenge virus replication, 4.3 $\log_{10}$ PFU/ml in the nasopharynx and 5.7 $\log_{10}$ PFU/ml in the trachea, and the primates shed challenge virus for 8 days in both the URT and LRT (Fig. 3C and D).

AGMs that were administered hMPV, a related paramyxovirus, on day 1 were not protected from RSV challenge and shed RSV challenge virus for 8 days in the URT and LRT (Fig. 3C and D). This treatment group represented a negative control group analogous to the placebo group and demonstrated that hMPV-infected AGMs were not protected from RSV, another human pneumovirus. Mean peak titers of 4.0 and 5.0 $\log_{10}$ PFU/ml in the URT and LRT, respectively, of AGMs were observed (Table 1).

AGMs immunized with b/h PIV3/RSV F2 or b/h PIV3/sol RSV F2 produced protective RSV serum antibodies. The efficacy of the b/h PIV3-vectored RSV vaccine candidates was further evaluated by the levels of RSV-neutralizing and RSV F IgG serum antibody titers produced 4 weeks postimmunization. The RSV-neutralizing antibody titers were determined with 50% PRNA (Table 2). AGMs infected with wild-type RSV A2 displayed high RSV neutralizing antibody titers of 9 $\log_2$ 4 weeks postinfection when an RSV subgroup A was used as the antigen in the PRNA. A 5 $\log_2$ reduction in RSV-neutralizing antibody titers was observed when RSV subgroup B was employed in the PRNA. The vaccine candidates b/h
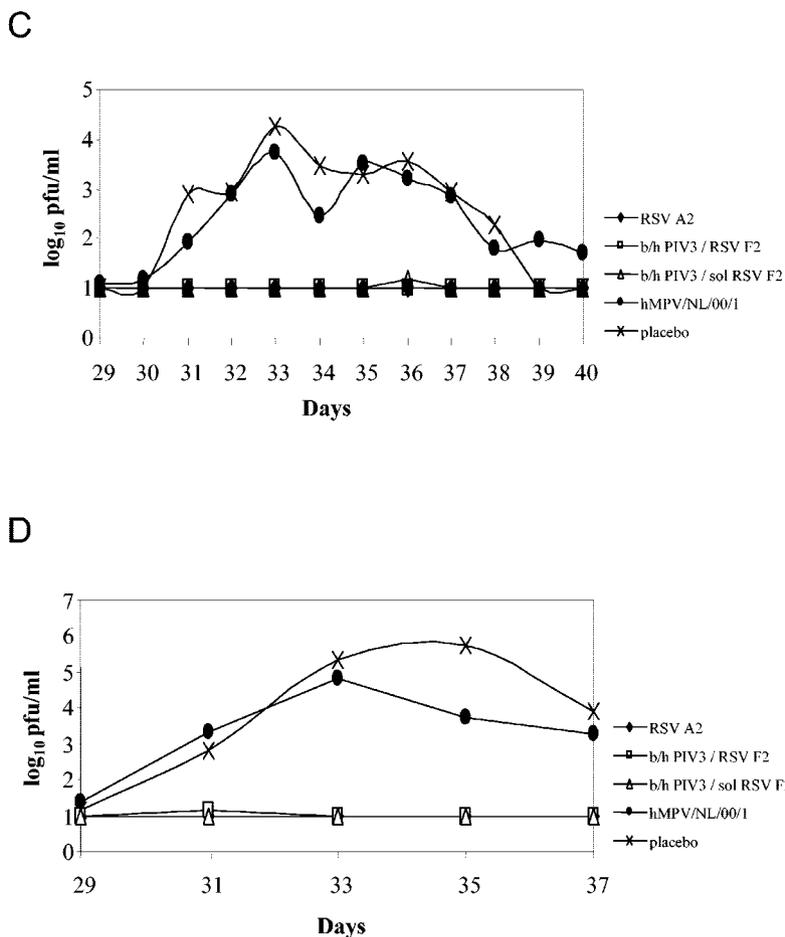
C

D

FIG. 3—Continued.

PIV3/RSV F2 and b/h PIV3/sol RSV F2 showed RSV-neutralizing antibody titers of ~4 log2 on day 28 postdose when RSV subgroup A or subgroup B was used as the antigen. In contrast, serum derived from animals that were administered placebo medium did not display RSV-neutralizing antibody titers for either RSV subgroup A or B.

The serum obtained on day 56, 4 weeks post-RSV challenge, was also tested for the presence of RSV-neutralizing antibodies (Table 2). Day 56 sera derived from AGMs infected with wild-type RSV A2 showed a 1.7 log2 increase in RSV-neutralizing antibody titer when subgroup A was tested, but the RSV-neutralizing antibody titer did not increase for subgroup B. A significant rise in neutralizing antibody titer for day 56 sera originating from b/h PIV3/RSV F2- and b/h PIV3/sol RSV

TABLE 2. Vaccination of AGMs with b/h PIV3/RSV F2 and b/h PIV3/sol RSV F2 produced RSV-neutralizing and RSV F-specific IgG serum antibody titers

Immunizing virus	Day of serum collection[a]	Mean RSV-neutralizing antibody titers[b] (50% reciprocal $\log_2$ ± SE)		RSV F IgG (U/ml) geometric mean $\log_2$ antibody titers[c]
		RSV A	RSV B	
RSV A2	28	9.0 ± 1.0	4.2 ± 1.0	8.6
	56	10.7 ± 0.6	4.3 ± 1.3	9.1
b/h PIV3/RSV F2	28	4.0 ± 1.0	3.4 ± 1.8	8.2
	56	4.1 ± 2.0	5.0 ± 1.4	9.0
b/h PIV3/sol RSVF2	28	4.1 ± 1.5	4.6 ± 1.4	8.0
	56	4.3 ± 1.0	5.0 ± 1.1	9.4
Placebo	28	<2.2 ± 0.3	<2.0 ± 0.0	2.3
	56	9.0 ± 0.1	2.0 ± 0.0	8.1

[a] All animals displayed RSV-neutralizing antibody titers of <2.4 log2 and RSV F IgG titers of <3.6 log2 U/ml on day 1. Serum was collected on day 1 (prior to immunization), day 28 (prior to RSV challenge), and day 56 (4 weeks post-RSV challenge).
[b] RSV A2 and RSV B 9320 were used as antigens in the neutralization assay.
[c] P > 0.05 (two-sided Wilcoxon exact P values). Pre- and postchallenge values were not significantly different for all three viruses.

TABLE 3. AGMs immunized with b/h PIV3/RSV F2 or b/h PIV3/sol RSV F2 induced hPIV3 neutralizing and HAI serum antibodies

Virus used for immunization	Date of serum collection[a]	hPIV3-neutralizing geometric mean reciprocal $\log_2$ antibody titer	Reciprocal geometric mean PIV3 HAI antibody titer[b]	
			hPIV3	bPIV3
b/h PIV3/RSV F2	Day 28	6.1	128.0	11.3
	Day 56	5.6	64.0	8.0
b/h PIV3/sol RSV F2	Day 28	5.8	128.0	16.0
	Day 56	5.7	64.0	8.0
Placebo	Day 28	<2.0	<4.0	<4.0
	Day 56	<2.0	<4.0	<4.0

[a] hPIV3 neutralizing antibody titers of <2.0 $\log_2$ and PIV3 HAI titers of <4.0 were present in day 1 sera.
[b] hPIV3/Wash/47885/57 and bPIV3/Kansas/15626/84 were used as antigens in the HAI assay.

F2-immunized primates for either subgroup A or B antigens was not observed. Placebo animal serum samples showed a 7 $\log_2$ increase in RSV-neutralizing antibody titer on day 56 for subgroup A RSV but only a low level of neutralizing antibodies for subgroup B.

To further measure the immune responses elicited by the vectored PIV3/RSV vaccines, RSV F protein-specific IgG levels were analyzed predose (day 1), 4 weeks postdose (day 28), and 4 weeks postchallenge (day 56) (Table 2). The predose primate sera from all treatment groups displayed values of less than 3.6 $\log_2$ IgG U/ml, indicating the absence of RSV F-specific IgG. In contrast, 4 weeks postvaccination, RSV F-specific IgG levels for sera derived from b/h PIV3/RSV F2 and b/h PIV3/sol RSV F2 showed titers of 8.2 and 8.0 $\log_2$, respectively. Similar levels of 8.6 $\log_2$ RSV F IgG titers were observed in day 28 sera originating from RSV A2-infected animals. As expected, only the day 28 sera of the placebo animals did not contain RSV F IgG. The RSV F IgG titers for day 56 sera from RSV A2-, b/h PIV3/RSV F2-, and b/h PIV3/sol RSV F2-immunized animals rose by 0.5 to 1.4 $\log_2$ in titer from the levels observed for day 28 sera. Day 56 sera obtained from the placebo animals challenged with RSV A2 showed a ~7 $\log_2$ rise in RSV F-specific IgG titer.

PIV3/RSV immunization of AGMs resulted in production of hPIV3 neutralizing and HAI serum antibodies. To evaluate whether the b/h PIV3/RSV vaccines could protect not only from RSV but also potentially from hPIV3 infection, primate sera were analyzed for the presence of hPIV3 neutralization and HAI serum antibodies (Table 3). Day 28 and 56 primate sera from animals immunized with b/h PIV3/RSV F2 and b/h PIV3/sol RSV F2 showed hPIV3 neutralizing antibody titers of ~6 $\log_2$. Human PIV3-specific HAI antibody titers of 128 and 64 were observed for b/h PIV3/RSV F2 and b/h PIV3/sol RSV F2 in day 28 and day 56 sera, respectively. Lower HAI antibody titers of 11.3 and 16.0 were displayed when the bPIV3 antigen was tested on day 28 sera. The day 56 sera displayed even lower bPIV3 HAI titers of 8.0. Since the surface glycoproteins F and HN of the b/h PIV3/RSV viruses were derived from human PIV3, a higher HAI serum antibody titer to the homologous antigen (hPIV3) was observed than to the heterologous bPIV3 antigen. hPIV3 neutralizing or PIV3 HAI serum antibodies were not detected in sera derived from placebo recipients.

DISCUSSION

A variety of approaches have been used in the past to immunize against disease caused by human RSV infection. To date, only inactivated, subunit, and live attenuated RSV vaccines have been evaluated in human clinical trials (22, 29, 34). Formalin-inactivated RSV did not protect vaccinees against RSV infection, and vaccinated individuals were more likely to develop severe RSV-associated disease than naïve individuals during subsequent RSV infection (22).

Following the failure of inactivated RSV vaccine, vaccine development focused on immunization with live attenuated RSV. The first live attenuated RSV vaccines tested in human trials were cold-passaged and/or chemically mutagenized viruses displaying temperature sensitivity. These vaccine candidates failed because they were either over- or underattenuated, and wild-type revertants were often isolated from vaccinees (23, 27, 28). Cold-passaged, temperature-sensitive viruses 248/955 and 530/1009, a more current series of RSV strains, were evaluated in RSV-seronegative children as young as 6 months. However, both of these vaccine candidates were insufficiently attenuated for further evaluation in infants (19). Cold-passaged, temperature-sensitive virus 248/404, the most attenuated live RSV tested in humans to date, caused mild to moderate congestion in the upper respiratory tract of infants 1 to 2 months old and therefore was still underattenuated as a vaccine for early infancy (40).

While there are currently no suitably attenuated live RSV vaccines for use in young infants, the clinical trials showed that immunization with a live RSV (i) did not result in enhanced disease during RSV reinfection, (ii) could elicit protective immunity against RSV infection in infants 1 to 2 months old, (iii) could be achieved in the presence of maternal antibodies, and (iv) might require two or more doses to achieve satisfactory infection rates and antibody responses.

Subunit vaccines consisting of purified RSV F were also evaluated as potential vaccines for immunization of the elderly (9, 10) and high-risk children (12, 31) and for maternal immunization (F. M. Munoz, P. A. Piedra, M. Maccato, C. Kozinetz, and W. P. Glezen, RSV after 45 Years, abstr., p. 45). In the elderly, purified RSV F was moderately immunogenic (6); 25 to 48% of the elderly vaccinees showed a rise equal to or greater than fourfold in RSV-neutralizing antibody titers. A phase 3 trial of 298 children with cystic fibrosis immunized with purified RSV F showed no statistically significant differences in

the frequency of LRT infections between the vaccinated and those receiving placebo (9).

Other RSV subunit vaccines that have been evaluated in clinical trials include BBG2Na, a fusion protein consisting of highly conserved residues 130 to 230 of the G protein from RSV conjugated to the albumin binding domain of streptococcal protein G (32). BBG2Na was well tolerated in healthy adults and moderately immunogenic; 33 to 71% of those immunized had a rise equal to or greater than twofold in neutralizing antibody titer. RSV subunit vaccines had minimum reactogenicity and did not cause enhanced disease; however, they were only moderately immunogenic (34).

The approach presented here utilizes a virus vector to deliver RSV F with the aim of inducing both humoral and cell-mediated immunity against RSV infection. Other virus vectors have been used in the past for delivery of RSV F and RSV G proteins. Vaccinia viruses F and G were separately able to induce long-term protection against wild-type RSV challenge in BALB/c mice (4). However, vaccinia viruses F and G failed to induce adequate levels of neutralizing antibody in seronegative chimpanzees. No protection was detected in the URT and incomplete protection was found in the LRT when the chimpanzees were challenged with RSV (3, 5). Adenoviruses expressing RSV F, RSV G, and RSV F and G have also been tested in RSV-seronegative chimpanzees and found to be poorly immunogenic (16).

Our vector delivery system did not elicit the level of neutralizing antibodies seen with wild-type RSV infection, presumably because only one RSV antigen was expressed. However, both the upper and lower respiratory tracts of AGMs immunized with both vaccine candidates were protected against RSV challenge 1 month postimmunization. It is not clear how long the immune response to RSV and/or hPIV3 will persist in AGMs. In hamsters, we were unable to detect any decay in RSV-neutralizing antibody and HAI antibody titer 53 days after immunization with 10^5 PFU of b/h PIV3/RSV F2 (R. Tang, unpublished data). Tao et al. showed that PIV3 immunity can last up to 4 months in hamsters (37).

The chimeric b/h PIV3/RSV F vaccines produced RSV-neutralizing antibodies specific for both RSV subgroups A and B. The high degree of conservation of the amino acid sequences between the RSV F proteins of subgroup A and B resulted in shared neutralizing epitopes. Not surprisingly, the levels of RSV-neutralizing antibody titers were lower by 5 $\log_2$ for b/h PIV3/RSV F than those observed for primate sera obtained from AGMs infected with wild-type RSV A2.

In the b/h PIV3/RSV vaccines, RSV neutralizing antibodies were produced only in response to the RSV F protein rather than to the whole RSV virus particle. The levels of RSV B cross-neutralizing antibody for sera obtained from AGMs infected with RSV A2 were reduced by 5 $\log_2$ compared to the antibody levels observed when the homologous RSV A2 antigen was tested. In contrast, a decrease in RSV B specific-neutralizing antibody titers produced by b/h PIV3/RSV F2 and b/h PIV3/sol RSV F2 was not observed. These results suggested that the serum neutralizing antibody levels induced by the RSV F protein were sufficient to protect primates from RSV challenge 1 month postvaccination.

Although the RSV-neutralizing antibody titers were lower for b/h PIV3/RSV F primate sera, the neutralizing activity for subgroup A and B RSV strains was essentially identical. Primate sera derived from wild-type RSV infection, displayed high RSV-neutralizing titers for the homologous RSV A antigen and lower levels for the RSV B antigen, which were similar in titer to those observed for the vectored PIV3/RSV F vaccines. A rise (>6 $\log_2$) in RSV F IgG antibody titers was observed for primates infected with RSV A2 or immunized with the b/h PIV3/RSV F vaccines. A further increase in either RSV-neutralizing or IgG antibody titers was not observed for animals vaccinated with b/h PIV3/RSV F or b/h PIV3/sol RSV F in response to the RSV challenge. Since the RSV neutralizing antibody titers measured for PIV3/RSV F vaccines were lower than those observed for sera obtained from primates infected with wild-type RSV, cellular immune responses may have played a role in generating such effective protection from RSV challenge. Future studies will address the contribution of the cellular immune system and of secretory IgA antibodies to the efficacy of the live attenuated PIV3/RSV vaccines.

The b/h PIV3 vector is expected to be attenuated in humans because the majority of the viral genome is derived from bPIV3, which was demonstrated to be safe in children (20). Skiadopoulos et al. clearly showed, using a rhesus monkey attenuation model, that the bPIV3 attenuation phenotype was polygenic in nature (35). While the bPIV3 F and HN genes contain some genetic determinants specifying attenuation, the greatest contribution to the attenuation phenotype was ascribed to the bPIV3 N and P proteins. Schmidt et al. evaluated a number of b/h PIV3-expressing RSV antigens from different PIV3 genome positions for replication in the respiratory tract of rhesus monkeys (33). All of the chimeric b/h PIV3-expressing RSV proteins replicated less efficiently than b/h PIV3 in the URT. Slightly higher titers (~0.5 $\log_{10}$ TCID$_{50}$/ml) were observed in the LRT of rhesus monkeys compared to the vector b/h PIV3. Taken together, these data further validate the expectation that b/h PIV3/RSV will be attenuated in humans.

Infants do not possess a well-developed immune system, and therefore multiple vaccine administrations may be necessary to develop long-lasting and protective immunity to RSV. Vaccination at 2, 4, and 6 months of age may be conceivable, ideally to be scheduled concurrently with other routine childhood vaccinations. PIV3 is highly immunogenic, and the first PIV/RSV vaccination induces high levels of PIV3 antibodies. This may result in vector immunity, such that subsequent immunizations with PIV/RSV may not produce a further rise in antibody titer. While we have not directly addressed this issue experimentally, a recent study by Karron et al. presented data showing that multiple doses of PIV3 will not result in vector immunity provided the dose administrations are spaced far enough apart (18).

The administration of a single dose of cp-45 PIV3 vaccine, a cold-passaged, temperature- sensitive virus, restricted the magnitude of vaccine replication after the second dose. However, the frequency of infection with a second dose of vaccine was clearly influenced by the dosing interval. Only 24% of infants shed virus when a second dose of vaccine was administered 1 month later. In contrast, 62% of infants shed virus when the second dose was administered 3 months after the first dose. These results suggested that to minimize PIV3 vector immunity effects, the interval between vaccinations should be >1 month but <3 months.

While the main goal of this study was to evaluate b/h PIV3/ RSV F2 and b/h PIV3/sol RSV F2 as potential RSV vaccines, we also wanted to determine whether hPIV3 serum HAI and neutralizing antibody titers were produced in response to vaccination. The levels of hPIV3 HAI and neutralizing antibodies observed for the primate sera obtained from animals immunized with both kinds of b/h PIV3/RSV F vaccines were similar to the titers displayed by rhesus monkeys vaccinated with b/h PIV3 (30). Rhesus monkeys immunized with b/h PIV3 were effectively protected from challenge with wild-type hPIV3. These results suggested that b/h PIV3 vectored RSV vaccines may be developed in the future as bivalent vaccines to protect infants from both RSV and hPIV3 infections and disease.

REFERENCES

1. **Chanock, R. M., and B. R. Murphy.** 1991. Past efforts to develop safe and effective RSV vaccines p. 35–42. *In* Meignier et al. (ed.), Animal models of respiratory syncytial virus infection. Merieux Foundation, Paris, France.
2. **Collins, P. L., and B. R. Murphy.** 2002. Respiratory syncytial virus: reverse genetics and vaccine strategies. Virology **296:**204–211.
3. **Collins, P. L., R. H. Purcell, W. T. London, L. A. Lawerence, R. M. Chanock, and B. R. Murphy.** 1990. Evaluation in chimpanzees of vaccinia virus recombinants that express the surface glycoproteins of human respiratory syncytial virus. Vaccine **8:**164–168.
4. **Connors, M., P. L. Collins, C. Y. Firestone, and B. R. Murphy.** 1991. Respiratory syncytial virus (RSV) F, G, M2 (22K) and N proteins each induce resistance to RSV challenge, but resistance induced by M2 and N proteins is relatively short-lived. J. Virol. **65:**1634–1637.
5. **Crowe, J. E., Jr., P. L. Collins, W. T. London, R. M. Chanock, and B. R. Murphy.** 1993. A comparison in chimpanzees of the immunogenicity and efficacy of live attenuated respiratory syncytial (RSV) temperature-sensitive mutant vaccines and vaccinia virus recombinants that express the surface glycoproteins of RSV. Vaccine **11:**1395–1404.
6. **Dudas, R. A., and R. A. Karron.** 1998. Respiratory syncytial virus vaccines. Clin. Microbiol. Rev. **11:**430–439.
7. **Durbin, A. P., W. R. Elkins and B. R. Murphy.** 2000. African green monkeys provide a useful nonhuman primate model for the study of human parainfluenza virus types-1, -2, and -3 infection. Vaccine **18:**2462–2469.
8. **Durbin, A. P., and R. A. Karron.** 2003. Progress in the development of respiratory syncytial virus and parainfluenza virus vaccines. Clin. Infect. Dis. **37:**1668–1677.
9. **Falsey A. R., and E. E. Walsh.** 1997. Safety and immunogenicity of a respiratory syncytial virus subunit vaccine (PFP-2) in the institutionalized elderly. Vaccine **15:**1130–2.
10. **Falsey, A. R., and E. E. Walsh.** 1996. Safety and immunogenicity of a respiratory syncytial virus subunit vaccine (PFP-2) in ambulatory adults over age 60. Vaccine **14:**1214–1218.
11. **Falsey, A. R., C. K. Cunningham, W. H. Barker, R. W. Kouides, J. B. Yuen, M. Menegus, L. B. Weiner, C. A. Bonville, and R. F. Betts.** 1995. Respiratory syncytial virus and influenza A infections in the hospitalized elderly. J. Infect. Dis. **172:**389–394.
12. **Groothuis, J. R., S. J. King, D. A. Hogerman, P. R. Paradiso, and E. A. Simoes.** 1998. Safety and immunogenicity of a purified F protein respiratory syncytial virus (PRP-2) vaccine in seropositive children with bronchopulmonary dysplasia. J. Infect. Dis. **177:**467–469.
13. **Hall, C. B.** 2001. Respiratory syncytial virus and parainfluenza virus. N. Engl. J. Med. **344:**1917–1927.
14. **Hall, C. B.** 1999. Respiratory syncytial virus: a continuing culprit and conundrum. J. Pediatr. **135:**2–44.
15. **Haller, A. A., T. Miller, M. Mitiku, and K. Coelingh.** 2000. Expression of the surface glycoproteins of human parainfluenza virus type 3 by bovine parainfluenza virus type 3, a novel attenuated virus vaccine vector. J. Virol. **74:**11626–11635.
16. **Hsu, K.-H. L., M. D. Lubeck, A. R. Davis, R. A. Bhat, B. H. Selling, B. M. Bhat, S. Mizutani, B. R. Murphy, P. L. Collins, and R. M. Chanock.** 1992. Immunogenicity of recombinant adenovirus-respiratory syncytial virus using Ad4, Ad5, and Ad7 vectors in dogs and a chimpanzee. J. Infect. Dis. **166:** 769–775.
17. **Jin, H., X. Cheng, V. L. Traina-Dorge, H. J. Park, H. Zhou, K. Soike, and G. Kemble.** 2003. Evaluation of recombinant respiratory syncytial virus gene deletion mutants in African green monkeys for their potential as live attenuated vaccine candidates. Vaccine **21:**3647–3652.
18. **Karron, R. A., R. B. Belshe, P. F. Wright, B. Thumar, B. Burns, F. Newman, J. C. Cannon, J. Thompson, T. Tsai, M. Paschalis, S.-L. Wu, Y. Mitcho, J. Hackell, B. R. Murphy, and J. M. Tatem.** 2003. A live human parainfluenza type 3 virus vaccine is attenuated and immunogenic in young infants. Pediatr. Infect. Dis. J. **22:**394–405.
19. **Karron, R. A., P. F. Wright, J. E. Crowe, Jr., M. L. Clements-Mann, J. Thompson, M. Makhene, R. Casey, and B. R. Murphy.** 1997. Evaluation of two live, cold-passaged temperature-sensitive respiratory syncytial virus vaccines in chimpanzees and in human adults, infants and children. J. Infect. Dis. **176:**1428–1436.
20. **Karron, R. A., M. Makhene, K. Gay, M. H. Wilson, M. L. Clements, and B. R. Murphy.** 1996. Evaluation of a live attenuated bovine parainfluenza type 3 vaccine in two- to six-month-old infants. Pediatr. Infect. Dis. J. **15:** 650–654.
21. **Karron, R. A., P. F. Wright, S. L. Hall, M. Makhene, J. Thompson, B. A. Burns, S. Tollefson, M. C. Steinhoff, M. H. Wilson, D. O. Harris, et al.** 1995. A live attenuated bovine parainfluenza virus type 3 vaccine is safe, infectious, immunogenic and phenotypically stable in infants and children. J. Infect. Dis. **171:**1107–1114.
22. **Kim, H. W., J. G. Canchola, C. D. Brandt, G. Pyles, R. M. Chanock, K. Jensen, and R. H. Parrott.** 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am. J. Epidemiol. **89:**422–434.
23. **Kim, H. W., J. O. Arrobio, C. D. Brandt, P. Wright, D. Hodes, R. M. Chanock, and R. H. Parrott.** 1973. Safety and antigenicity of temperature-sensitive (ts) mutant respiratory syncytial virus (RSV) in infants and children. Pediatrics **52:**56–63.
24. **Krilov, L. R.** 2002. Palivizumab in the prevention of respiratory syncytial virus disease. Expert Opin. Biol. Ther. **2:**763–769.
25. **Lee, M. S., D. P. Greenberg, S.-H. Yeh, R. Yogev, K. S. Reisinger, J. I. Ward, M. M. Blatter, et al.** 2001. Antibody responses to bovine parainfluenza virus type 3 (PIV3) vaccination and human PIV3 infection in young infants. J. Infect. Dis. **184:**909–913.
26. **MacPhail, M., J. H. Schickli, R. S. Tang, J. Kaur, C. Robinson, R. A. M. Fouchier, A. D. M. E. Osterhaus, R. R. Spaete, and A. A. Haller.** 2004. Identification of small animal and primate models for evaluation of human metapneumovirus (hMPV) vaccine candidates and implications for hMPV vaccine design. J. Gen. Virol. **85:**1655–1663.
27. **McIntosh, K., A. M. Arbeter, M. K. Stahl, I. A. Orr, D. S. Hodes, and E. C. Ellis.** 1974. Attenuated respiratory syncytial virus vaccines in asthmatic children. Pediatr. Res. **8:**689–696.
28. **McKay, E., P. Higgins, D. Tyrrell, and C. Pringle.** 1988. Immunogenicity and pathogenicity of temperature-sensitive modified respiratory syncytial virus in adult volunteers. J. Med. Virol. **25:**411–421.
29. **Murphy, B. R. and P. L. Collins.** 2002. Live-attenuated virus vaccines for respiratory syncytial and parainfluenza viruses: applications of reverse genetics. J. Clin. Investig. **110:**21–27
30. **Pennathur, S., A. A. Haller, M. MacPhail, T. Rizzi, S. Kaderi, F. Fernandes, L. Bicha, J. H. Schickli, R. S. Tang, W. Chen, S. Nguyen, S. Mathie, H. Mehta, and K. L. Coelingh.** 2003. Evaluation of attenuation, immunogenicity and efficacy of a bovine parainfluenza virus type 3 (PIV-3) vaccine and a recombinant chimeric bovine/human PIV-3 vaccine vector in rhesus monkeys. J. Gen. Virol. **84:**3253–3261.
31. **Piedra, P. A., S. Grace, A. Jewell, S. Spinelli, D. Bunting, D. A. Hogerman, F. Malinoski, and P. W. Hiatt.** 1996. Purified fusion protein vaccine protects against lower respiratory tract illness during respiratory syncytial virus season in children with cystic fibrosis. Pediatr. Infect. Dis. J. **15:**23–31.
32. **Power, U. F., T. N. Nguyen, E. Rietveld, R. L. de Swart, J. Groen, A. D. Osterhaus, R. de Groot, N. Corvaia, A. Beck, N. Bouveret-Le-Cam, and J. Y. Bonnefoy.** 2001. Safety and immunogenicity of a novel recombinant subunit respiratory syncytial virus vaccine (BBG2Na) in healthy young adults. J. Infect. Dis. **184:**1456–1460.
33. **Schmidt, A. C., D. R. Wenzke, J. M. McAuliffe, M. St. Claire, Huang, W. R. Elkins, B. R. Murphy, and P. L. Collins.** 2002. Mucosal immunization of rhesus monkeys against respiratory syncytial virus subgroups A and B and human parainfluenza virus type 3 by using a live cDNA-derived vaccine based on a host range-attenuated bovine parainfluenza vurs type 3 vector backbone. J. Virol. **76:**1089–1099.
34. **Simoes, E. A., D. H. Tan, A. Ohlsson, V. Sales, and E. E. Wang.** 2001. Respiratory syncytial virus vaccine: a systematic overview with emphasis on respiratory virus subunit vaccines. Vaccine **20:**954–960.
35. **Skiadopoulos, M. H., A. C. Schmidt, J. M. Riggs, S. R. Surman, W. R. Elkins, M. St. Claire, P. L. Collins, and B. R. Murphy.** 2003. Determinants of the host range restriction of replication of bovine parainfluenza virus type 3 in rhesus monkeys are polygenic. J. Virol. **77:**1141–1148.
36. **Tang, R. S., J. H. Schickli, M. MacPhail, L. Bicha, J. Spaete,**

R. A. M. Fouchier, A. D. M. E. Osterhaus, R. Spaete, and A. A. Haller. 2003. Effects of human metapneumovirus and respiratory syncytial virus antigen insertion in two 3′ proximal genome positions of bovine/human parainfluenza virus type 3 on virus replication and immunogenicity. J. Virol. **77:** 10819–28.

37. Tao, T., F. Davoodi, C. J. Cho, M. H. Skiadopoulos, A. P. Durbin, P. L. Collins, and B. R. Murphy. 2000. A live attenuated recombinant chimeric parainfluenza virus (PIV) candidate vaccine containing the hemagglutinin-neuraminidase and fusion glycoproteins of PIV1 and the remaining proteins from PIV3 induces resistance to PIV1 even in animals immune to PIV3. Vaccine **18:**1359–1366.

38. Van Wyke Coelingh, K. L., C. C. Winter, E. L. Tierney, W. T. London, and B. R. Murphy. 1988. Attenuation of bovine parainfluenza virus type 3 in nonhuman primates and its ability to confer immunity to human parainfluenza virus type 3 challenge. J. Infect. Dis. **157:**655–662.

39. Welliver, R. C. 2003. Review of epidemiology and clinical risk factors for severe respiratory syncytial virus (RSV) infection. J. Pediatr. **143**(Suppl. 5)**:**S112–S117.

40. Wright, P. F., R. A. Karron, R. B. Belshe, J. Thompson, J. E. Crowe, Jr., T. G. Boyce, L. L. Halburnt, G. W. Reed, S. S. Whitehead, E. L. Anderson, A. E. Wittek, R. Casey, M. Eichelberger, B. Thumar, V. B. Randolph, S. A. Udem, R. M. Chanock, and B. R. Murphy. 2000. Evaluation of a live, cold-passaged, temperature- sensitive, respiratory syncytial virus vaccine candidate in infancy. J. Infect. Dis. **182:**1331–1342.